DRUG TARGETING WITH PHAGOCYTIC POLYMORPHONUCLEAR LEUCOCYTES

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To

my MUM

my inspiration

to

my family

and friends

my support

To

Them all

my love.

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DRUG TARGETING WITH PHAGOCYTIC POLYMORPHONUCLEAR LEUCOCYTES

A thesis submitted by Robert Hyde BSc. for the degree of
Doctor of Philosophy

SUMMARY

1. Phagocytic polymorphonuclear leucocytes (PMNLs) or neutrophils have a marked avidity for the uptake of particulate material and are the first cell type to respond to inflammatory stimuli *in vivo*.
2. By harnessing these pathophysiological characteristics the inherent targeting capacity of the PMNL could be exploited to carry drug loaded particles to these sites.
3. *In vitro* chemotaxis of PMNLs through cellulose nitrate filters (pore size 5μm diameter) was studied in response to N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) in the Blindwell chamber assay
4. After phagocytosis of 1.1μm polystyrene latex (PSL) beads at a range of incubation concentrations (5,10,20,and 30 beads/cell) the migration of the PMNL population was not significantly different from control, without beads.
5. The distribution of the beads within the filter showed that a disproportionately large number of PSL (~50%) were associated with the cells on the surface of the filter that had not penetrated the filter. Eighty percent of the PMNL population migrated and despite containing less PSL beads/cell, 50% of the dose was carried into the filter. Between 5 and 10% of these PSL were carried beyond 60μm in the assay.
6. These results suggested heterogeneity of the PMNL population and to achieve efficient targeting with these cells preferential selection of the migratory sub-population would be needed.
7. The air-pouch model was then developed to study the focal accumulation of PMNLs *in vivo* . The PMNL isolated did not survive long enough in the circulation due to the trauma of the isolation procedure used an alternative method will have to be employed.

KEYWORDS: Polymorphonuclear leucocytes (PMNLs), Drug Targeting, Chemotaxis, Phagocytosis,

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LIST OF ABBREVIATIONS

PMNL	Phagocytic polymorphonuclear leucocyte or neutrophil
MNL	Mononuclear leucocyte
MPS	Mononuclear phagocytic system
FMLP	N-formyl-L-methionyl-L-leucyl- L-phenylalanine
HBSS	Hanks balanced salt solution
DMSO	Dimethyl sulphoxide
FACS	Fluorescent activated cell sorting
I.P.	Intraperitoneal
PSL	Polystyrene latex beads
L.I.	Leucotactic index
L.F.	Leading front
S.C.	Subcutaneous
I.V.	Intravenous
MIF	Macrophage inhibitory factor
TNF	Tissue necrosis factor
MDP	Muramyl dipeptide
MAF	Macrophage activating factor
ITP	Idiopathic thrombocytopenic purpura
AIHA	Autoimmune haemolytic anaemia
MGP	Marginating granulocyte pool
CGP	Circulating granulocyte pool
LTB	Leucotriene B_4
HEPES	N-2-hydrxyethylpiperazine-N-2-ethanesulphonic acid

CHAPTER 1.

CHAPTER 1. INTRODUCTION.

1.1. AN INTRODUCTION TO DRUG TARGETING

Drug delivery is moving rapidly into a new phase - that of targeting drugs to specific sites in the body. In recent years knowledge of natural membranes, cell receptors and receptor recognising molecules have brought the vectoring of drugs as forseen by Erlich and his 'Magic Bullet' to the forefront of developmental research. By the use of carrier systems it is hoped that the side-effects associated with drugs may be circumvented. The carrier system will serve to protect the body from the drug and vice versa and tissue specific targeting could be achieved using ligands which are attracted to the desired site. This new generation of drug delivery systems, as described by Gregoriadis (1977), Tomlinson (1989, 1987, 1983), Poznansky and Juliano (1984), Pouton (1985) and Trouet (1985) has involved the development and use of many natural and synthetic carriers.

The potential of the drug targeting concept is immense and, in the ideal situation, the drug carrier unit would preserve its integrity in the circulation, avoid interaction with normal non-target cells, penetrate tissue barriers at the appropriate site and selectively recognize and associate with the diseased cells. After localisation, release of the active drug would follow and the carrier would be safely disposed of. Successful targeting therefore depends on the carrier drug unit, the target site and the biological barriers that separate the two. The first consideration in drug delivery research must be the disease condition where subtle or gross changes in the anatomy and pathophysiology of the

tissue are manifested. These changes could be exploited by careful design of the carrier system to achieve enhanced localization.

Site-specific drug delivery systems utilising various carrier systems, such as colloidal particles (Tomlinson, 1987, Davis and Illum, 1986, Illum and Davis, 1985), liposomes (Senior, 1987, Fidler, 1986, Van Berkel *et al* , 1986, Schneider, 1985, Gregoriadis, 1981, Alving, 1983.), monoclonal antibodies (Pimm, 1988, Weinstein *et al* , 1986, Rowland, 1987, Vitetta *et al* , 1986), soluble polymers (Lloyd, 1987) and biological macromolecules (e.g. proteins) (Fiume *et al*, 1988) have been developed over the years though successful targeting has been limited by the inherent complexities of the biological milieu. The advantageous characteristics of antibodies, lectins and proteins for specific interactions with cell or tissue receptors has to be offset against their nonspecific localization in nontarget tissue. Survival of particulate material in the circulation is limited by avid and relatively indiscriminate clearance by cells of the mononuclear phagocytic system (MPS) and, as such, the MPS can be regarded as either a constraint to site specific delivery, or an opportunity for targeting .

1.2. THE MONONUCLEAR PHAGOCYTIC SYSTEM AND DRUG TARGETING

The MPS is a connective tissue system of cells, distributed throughout the body, which includes the cell types listed in table 1.01. The principle functions of these cells are the clearance of a large variety of potentially harmful substances from plasma, catabolism of ingested macromolecules, participation in the immune response and synthesis and secretion of various effector molecules. Materials cleared include effete red blood cells, circulating tumor cells, inert colloids, autologous tissue debris,

immune complexes, denatured proteins, specific glycoproteins, bacterial endotoxins, steroids and lipoproteins (Brouwer and Knook, 1983). The phagocytic capacity of the MPS, coupled with its widespread distribution in the body (table 1.01), make it a considerable barrier to drug targeting particularly if the intended site of drug action is not immediately adjacent to the site of administration (Posnansky and Juliano, 1984).

When foreign particles are introduced intravenously, about 90% are extracted by the Kupffer cells, 5% by the spleen and less than 5% by the bone marrow (Tomlinson, 1987). The predominance of uptake in the liver does not only reflect the high concentration of macrophages but also their accessibility. After 100% transit through the lungs, about 28% of the blood from the heart goes through the liver and then the spleen, with less than 10% going through the skeleton (Tomlinson, 1987).
Macrophages are able to bind and engulf a variety of particles from the blood and adherence to the macrophage is often facilitated by the adsorbtion of opsonins including immunoglobulin G, complement C3b, and fibronectin, and also by independent interaction with the corresponding receptors on the macrophage. Non-specific processes are also recognised as contributing to clearance of latex particles (Lehnert and Tech, 1985, Hsu and Juliano, 1982) and it seems that the net surface charge, hydrophobicity and size of the particle also play an important part in determining the carriers' fate (Schwendener *et al.* , 1984, Abbracchio *et al.* , 1982, Van Oss, 1978).

In this very brief resumé of the MPS the problems and opportunities of targeting with particles are evident: targeting to the MPS is relatively easy but avoidance of the MPS, and hopefully targeting elsewhere in the body (e.g. to inflammation, infection, or tumors) is difficult.

Table 1.01. The cells which comprise the Mononuclear Phagocytic System and their tissue location

TISSUE	CELLS
NORMAL STATE	
Blood	Monocyte
Connective tissue	Histiocyte
Liver	Kupffer cell
Lung	Alveolar macrophage
Lymph nodes	Free and fixed macrophages
Spleen	Free and fixed macrophages
Bone marrow	Fixed macrophages
Serous cavities	Pleural and peritoneal Macrophages
Bone	Osteoclasts
Central nervous system	CSF macrophages
Skin	Histiocytes
Synovia	Type A cell
INFLAMMATION	
Any inflamed tissue	Exudate macrophage
	Exudate resident macrophage
	Epithelioid cell
	Multinucleated giant cell

1.2.1. TARGETING TO THE MPS

Clearance of drug carrier from the circulation by cells of the MPS has proved to be advantageous in the treatment of conditions involving these cells. The selective delivery of drugs encapsulated in liposome particles in various disease states, has been reviewed by Schroit *et al* (1983) (table 1.02).

Table 1.02. Macrophage-associated disease amenable to treatment with particulate drug delivery systems

DISEASE	DRUG
INTRACELLULAR PARASITES	
Leishmaniasis	Antimony drugs
Histoplasmosis	Amphotericin B
Leprosy	Sulphones
NEOPLASMS	
Histiocytosis X	Doxorubicin, vincristin
ENZYME STORAGE DISEASE	
Gaucher's disease	Glucocerebrosides ß-glucosidase

The MPS is the cell population involved in the progression of various diseases and coupled with its affinity for particulate material has been a popular tissue for targetted therapy. Targets include the facultative parasites that are able to survive and proliferate within the endocytic vacuole of the cells of the MPS and are not destroyed by lysosomal enzymes (Alving, 1983). Anti-infective therapy in the treatment of Leishmaniasis with antimonials or Candidiasis with amphotericin B were far more effective when the drug was delivered in colloidal carriers than given free (Poznansky and Juliano, 1984, Davis and Illum, 1986). Attempts have also been made to treat lysosomal storage diseases using colloidal carriers. In these disorders the lysosomes of the cell lack a particular enzyme with the result that undegraded material can build up in the lysosomal compartment. The lysosomal enzyme deficiency in Gaucher's disease (Gregoriadis, 1981), mucolipases type II and III (Tomlinson, 1987) and murine globoid leukodystrophy (Umezawa *et al*, 1985) are such disorders. Direct enzyme supplement or replacement is inappropriate due to enzyme breakdown and immune responses when given systemically. Encapsulation of the enzymes in liposome carriers and knowledge of liposome processing in these enzyme deficient phagocytic cells has facilitated deposition of the enzyme within the cellular lysosomes to restore some function (Poznansky and Juliano, 1984). Passive localisation of colloidal carriers in phagocytic cells could also be exploited as a method of delivering cytotoxic agents to the cells in therapy of neoplasms of the MPS. These conditions are quite rare and include histiocytic medullar reticulocis, monocytic leukaemia, hairy cell leukaemia and certain forms of Hodgkin's disease (Davis and Illum, 1986).

Much attention has been given to the question of macrophage activation by bacterial cell wall components and their synthetic analogues. These molecules (e.g. muramyl-dipeptide (MDP)) and the lymphokines (MIF, MAF, C-reactive protein, gamma-interferon and TNF) tend to have poor delivery to macrophages and are rapidly excreted and catabolised by the liver (Davis and Illum, 1986). When encapsulation of these compounds into colloids was investigated they were highly effective at activating macrophages *in vitro* and *in vivo* (Fidler, 1986). Targeting of these compounds activates the macrophage in situ and boosts their tumor cell surveillance and killing activity (Peil, 1985, Fidler, 1980, 1986, and Gregoriadis, 1980). While success in this area is limited to those situations where the tumour burden is not too high it will work against a wide variety of different tumour cells and could form a part in combination therapy. Fidler (1986) suggested that liposomal encapsulated drugs may have potential for immunomodulation in antiviral therapy and also for direct treatment of virus infection of the MPS cells.

1.2.2. AVOIDANCE OF THE MPS

Targeting to the MPS poses few problems however, it is the rest of the body that is difficult to access specifically. Because of the inability of particulate carriers to leave the circulation they can only have a use that is restricted to either biochemical or cellular targets within the vasculature. In addition discrete extravascular targets can be reached when the pathological state permits it. Access of particles to vascular or extravascular targets is dependent upon avoidance of the cells of the MPS. Various methods have been used to achieve this for example, by MPS

blockade using polystyrene latex particles or dextran (Illum*et al*, 1986), altering opsonisation in such a way that the particles adhere to other surfaces or exploit passive and receptor mediated processes to access other compartments. It has been shown that particles coated with either negative or positive macromolecules can have altered organ uptake (Tomlinson, 1987). Steric stabilization by introduction of hydrated (hydrophilic) polymer layer to the surface of the particles has reduced opsonisation and hence altered distribution. Poloxamers of different hydrophilicity/hydrophobicity have been grafted on to particles (Davis and Illum, 1986) reducing liver uptake and diverting non-extravasating particles to other vascular targets, including monocytes and bone marrow.

Current research into avoidance of the MPS to prolong survival of carriers in the circulation seems to diverge from the normal targeting concept. The localisation of the drug-carrier will then rely on physiological changes at the focus of the disease, for example increased permeability, and passive accumulation rather than specific interaction. Once prolonged survival in the circulation is achieved an important question arises; will the introduction of a tissue specific ligand, for homing to the target, reprogram the complex for clearance by the MPS?
To avoid the majority of problems involved in designing a targetable carrier need we look any further than the cells in the circulation?

1.3. BLOOD CELLS IN DRUG DELIVERY

1.3.1 ERYTHROCYTES

Erythrocytes have been extensively studied as drug carriers (Ihler, 1979, 1982, Gregoriadis, 1981, Pitt, 1982, Offord, Pitt & Lewis, 1985, Poznansky & Juliano, 1984). Encapsulation of molecules into erythrocytes has been attempted using various methods, including interaction of the cells with liposomes (Jung, 1987), electric modification (Tsong, 1987), osmotic loading (Offord *et al* , 1985) and continuous flow dialysis (Ropars *et al* , 1987). The best results have been obtained using reverse hypotonic lysis (Offord *et al*, 1985) . During hypotonic haemolysis large holes or pores opened in the cell membrane through which low-molecular-weight compounds, macromolecules and even viruses could pass (Ihler and Tsang, 1987). When the original osmotic conditions were restored the pores closed trapping substances added to the external medium within the erythrocyte. By these gentle loading techniques the cells can survive in the circulation after reinfusion with near normal life span (Lewis & Alpar, 1984, Ihler and Tsang, 1987.). Early work with erythrocyte carriers focused on their utilization in enzyme-replacement-therapy for inborn errors of metabolism. The proposed uses have expanded to encompass slow release depots for enzymes, such as asparaginase (Alpar and Lewis, 1983) to remove tumour-promoting amino acids. In addition erythrocye carriers have been used as capsules for drugs like the chemotherapeutic agents cyclophosphamide or methotrexate (Alpar and Lewis, 1987) or anti-inflammatory agents cortisol-21-phosphate or alpha-1-antitrypsin (Pitt 1982, Pitt et al, 1983) to prolong the therapeutic efficacy of the drug. An alternative to slow release delivery is rapid targeting to the MPS. By heat damage, desialylation or coating with Rhesus factors the

encapsulated erythrocytes are cleared by macrophages like liposomes and any foreign particle in the circulation. They could be used for delivery of drugs to treat Leishmaniasis or to introduce lysosomal enzymes, such as glucocerebrosidase, to the erythrophagocytic cells of patients with lysosomal storage diseases (e.g. Gaucher's disease).

1.3.2. PLATELETS

The platelet also serves as carrier of biological substances and drugs (Yeon et al , 1987). By active transport it can selectively accumulate 5-hydroxytryptamine, histamine, dopamine, amino acids, adenine, adenosine, serotonin catecholamines and it binds angiotensin, hydrocortisone and other hormones. They also bind various drugs which include the vinca alkaloids, Vinblastin and vincristin, which have been used widely in the treatment of leukaemia, lymphoma and other solid tumors. These drugs bind specifically to tubulin in the cytoskeleton of the platelet and can be loaded and delivered in this form. Macrophages of the MPS were the primary target in the treatment of idiopathic thrombocytopenic purpura (ITP), an autoimmune disorder in which platelets react with an autoantibody and are cleared from the circulation by macrophages. Infusion of Vinblastin loaded platelets to the MPS can cause long lasting remission by selectively killing the phagocytic cells. This therapy was also applied in autoimmune haemolytic anaemia (AIHA) the red cell equivalent of ITP and in malignant disorders of the MPS as a combination treatment with chemotherapy and radiotherapy .

.3.3. LEUCOCYTES

Leucocytes, in respect of their pathophysiology, lend themselves spontaneously to targeting as infiltration of polymorphonuclear leucocytes (PMNL) , mononuclear leucocytes (MNL), and lymphocytes is a fundamental feature of many disease states and in damaged tissue (Ryan & Majno, 1971). Their localization has been demonstrated in various infectious conditions and also in noninfectious inflammatory lesions, for example in rheumatoid arthritis, myocardial infarction and malignant tumors (Reba & Chandeysson, 1980). The cell types involved originate from the blood, and adhere to the vascular endothelium before emigrating from the vessel (diapedesis) in order to dispose of pathogens, toxins and cell debris. They also carry out immune reactions to antigenic material and contribute to the repair of the injured tissue . By the nature of their function the inflammatory cells, mentioned above, offer great potential as carriers for drugs to specific sites in the body.

1.4. PMNL'S AS POTENTIAL VECTORS FOR DRUGS

The phagocytic PMNL's or neutrophils are the most promising candidates as drug targeting carriers since they have a marked avidity for the uptake of particulate material, and are able to respond quickly and in large numbers to the appropriate inflammatory stimuli. If these characteristics could be harnessed the inherent problems in avoidance of natural clearance mechanisms and the penetration of selected biological barriers encountered using other carriers (in particular particles) could possibly be overcome. To date, exploitation of these cells has been minimal though encouraging work has been conducted in the field of diagnostic radiology

in which workers have demonstrated focal accumulation of radiolabelled PMNLs into inflammatory lesions using a variety of isolation and labelling techniques (McAfee, Subramanian and Gagne, 1980, Thakur and McKenney, 1980, Rayuda, 1983,).

Certain soluble (McAfee & Thakur, 1976a. Zakhireh *et al* , 1979) and particulate (McAfee & Thakur 1976b, English & Andersen 1974) isotope complexes have been widely used as even after the trauma of isolation and radiolabelling the cell's viability , random migration , bactericidal capacity and most importantly chemotaxis were unimpaired in vitro . Therefore, the possibility for loading PMNLs *in vitro* without damaging their pathophysiological function presents encouraging evidence for the use of these cells in the drug targeting field. Further encouraging evidence was presented by Boggs (1974) who transfused PMNLs for the prevention and treatment of infection in patients with neutropenia. The migration of normal isogenic transfused PMNLs into induced inflammatory exudates in normal and neutropenic subjects was demonstrated (Boggs, 1974) and were shown to be able to survive longer periods of neutropenia with PMNL transfusion than without.

1.4.1 THE PATHOPHYSIOLOGY OF THE PHAGOCYTIC PMNL

In order to be used as a vector for drugs the ability of cells to respond normally to chemotactic stimuli is of paramount importance. The evidence presented earlier (section 1.4) provides a promising start but to understand more fully the potential in this field, as well as the problems, it is important to outline the fundamentals of the *in vivo* kinetics, the function of the PMNL and the relevant techniques for assessing them.

1.4.2 THE *IN VIVO* KINETICS OF THE POLYMORPHONUCLEAR LEUCOCYTE

The neutrophil or phagocytic PMNL is termed the professional phagocyte because it is specially designed to seek and destroy pathogenic organisms or foreign material in the body. PMNLs are formed in the bone marrow at the rate of approximately 10^{11} mature cells/day (Wade and Mandell, 1983). When released into the circulation their kinetics are complex and this is further complicated by the health and metabolic state of the individual. Peters *et al* (1980) have outlined the distribution of PMNLs between the "circulating" granulocyte pool (CGP) and the "marginating" granulocyte pool (MGP). From these studies the kinetics of the PMNLs are seen to be quite different from those of red blood cells and platelets. In healthy subjects PMNLs spend an average of 10 hours in the blood and the rate of loss of mature cells is primarily a random age dependent function. About 50% of the PMNLs are adherent to the walls of the capillaries or post-capillary venules (marginated pool) rather than circulating free (Boggs, 1974; Peters *et al* , 1980). Thus the PMNL concentration as determined in a sample of venous blood represents an average of half the intravascular population. Exchange between circulating and marginating pools is rapid and continuous under normal conditions. This reflects the protective and inflammmatory function of the PMNLs which are conducted primarily in exudates or tissues rather than in the blood. Therefore, since a PMNL must marginate before it can diapedese and enter the tissues, the preferential filling of the marginated pool at the expense of the circulating pool is advantageous in infectious conditions. Adrenaline and exercise cause the circulatory blood pool of

PMNLs to rise to 79% (Peters *et al* , 1980) in order to prepare the body for possible tissue damage. In the event of infection or damage, however, the balance is rapidly reversed and in spite of mobilization of vast reserves from the bone marrow a serious infection or inflammatory lesion can consume up to 12 times the number of cells normally present in the blood in 24 hours (Boggs, 1974).

A further complication to this picture of PMNL kinetics in the circulation is the apparent heterogeneity of the PMNL population. Observations of PMNL heterogeneity can be traced back to the 1920s when Florence Sabin (1923) described poor locomotory capacity in immature PMNLs. Thus heterogeneity of these cells for many years was felt to reflect variances in cell maturity rather than different sub-populations of cells. Also in the 1920s, McCutcheon (1923), who was the first to quantitate individual PMNL locomotion showed wide variation in rates of locomotion and more recently it has been shown that individual cells may vary upto tenfold in their rates of locomotion (Allan & Wilkinson, 1978). This variability was not felt to reflect differences in cell maturity but the endogenous differences within the cells. Numerous other observations suggest heterogeneity of PMNLs (Gallin, 1984) for example; infected sputum, even in the setting of overwhelming pneumonia, have bacteria associated with only some PMNLs and *in vitro* studies provide similar evidence suggesting heterogeneous phagocytosis as about 20 percent of adherent PMNLs will not ingest opsonised micro-organisms (Gallin, 1984). These findings prompted more intesive studies which have demonstrated that only 80 percent of the PMNLs readily express the Fc receptor (Klempner & Gallin, 1978). These cells had superior adhesiveness, aggregation and chemotaxis to the chemotactically active fragments of the fifth complement component, they also phagocytosed

and killed opsonised bacteria more efficiently. It has also been demonstrated that the PMNL population exhibit differential binding of chemoattractant peptides (Seligman et al, 1984). After stimulation with N-formylmethionylleucylphenylalanine (FMLP) approximately 65 percent of the cells transiently depolarize while the remaining 35 percent either do not respond or hyperpolarize slightly. Further studies with mouse monoclonal antibodies have led to the development of immunoglobulins that are specific for PMNL sub-populations (Gallin, 1984). 31D8 is one example developed by Dr H Malech and in collaborative studies this IgG (Seligman et al, 1985) was shown to recognise a sub-population (70 ± 10%) of human PMNLs. Selligman et al (1985) also demonstrated that the population of cells that bind 31D8 are the same cells that avidly bind formyl peptides and exhibit membrane depolarization following cell activation. The implications of the structural and functional heterogeneity of the PMNL population have not been fully elucidated but enrichment of cells expressing Fc receptors occurs in abscesses suggesting preferential mobilisation of these responsive cells.

1.4.3 PMNL RESPONSE TO INFLAMMATORY STIMULI

After considering PMNL kinetics in the body it is important to outline the avid localization of these cells in the target tissue. The mechanisms underlying PMNL accumulation in a tissue are complex and are fundamentally dependent on the interaction between the PMNL and the vascular endothelial cell, an interaction triggered by a chemical signal generated in the tissue. The mechanism involved can be best considered if the acute series of events following infection of a tissue with

microorganisms is outlined. The microbe has to be recognised as foreign before the relevant leucocyte recruitment signals are generated. If antibodies to the microbe or to its products are present, immune complexes can form and induce the "classical pathway" complement activation. In addition microbial cell wall or microbial products, for example endotoxin, can induce complement activation by the alternative pathway in the absence of antibody (Sim *et al* , 1986, Turk, 1983). Complement activation results in the coating (opsonisation) of the microbes with C3b. This facilitates subsequent phagocytosis by PMNLs which have the C3b receptors. The terminal product of complement activation is the macromolecular complex (C5b-9) thought to be in the form of a cylinder with a lipophylic end which causes lysis of the foreign cell by insertion into the membrane (Tschopp et al, 1982, Tranum-Jensen et al, 1978). Complement activation leads to the release of cleavage by-products. The most important is C5a after the cleavage of the fifth complement component. C5b combines spontaneously with C6, C7, C8 and C9 to form the lytic pore. The smaller by-product C5a exibits potent chemotactic activity (directed movement along a concentration gradient) and chemokinetic activity (increased random movement) *in vitro* . Other endogenous substances secreted by microbes can act in the same way (eg formyl peptides) and PMNLs can themselves modulate their environment with products of stimulated arachidonate metabolism (e.g. LTB_4) (Ford-Hutchinson *et al* , 1980).

The release of these factors in the tissue results in a reaction in the circulation and the key phase in PMNL recruitment, upon which all subsequent events depend, which is PMNL adherence. In normal conditions blood cells travel in single file through capillaries a common observation being columns of erythroctyes preceded by single leucocytes

(Williams et al, 1984). On entering wider diameter post-capillary vessels the leucocytes tend to be pushed toward the vessel wall by the column of smaller erythrocytes. The apparent predilection of PMNL adhesion (margination) in postcapillary vessels may be related primarily to shear forces since this is the first site of major decrease in vessel shear wall stress (Harlan, 1985). In response to an inflammatory stimulus or when a chemoattractant is applied, increased adherence between PMNLs and the vascular endothelial wall is evident (Williams et al, 1984). This results in PMNLs rolling along the wall and becoming fixed with their surfaces adjacent to the wall becoming flattened (Atherton & Born, 1972). It is possible that during their passage along capillaries and venules PMNLs experience a surface change because of the diffusion or active transport of chemoattractant molecules across the endothelial barrier to the microvessel lumen. Recent observations support an active role for the vascular endothelial cell in the induction and evolution of the inflammatory response. Rotrosen *et al* (1987) demonstrated formyl peptide processing by cultured human umbilical vein endothelial cells. These cells bind and internalise FMLP in a specific and saturable fashion and release a significant portion of the internalised FMLP in a biologically active form. Supporting a potential role for the endothelial cell in the modulation of leucocyte infiltration and activation in the inflammatory microenvironent. It has also been demonstrated that PMNLs express a 5 to 10 fold increase in specific surface ligands (Bainton et al, 1987) which mediate adhesion to endothelial cells after stimulation with FMLP. These structurally related glycoproteins include Mac-1, p150,95, lymphocyte function-associated antigen (LAF-1) and CDw18 (Buyon et al, 1988; Bainton et al, 1987; Harlan et al, 1987; Wallis et al, 1986) and although present on the PMNL cell surface early in the maturation stage they are present in an intracellular pool which are mobilised at the inflammatory

stimulus (Wallis *et al*, 1986). Adherence is followed by emigration or diapedesis through the interendothelial cell junctions (Ryan and Majno, 1977). The passage through the vessel wall was studied in detail (Marchesi and Florey, 1960) and clear pseudopodia were seen emerging from the flattened surface of the PMNL adjacent to the endothelial cell junction and once through the pseudopodium was followed by the rest of the cell. How chemoattractants induce this PMNL extravasation is not known *in vivo* . Once the PMNLs are adherent to the endothelium it is propossed that receptor mediated reactions stimulate migratory activity in response to chemoattractants (Wilkinson, 1982) and specific migration (chemotaxis) into the tissue. The evidence of the mechanism of chemotaxis in tissues is very limited and much of the knowledge in this field has to be extrapolated from *in vitro* assays to the complex situation in the tissues.

1.4.4. PMNL CHEMOTAXIS

The directional movement towards the source of a chemical gradient is termed chemotaxis. This process causes cells to accumulate at the source of the chemical signal. The factors that induce this reaction are defined as chemotactic factors or chemoattractants. The mechanism of leucocyte locomotion has been extensively studied in recent years and both Stossel (1978a) and Wilkinson (1982) visualize chemotaxis as having three phases: an initial sensory or recognition phase; a transduction phase in which the signal is translated and triggers the effector phase in which the cells' motility apparatus is activated to produce directional migration.

1.4.4.1 RECOGNITION

A wide range of substances attract leucocytes (Wilkinson, 1982). Of particular biological significance are the complement derived peptides (C5a and C5a des Arg) and the lipoxygenase products of arachidonic acid metabolism (e.g. leukotriene B_4). Also important, particularly in chronic inflammation, are the lymphokines predominantly of T lymphocyte origin. Since the finding that human PMNL's are attracted chemotactically by synthetic N-formylmethionyl peptides (Schiffman *et al* , 1975. Boxer *et al* , 1979.). N-formylmethionyl-L-leucyl-L-phenylalanine (FMLP) and its analogues have been used extensively as probes to study molecular events in chemotaxis. A formyl peptide receptor has been demonstrated on PMNLs (Schiffman, Corcoran and Aswanikumar, 1978.). C5a and leucotriene B_4 (LTB_4) also interact through specific receptor sites to evoke chemotaxis.

To supplement the specific interaction through receptors a more general form of recognition may be advantageous to leucocytes, since these cells are required to respond to disordered structure at the site of tissue damage in inflammation and also in normal clearance activity. It has been demonstrated that denaturation of globular proteins such as haemoglobin, myoglobin and serum albumin produces a conformational change within the molecule which leads to exposure of non-polar groups which are normally concealed (Wilkinson, 1982). This change which is associated with the acquisition of chemotactic activity suggests that leucocytes may be able to recognize broad, physicochemical properties of attractant molecules, for example hydrophobicity.

1.4.4.2 TRANSDUCTION

The binding or interaction of chemoattractant ligands with the cell surface actually initiates chemotaxis through biochemical mechanisms which are as yet poorly defined. However, certain measurable receptor-mediated changes are known to occur (Wilkinson, 1982) and may be involved in the translation of receptor binding to motility. The requirement of ions for chemotactic responsiveness has long been disputed. Calcium has been proposed as a second messenger in the activation sequence as changes in the cytosolic free calcium is one of the first measurable events subsequent to receptor occupancy (Korchak et al, 1984.). Increase in calcium influx and intracellular mobilization have been reported though probably only internal redistribution of calcium is necessary (Weening and Roos, 1980. Maresco, Becker and Olivier, 1979.). This may be related to the assembly of microtubules and/or microfilaments. Influx of potassium (K^+) and the efflux of sodium (Na^+) caused by activation of the plasma membrane (Na^+- K^+)ATPase have been reported (Showell and Becker, 1976.). These ionic movements induced by chemotactic stimuli, most probably lead to a decrease in the charge of the cell surface which has been proposed as the signal for the initiation of cell movement. There are a number of reports that deal with the role of cyclic nucleotides in leucocyte motility (Hill, 1978. Weening and Roos, 1980. Korchak *et al* , 1984.). Agents that increase intracellular cyclic AMP levels inhibit chemotaxis and conversely agents that increase the intracellular cyclic GMP concentration enhance cell motility. It appears that these mediators exert their effects by modulating microtubule assembly.

1.4.4.3 THE EFFECTOR PHASE

When PMNLs are exposed to a synthetic chemoattractant such as FMLP they undergo striking morphological change. The stimulated PMNLs begin to change shape and ultrastructural studies have indicated that orientation of intracellular components include localization of the centriole and its radial array of microtubules between the nucleus and advancing pseudopods (Stossel, 1978). Locomotion itself is thought to be the result of the sliding of interdigitating actin and myosin filaments (Stossel, 1978). Active binding protein, a Ca^{2+} ion dependent regulator protein (gelsolin) and some other proteins (acumentin and profilin) regulate the assembly and gelation of actin, and myosin contracts these gels during orderly making and breaking of cell-substratum contacts during locomotion (Weening and Roos, 1980).

1.4.5. PMNL PHAGOCYTOSIS

Phagocytosis is the cellular function essential for host defense against infection and also turnover of old body tissues. Phagocytic PMNLs are therefore required to recognize and respond to a plethora of different antigens. It is not conceivable that phagocytic cells are endowed with an infinite number of receptors to selectively recognize a wide range of foreign and damaged material. It has been demonstrated however, that receptors to the common serum opsonins C3b and to the Fc portion of the IgG molecule do exist (Weening and Roos, 1980, Stossel, 1974a, 1974b, 1978b). These receptors do not account for the recognition of all materials and Van Oss (1978) has proposed that the degree of surface hydrophobicity and the overall surface charge of the particle are important in recognition

and adherence; so only particles more hydrophobic and or more positively charged than the phagocyte will be ingested. Van Oss (1978) also proposed that opsonisation promotes phagocytosis by increasing the hydrophobicity of the particle surface. The actual process of recognition is therefore still a relative mystery as is the mechanism of transduction of adherence to ingestion. The moment particles adhere to the plasma membrane of PMNLs these cells are activated by an unknown mechanism. Redistribution of intracellular calcium as in chemotaxis seems to be important for transmitting the activation signal (Weening and Roos, 1980). Divalent cations (Ca^{2+} and Mg^{2+}) in the extracellular medium have an important effect on ingestion, as they do in migration and spreading (Stossel, 1974b). The involvement of methylation of phospholipids and the role of cyclic nucleotides is uncertain. After adherence to the plasma membrane pseudopods circumferentially surround opsonised hydrophobic particles by creeping from one opsonising molecule or hydrophobic region to another. This process is called the "zipper" mechanism of phagocytosis. The pseudopodia fuse at the distal side of the particle which thereby become enclosed within the phagocytic vesicle or the phagosome (Stossel, 1974b). Ingestion of particles is an active energy dependent event. Engulfment therefore activates an ATP generating processes, specifically glycolysis and glycogenolysis in PMNLs. These facts are consistent with the idea that an energy utilizing mechanism such as actin-myosin interactions drives ingestion as well as migration. The anatomy of engulfment supports this as the network of microfilaments that comprise the lamellipodium of migrating phagocytes is prominent in the pseudopods that surround the particle in phagocytosis (Stossel, 1974b).

In studies of phagocytosis and pinocytosis Pratten and Lloyd (1986) and Roberts and Quastel (1963) have demonstrated, using polystyrene latex (PSL) that the optimal size range for the phagocytosis of particles by PMNLs lies between 0.3μm and 3μm in diameter. Particles smaller than these are taken up in large numbers though not appreciably by weight as nonspecific pinocytotic uptake is taking place. Relatively little activation of the cell membrane is achieved as oxygen consumption is only marginally raised above normal (Roberts and Quastel, 1963).

Adherence of particles and hence perturbation of the plasma membrane triggers the fusion of cytoplasmic granules with that part of the plasma membrane surrounding the phagocytic vesicle and deliver their enzyme contents into the phagosome (degranulation) (Stossel, 1974b). Also during phagocytosis PMNL's are metabolically stimulated and within 30 seconds of initiation of phagocytosis a sharp increase in oxygen uptake by the cells occurs (the oxygen or respiratory burst). The consumed oxygen is converted enzymatically to hydrogen peroxide (H_2O_2) and other reactive oxygen metabolites (e.g. superoxide (O_2^-) and hydroxyl radicles (OH^-)) as a result of glucose oxidation by the hexose monophosphate shunt (HMP shunt) which is stimulated several fold during phagocytosis (Weening and Roos, 1980, Stossel, 1974b). As a result of degranulation and the respiratory burst before closure of the phagocytic vesicle products are spilled out extracellularly. This extracellular leakage has been attributed to the development of tissue erosion seen in certain inflammatory conditions; for example in rheumatoid arthritis. This phenomenon facilitates quantitative measurement of phagocytosis *in vitro* using the release of free radicles during the respiratory burst as an index of phagocytic activity.

1.5. AIMS AND OBJECTIVES

The aim of this investigation was to examine the potential of phagocytic cells, namely PMNLs, derived from the circulation as carriers of particles to sites of inflammation. The primary objective was to study the migration of a population of isolated and purified cells, and elucidate their active carrying capacity when loaded with particles *in vitro* . In addition it was hoped to extend these studies *in vivo* and determine whether the same cells containing particles would actively accumulate in an inflammatory lesion.

The investigation involved the development and routine use of a variety of techniques which included:-

(1) The reproducible isolation and purification of a large viable population of PMNLs.

(2) The development of a reliable method of measuring the chemotaxis of the isolated PMNL population.

(3) Establishment of a suitable phagocytosis assay.

(4) Investigation of the effect phagocytosis of different numbers of microspheres has on the chemotaxis of the population of PMNLs using the established protocols developed in (1), (2) and (3).

(5) Development of a model of inflammation to study the *in vivo* localisation of PMNLs loaded with particles.

CHAPTER 2.

CHAPTER 2. ISOLATION AND PURIFICATION OF RAT PMNLS

2.1 INTRODUCTION

The isolation of PMNLs in large numbers that were viable and exhibited normal pathophysiological functions was essential for these investigations. PMNLs could be purified from whole blood (Hardeman, 1980). Isolation of PMNLs from whole blood is a widely employed technique. Generally the maximal yield of a million cells per ml of whole blood, would necessitate the use of many rats to purify enough cells for study (Schmitt et al, 1986). Alternatively they may be obtained from an inflammatory exudate where the PMNLs would be in a relatively pure suspension providing the cells were harvested during the acute phase of the infiltration (Patel, 1985). The latter approach was that adopted as it had been shown to yield larger numbers of functional PMNLs as investigated by Patel (1985) in this laboratory. Some minor adaptations were employed (Cambell and Hallet, 1983) to improve the established technique.

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS

Adult male Wistar rats (average weight 200-250g) were purchased from Banting and Kingman (Hull, U.K.) and were used to raise PMNLs throughout these investigations. Animals were housed in the University animal house at 20°C and maintained on Rat and Mouse Breeding Diet, (Pilsbury's Limited, Birmingham, U.K.) with water *ad libitum*.

2.2.2 CHEMICALS AND REAGENTS

Analar grade reagents and solvents were supplied by BDH Chemicals, Poole, U.K and Sigma, Poole U.K. Sterile distilled water and saline (0.9% w/v) were supplied by West Midlands Regional Sterile Supply Unit,Wolverhampton, U.K. Medical grade oxygen (95% 0_2 / 5% CO_2) and Nitrous oxide (NO_2) were supplied by British Oxygen Company, Wolverhampton, U.K. Fluothane was supplied by ICI Pharmaceuticals Division, Macclesfield, Cheshire, U.K.

2.2.3 STERILISATION OF GLASSWARE AND REAGENTS

To minimise bacterial contamination all glassware, pipette tips, eppendorf tubes, millipore filters and the NaOH and HCl solutions were sterilised by heating in an autoclave at 15 pounds per square inch (PSI) for 20 minutes. The Hanks balanced salt solution (HBSS) (Gibco Ltd, Paisley, Scotland) was sterilised by filtration through a sterile millipore filter (0.22μm pore size) and stored at 4° C before use.

2.2.4 ANAESTHESIA

Halothane (Fluothane) anaesthesia was induced in the experimental animals prior to injection of the irritant and lavage of the inflammatory exudate. Oxygen (O_2/CO_2) and nitrous oxide were mixed in a 1 : 1 ratio at a flow rate of 500cc/min through a calibrated vapouriser on the Boyle's apparatus. To anaesthetise the animal halothane was delivered at 3-4%, which was reduced for maintainence during surgery or injection to 1-1.5%. Animals were sacrificed with an overdose of halothane and cervical dislocation following leucocyte collection.

2.2.5 OPERATION PROCEDURE FOR CELL ISOLATION

A PMNL population was raised according to the method of Cambell and Hallet (1983). A male Wistar rat was anaesthetised according to the procedure outlined in section 2.2.4. Peritonitis was induced by injection of 10mls of sodium caseinate (12% w/v) NaCl (0.9% w/v) into the peritoneal cavity. The animal was sacrificed after 12 hours. The belly fur was soaked with ethanol and removed exposing the abdominal muscle. A small incision was made along the ventral midline and the exudate recovered by lavaging with four 5ml aliquotes of cold (4°C) HBSS containing 10 U/ml heparin. The leucocyte rich fluid was harvested using a plastic pasteur pipette and collected in sterile universals (Sterilin, Teddington, Middlesex, U.K.) and kept on ice. The cells in the lavage fluid were recovered by centrifuge at 100g for 5 minutes at 4°C in a cool spin. The pellets were then pooled and the contaminating erythrocytes lysed by osmotic shock with ice cold distilled water (9 volumes) for 30 seconds. Isotonicity was restored by subsequent addition of 10 times concentrated HBSS (1 volume). After washing at 200g for 5 minutes the cell pellet was resuspended in 8mls HBSS. The cell suspension was then layered gently onto 3mls Histopaque-1077 (1.077g/ml), (Sigma Chemical Company) in a 15ml conical plastic tube and centrifuged at 400g for 30 minutes. The opaque interface containing predominantly mononuclear leucocytes (MNLs) was removed leaving a pellet of polymorphonuclear leucocytes.The resulting pellet was washed three times at 100g for 5 minutes in cold heparin HBSS before performing total, differential and viability counts.

2.2.6 TOTAL LEUCOCYTE COUNTS

Total leucocyte counts were made using an improved Neubauer counting chamber (Scientific Supplies Co.).

2.2.7 VIABILITY COUNTS

Assessment of PMNL viability was based on their ability to exclude trypan blue (1% w/v) (Sigma Chemical Co.). Viability was assessed after incubation for 5 minutes at 37° C prior to use in the chemotaxis assay. Unless otherwise stated the viability all cell populations used was greater than 95%.

2.2.8 DIFFERENTIAL LEUCOCYTE COUNTS

Assessment of the proportion of PMNLs in the isolated cell population was conducted using a histological technique. The cell preparations ($1x10^6$ cells) were centrifuged onto microscope slides using the cytobuckets developed for the Beckman (TJ6) centrifuge (see section 3.3.2). The slides were air-dried and were stained using Haema-Gurrs stain (BDH Diagnostics, Poole, U.K). Greater than two hundred cells were examined under light microscopy (times 100 magnification) and cells assigned as either polymorphonuclear or mononuclear.

2.2.9 COMPARISONS OF THREE METHODS USED TO RAISE A PMNL POPULATION.

Before the technique detailed in 2.2.5 for raising a population of PMNLs was employed, the method of Patel (1985) was used. Peritonitis was induced by injection of 10mls oyster glycogen (1% w/v). After 4 hours the animal was sacrificed and the exudate harvested by one of two methods: (1) 20mls of HBSS (10 U/ml Heparin) was injected into the peritoneum and the cells were drained through (1) a large gauge needle (Lewis, 1986); or (2) the exudate was collected from the open peritoneal cavity by washing with 4 x 5mls of HBSS (10 U/ml heparin) using a plastic pasteur pipette (section 2.2.5). In each case the cells were harvested, washed and total, viability and differential cell counts conducted (see section 2.2.6, 2.2.7 & 2.2.8).

PMNL chemotaxis can be stimulated by casein (Schmitt *et al* , 1986) without induction of degranulation or stimulation of the oxidative burst. Casein can therefore be used effectively as an irritant for induction of peritonitis to raise PMNL's (Talbot, 1985; Cambell and Hallet, 1983). Sodium caseinate (12% w/v in sterile NaCl (0.9% w/v)) was then compared with the use of oyster glycogen as the irritant of choice to elicit PMNLs.

2.2.10 DETERMINATION OF CELL INFILTRATION AND OPTIMAL HARVESTING TIME USING SODIUM CASEINATE AS THE ELICITING AGENT.

The method employed by Patel (1985) meant that 4 hours of the working day were lost whilst the cells were elicited. In view of the previous findings (see section 2.3.1) sodium caseinate was found to produce a

larger cell infiltration, at 4 hours after challenge. This study was therefore conducted to determine the time course of infiltration of inflammatory cells elicited with sodium caseinate, with the aim of optimising the cell yield and improving the time management. Peritonitis was induced according to the procedure detailed in 2.2.5 and the exudate was recovered from each group of animals (n=4) at 0, 1, 2, 4, 6, 12, and 24 hours. The cells were washed and total, viability and differential cell counts conducted.

2.3 RESULTS

2.3.1. COMPARISON OF THREE METHODS USED TO RAISE A PMNL POPULATION.

Harvesting of the peritoneal exudate by drainage through a large guage needle proved inappropriate because the lavage fluid could not be recovered in full. During the drainage insertion or manipulation of the needle resulted in extensive erythrocyte contamination and the yield of PMNL's was poor (17.5 ± 5.4 million cells (n=6). The alternative procedure facilitated recovery of the exudate by washing the peritoneum with the lavage fluid. Careful operative technique reduced erythrocyte comtamination to levels easily eliminated by hypotonic lysis. Using oyster glycogen and this new technique the cell yield was increased to 55.6 ± 5.9 million cells (n=10) underlining the advantage obtained by opening the peritoneum to wash out the cells. Inspite of this the recovery was very variable which was possibly due to hydrolysis of the glycogen during storage and loss of irritant properties.

After these initial investigations I.P. injection of sodium caseinate was used to raise PMNL. The cell yield was three times that obtained using

oyster glycogen (169.1 ± 15 million cells (n=4)). There was an increased proportion of PMNL using this irritant (see table 2.01).

Table 2.01 Recovery of PMNL's using three different methods. All values represent the mean ± s.e.m.. The number of animals is shown in parentheses.

Irritant	Harvesting method	Yield ($\times 10^{-6}$)	%PMNL
Oyster Glycogen	drainage needle	17.5 ± 5.4 (6)	68.4
Oyster Glycogen	surgery/lavage	55.6 ± 5.9 (10)	81.6
Sodium caseinate	surgery/lavage	169.1± 15 (4)	91.5

2.3.2. DETERMINATION OF CELL INFILTRATION AND OPTIMAL HARVESTING TIME USING SODIUM CASEINATE AS THE ELICITING AGENT.

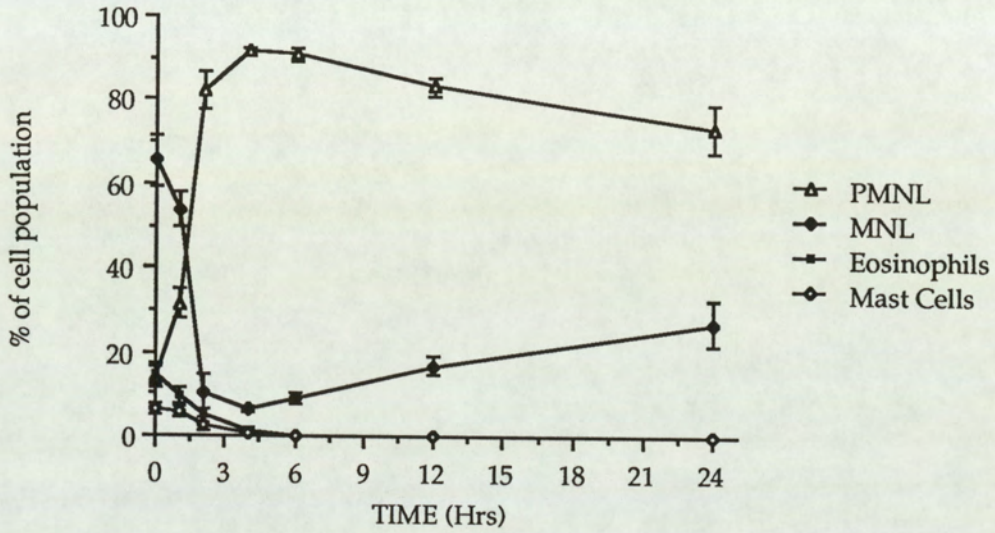
The accumulation of inflammatory cells after I.P. challenge with sodium caseinate followed that of an acute inflammatory reaction. The infiltration of cells can be see in table 2.02. and fig 2.01. There is little cell infiltration during a lag period of about 2 hours. This was followed by massive infiltration of PMNL's which started between 1 and 2 hours following challenge and peaked 6 hours after the onset of peritonitis. Later than 6 hours there was a slow decrease in the total cell number harvested and as monocytes began to infiltrate a concurrent fall in the proportion of PMNL's to MNL's (see figure 2.01) . From fig 2.01 it is

evident that the number of PMNL's recovered after 4 hours (154.7 ± 13.6 million PMNLs (n=4)) was similar to that recovered after 12 hours, (176.9 ± 16.6 million PMNLs (n=4)). The implication of the 12 hour incubation period facilitated lavage of the cells the morning after challenge and efficient utilisation of time. The yield of PMNLs was not diminished but purification was important as there were proportionally more MNLs in the population after 12 hours.

Table 2.02. The number of cells harvested from the rat peritoneum after I.P. challenge with 10ml sodium caseinate (12% w/v in sterile NaCl (0.9% w/v)). The animals were sacrificed following elicitation for 0, 1, 2, 4, 6, 12, and 24 hours. All values represent the mean ± sem.. The number of animals is shown in parentheses.

Time (hours)	Number of Cells (x 10^{-6})	
0	12.6 ± 3.7	(4)
1	12.6 ± 1.7	(4)
2	38.0 ± 2.9	(4)
4	169.0 ± 15.0	(4)
6	297.1 ± 16.2	(4)
12	212.5 ± 19.4	(4)
24	225.3 ± 22.5	(4)

Figure 2.01. Shows the percentage composition of the infiltrating cell population after I.P. challenge with sodium caseinate (12% w/v in sterile NaCl (0.9% w/v)). The animals were sacrificed following elicitation for 0, 1, 2, 4, 6, 12, and 24 hours. Each point represents the mean ± sem (n = 4).



2.5. CONCLUSIONS

The investigations described in this chapter resulted in the development of a reproducible technique for the isolation and purification of a large population of PMNL's. Using this technique a cell yield of greater than 150×10^6 cells was routinely obtained.

CHAPTER 3.

CHAPTER 3. DEVELOPMENT OF THE PHAGOCYTOSIS ASSAY

3.1 INTRODUCTION

The most frequently used assay method for the quantification of phagocytosis is the direct counting of the particles or bacteria per cell under a microscope (Giordano & Lichtman, 1973, Van Furth & Deisselhoff-Den Dalk, 1980, Dunn & Tyrer, 1981, Dunn *et al* ,1983, Celada *et al* , 1983, Oda & Maeda, 1986,) Assessment by this method in many studies is hampered by the inability to distinguish whether particles have actually been ingested by cells or are adherent to the cell surface. The application of electron microscopy to this type of study cannot solve this problem, since particles trapped between folds of a cell membrane can seem in tangential sections to lie inside the cell. Encouraging scanning electron micrographs have shown evidence of internalisation, however these are not satisfactory for quantitative determinations (Kawaguchi *et al*, 1986). Improvements to this assay procedure involve the specific removal of adherent particles using EDTA washing (Beukers *et al* . 1978, Diederkauf *et al* . 1977), dissolution of latex particles using xylene (Oda & Maeda, 1986, Giordano & Lichtman, 1973) , quenching of fluorescent labelled bacteria with crystal violet (Hed, 1977), erythrocyte lysis using osmotic shock and digestion of *Staphylococcus aureus* with lysostaphin (Van Furth & Diesselhoff, 1980). The success of these techniques does not escape from the tedious nature of the counting, and cells packed with particles will be impossible to evaluate accurately since the particles may be obscurring each other.

Polystyrene latex particles (PSL) have been the model particle of choice in many studies of phagocytosis and as such they have enabled

quantification by other methods. The absorbance of polystyrene at 255nm has been used to develop assays for phagocytosis (Roberts & Quastel, 1963, Beukers, *et al* , 1978, Diederkauf, 1977). The incorporation of fluorescent dyes into these particles has enabled quantification by fluorescence microscopy (Giordano & Lichtman, 1973, Hed, 1977, Dunn & Tyrer, 1981, Dunn et al , 1983, Celada et al , 1983, Van Furth & Diesselhoff-Denn Dalk, 1980, Lehnert & Tech, 1985), spectrofluorimetry (Schroeder & Kinden, 1983, Oda & Maeda, 1986, Muller & Schuber, 1986) or flow cytometry (fluorescence activated cell sorting (FACS)) (Dunn & Tyrer, 1981, Steinkamp et al , 1982, Bjerknes, 1984, Ogle et al , 1985, Sveum et al, 1986, Blair et al , 1986). The latter has been the method of choice in recent years and has become an important tool in the assessment of phagocytosis and many other cell functions (Shapiro, 1984). Flow cytometry has given the advantage of speed, greater statistical accuracy (with $n \geq 10,000$) and the ability to accumulate information on the cell population such as percent of phagocytic cells, the distribution of intracellular fluorescent beads per cell as well as the total particle uptake. Dunn and Tyrer (1981) have also shown good correlation between cells observed and analysed by fluorescent microscopy and flow cytometry.

In order to conduct this study the isolated population of PMNL's had to be loaded with particles and their degree of loading assessed routinely. Much of the developmental work with this assay was conducted at A.D.D.R, Ciba-Geigy Pharmaceuticals, where a FACS was routinely available, and return to Aston University required major amendment to the final assay due to the nonavailability of the equipment used (this will be explained in section 3.2.3.3).

3.2. MATERIALS AND METHODS

3.2.1. PREPARATION OF PMNLS

PMNLs were prepared according to the procedure outlined in section 2.2. After counting, the PMNLs were resuspended to a concentration of 2×10^7 cells per ml in HBSS at 37°C and placed in an incubator at the same temperature prior to addition of particles.

3.2.2. PREPARATION OF POLYSTYRENE LATEX PARTICLES

The model particles of choice for this project were fluoresbrite carboxylated polystyrene latex microspheres (2.5% solids-latex) (Polysciences Inc, Warrington.) with a batch diameter of 1.1μm (SD = 0.02). The stock suspension of microspheres were dispersed by immersion in a sonic bath for 10 seconds prior to dilution to the required particle concentration.

The particle diameter quoted by the manufacturer was tested using electron microscopy. The particles were diluted 1: 10 in prefiltered sterile distilled water. A 3μl drop was then placed on a formvar coated nickle 200 hexagonal grid. The grid was covered and allowed to stand for 3 minutes. The excess liquid was drained off using filter paper and left to dry completely. The particles were viewed in a Philips CM10 Transmission Electron Microscope at 80kV. Electron micrographs were taken at a magnification of 7000. The resulting negatives were enlarged using an overhead projector and the diameter of each particle recorded.

The concentration of the stock particle suspension was determined by calculation using the formula (Polyscience, 1987): -

$$N = \frac{6\,W \times 10^{12}}{\pi \times p \times d^3}$$

Where:

W = % solids
d = diameter (μm)
p = density of bulk polymer (g / ml)

When:

W = 2.5% solids
d = 1.1μm
p = 1.05 g / ml polystyrene

Hence

$$N = \frac{4.55 \times 10^{10}}{d^3}$$

$N = 3.42 \times 10^{10}$ microspheres per ml in the stock solution.

Each microsphere dilution, like the cells, were prepared in HBSS at 37°C and placed in an incubator at the same temperature.

3.2.3. PHAGOCYTOSIS ASSAYS

3.2.3.1 INCUBATION

The cell suspension and the required bead suspension were mixed in equal volumes, to a final volume of 5ml in mini biju bottles (Sterilin, Teddington, Middlesex, U.K.). These were sealed with parafilm and clipped on to the wheel of the blood cell mixer (Type BCM, Voss of Malden, Essex.) and were incubated at 37°C while mixing at 30 revolutions per minute for 1 hour. After incubation the cell suspension was transfered to a conical plastic centrifuge tube and the biju bottle washed with 5mls of fresh HBSS. The cells were washed three times by centrifugation at 100g for 5 minutes to remove any non-cell-associated microspheres. After the second wash each cell sample was counted to correct the resuspension volume to account for any cell loss. Each sample was resuspended after the final wash to 10^7 cells per ml in SHBSS for phagocytosis assays and assessment of chemotaxis.

3.2.3.2 ASSESSMENT OF PHAGOCYTOSIS USING FLUORESCENT MICROSCOPY ON PREPARED CELL SMEARS

Following incubation, washing and resupending (see section 3.2.3.1) 5Oμl of the final cell suspension was dropped on to one end of a microscope slide using a clean cover slip the drop was drawn across the slide. The smear was allowed to air dry before staining (section 2.2.9). The cells were examined under the oil emersion (x10 eyepiece and x100 objective) on the UV-visible microscope (Zeiss, Carl Zeiss, Oberkochen, W, Germany). For each sample greater than 200 cells were counted, the number of

particles in those cells was recorded and the number of cells without particles assessed.

3.2.3.3 ASSESSMENT OF PHAGOCYTOSIS USING FLUORESCENT MICROSCOPY ON CELLS PREPARED USING THE SHANDON CYTOSPIN AND THE SPECIFICALLY DESIGNED CYTOBUCKETS

Following incubation, washing and resupending (see section 4.2.3.) the cells were further diluted to a concentration of 5×10^6 cells per ml. 200µl aliquots of this suspension were then pipetted into the polypropylene slide holders in the Shandon cytospin (Shandon) a specifically designed centrifuge for the preparation of haematological slides (see figure 3.01). The rotor was spun for 5 minutes at 100g the slides were carefully removed and airdried before staining (section 2.2.8). Phagocytosis was assessed by counting greater than 200 cells, scoring the number of particles in the cells and the proportions with and without microspheres.

For routine access to this method of phagocytosis analysis at Aston a specifically conceived cytospin bucket had to be designed for use in the bench centrifuge in the laboratory. The design was based on the principle set-up of the cytospin rotor. It was constructed as an integral unit which, once assembled, would fit inside the buckets of the TH4 swingout rotor of the Beckman centrifuge (model TJ6) (see section 2.2.5. for details). The cell suspension, 100µl (5×10^6 cells) was spun onto the slides at 100g for 5 minutes. The slides were then carefully removed from the cytobucket, airdried and processed according to the details above. Specific correlation experiments were conducted to establish that both methods gave comparable phagocytic indices for the same population of cells.

3.2.3.4 ASSESSMENT OF PHAGOCYTOSIS USING FLOW CYTOMETRY

After incubation, washing and resuspension (see section 4.3.2.) 0.5ml of the final cell suspension ($5x10^6$ cells/ml) was fixed by addition of 0.5ml of paraformaldehyde (1%w/v) in a sterile Eppindorf tube. These samples were stored at 4^oC for analysis. Flow cytometry was carried out using the Becton and Dickinson FACS 440 the cells were analysed for fluorescence (phagocytosed microspheres) and light scatter (cell size) as they flowed through a flow cell intersecting the 488nm line from an argon ion laser excitation source. Optical sensors measured fluorescence and size on a cell-by-cell basis for each population (n=20,000). Signals were collected on the micro-vax computer and the data analysed using Consort 40 software from frequency-distribution histograms.

The phagocytosis of beads by the population of PMNL's was calculated from the mean fluorescence associated with the cells. This value was divided by the mean fluorescence of the singlet bead peak. Parameters were also set to record the proportion of cells that contained beads, by gating the cells in channel 0 - 15 which fell below the singlet bead peak.

3.2.4. INVESTIGATION OF FLUORESBRITE POLYSTYRENE LATEX PHAGOCYTOSIS BY PMNL AS ASSESSED BY BOTH MICROSCOPY AND FACS.

The method initially employed to quantify the uptake of Fluoresbrite PSL by PMNL's was fluorescence microscopy on cells prepared by smearing (section 3.2.3.2). This was adapted during a spell of work at A.D.D.R. (Ciba-Geigy Pharmaceuticals, Horsham) where the resolution of cell definition

and beads was improved using the Shandon Cytospin to prepare the slides (section 3.2.3.3.). The FACS was also available at A.D.D.R. and was used as an alternative method for quantifying phagocytosis. This study was undertaken to compare both methods and determine the optimum procedure for routine quantification of phagocytosis.

PMNL phagocytosis was compared at incubation concentrations of 5, 10, 20, 30, and 50 beads per cell in different populations of isolated PMNL's (10^7 cells/ml).

3.2.5. THE DESIGN AND TESTING OF THE CYTOSPIN BUCKET FOR THE BECKMAN (TL6) CENTRIFUGE

The Shandon cytospin (Shandon Southern Products Ltd, Runcorn, Cheshire, U.K.) was designed for the preparation of haematological samples. This method was found to be better for preparation of cells for differential counts and also for use in quantifying bead uptake by PMNL's in this study (section 3.2.4). The routine use of this method at Aston University required the development of a bucket which would mimic the principles of the cytospin in a swingout bench centrifuge (Beckman TL6). The Shandon cytospin rotor comprised of: (1) a plastic slide holder which was also the cell suspension reservoir, (2) a filter card, (3) a slide and the centrifuge which was designed to hold 1,2,and 3 whilst spinning . The unit was assembled (see figure 3.01. a&b) and the cell suspension loaded into the verticle reservoir (1). When spun the cell suspension was ejected by centrifugal force on to the slide via the drainage hole (4). The filter card (2) which was positioned between the slide and the holder creating a seal at the lip (5), soaked up the suspending buffer and the cells were deposited onto the slide.

Figure 3.01 The cytospin slide holder which fits, as it is drawn, into the rotor of the cytospin. The numbers refer to the parts refered to in the text:- 1. slide holder and cell reservoir, 2. the filtercard, 3. slide, 4. lip creating the seal at the exit of 5. the drainage hole.

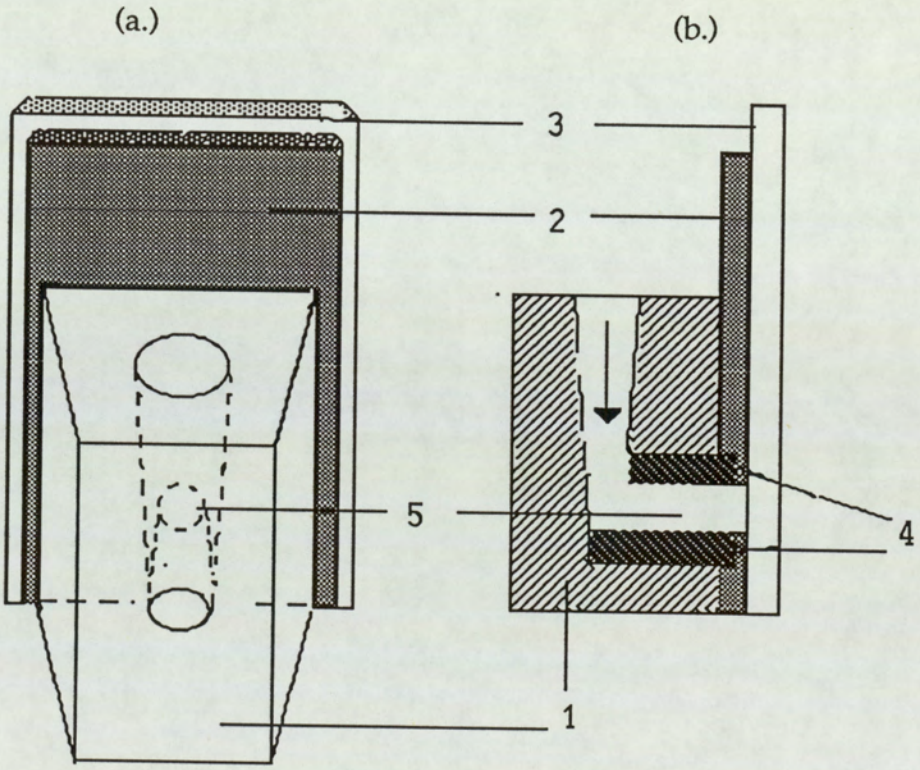


Figure 3.02 Cross section through one chamber of the cytospin bucket. The letters correspond to the parts referred to in the text:- A. top cell reservoir holder, B. bottom slide holder, C. cell reservoir (1ml Gilson tip), D. lip to seal filter card to slide (3 x cut off 200µl gilson tip), E. filtercard, and F. slide. The arrows designate how the bucket swings out and the cells are spun onto the slide.

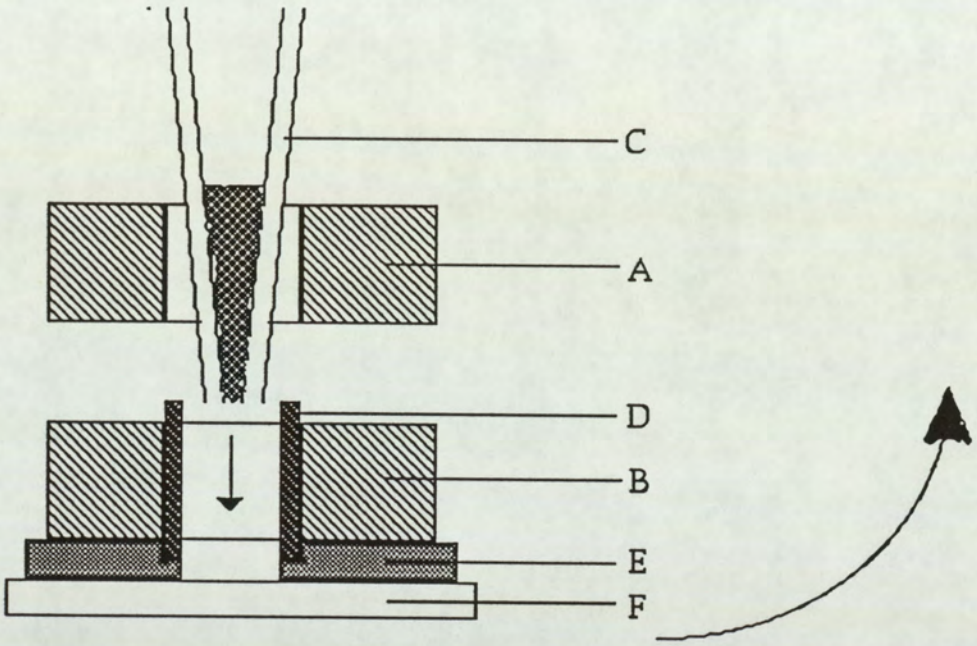
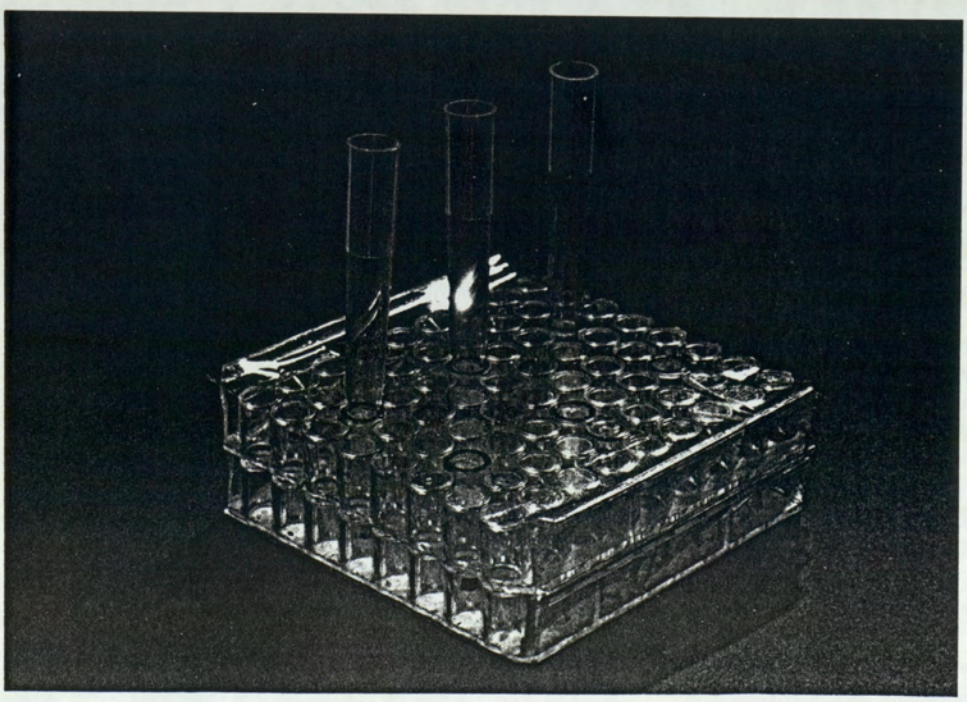


Plate 3.1. The assembled cytospin bucket



The alternative to this 'The cytobucket' was constructed from two microtiter plates (a&b), 3 x 1ml Gilson pipette tips (c), 3 x 200µl Gilson pipette tips (d), filter cards (e), slides (f) and sellotape. These were assembled as in figure 3.02 b and plate 3.01. The microtiter plates were cut to fit into the centrifuge bucket and the bottom of three wells were drilled out (in a and b) so that 3 slides could fit onto each holder (b). To ensure the same seal was achieved between the slide and the holder a lip was introduced by insertion of a trimmed 200µl pipette tip into each hole on (b). The slide and filter card were aligned and fixed to (b) using sellotape. The cell reservoir (c)(the 1ml pipette tip) was held in place over the assembed slide holder by (a). 100µl of cell suspension was gently delivered into (c) (x3) and then loaded into the centrifuge. By the same principle as the cytospin the cells are spun onto the slides.

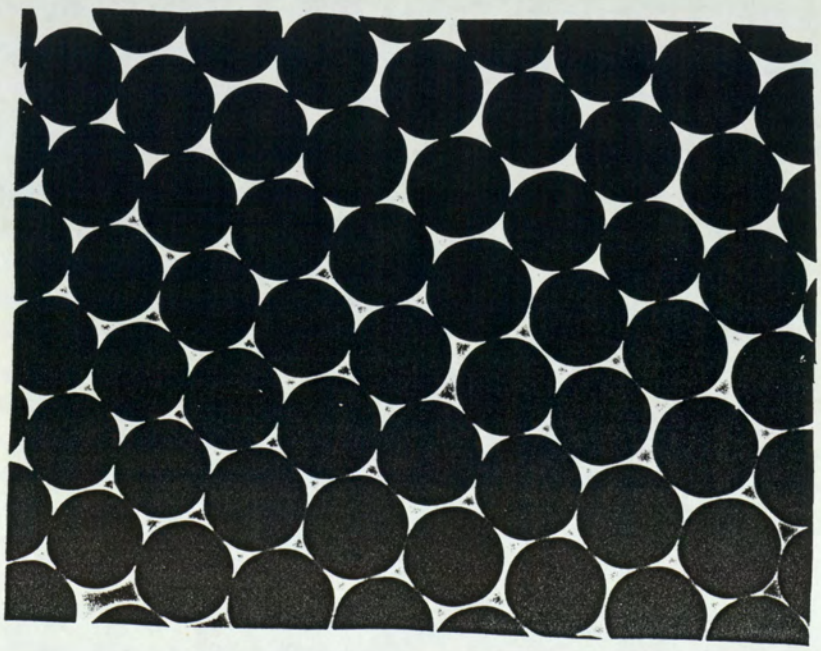
An experiment was conducted to establish that cell prepared by each method gave the same phagocytic values for the same cell population. A population of PMNL's were isolated (section 2.2.4-2.2.7) and incubated at a concentration of 10^7 cell per ml and 10 beads per cell. After incubation the cells were washed, diluted to $5x10^6$ cells/ml and slides prepared by both methods descibed above.

3.3. RESULTS

3.3.1 DETERMINATION OF THE SIZE OF THE FLUORESBRITE POLYSTYRENE LATEX USED

The diameter of the polyscience polystyrene latex particles used in this investigation was 1.0 ± 0.06µm (mean ± s.d.). This result was determined from analysis of 200 beads from photoelectron micrographs (plate 3.02). The experimental diameter was lower than the manufacturers documented figure of 1.1 ± 0.03µm a finding which agreed with the trend demonstrated by Johnson *et al* (1986). This parameter is an important variable when taking into account the drug carrying capacity of the beads and in determining an appropriate dose. In this case the bead had a diameter which was 10% less than the documented size, therefore the bead volume was 25% less than for a 1.1µm bead. If the drug was contained within the matrix of the bead the carrying potential would be reduced significantly. This question would however be addressed in later studies if targeting of the beads is possible.

Plate 3.02 Photoelectron micrograph of 1.1µm Polyscience PSL beads



3.3.2. THE COMPARISON OF FLUORESBRITE POLYSTYRENE LATEX PHAGOCYTOSIS BY PMNL AS ASSESSED BY MICROSCOPY AND FACS.

The development of a method for the assessment of phagocytosis of fluoresbrite PSL began using fluorescent microscopy of smeared cells. The same principle was used to prepare the slides as for a standard haematological differential count. On examination of these cells it was difficult to assign them as being polymorphonuclear or mononuclear and consequently it was difficult to accurately determine the PSL bead content. If the PMNL's had taken up a significant number the beads appeared to overlap, possibly grouped in phagolysosomes. This is illustrated in the plates 3.03a which is a fluorescence photomicrograph of PMNLs observed under high power and plate 3.03b which is a scanning electron micrograph of the same sample of cells. Plate 3.04a and 3.04b were the same sample of cells prepared with the cytospin. During routine use of the cytospin it was found that the centrifugal force applied effectively flattens the cells onto the slide (plate 3.04b). As the cells are flattened, the nucleus is spread and the beads contained within the cells form a monolayer. This made the cells easy to distinguish and facilitate more accurate counting because the beads were in one focal plane (plate 3.04a and 3.04b).

Plate 3.03.a. Photomicrograph of smeared PMNLs illuminated with visible and uv light.

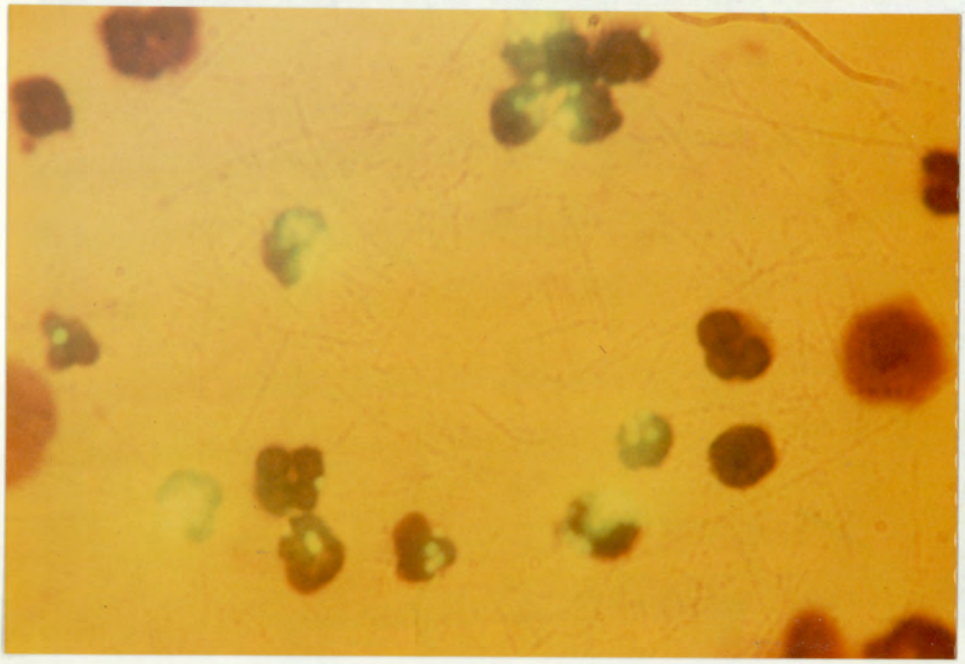
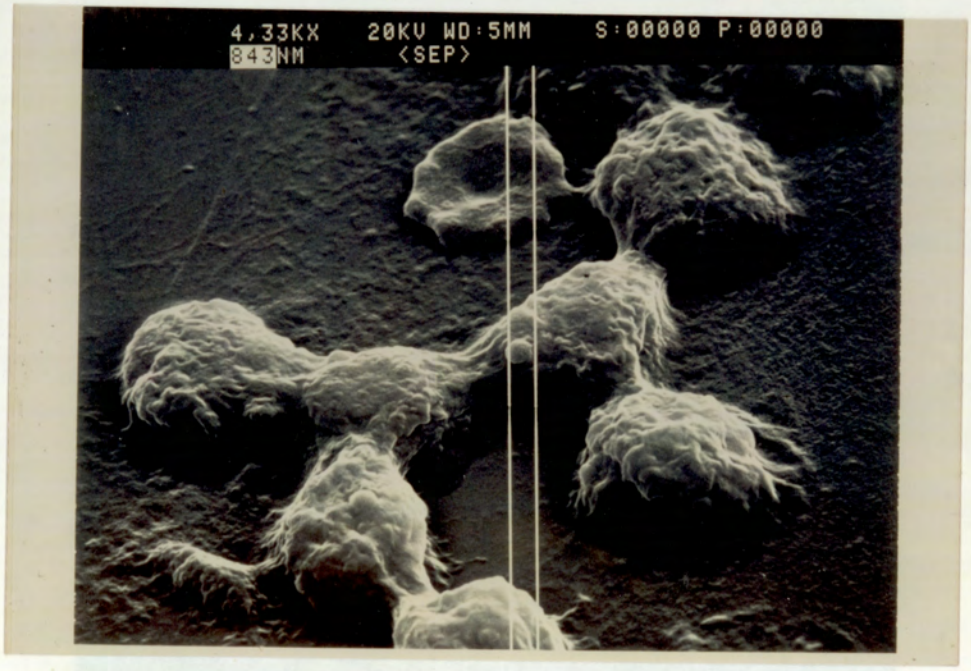


Plate 3.03.b. Scanning electron micrograph of smeared PMNLs. The white space band was added to indicate the bead size.

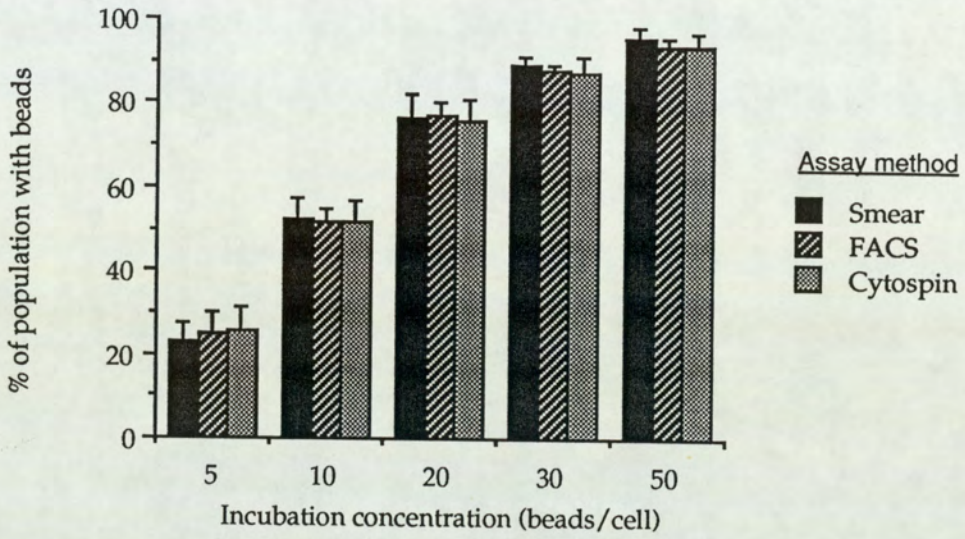


The phagocytosis data confirmed the observation that spinning the cells on to slides made analysis of phagocytosis easier and more accurate. At the lowest incubation concentration ratio (5 beads per cells) there was no significant difference between the uptake assessed by each method. At each subsequent concentration the cytospin gave significantly higher phagocytic uptake than the cell smears (at 10 beads per cell $P< 0.05$, at 20, 30 and 50 beads per cell $P<0.001$) . The number of beads per cell expressed as a percentage of the cytospin result (table 3.01. in parentheses) showed that the discrepancy between the methods increases as the bead concentration increases. This emphasised the loss in counts using smears as more beads were taken up by the cells. Despite the difficulty in quantifying the phagocytosis using smeared cells it was still possible to score the cells as fluorescence positive or negative. It was found that the percentage of cells containing beads was the same at each concentration (figure 3.03).

Table 3.01. Comparison of phagocytosis of 1.1μm diameter fluoresbrite PSL by PMNL's as assessed by the Cytospin, Smear and FACS methods. Each bead concentration represents the mean beads/cell ± sem from 6 phagocytic cell populations. The values in parenthesises represent the % of the Cytospin results. Significance between the cytospin and smear, and cytospin and FACS were conducted using the unpaired 't' test (*P< 0.05, **P< 0.01, ***P< 0.001.)

	ASSAY METHOD		
Incubation Beads : cells	Cytospin	Smear	FACS
5 : 1	0.78 ± 0.11	0.67 ± 0.09 (86)	0.7 ± 0.10 (90)
10 : 1	2.18 ± 0.11	1.70 ± 0.11* (78)	2.23 ± 0.20 (102)
20 : 1	7.89 ± 0.54	4.97 ± 0.30***(64)	6.27 ± 0.36* (79)
30 : 1	17.18 ± 1.45	9.23 ± 0.46***(53)	9.65 ± 0.47***(56)
50 : 1	25.92 ± 1.9	12.18 ± 0.52***(47)	13.81±0.65***(53)

Figure 3.03. Percentage of the cell population containing fluoresbrite PSL at each bead concentration. Each bar represents the mean ± sem from 6 phagocytic cell populations for each method.



The FACS was an alternative method used to quantify phagocytosis. It is an accurate, simple and quick method, and the correlation between the FACS and fluorescence microscopy was good when Dunn and Tyrer, (1981) investigated phagocytosis of PSL by macrophages in culture. However, there are some practical problems with analysis using the FACS in that the use of calibrated beads (i.e uniform size and uniform fluorescence) was normally required to produce a good histogram of the beads (figure 3.04) giving discernable sharp singlet, doublet and triplet peaks. These beads were however very expensive and the large numbers needed for the experiments in this thesis made uncalibrated beads preferable. The uncalibrated beads gave a sharp singlet peak on the FACS but the peaks after this merged together due to the wider variation in size and fluorescence intensity (figures 3.04. and 3.05.a to e.). Uncalibrated beads therefore could not be used for quantitative determination of how many cells had 1, 2, 3, 4, or more beads in but for determination of total

uptake where the important perameters were total fluorescence and the mean fluorescence of the singlet peak this technique was appropriate.

Figure 3.04 Histogram produced by FACS 440 analysis of calibrated 1.1μm PSL beads. The fluorescence histogram demostrates decernable peaks for singlet, doublet and triplet bead clusters. This analysis was conducted before addition of the microvax system which enhanced resolution, increasing the channel number from 225 to 1024. The new system was employed in the later analyses (figure 3.04)

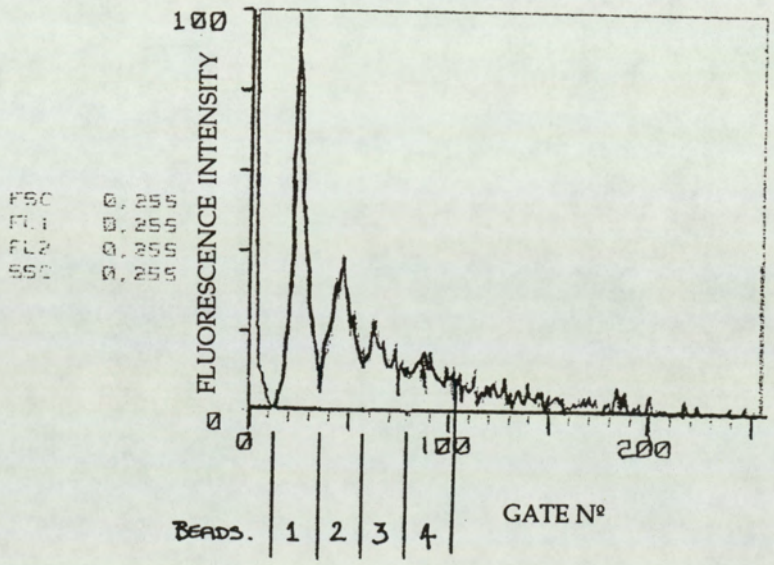


Figure 3.05a FACS analysis of phagocytosis of uncalibrated 1.1µm Fluoresbrite PSL at a incubation concentration of 5 beads per PMNL.

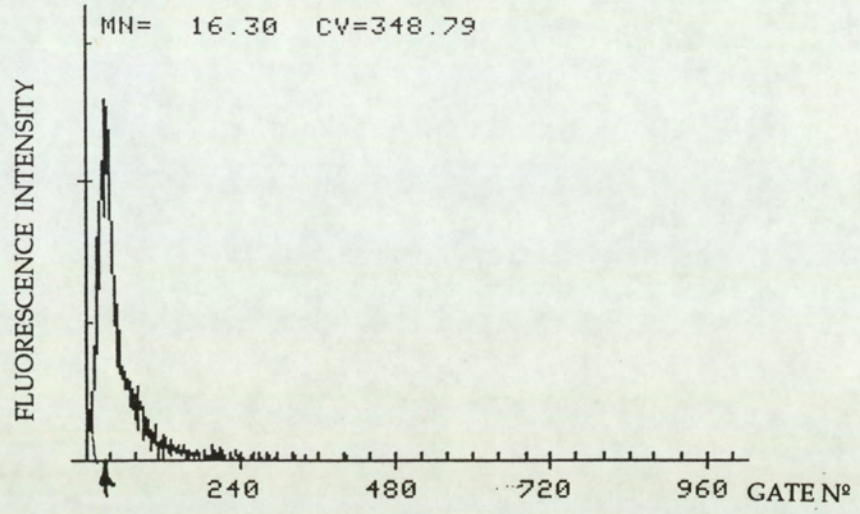


Figure 3.05b FACS analysis of phagocytosis of uncalibrated 1.1µm Fluoresbrite PSL at a incubation concentration of 10 beads per PMNL.

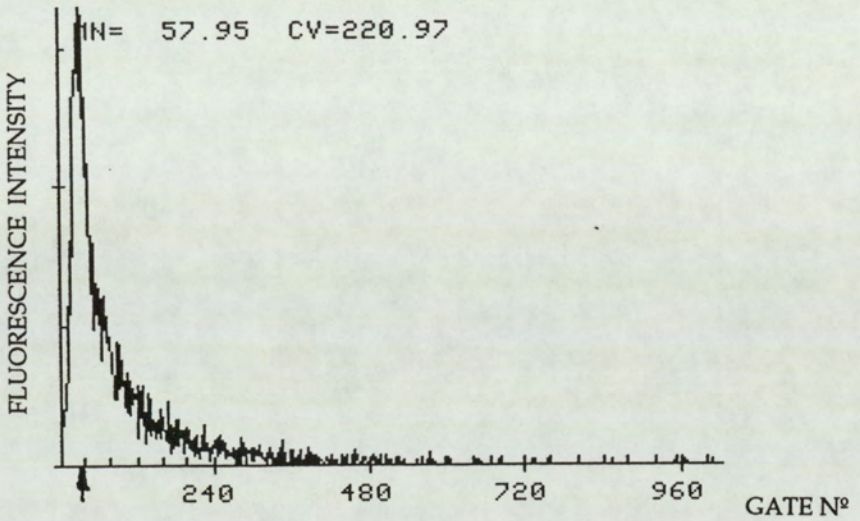


Figure 3.05c FACS analysis of phagocytosis of uncalibrated 1.1µm Fluoresbrite PSL at a incubation concentration of 20 beads per PMNL.

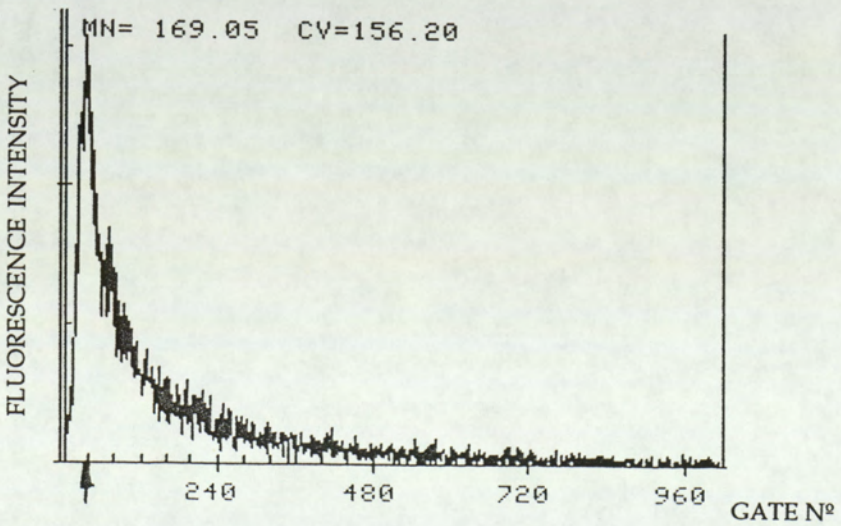


Figure 3.05d FACS analysis of phagocytosis of uncalibrated 1.1µm Fluoresbrite PSL at a incubation concentration of 30 beads per PMNL.

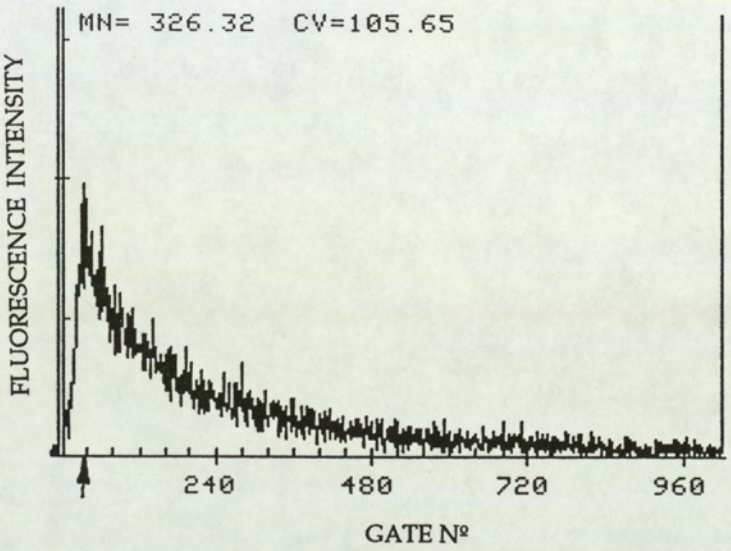
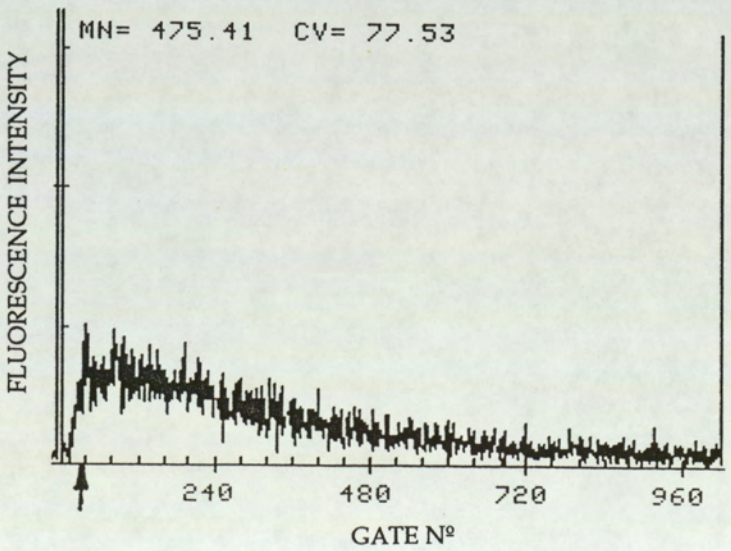


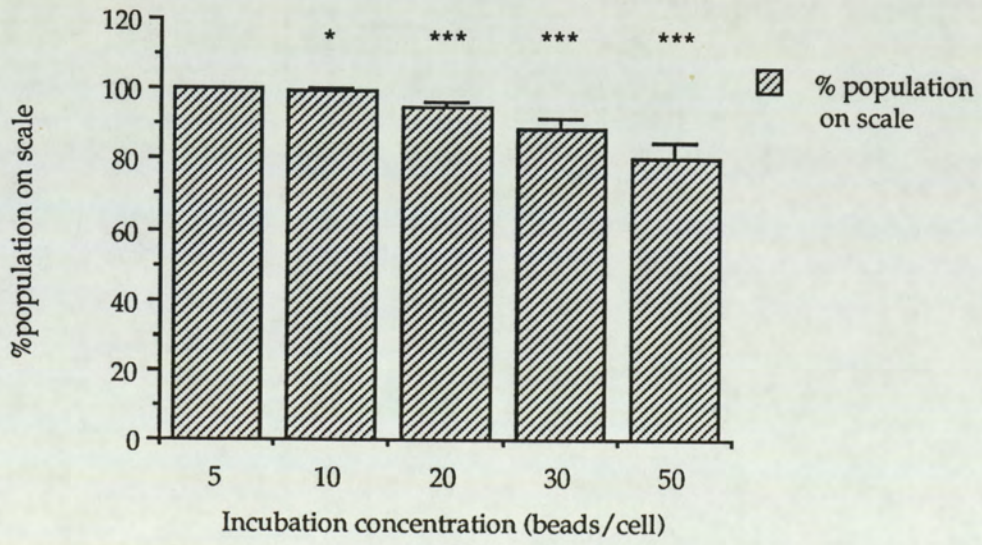
Figure 3.05e FACS analysis of phagocytosis of uncalibrated 1.1µm Fluoresbrite PSL at a incubation concentration of 50 beads per PMNL.



The comparison between bead phagocytosis assessed by the cytospin and the FACS showed a similar pattern as with the smears. At the low concentrations of 5 and 10 beads per cell there was no significant difference in uptake. At the higher concentrations 20, 30 and 50 beads per cell the FACS gave significantly lower phagocytosis than the cytospin ($P<0.05$, $P< 0.001$ and $P< 0.001$ respectively). This was accounted for by a limitation in the analytical power of the FACS. The data for all parameters is accumulated in a series of channels, this particular machine had 1024. Consequently there were a finite number of beads per cell that could be split between those channels. The singlet peak in this study had a median value of 32 (therefore peak width = 64 channels) so not taking into account the cumulative fluorescence variation the maximum beads per cell that could be recorded on scale was 16 (i.e. 1024/64). If a PMNL phagocytosed more than this number of beads the cell fluorescence was recorded in the last channel in analysis assigning it a fluorescence value comensurate with that of a cell with 16 beads and not the true quantity which may have been far greater. This finding was confirmed by gating the last channel (1024) to determine the number of cells that actually went offscale at each bead concentration. Figures 3.05a and b there is no peak at channel 1024 therefore an insignificant number of PMNLs were off scale in this population. Figures 3.05c, d and e show a significant rise in the channel 1024 peak. In the combined results from six PMNL populations are shown in figure 3.06. Which shows that concentrations greater than 5 beads per cell give a significant number of the cell population in channel 1024, with as many as 10% at 30 and 20% at 50 beads per cell. It was evident from these results that the flaw in this technique was the limitation of the FACS with this size of bead (1.1μm diameter). The resolution was such that the complete range of beads per cell could not be expressed in 1024 channels. A previous study (Dunn and

Tyrer, 1981) comparing microscopy and the FACS involved the use of 2µm diameter beads which were not taken up in such large numbers and were easier to count did not present the same problems with quantification of bead uptake and also demonstrated good correlation between the two techniques. Determinations during these investigations emphasised that the discrepancy in uptake between the FACS and the cytospin method was the result of greater loading of the PMNLs with 1.1µm beads and FACS not being powerful enough tool to collect the complete data within the constraints of this assay.

Figure 3.06 This graph shows the percent of the population of cells analysed by the FACS that were contained in the channels 0 to 1023. The departure from 100% representing the cells with approximately 16 beads per cell or more. Significance was assessed using the unpaired 't' test comparing each group with 5 beads/cell which had 99.9% of the population onscale (* P< 0.05, **P< 0.01 and ***P< 0.001)



3.4.2 THE DESIGN AND TESTING OF THE CYTOBUCKET FOR THE BECKMAN (TL6) CENTRIFUGE

Although the prototypes of the cytobucket were unsuccessful in preparing cell slides it was soon realised that the important part of the whole design was the lip (d) (fig 3.02) which created the seal between the slide and the holder. Without this the cells are washed off the slide into the filter by the centrifugal force generated. The addition of a cut-off 200µl pipette tip created the slight lip reqired to seal the well so that the cells could spin down and not be washed away.

In the comparison study 200 cells were counted on 10 slides produced by the cytospin and the 10 by the cytobucket method. The was no difference between the phagocytosis assessed by the two methods and the cell's morphology was the same.

Table 3.02. Comparison of phagocytosis assessed from slides prepared by the cytospin and the cytospin bucket. There was no significant difference between the results obtained from determination of phagocytosis on replicate slides prepared using the two different cytospin methods (n=10)

Slide preparation method	Mean Phagocytosis beads/cell (n)
Cytospin	2.04 ± 0.11 (10)
Cytobucket	1.98 ± 0.08 (10)

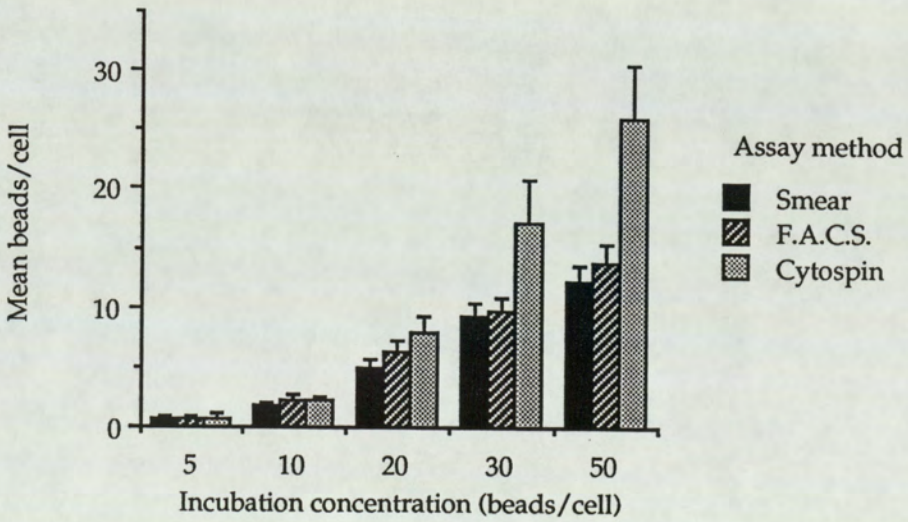
3.4. DISCUSSION.

The investigations in this chapter led to the development of a reproducible method of quantifying PMNL phagocytosis of 1.1μm diameter fluoresbrite PSL. Free particles were not evident in great numbers in the final total counts or differential cell count. It was assumed that beads adherant to the cells after three washes would be taken up if not already internalised. The PSL beads were avidly phagocytosed and the number of beads taken up per cell increased in proportion to the incubation concentration.

The techniques for the analysis of phagocytosis were compared in table 3.01 and are illustrated in figure 3.07 above. The analysis of phagocytosis using microscopy on cell smears and the results from the FACS gave lower values for beads per cell than the cytospin. The cytospin flattened the cells onto slides so that the beads were laid in the same focal plane making them easy to count. From the photograghs of cells prepared by this method (plate 3.04a and 3.04b) it is possible that the cells spin down over free beads, resulting in an artifactually high phagocytic index. The low concentration of free beads observed after washing the cells suggests that this was not the case. Evidence for the under estimation of phagocytosis by the other two methods was more convincing. The three methods gave similar results at the low incubation concentration, 5 : 1, and at 10 : 1 for FACS and cytospin (table 3.01) but the smear gave a lower value ($P<0.05$) . At the incubation concentration of 20 beads per cell the FACS and smear gave a lower value ($P<0.05$ and $P<0.001$ respectively) than the cytospin. At the higher concentrations (30 : 1 and 50 : 1) the FACS and smear gave a significantly lower bead uptake than the cytospin

($P<0.001$). The smears gave low results at high bead concentrations because it was impossible to count all of the beads in a three dimensional

Figure 3.07 The comparison of PMNL phagocytosis of 1.1μm fluorescent latex beads as assessed by three different methods (1) by fluorescence microscopy on cell smears (smear), (2) Analysis using the Fluorescent activated cell sorter (F.A.C.S.) and fluorescence microscopy on cells prepared by centrifugation using the cytobucket. Each bar represents the mean ± s.d (n= 6) PMNL populations. Analysis of the data using Analysis of variance (2x2 factorial ANOVA) which compared the three methods at each bead concentration and demonstrated significant difference $P<0.01$ between (A) the different methods, (B) the phagocytosis at each bead concentration, and also the interaction of these two perameters.



cell (see plate 3.03a) where the beads appeared to be clumped in phagocytic vacuoles. The phagocytosis assessment was low when the incubation concentrations were such that cells had taken up sufficient beads to obscure each other. The FACS gave lower results through a

constraint in the resolution of the equipment and because some of the PMNL's phagocytosed a very large number of 1.1μm Fluoresbrite beads. Increasing the sensitivity could not get all of the cells to fall inside the 1024 channels available. FACS is a very powerful tool in all spheres of cell biology and physiology (Shapiro, 1984) although advantages of speed, accuracy and reproducability have to be weighed against the physical constraints of the apparatus. As in this case the size of beads and the potential loading of these PMNL cells with beads at the concentrations used was out of the range for analytical use of the FACS.

CHAPTER 4.

CHAPTER 4. *IN VITRO* ASSESSMENT OF CHEMOTAXIS BY MICROFILTER ASSAY.

4.1. INTRODUCTION

Cell locomotion and chemotaxis are complex phenomena in which cells are moving through space and time, and in which the movement responds and changes continuously as the environment changes. A chemotactic gradient is usually a changing environment. Thus to assay these phenomena accurately, or to determine any single parameter that gives a useful measure of them is not straightforward. The different assays that are available at present are useful in different contexts, and the choice of an assay depends on what the investigator wants to find out. It is important to remember that when considering the response to a single attractant *in vitro* whether it can only be tentitively extrapolated to the intricate interactions that occur *in vivo* . The various assay systems in current use and the principles involved in the measurement of leucocyte locomotion and chemotaxis are outlined in the opening chapters of 'Leukocyte Chemotaxis' by Gallin and Quie (1978) and by Wilkinson (1982), Cates (1981) and more recently in 'Methods in Enzymology' (Di Sabato, 1988). In general, these assays can be separated into two categories. The first includes those in which a large population of cells is allowed to move in the presence or absence of locomotor stimulants or chemotactic factors. After a preset time period the movement is stopped and the distribution of the population examined, these are end point assays. They are the most widely used and include the micropore filter assay and the agarose assay. The second category includes those in which the behaviour of individual cells exposed to locomotor or chemotactic stimuli is examined. These are visual analyses and include time-lapse

cinematography and other visual assays such as the orientation assay. In these assays, the behaviour of individual cells can be followed through the whole experimental time; thus much detail can be studied. However, it is only practicable to make such detailed analyses on small populations of cells.

The most popular assay system used by workers in the field of chemotaxis is the micropore filter assay introduced by Boyden (1962) or one of its many modifications. Wilkinson and Allen (1978) have extensively reviewed the system for measuring leucocyte locomotion and have attributed the popularity of the microfilter assay to its simplicity, sensitivity and reproducability. The standard chamber developed by Boyden (1962) comprises an upper compartment which holds the leucocyte suspension and a lower compartment into which the chemoattractant or control media is placed. The two chambers are separated by a plain porous filter with well defined pore size. Gradients of small chemoattractant molecules and even large proteins can be set up across the filter rapidly (Wilkinson, 1982) and during incubation at 37°C the leucocytes actively migrate through the pores towards the chemotactic stimulus. A variety of methods have been documented for the assessment of directed migration. These involve the counting of the cells that migrate through to the attractant surface of the filter, radioactivity measurements of labelled leucocytes that migrate into a second filter (Gallin, Clark and Goetzl, 1978) and determination of associated cells by fluorometrically measuring the DNA content of a second filter (Shah and Larson, 1984). The assessment techniques pertinent to the present study are those which actually quantify the migration of cells within the filter. Zigmond and Hirsch (1973) have used the distance travelled by the 'leading front' to quantify cell migration. For such measurements the

cells are not allowed to traverse the filter, thus avoiding cell loss, and microscopic measurement of the distance between the upper surface of the filter (or monolayer) and the furthest point in the filter at which the nuclei of two or more cells are in focus together which gives the leading front distance. Criticism of this method has been levelled, (Wilkinson, 1982; Maderazo and Woronick, 1978) as under certain circumstances the leading front of cells may represent a priviledged population which is able to respond to a chemotactic stimulus but it is not representative of the whole population of cells. For example in PMNLs pretreated with aggregated IgG then placed in a chemotactic gradient. The IgG treatment reduces considerably the number of locomoting cells (Wilkinson, 1982). Nevertheless the minority of cells which did migrate were able to penetrate considerably and the leading front value was not decreased significantly. Maderazo and Woronick (1978) showed that counting the total number of cells entering the filter without prior knowledge of their distribution is a good indicator of chemotactic response. Hence development of a 'leucotactic index' which involves calculation of the mean distance migrated by the whole population within the filter. As the PMNLs are essentially a heterogeneous cell population it will be necessary when determining the effect of particle uptake that assessment of migration incorporates all of the cell groups and not just the fastest moving cells.

The appropriate assay system for investigation of the chemotaxis of PMNLs had to fulfil several important criteria. The assay of choice had to provide a matrix through which the cells could migrate actively (chemotaxis) and passively (random migration). The cells also had to deform to squeeze through gaps or pores, in analogy to the conditions encountered during diapedesis and migration in the inflammatory

condition. It was also important that the migration of the whole population should be measured as the PMNL population isolated was a heterogeneous mix of cells of different ages and activation states. The assay chosen was a modification of the micropore filter assay introduced by Boyden (1962) which fulfilled all of these criteria.

4.2. MATERIALS AND METHODS

4.2.1. CHEMOATTRACTANT

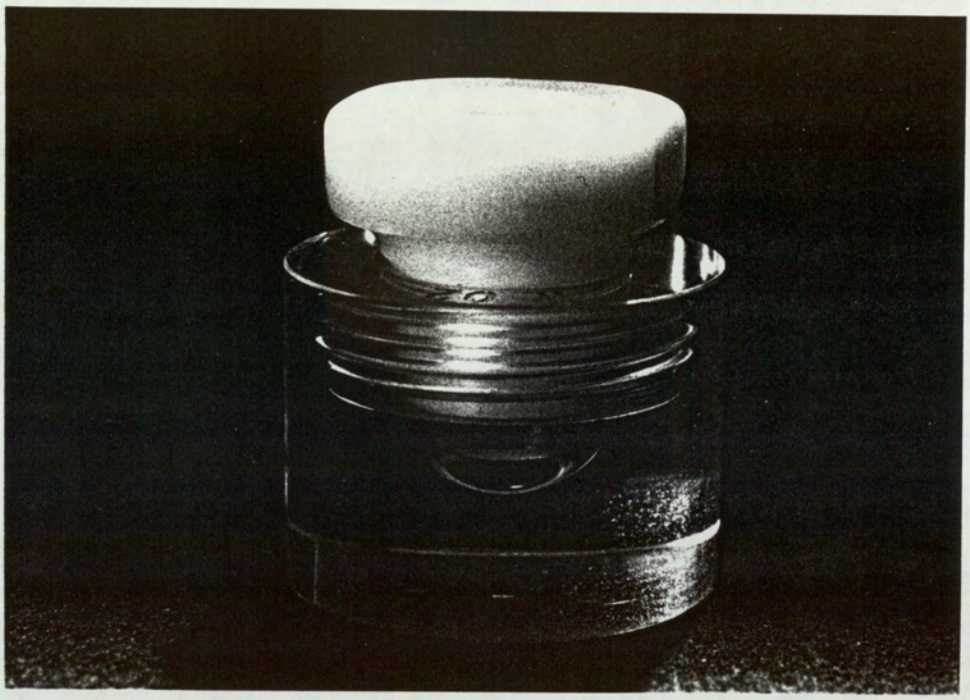
The synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) was obtained from Sigma Chemical Company (Fancy Road, Poole, Dorset, U.K.). Stock solution was made up in DMSO at a concentration of 10^{-2}M. The peptide was further diluted with buffered HBSS, 1mg/ml BSA to the required concentration for a set of experiments and 2.5ml aliquotes of this were stored at -20^{o}C in sterile Eppindorf tubes (Anderman & Co Ltd, Central Ave, East Molesey, Surrey, U.K.). It was important to prepare fresh FMLP for each set of experiments as it tends to decay with time due to oxidation of the methionine sulphur (Wilkinson, 1988).

4.2.2. BLIND WELL CHAMBERS

Blind well chambers were purchased from Buckley scientific Ltd. (Prestwood, Great Missenden, Bucks.). The lower chemoattractant chambers were constructed of polished acrylic whilst the upper filter retainers were of white Delrin. The wells had a diameter of 8mm, were 8mm deep and accomodated 200µl of fluid (Plate 4.01). Cellulose nitrate filters (13mm dia.) (Sartorius GmbH, Gottingen, W.Germany.) with a

5μm pore size and an average thickness of 140μm were used to assess PMNL migration.

Plate 4.01. The assembled blind well chamber.



4.2.3. PRETREATMENT OF PMNLS PRIOR TO ASSAY

The PMNLs were prediluted to a concentration of 10^7 cells ml^{-1} with HBSS (1mg/ml BSA) at 37°C. After dilution the cell suspensions were incubated at 37°C for 30 minutes in an incubator.

4.2.4 ASSEMBLY OF BLIND WELL CHAMBERS.

The chambers were prewarmed in an incubator at 37°C as were the chemoattractant (10^{-8}M FMLP), buffered HBSS (1mg/ml BSA, pH 7.4) and the cell suspension. The chambers were assembled quickly and carefully to ensure minimal cooling. A 200µl aliquot of chemoattractant or buffered HBSS was delivered into the lower well of the chambers using a precalibrated automatic Gilson pipette. One filter was placed above each well using a suction line and careful manipulation with a pair of blunt forceps. It was important to ensure uniform absorption of fluid over the whole filter area and filters absorbing fluid unevenly or having air-bubbles were discarded and the process repeated with dry chambers and fresh reagents. When using these filters it was not possible to distinguish the upper and lower surfaces so they were placed over the wells as they were packed by the manufacturers (i.e. the upper surface of the packed filter became the cell side and the lower became the chemoattractant side). Filters from the same batch were used throughout a set of experiments. The filter retainers were screwed down firmly and 200µl of the cell suspension (2 x 10^6 cells) delivered to the upper half of the chamber. Labelled glass cover slips (22 x 22 mm) (Chance Propper Ltd.) were placed over the chambers for identification and to prevent evaporation of the medium. The chambers were then placed in an incubator at 37°C for the required time period. For a diagram of an assembled blind well chamber see Plate 4.01.

4.2.5. THE PROCESSING OF THE FILTERS.

4.2.5.1. PREPARATION OF HARRIS'S HAEMATOXYLIN

Harris's haematoxylin is a powerful selective nuclear stain which gives sharp delineation of nuclear structure, as such it was ideal for staining the multi-lobed nuclei of the PMNL in the filters. It was prepared by dissolving the haematoxylin (1g.) in absolute alcohol (10ml), this was added to ammonium or potassium alum (20g.) which had previously been dissolved in hot distilled water (200ml.). This was brought quickly to the boil and mercuric oxide (0.5g.) was added, and the solution turned purple. The stain was cooled rapidly under a running tap. After cooling, glacial acetic acid (8ml.) was added to the above to sharpen the nuclear staining when used. The stain was prepared in a flask of ample size as some frothing takes place on addition of the mercuric oxide. The stain was always filtered before use.

4.2.5.2. THE STAINING AND MOUNTING OF FILTERS.

After incubation the filters were carefully removed from the blind well chambers using sharp syringe needles and forceps. Each one was mounted on its own needle by piercing the edge the filter and restraining with a ball of parafilm. The filters were fixed in methanol for 1 to 2 seconds and after drying they were stained according to the method of Cates *et al* (1978).

Table 4.01. Staining procedure for PMNL's in cellulose nitrate filters following the blindwell chamber assay.

REAGENT	TIME IN REAGENT
Harris's haematoxylin	2 minutes
Ethanol (70%)	30 seconds
Water rinse	2-3 seconds
Bluing reagent (20g. $MgSO_4$ + 2g. $NaHCO_3$ in 1 litre H_2O)	2 minutes
Water rinse	2-3 seconds
Propanol (70%)	30 seconds
Propanol (95%)	30 seconds
Propanol (100%)	2 minutes
Propanol (100%)	2 minutes
Propanol (100%)	3 minutes
Xylene	2 minutes
Xylene	2 minutes

When stained, the filters were carefully removed from their needles and mounted on a microscope slide, cell side upwards, in a drop of DPX mountant (BDH Chemicals, Poole, Dorset.). Another drop of DPX was added to the top of the filter and a cover slip placed over the filter. The

cover slip was pressed very gently and evenly to eliminate any air bubbles and to flatten the filter for examination.

4.2.6. ASSESSMENT OF MIGRATION.

The migration response was assessed using 'the Leading Front' method Zigmond and Hirsch (1973) and also by means of the 'Leucotactic Index' (Maderazo and Woronick, 1978)

4.2.6.1. 'THE LEADING FRONT TECHNIQUE'

The depth of PMNL migration was determined by examining the filters under oil emersion (x1000). The fine adjustment of the microscope was racked down past the leading front of cells. It was then racked back to the place where the nuclei of two or more cells were in focus together and a micrometer reading taken. The fine adjustment was racked back to the top of the filter to 'the monolayer' and another reading taken. The difference between the two readings gives the leading front distance of migration. This procedure was repeated in six random fields in each filter.

4.2.6.2. 'THE LEUCOTACTIC INDEX'

Unlike the 'Leading front method' which only encompasses the cells that are moving fastest and those not moving at all the 'Leucotactic Index' is a measure of the migration of the whole population of cells. The 'Leucotactic Index' was derived by counting the number of cells at 10μm intervals from the upper surface to the distal surface. The number of cells at each level [B] was then multiplied by the distance from the monolayer

[A]. The products of [A]x[B] were added and the sum divided by the total number of cells counted (i.e. the sum of [B]) to obtain the 'Leucotactic Index' six randomly selected fields were examined and the mean 'Leucotactic Index' calculated from six animals for example:–

Table 4.02. An example of the calculation of the 'Leucotactic Index'

DISTANCE FROM ORIGIN [A]	NO. OF CELLS PER LEVEL [B]	[A]x[B]
0	X_1	0
10	X_2	$10X_2$
20	X_3	$20X_3$
30	X_4	$30X_4$
40	X_5	$40X_5$
–	–	–
–	–	–
–	–	–
140	X_{15}	$140X_{15}$
	$\Sigma[B]$	$\Sigma([A]x[B])$

$$\text{'Leucotactic Index'} = \frac{\Sigma([A]x[B])}{\Sigma[B]}$$

4.2.7. DETERMINATION OF THE OPTIMAL CONDITIONS FOR CHEMOTAXIS STUDIES.

4.2.7.1 DOSE RESPONSE OF PMNL's TO FMLP IN THE BLIND WELL CHAMBER ASSAY

A stock solution of FMLP (10^{-2}M) was diluted with buffered HBSS (1mg/ml BSA, pH 7.4) to 10^{-6}M, 10^{-7}M, 10^{-8}M, 10^{-9}M, 10^{-10}M and 10^{-11}M. Aliquotes of each concentration of chemoattractant (200µl) were delivered to the lower compartment of each of triplicate blind well chambers. Control chambers received 200µl of buffered HBSS (1mg/ml BSA, pH 7.4). The remainder of the assay was performed as described earlier (see section 4.2.4.). For the dose-response experiments the migration was assessed by both the leading front method and also by calculating the 'Leucotactic Index' to compare the two methods.

4.2.7.2. THE EFFECT OF INCUBATION TIME ON PMNL MIGRATION IN THE BLIND WELL CHAMBER ASSAY.

PMNL migration in response to FMLP (10^{-8}M) was assessed after 30 mins, 45 mins, 60 mins, 75 mins, 90 mins, 120 mins and 150 mins in order to find the appropriate incubation time for the subsequent investigations. The optimal time would be such that the leading front had not reached the distal surface but the population had significantly penetrated the filter. As in 4.2.7.1. both the leading front and 'Leucotactic Index' were determined.

4.2.8 INVESTIGATION OF THE EFFECT PHAGOCYTOSIS OF FLUORESBRITE CARBOXYLATE PSL HAS ON THE MIGRATION OF PMNL'S.

The PMNL population was isolated by the method described in 2.2.5. The cells were washed and purified. Polystyrene latex microspheres were prepared (see section 3.2.2) to final concentrations of 0, 5, 10, 20, and 30 beads per cell in a cell suspension of 10^7 cells/ml for each population of cells investigated. Following incubation and removal of excess beads (see section 3.2.3.1) the cells were resuspended to a concentration of 10^7 cells/ml in HBSS (1mg/ml BSA, pH 7.4) in preparation for the assessment of chemotaxis. The chemotaxis assay was conducted by the methods detailed in section 4.2. The only amendment to the technique was the use of oil of cedar wood (BDH Chemicals, Poole, U.K) to mount the filters after fixation and staining. It was found that the DPX mountant dissolved the fluoresbrite latex beads that were contained within the cells and consequently no fluorescence could be seen when observed under the fluorescence microscope. The discovery that oil of cedar wood had been used in the processing of cellulose nitrate blocks when embedding tissue for sectioning and histology (Disbray and Rack, 1970) prompted its use in mounting the filters. This enabled clarification of the filters without dissolution of the fluorescent latex and facilitated counting of PMNLs and fluorescent particles at all levels of the filters.

Directed (in response to FMLP, 10^{-8}M in HBSS, 1mg/ml BSA) and random migration (in response to HBSS, 1mg/ml BSA) were assessed for each bead concentration by calculation of the Leucotactic Index. Additional information was obtained by counting the number of visible

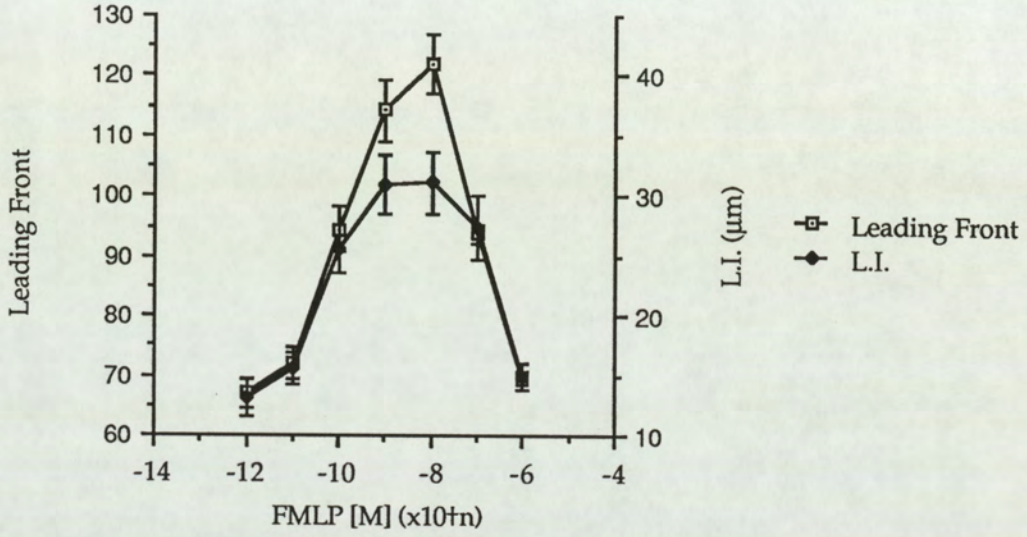
fluorescent latex beads at 10μm increments through the filter. The random and directed migration patterns and number of beads transported by cells were determined at 5 different bead concentrations for 6 populations of PMNL's.

4.3. RESULTS.

4.3.1. DOSE RESPONSE OF PMNL's TO FMLP IN THE BLIND WELL CHAMBER ASSAY.

The chemotaxis of PMNL into micropore filters in response to varying concentrations of FMLP (10^{-6} to 10^{-11}M) is shown in figure 4.01. Directed migration was assessed by both the 'Leading Front' method and the 'Leucotactic Index'. The optimal chemotactic concentration for FMLP was found to be 10^{-8}M. both higher and lower concentrations of FMLP were seen to inhibit migration. The chemotactic response assessed by each technique gave dose response profiles which were very similar.

Figure 4.01. The reponse of PMNL's to a range of concentrations of FMLP (10^{-6}M to 10^{-12}) in the blindwell chamber assay. The migration response was assessed by both the leading front method and by calculation of the leucotactic index. Each point represents the mean ± s.e.m. of the migration measured in 6 replicate filters.



4.3.2. THE EFFECT OF INCUBATION TIME ON PMNL MIGRATION IN THE BLIND WELL CHAMBER ASSAY.

Figure 4.02 illustrates the development of the PMNL chemotactic response to an optimum concentration of FMLP (10^{-8}M). These 'Leading Front' results showed that incubation for 90 minutes resulted in the appearance of cells on the chemoattractant side of the filter ('Leading Front' = 140µm). In this case the 'Leading Front' could not be used and the 'Leucotactic Index' could not be calculated. The optimal incubation time was taken to be 60 minutes, which gave marked difference between random and directed migration, for the 'Leading Front'(figure 4.02) and

the 'Leucotactic Index' (figure 4.03), and sufficient filter depth to observe enhanced or inhibited migration in later studies. Figure 4.04. shows that there was excellant correlation (R= 0.99) between the directed migration (Directed(D) - Random(R)) when the 'Leading Front' was compared with the 'Leucotactic Index'. Dispite this in all subsequent studies the 'Leucotactic Index' was the method of choice as the whole population had to be assessed in view of the heterogeneity of PMNL cell populations.

Figure 4.02. The effect of incubation time on PMNL directed and random migration assessed by the Leading Front technique in the blindwell chamber assay. FMLP (10^{-8} M) was used as the chemoattractant. Each point represents the mean ± s.e.m. of measurement in 6 replicate filters.

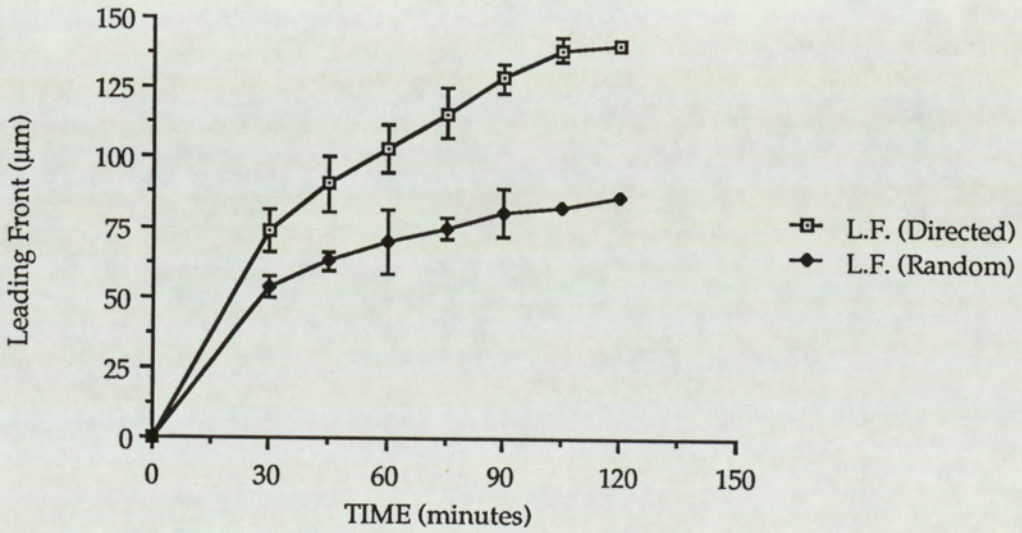


Figure 4.03. The effect of incubation time on PMNL directed and random migration assessed by the Leucotactic Index in the blindwell chamber assay. FMLP (10^{-8} M) was used as the chemoattractant. Each point represents the mean ± s.e.m. of measurement in 6 replicate filters.

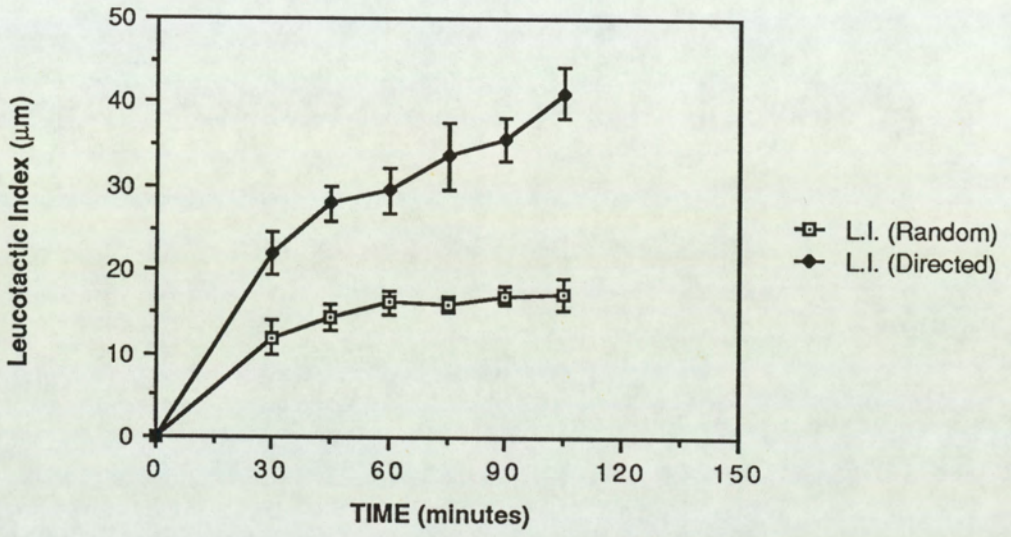
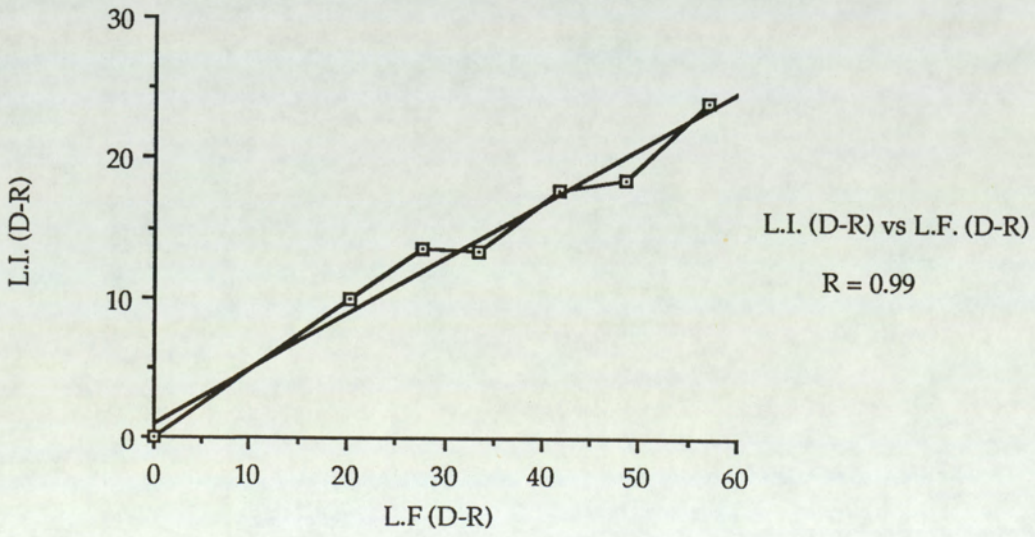


Figure 4.04. The correlation between the directed - random migration as assessed by the Leading Front technique and the Leucotactic Index (R= 0.99)



4.3.3. THE EFFECT OF PHAGOCYTOSIS OF FLUORESBRITE CARBOXYLATE PSL ON THE MIGRATION OF PMNL'S IN THE BLINDWELL CHAMBER ASSAY.

The PMNL's were preincubated with PSL beads at 5, 10, 20 and 30 beads per cell and the number of beads per cell calculated from fluorescent microscopy on slides prepared by the cytobucket technique (section 3.2.5) were 1.22, 3.42, 8.6, and 18.2 beads per cell respectively (table 4.03).

Table 4.03. Phagocytosis expressed as mean beads per cell ± sem after incubation at concentrations of 5, 10, 20,and 30 beads per cell. Number of PMNL populations in parentheses.

Incubation concentration %population beads/cell	Bead concentration	% with beads
5:1	1.22 ± 0.09 (6)	27.8 ± 2.4
10:1	3.42 ± 0.33 (6)	58.8 ± 4.0
20:1	8.60 ± 0.88 (6)	77.1 ± 3.2
30:1	18.2 ± 1.08 (6)	87.9 ± 5.0

These cells were then used in the blindwell chamber to investigate their migratory properties compared to unloaded cells. The 'Leucotactic Index' for each bead concentration was determined for directed migration in

response to FMLP and random migration for each population of cells. The results are summarised in table 4.04 which shows that there is no significant difference between the migration (random and directed) of PMNL populations with or without particles at all concentrations used.

Table 4.04. The migration of PMNL with and without beads in response to FMLP (10^{-8}M) (Directed) and to the buffer (HBSS) (Random) in the blindwell chamber assay. All values represent the mean 'Leucotactic Index' ± SD assessed in six populations of PMNLs after incubation with 0. 5, 10, 20 and 30 fluoresbrite polystyrene latex beads (1.1µm diameter).

Incubation beads : cell	Directed migration	Random migration
0	32.77 ± 2.10 (6)	14.76 ± 1.30 (6)
5 : 1	32.20 ± 2.74 (6)	14.87 ± 1.12 (6)
10 : 1	32.02 ± 2.75 (6)	14.86 ± 1.28 (6)
20 : 1	32.32 ± 2.60 (6)	14.39 ± 1.25 (6)
30 : 1	32.36 ± 3.85 (6)	14.57 ± 1.06 (6)

The 'Leucotactic Indices' included in table 4.04 are further illustrated in figures 4.05, 4.06a, 4.07a, 4.08a, and 4.09a the data plotted are the mean number of cells at each level in the filter after directed migration and random migration. The cell distribution after random migration shows

an exponential decrease in the number of cells with an increase in the depth of penetration of the filter. The cells migrating in response to FMLP (10^{-8}M) demonstrated a markedly different distribution within the filter. The cells have penetrated deeper into the filter and in greater numbers in response to FMLP. At each bead concentration the distribution profile demonstrates the similarity reflected in the 'Leucotactic Indices' calculated from this data (table 4.04). Photographs of the cells migrating in response to FMLP at the incubation concentration of 30 beads per cell are shown in plates 4.02a, 4.02b, 4.03a, 4.03b, 4.04a and 4.04b. These illustrate PMNLs containing PSL beads at 0, 50 and 100μm depth in the filter. Plate 4.02b demonstrates clearly the concentration of beads on the surface and plate 4.03b and plate 4,04b the decrease in the number of beads and cells with increase in depth.

Plate 4.02a PMNLs (30 beads/cell) seen under normal light on the surface of the filter

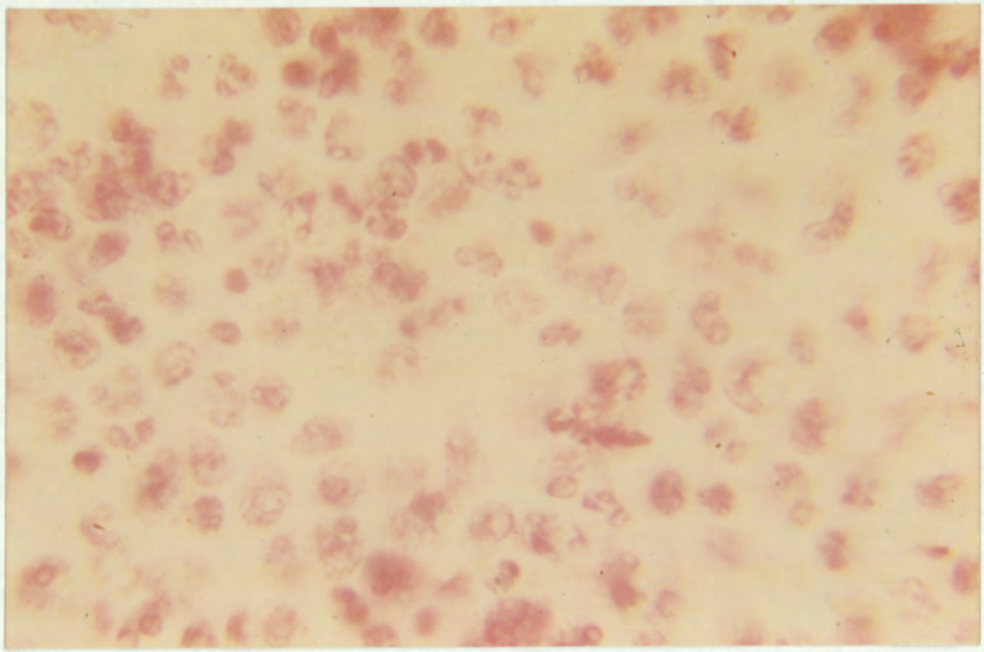


Plate 4.02b PMNLs (30 beads/cell) and beads seen under UV light on the surface of the filter

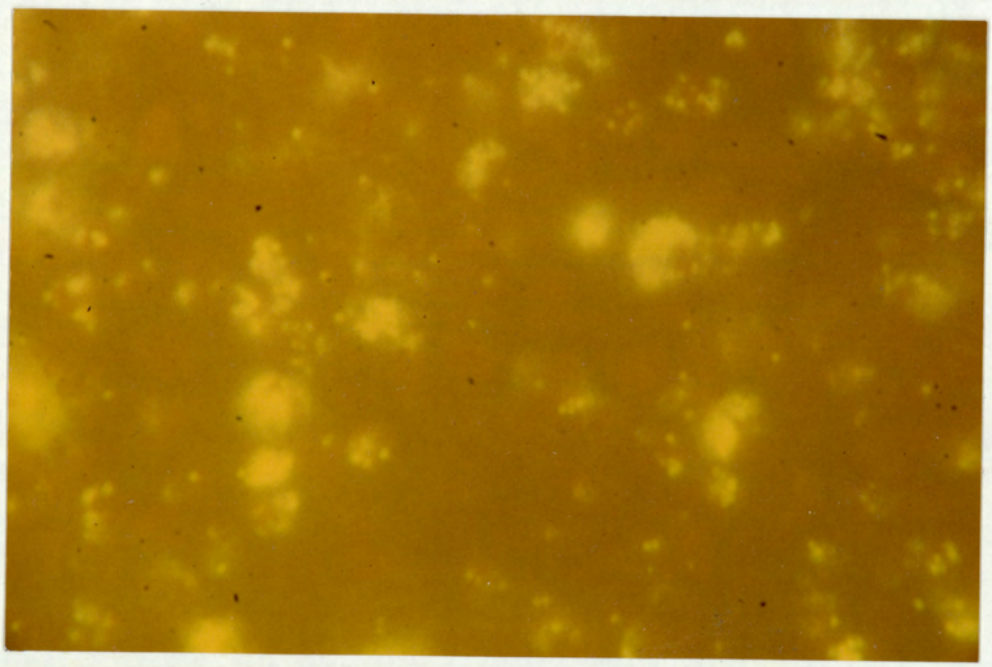


Plate 4.03a PMNLs (30 beads/cell) seen under normal light at 50µm through the filter.

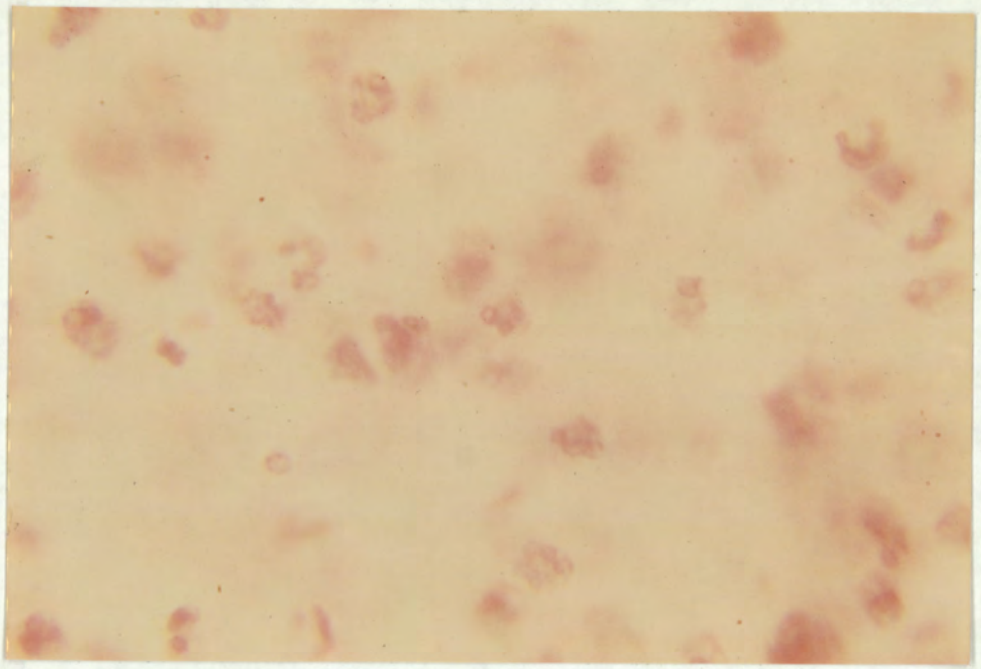


Plate 4.03b PMNLs (30 beads/cell) seen under UV light at 50µm through the filter.

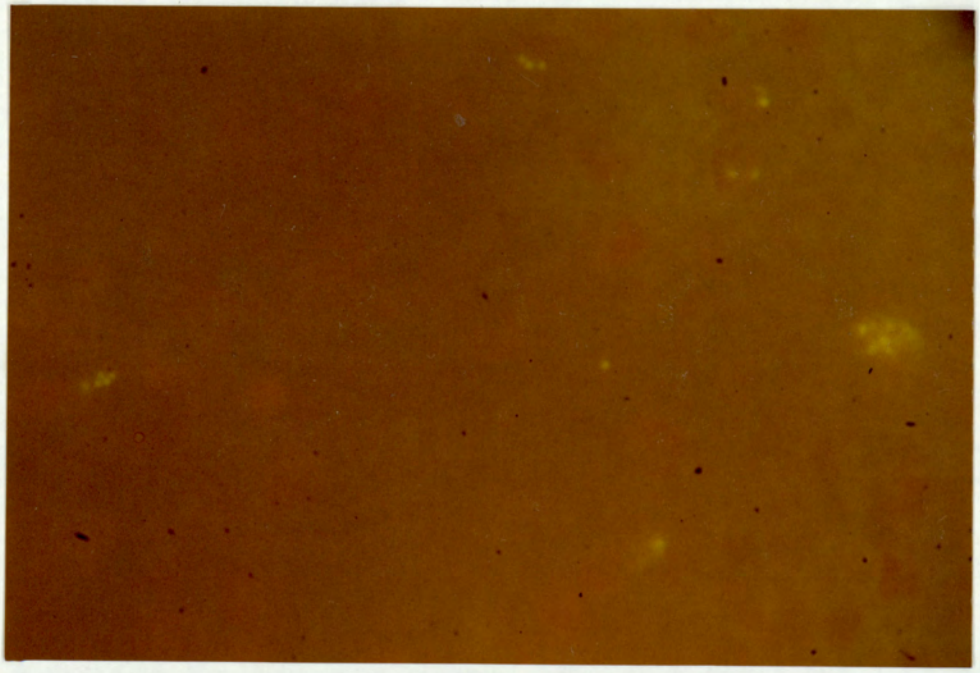


Plate 4.04a PMNLs (30 beads/cell) seen under normal light at 100µm through the filter.

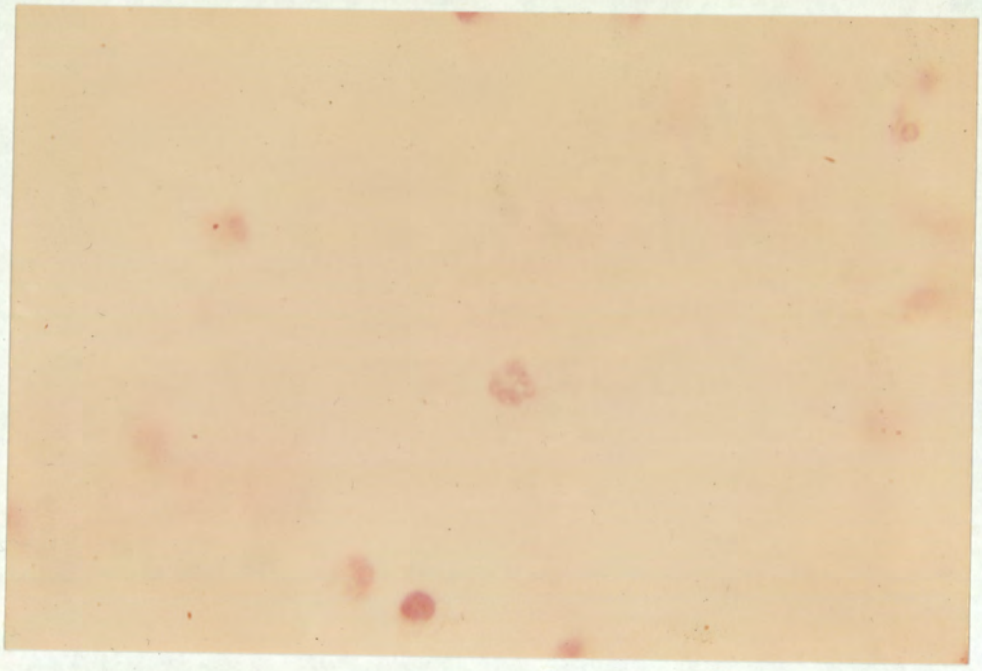


Plate 4.04b PMNLs (30 beads/cell) seen under UV light at 100µm through the filter.

The additional figures 4.06b, 4.07b, 4.08b and 4.09b represent the distribution of polystyrene latex beads expressed as beads per cell at each level of the filters at the incubation concentrations 5, 10, 20 and 30 beads per cell respectively. The practical problems in obtaining this data were comparible to those encountered using cell smears to assess phagocytosis. The cell counts were conducted at discrete levels in the filter to determine the 'Leucotactic Index' and hence the particles contained within the cells were assessed in the same fields of view. A two dimentional interpretation of a three dimensional situation meant that indices plotted were underestimates of the actual number of beads per cell. The number of beads per cell plotted against the depth in the filter (figures 4.06b, 4.07b, 4.08b and 4.09b) illustrate that the cells containing the most beads per cell at each incubation concentration are the cells that remain on the surface of the filter. This is demonstrated in both random and directed migration at each bead concentration used. The PMNL's that penetrate the filter contain a lot less beads than those on or near the surface of the filter. The number of beads in the cells that migrate appears to increase as the incubation concentration increases without any significant effect on the 'Leucotactic Index'. The bead concentration of the cells that have migrated into the filter in response to FMLP have approximatelty the same loading of cells irrespective of the distance they have moved. This is illustated by the plateau of the phagocytic indices from a depth of 20 to 30μm to 100μm in the filter at each bead concentration (figure 4.06b, 4.07b, 4.08b and 4.09b).

Figure 4.05. The distribution of cells without beads in cellulose nitrate filters after migration in response to FMLP (10^{-8}M) (Directed) and to the buffer (HBSS) (Random).

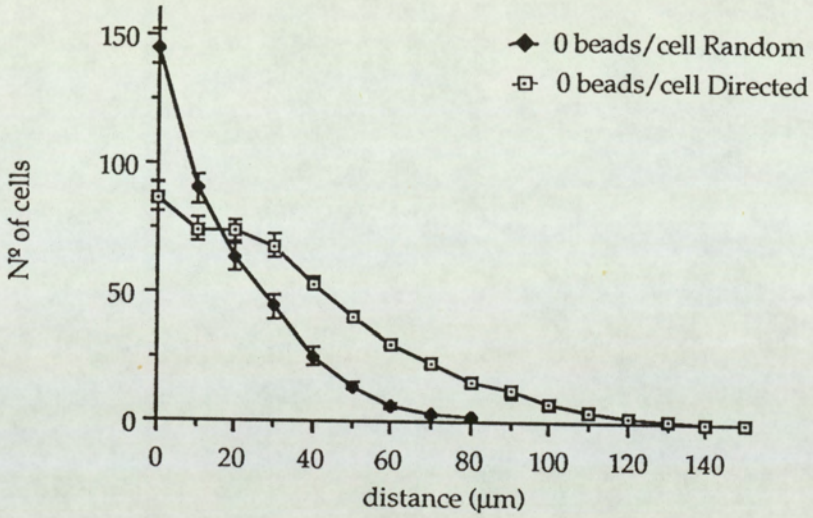


Figure 4.06.a. The distribution of cells in cellulose nitrate filters after incubation with 5 beads/cell after migration in response to FMLP (10^{-8}M) (Directed) and to the buffer (HBSS) (Random).

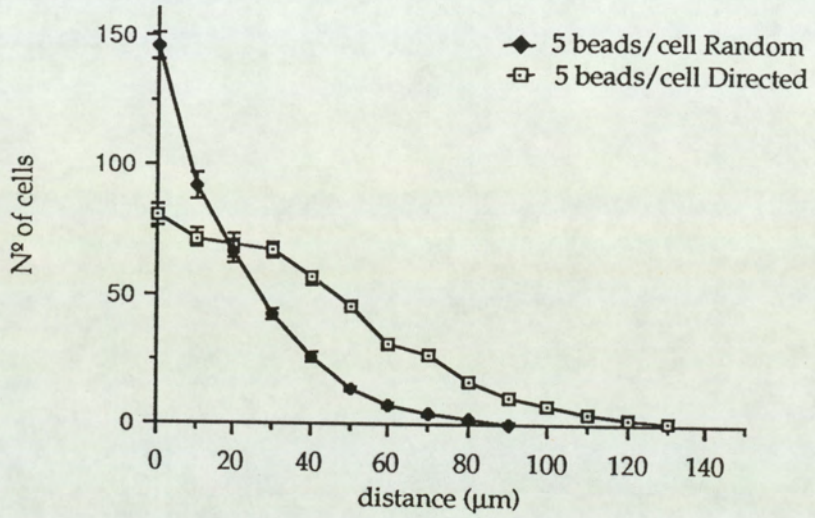


Figure 4.06.b. Results expressed as the number of beads per cell at each level of the filter after incubation with 5 beads/cell and migration in response to FMLP(10^{-8}M) (Directed) and to the buffer (HBSS) (Random).

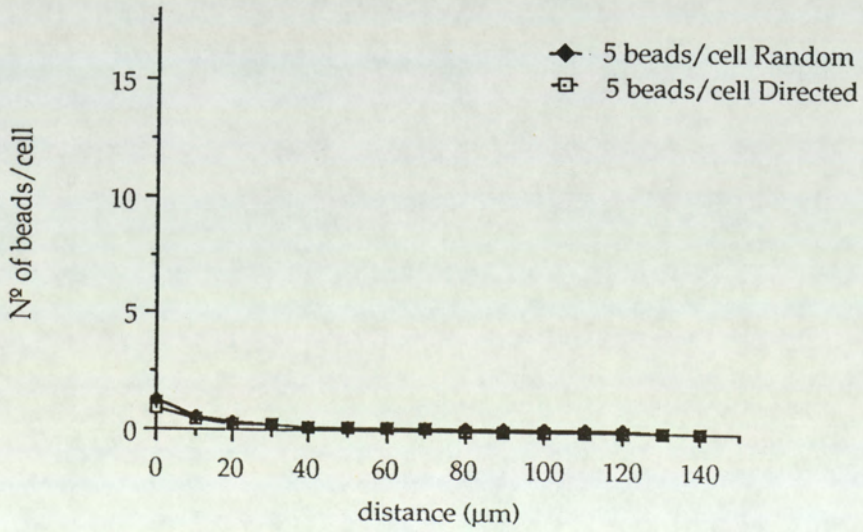


Figure 4.07.a. The distribution of cells in cellulose nitrate filters after incubation with 10 beads/cell and migration in response to FMLP (10^{-8}M) (Directed) and to the buffer (HBSS) (Random).

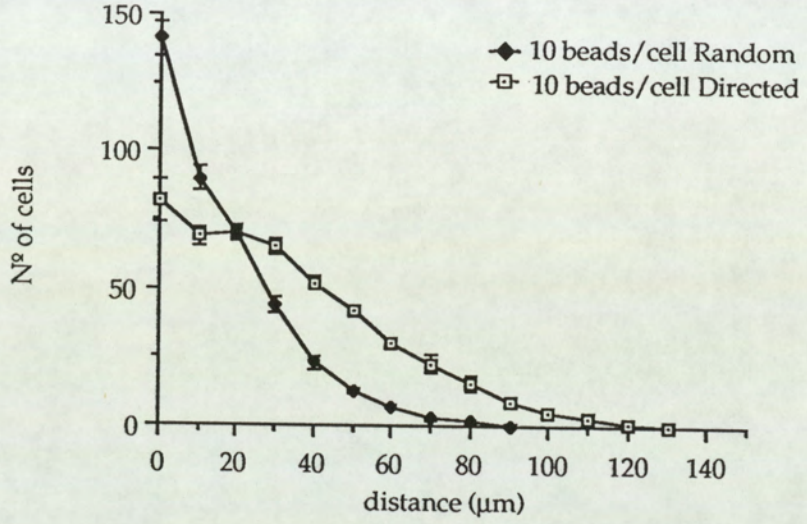


Figure 4.07.b. Results expressed as the number of beads per cell at each level of the filter after incubation with 10 beads/cell and migration in response to FMLP(10^{-8}M) (Directed) and to the buffer (HBSS) (Random).

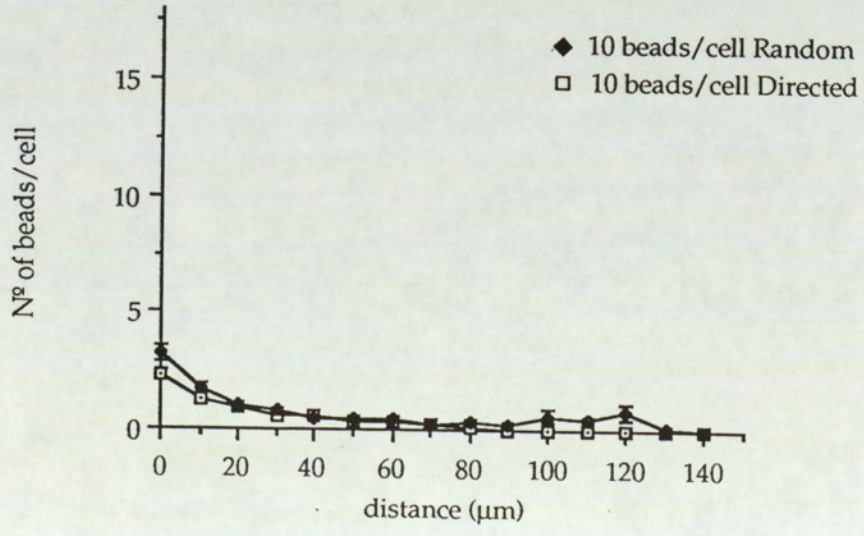


Figure 4.08.a. The distribution of cells in cellulose nitrate filters after incubation with 20 beads/cell after migration in response to FMLP (10^{-8}M) (Directed) and to the buffer (HBSS) (Random).

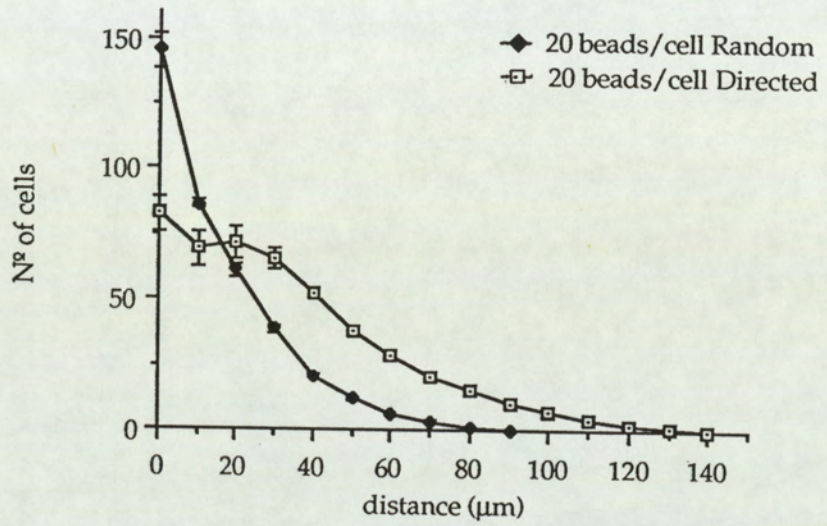


Figure 4.08.b. Results expressed as the number of beads per cell at each level of the filter after incubation with 20 beads/cell and migration in response to FMLP(10^{-8}M) (Directed) and to the buffer (HBSS) (Random).

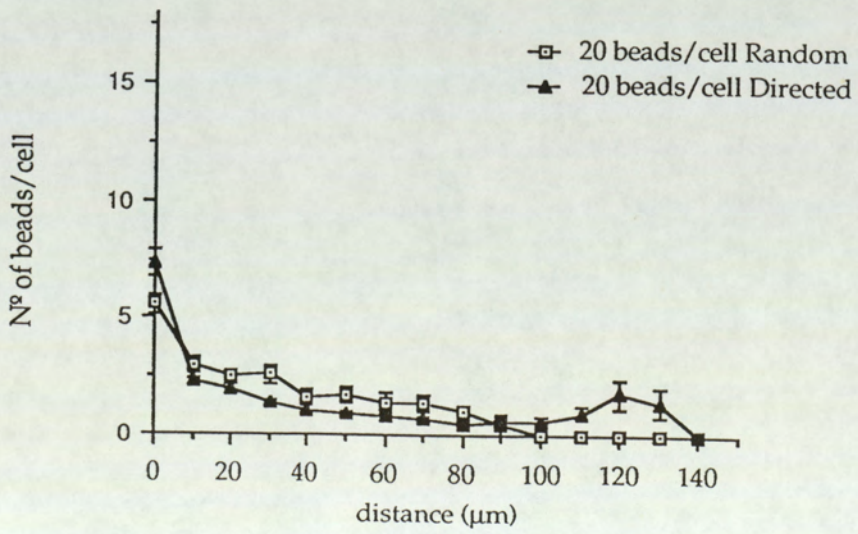


Figure 4.09.a. The distribution of cells in cellulose nitrate filters after incubation with 30 beads/cell after migration in response to FMLP (10^{-8}M) (Directed) and to the buffer (HBSS) (Random).

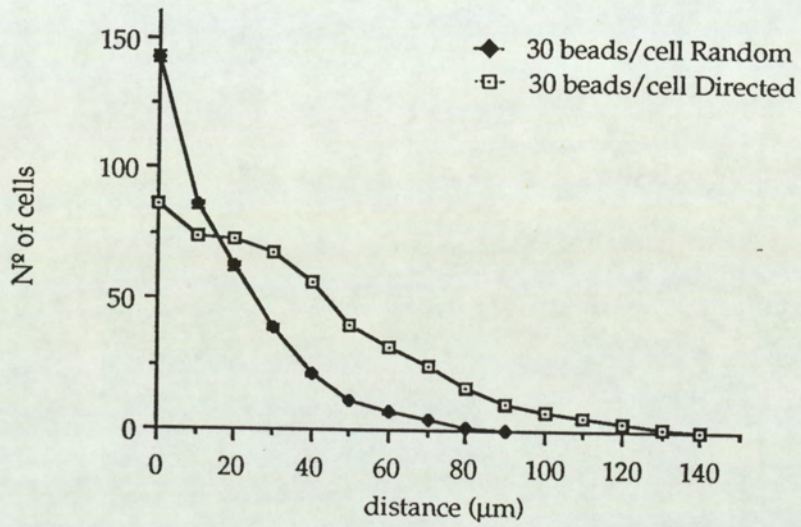
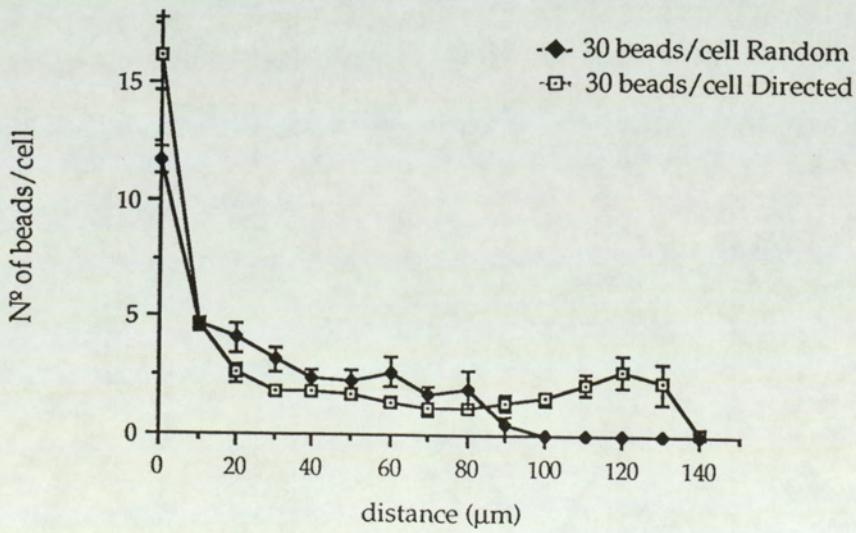


Figure 4.09.b. Results expressed as the number of beads per cell at each level of the filter after incubation with 30 beads/cell and migration in response to FMLP(10^{-8}M) (Directed) and to the buffer (HBSS) (Random).



4.4 DISCUSSION

It has been demonstrated that the migration pattern of a PMNL population is the same with or without loading with beads (table 4.02). This is encouraging as it suggests that the cells will carry beads through the filter and also that incubation with up to 30 beads per cell will not impair their chemotactic response to FMLP. This is further illustrated in figure 4.10a which show that the distribution of the cells expressed as a percentage of the population within specific areas of the filter were the same with each bead concentration.

In the simplest situation the concentration of beads per cell at each level in the filter would be the same, which means that the phagocytic capacity of PMNLs was not related to the chemotactic responsiveness. The transportation of beads through the filter is however, complicated by the heterogeneity of the PMNL population (Seligmann et al, 1984; Gallin, 1984). This is illustrated in figures 4.06b, 4.07b, 4.08b and 4.09b where PMNLs on or near the surface of the filter contain a disproportionately large number of beads per cell compared to those that have migrated into the filter. The overall picture is emphasised most clearly at the highest incubation concentration (30 beads/cell). The inherant problems with bead counts in the filters were similar to those encountered on smeared cells which meant the actual beads per cell determined at each level would be an underestimate of the actual phagocytic loading of 18.2 ± 2.65 beads per cell (table 4.01) but the results still suggest that the PMNL population is heterogeneous and comprises at least two different sub-groups. One fraction is active phagocytically but does not respond to FMLP in the blindwell chamber assay, the second fraction exhibit random and directed migration but do not phagocytose large quantities of

beads. This second sub-population show evidence of homogeneity in their beads carrying capacity which plateaus from 30μm and the bottom of the filter at each concentration investigated (figure 4.06b, 4.07b, 4.08b and 4.09b). The heterogeneity of the PMNL population is further illustrated in figure 4.10b which shows the percentage distribution of the total beads counted. If the population were homogeneous the percentage bead distribution would be the same as figure 4.10a for the cells. From previous discussion this is obviously not the case and figure 4.10b shows that at incubation concentrations of 5, 10, 20 and 30 beads per cell 47.7, 46.6 52.8 and 59 percent of the beads phagocytosed were contained within cells that did not infiltrate the filter respectively, these cells were representative of 16.5, 17.5, 17.4 and 17.2 percent of each cell population. More encouraging however is the fact that the remaining 52.3 (at 5 beads per cell), 53.4 (at 10 beads per cell), 47.2 (at 20 beads per cell), and 41 (at 30 beads per cell) percent of the beads were actively carried into the filter. These beads were, however, contained within a much larger proportion of the cells; i.e. 83.5, 82.5 82.6 and 82.8 percent of the respective cell populations.

Figure 4.10.a. The percentage of the PMNL population after directed migration present on the surface of the filter (0), between 10 and 50μm, between 60 and 100μm and beyond 100μm (>100μm) counted in the filter; where each column represents the mean ± SD of 6 PMNL populations at each bead concentration.

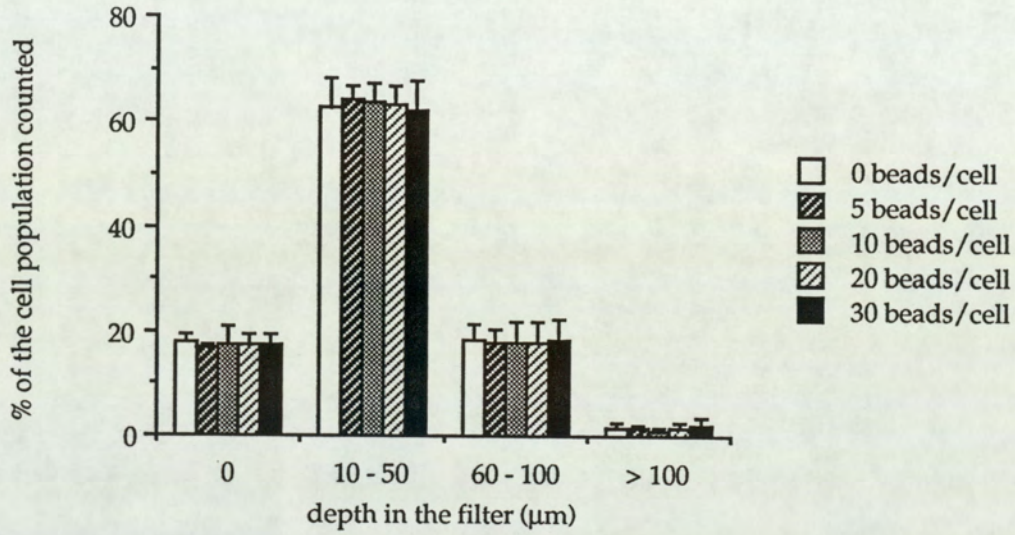
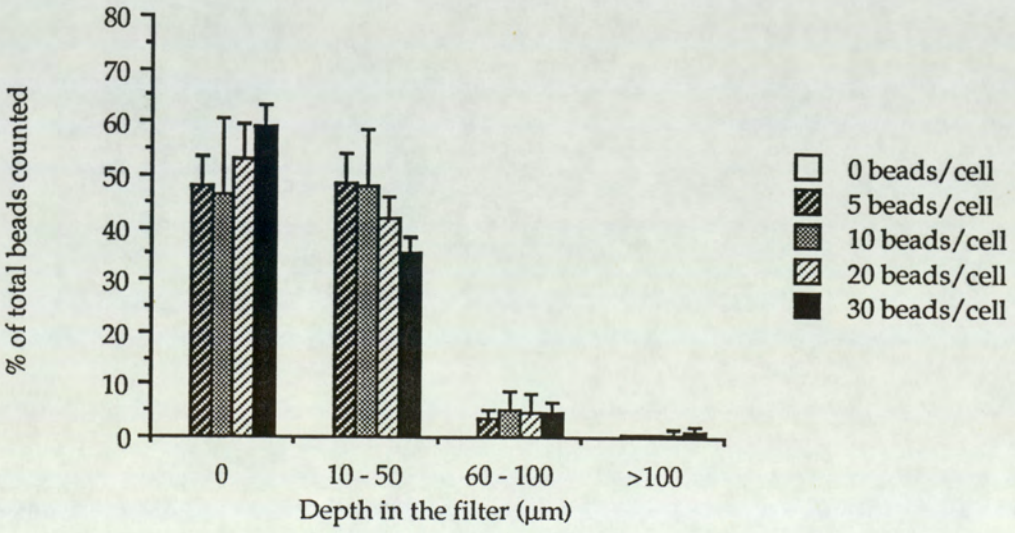


Figure 4.10.b. The percentage of the total number of counted PSL beads, carried by PMNL's by directed migration, present on the surface of the filter (0), between 10 and 50μm, between 60 and 100μm and beyond 100μm (>100μm) counted in the filter; where each column represents the mean ± SD of 6 PMNL populations at each bead concentration.



It is also evident from figure 4.10b that there is an apparent increase in the percentage of beads at the filter surface as the incubation concentration increases but was only significantly different from 5 beads per cell at the highest concentration, 30 beads per cell ($P<0.005$). Conversely the percentage of beads carried to depths between 10 and 50μm in the filters demonstrate a slight decrease as the incubation concentration was increased. At the bead concentrations 10 beads per cell the difference was insignificant from the percentage bead content at 5 beads per cell. At 20 and 30 beads per cell a significant decrease in the percentage bead content was demonstrated ($P< 0.05$ and $P<0.005$ respectively). The evidence presented suggests that there are no quantitative differences between distribution of the population through the filter. The observed difference in the distribution of beads must therefore be a characteristic of the phagocytic loading of the PMNL population. It could be hypothesised from fig 4.10b that the PMNLs which ingest the most particles phagocytose beads more avidly at the higher incubation concentrations which would explain the increase in proportion of beads not carried and a relative decrease in the proportion carried by the PMNLs. To elucidate this further the phagocytic kinetics of the PMNL subpopulations would have to be investigated in relation to their chemotactic response. Although this would appear to put a negative inflection on the bead carrying potential of the PMNL population the total number of beads transported into the filter increases considerably (table 4.05) with the increase in loading of the PMNL population.

Table 4.05. The number of PSL beads carryed to diifferent regions of the cellulose nitrate filter in the blindwell chamber assay. Each value represents the mean ± sem of 6 populations of PMNLs migrating in response to FMLP (10^{-8}M).

Depth in the filter (μm)	Incubation concentration (Beads/cell) 5	10	20	30
	Number of PSL beads(±sem)			
0	102.5 (11.4)	254 (28.1)	590 (34)	1370 (111)
10 - 50	102 (6.5)	282 (45.7)	469 (30)	811 (53)
60 - 100	8.7 (2.7)	31 (9.8)	82 (43)	116.2 (25.7)
>100	0.7 (0.2)	2.3 (0.5)	13.6 (5.6)	25 (11)

This potential carrying capacity is theoretical at this stage of the project. However if it is assumed that the percentage of the population that actively migrate in the blindwell chamber assay would actively accumulate at an inflammatory site after loading. The results suggests that upto 50 percent of the potential dose could be targeted (figure 4.10b). Whether it is realistic to extrapolate these preliminary *in vitro* finding have to be addressed at this point. The PMNLs used in these

investigations were isolated from inflammatory peritoneal exudate and despite demonstrating normal pathophysiological function in terms of phagocytosis and chemotaxis *in vitro* this does not indicate that these PMNL populations would demonstrate the same characteristics if reinfused into the circulation of a rat.

CHAPTER 5.

CHAPTER 5. *IN VIVO* MODEL OF PMNL CHEMOTAXIS

5.1.1. INTRODUCTION TO THE MEASUREMENT OF LEUCOCYTE CHEMOTAXIS *IN VIVO* .

Several methods have been developed to study the vectorial migration of purified cell populations *in vitro* (section 4.1.1). These methods have established the existence of a number of different chemotactic agents and have demonstrated the presence of specific receptors for these agents on the surface of migrating cells. Since all of these methods use isolated chemotactic agents, and highly enriched sources of migrating cell populations, directed migration can be readily demonstrated. By contrast, measurements of chemotactic responses under physiological conditions *in vivo* are more complex.

The response of an animal to a chemotactic agent represents the net effect of (1) the direct chemotactic activity of the agent itself and (2) any physiological chemotactic factors that may be generated by this agent. In addition to these factors the *in vivo* response will also be influenced by complex local inhibitory or amplification signals and the tissue structure at the inflammatory site (e.g. lymphatic drainage and vascularity). Enhanced adhesive properties and random motion of cells contribute to the inflammatory response but it is difficult to prove that the accumulation of cells at any site is a consequence of chemotaxis. Preferential accumulation at the inflammatory locus does however, suggest that PMNLs exhibit targeted directional migration.

A variety of animal models have been used to examine cellular localisation and to evaluate the role of chemotaxis and chemotaxins in inflammatory reactions (Boyle et al, 1988). These methods can be grouped into four main categories: (1) histopathological approaches (Boyle et al, 1988) (2) use of radioactively labelled leucocytes to monitor cellular localisation (Thakur, 1982), (3) the skin window technique (Rebuck & Crowley, 1955) and various modifications of this procedure, including inplantation chambers and sponges (Bailey, 1988), and (4) the air-pouch model. In addition to these there are many, many models used to study the mechanisms underlying the progress of inflammation in infections (Zeiler & Endermann, 1986; Bailey & Sturm, 1983) and rheumatoid arthritis (Billingham, 1983; Rainsford, 1982; Toivonen *et al* , 1982)

From a review of the techniques that have been used in the study of *in vivo* chemotaxis of PMNLs and the progress of inflammatory disease the air-pouch model is the most versatile and offers great potential in the investigation of a broad spectrum of inflammatory conditions. The major advantage for the progress of this study was quantification, in that the exudate could be collected and the cell numbers evaluated. Similar advantages could be proposed for the models that use the implanted chambers. Further processing would make it possible to analyse the response in the membrane lining the pouch. Hence the air-pouch could be developed to incorporate the use of radio-labelled cells and histology to analyse the targeted response of PMNLs.

5.1.2. AIR-POUCH PROCEDURE

The 'granuloma pouch' technique was developed for studying inflammatory responses in rats (Selye, 1953a, 1953b). Air is injected subdermally to generate a symmetrical air-pouch on the back of the animal. This results in the disruption of the normal architecture of the skin and creating a space into which a chemotactic factor, or putative inflammatory stimulus can be introduced. On its ventral surface the air-pouch membrane is thin and can be removed, stained and microscopically examined for infiltrating cells. More importantly the inflammatory cells can be harvested from the pouch, counted and the morphology determined. The major advantage of the air-pouch model is its simplicity and the accomodation of large numbers of samples. The creation of the connective tissue air-pouch is simple. Extensive surgical manipulation is not required, and it is easy to generate sterile air blebs on a large number of experimental animals. The simplicity of this technique and its ability to provide quantitative assessments of cellular infiltrates makes it particularly valuable for measuring chemotaxis *in vivo* . By comparison with skin window and chamber implantation techniques, the air-pouch is generated with minimal disruption of the surrounding tissue and thus can be achieved reproducibly without infection.

The major limitation of the technique is the subjective nature of the quantitation. Unlike studies using labelled leucocytes it is not possible to obtain a totally objective measure of cell migration. In many studies, histological determination of the inflammatory response is difficult due

to patchy localisation around blood vessels. This problem can usually be minimised by increasing the concentration of the chemoattractant, which increases the response and gives a uniform infiltrate.

5.1.3. PRINCIPLE USES OF THE AIR-POUCH PROCEDURE

In addition to use as an *in vivo* model for studying chemotaxis the air-pouch has been developed for understanding the progression of inflammatory disease. Drachman and Solokoff (1966) showed that the synovial membrane does not develop in embryos in the absence of movement suggesting that the synovial lining is simply an accretion of macrophages and fibroblasts stimulated by mechanical disruption of the connective tissue. Edwards *et al* (1981) demonstrated that formation of the air-pouch produced granuloma linings which bear a superficial resemblance to synovial linings. As a consequence of these findings it was proposed that the air pouch should be exploited as a model of synovial lining tissue (De Brito et al, 1987; Sedgwick et al, 1983; Edwards et al, 1981). It has also been shown that the inflammatory reactivity increases markedly with the establishment of the lining (Sedgwick et al, 1983) reflecting the development of structural features. These include (a) new blood vessels; (b) the formation of an organised layer of mononuclear phagocytes and fibroblastic cells and (c) the formation of an increasingly effective mechanical barrier that retains the the irritant and the products of the inflammatory response. The model has been used as a facsimile synovia to study the development of acute and chronic inflammation induced by a variety of factors. Immune inflammation has been induced by single or multiple antigenic challenge into the air-pouch of animals presensitised with Freund's adjuvant and the immunogenic antigen, for

example BSA (Sin *et al* , 1984; Yoshino *et al* ,1984), azobenzene-arsonate-conjugated acetyl bovine serum albumin (Ohuchi *et al* , 1982, 1983 and 1984; Tsurufugi *et al* , 1982; Kurihata *et al* , 1983; Hirasawa *et al* , 1986) or Pertussis vaccine (Sedgwick *et al* , 1985a). The involvement of other contributary factors have been investigated using zymosan (Konno & Tsurufugi, 1983) and carrageenan (Sin *et al* , 1986; Watanabe *et al* , 1985; Sedgwick *et al* , 1985b) which activate the alternative complement pathway; monosodium urate and calcium pyrophosphate dihydrate crystals (Sedgwick *et al* , 1983; Brooks *et al* , 1987) whose synovial deposition is associated with gout; croton oil (Dalhoff *et al* , 1982); *Escherichia coli* (Dalhoff *et al* , 1983; Zeiler & Endermann, 1986); casein (Konno & Tsurufuji, 1983) and FMLP (Lawman *et al* , 1984). Induction of polyarthritis in the experimental animal by subdermal inoculation of *Mycobacterium tuberculosis* (DeBrito et al, 1987) has demonstrated spontaneous changes in the air-pouch lining comparable to and co-incident with the proliferating synovitis that developed in the peripheral joints of the same animal. The air-pouch is therefore a versatile technique for use in 'simple' studies of *in vivo* chemotaxis to the complex interactions in chronic inflammation.

5.1.3. USE OF RADIOLABELLED LEUCOCYTES

A number of methods have been described that use radiolabelled cells to study directed movement to a site of inflammation or to a chemotactic agent. A variety of *in vivo* and *in vitro* methods for labelling cells have been used (Thakur and McKenney, 1980). *In vivo* labelling can be achieved by injection of a radioisotope, e.g. [^{3}H] thymidine, into the animal, allowing the isotope to be incorporated into cells during cell

division. Cells labelled in this way can then be transfered to an experimental animal, either by isolation and injection or by surgically joining the circulations of the 'labelled' and experimental animals (Thakur and McKenney, 1980). The fate of the labelled cells is then determined by autoradiographic examination of tissue sections of the inflammatory site where the labelled cells will localise.

A second approach is to label cells *in vitro* . In this approach, blood is removed either from an experimental animal or from a allogenic donor, and the leucocyte population is isolated and labelled with a suitable radioisotope. In most cases , the isotopes of choice are gamma emitters such as ^{51}Cr, ^{111}In, ^{133}Xe, or ^{99}Tc (McAfee *et al*, 1980). These isotopes have the advantages that they are readily incorporated into the cytoplasm of leucocytes and that the distribution in experimental animals can be monitored by a variety of methods, either with or without sacrificing the animal. Using gamma counting tissue distribution, blood clearance and survival in the circulation can be determined. Using more sophisticated counting instruments, e.g. the gamma camera. This approach enables the distribution of injected cells to be followed efficiently, and it permits accurate quantification of the accumulation of cells at different sites in the animal.

The validity of extrapolating the results of these studies to normal physiological responses assumes that the isotope remains associated with the labelled cells following injection, and that the cell type labelled was not modified in any way during the isolation and labelling procedures. These *in vitro* labelling techniques have proved successful for locating abscesses and other sites of inflammation in man and have confirmed in animal models that the PMNL is the first cell type to respond to

inflammatory stimuli. The approach, however, is of limited value for long-term studies, and it can be difficult to demonstrate accumulation of large numbers of counts at an inflammed site verses a control site and also cells are rapidly turned over, having a short half life.

5.2 MATERIALS AND METHODS.

5.2.1. ANIMALS

Adult male Wistar rats (150g.-175g.) were purchased and maintained according to the details in section 2.2.1. Rats of this weight were used in order to avoid injection of air into subcutaneous fat tissue which was found to form inappropriate pouches. Larger animals with more subcutaneous fat formed air-pouches with air-filled fat bubbles.

5.2.2. PREPARATION OF THE AIR-POUCH.

The rats were anaesthetised using halothane (see section 2.2.4) then the back of the rat was shaved, removing all the fur on the area between the scapulae and the rump, using electric clippers. To form the pouch 20mls of air was injected slowly via a 25 gauge needle which was inserted subcutaneously in the area just behind, but between the scapulae. The thumb and first finger of the other hand were placed in a "V" over the head during injection to form an ellipsoid or oval shaped air-pouch on the dorsum, and prevent the air-pouch forming over the head and neck as well as the back. The extent of the pouch was delineated with a marker pen before the animal revived. To maintain an open cavity 10mls of air

was injected into each pouch after 3 days. The animals were challenged 6 days after formation of the air-pouch, which is the time established for the development of an organised lining and blood supply (Sedgwick et al, 1983; Sin et al, 1984).

Handling the animals after development of the air-pouch was difficult due to its location and it was thought inappropriate to scruff the animal in this condition. The animals, where possible, were encouraged into a small perspex restrainer for the purpose of anaesthesia.

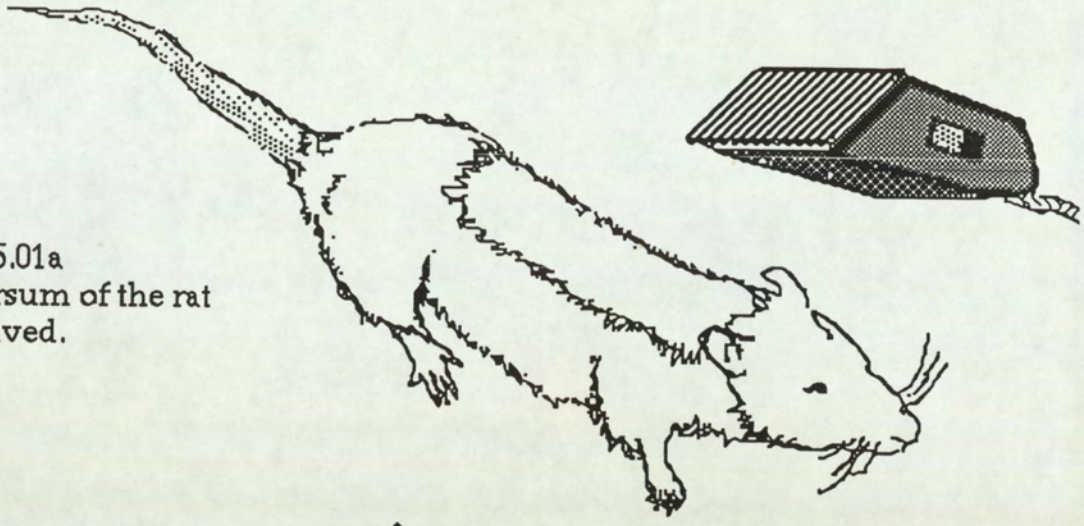


Figure 5.01a
The dorsum of the rat was shaved.

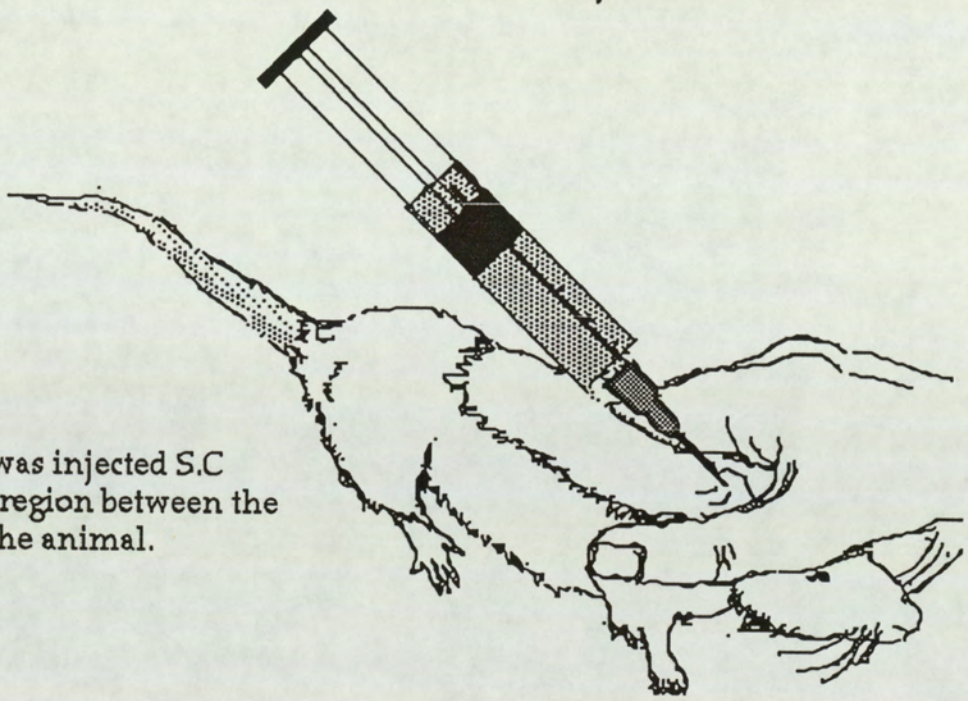


Figure 5.01b
20 ml of air was injected S.C in the scruff region between the scapulae or the animal.

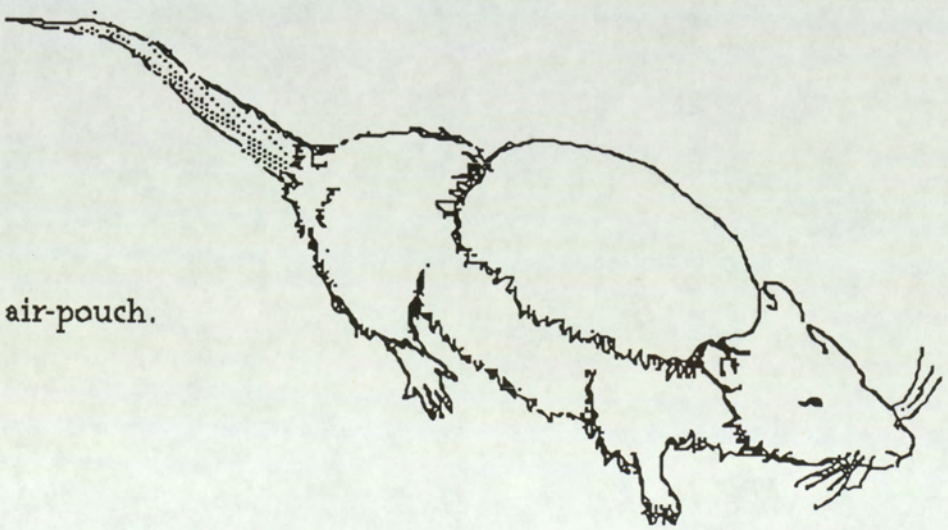


Figure 5.01c
The formed air-pouch.

5.2.3. STUDY OF CELL INFILTRATION INTO THE AIR-POUCH IN RESPONSE TO FMLP

To study the *in vivo* chemotaxis of PMNL's the normal inflammatory response of PMNL in the air-pouch model had to be established. The chemoattractant FMLP (10^{-8}M in HBSS, 1mg/ml BSA, pH 7.4) was used as the irritant in the inflammatory air-pouches to enable comparison between *in vitro* and *in vivo* results. FMLP and the control buffer HBSS were prepared according to the procedure described previously (section 3.2.1) as was . Before challenge the animal was anaesthetised using halothane then 2ml of FMLP or control buffer (HBSS) was injected carefully through the centre of the air-pouch. The animals were sacrificed at 0, 2, 4, 6, 8 and 24 hours by overdose of halothane then very careful cervical dislocation. The air-pouch was gently dissected out without bursting the underlying membrane. Once excised, a small incision was made at one end of the pouch and the cells and exudate lavaged with 2 aliquotes of HBSS (5mls) using a plastic pasteur pipette to wash the pouch. The inflammatory cells were then washed by centrifugation at 200g for 5 minutes then total and differential counts were conducted (see sections 2.2.7 and 2.2.9).

5.2.4. BLOOD DISTRIBUTION STUDY

Prior to the study of the distribution of radiolabelled PMNL's, after infusion into rats, it was necessary to determine the blood volume of the rat and the volume of blood contained within the vasculature of each tissue. This information was essential in the studies of the tissue distribution of indium-111 labelled PMNL's and enabled determination

of the counts associated with infiltrating cells or cells marginated in the tissue vasculature and those circulating free in the blood.

5.2.4.1. ANAESTHESIA

The anaesthetic technique used for this set of experiments was different to that used in previous studies. Halothane anaesthesia was used to establish the air-pouches but because it causes a progressive fall in the systemic blood pressure (Green, 1982) it was thought to be inappropriate for I.V. injection and studies of blood distribution and PMNL localisation. The agent used was fentanyl-fluanisone (Hypnorm; Janssen Pharmaceuticals Ltd, Wantage, Oxon, U.K.) which was given I.M., into the thigh muscle, at a concentration of 0.3 ml/kg (Green, 1982). This dose has little effect on cardiac output or blood pressure (Green, 1982), and has a duration of effect lasting for between 15-30 minutes.

5.2.4.2. DETERMINATION OF THE TOTAL AND TISSUE BLOOD VOLUMES IN THE MALE WISTAR RAT IN CONTROL AND AIR-POUCH ANIMALS.

Using radioiodinated serum albumin as a blood marker this study enabled a determination of the total blood volume and the individual tissue blood volumes in male wistar rats for; (1) control animals, (2) air-pouch animals without inflammation, and (3) air-pouch animals with inflammation. Six days prior to the distribution study air-pouches were inflated (see section 5.2.2) . Plastic scintillation vails (Packard, Pangbourne, Berks, U.K.) with caps were preweighed and numbered.

The radioiodinated albumin (^{125}I albumin; Amersham International plc, Amersham, U.K.) was supplied in 1ml of sterile saline (50μCi/ml). This was diluted further in sterile saline to give a concentration of 5μCi/ml. The dose injected was 1μCi in 200μl.

The animals were anaesthetised by I.M. injection of Hypnorm (see section 5.2.4.2). After 5-10 minutes the tail was rinsed under warm running water to clean it and cause mild vasodilatation thereby facilitating visualisation of the tail vein. The tail vein injection was difficult but useful, in that the dose could be uniformly corrected by subtracting the counts remaining in the tail from the dose in case of unsuccessful injection . The 25 gauge needle was bent slightly and introduced just below the surface of the skin along the line of the tail vein. Once in the vein blood can be massaged gently back into the needle. The syringe could then be introduced and the dose delivered carefully. The dose, was delivered through an unprimed needle, so the counted dose for each animal was injected into the vial through an unprimed syringe needle.

After injection, the dose was allowed to equilibrate in the circulation for 10 minutes then a blood sample was taken by cardiac puncture and the animal sacrificed by cervical dislocation. In those cases where the whole organ or tissue was not excised the following procedures were followed: (a) for skin a sample was stripped from the abdomen; (b) for skeletal muscle the Vastus Lateralis muscle of the thigh was separated from the femur; (c) for bone the central portion of the femur comprised the sample, no attempt was made to separate bony tissue from the marrow; (d) a sample of the small intestine was excised. The vials containing the tissues were then weighed and the samples were analysed for

radioactivity using an L.K.B.-Wallac 1282 Compugamma (L.K.B.-Pharmacia, Milton Keynes. U.K.). Each sample was counted for 1 minute with the emission windows set to record the counts between 20 and 90 KeV.

To make the required corrections when calculating the results the total tissue weights were required for those organs where only a small sample was excised. The assumption that the tissue weight increases in a linear relation with time has to be made as the data was available for a 300g rat (Lowry and Hawkins, 1986) and all animals used were between 150 and 250g at the time of experiment.

Organ weights for a 300g rat (Lowry and Hawkins, 1986): -

Skin	31.9g
Muscle	130.5g
Small intestine	24.9g
Fat	114.84g
Bone	33g

5.2.5. STUDY OF SURVIVAL AND INFILTRATION OF INDIUM-111-LABELLED PMNLS

5.2.5.1. RADIOLABELLING PMNLS WITH INDIUM-111-OXINE

The cells were elicited and harvested according to the procedure detailed in section 2.2.5 and 2.2.6. Total, differential and viability counts were determined and the cells suspended to 5×10^7 cells/ml in HBSS (pH 7.4)

for labelling. The recommended dose to study PMNL localisation at sites of inflammation in human adults was between 500 and 1000μCi (Williamson et al , 1986; Bell *et al* , 1986). In many of these studies the radioactivity was monitored using gamma scintigraphy necessitating the administration of high doses of radioactivity to achieve focal accumulation at the inflammatory lesion and to discriminate the lesion from background. Later studies of PMNL kinetics and distribution found 200-500μCi to be a satisfactory dose (Weiblen et al, 1979). For distribution studies in the rat this was scaled down to a dose of 1-2 μCi per animal. The activity required for labelling is calculated from 1.25 times the expected dose to be delivered (Amersham technical bulletin, Indium (^{111}[In]) oxine solution, code: IN.15P) this was added dropwise from a 1ml syringe to the cell suspension. The tube was capped and incubated, undisturbed, for 15 minutes at room temperature. The labelled cell suspension was then transfered to a plastic centrifuge tube leaving a residual volume of 0.2 - 0.5mls in the tube (this contained any clumps of PMNLs which had settled during incubation and were discarded). The cell suspension was diluted with an equal volume of platelet-depleted plasma which was prepared by centifugation of 10ml whole blood at 1000g for 5 minutes. The cells were then washed by centrifugation at 100g for 5 minutes. The cells were washed three times in 50% plasma. Samples of the cells and supernatant were taken at each stage to determine the efficiency of labelling and to ensure the free ^{111}[In] was not excessive. The cells were then resuspended to a concentration of $10x10^6$ cell/ml before labelling. Allowing for cell loss during labelling the dose, for each animal, contained 1-2 $x10^6$ cells and 1-2 μCi of radioactivity.

5.2.5.2. THE DISTRIBUTION OF INDIUM-111-LABELLED DONOR RAT PMNLS AFTER REINFUSION INTO RECIPIENT ANIMALS.

To investigate the survival of PMNL's raised by the method detailed in section 2.2.5 and 2.2.6. the cells were labelled with indium-111-oxine (section 5.2.5.1). The animals were anaesthetised using hypnorm (section 5.2.4.2) and 200µl of the labelled cell suspension was injected slowly through a 25 gauge needle, into the tail vein. The animals were sacrificed at 10 minutes, 30 minutes, 3 hours, 6 hours and 24 hours after a blood sample had been taken by cardiac puncture. The tissues were dissected, weighed and counted (section 5.2.4.3.). The program for counting ^{111}In was similar to that for ^{125}I the samples were counted for 1 minute and the emission windows were set to record radiation at between 150 and 600 KeV.

5.2.5.3. STUDY OF THE INFILTRATION OF REINFUSED PMNL INTO THE INFLAMMATORY AIR-POUCH.

To determine the infiltration of these labelled cells the 6 day air-pouch was used (see 5.2.1 and 5.2.2). The anaesthetic technique was the same as in section 5.2.4.2. The air-pouches were challenged at time 0 with 2ml FMLP ($10^{-8}M$) following this 200µl of labelled cell suspension was injected into the tail vein. After 6 hours a blood sample was obtained by cardiac puncture and the animal sacrificed by cervical dislocation. The tissues were dissected and placed in preweighed vials

5.3. RESULTS

5.3.1. STUDY OF CELL INFILTRATION INTO THE AIR-POUCH IN RESPONSE TO FMLP AND HBSS

Prior to the study of PMNL distribution and survival after reinfusion the infiltration of host PMNLs into the control (2ml HBSS) and the inflammatory (2ml 10^{-8}M FMLP) air-pouch was conducted. The infiltration of PMNLs and MNL in response to FMLP are shown in figures 5.01a and to HBSS in figure 5.01b. Greater than $5x10^7$ PMNLs infiltrated the air-pouch in 6 hours with FMLP. After this time the PMNL number decreased and the MNL began to increase (figure 5.01a). This was a pattern indicative of the acute inflammatory response and comparable to the peritoneal, infiltration in response to sodium caseinate (section 2.3.2). The infiltration in response to HBSS was negligable. There was a small PMNL infiltration ($<5x10^6$ PMNL) that mirrored that with 2ml 10^{-8}M FMLP but the resident MNL population remained constant (figure 5.01b).

Figure 5.02a. The infiltration of PMNLs and MNLs into the air-pouch after challenge with 2ml 10^{-8}M FMLP. Each value represents the mean ± sem (n=4)

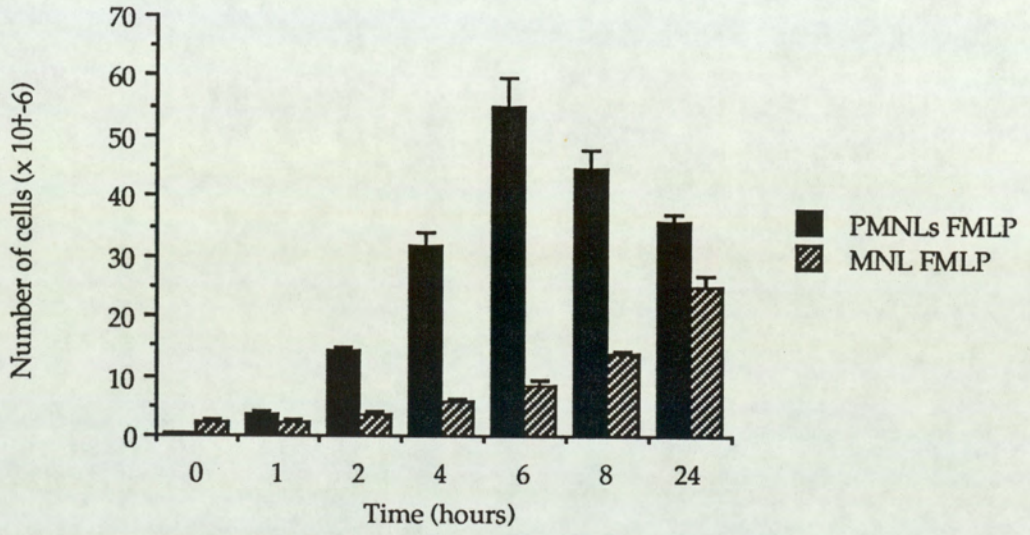
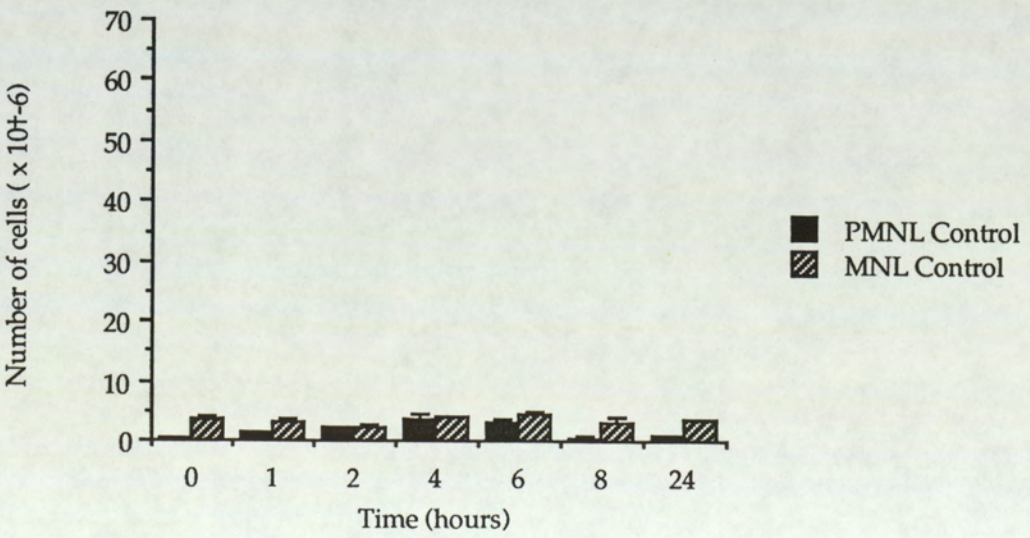


Figure 5.02b. The infiltration of PMNL and MNL into the air-pouch after challenge with 2ml HBSS. Each value represents the mean ± sem (n=4).



5.3.2. BLOOD DISTRIBUTION STUDY

To study the distribution of PMNLs in the rat it was essential to determine the blood volumes contained within each organ (table 5.01). The total blood volumes determined in control animals (7.48 ± 0.26 ml/100g), animals with control air-pouch (7.41 ± 0.76 ml/100g) and animals with inflamed air-pouch (7.84 ± 0.76 ml/100g) were not signficantly different. They also agreed closely with the value determined in normal control animals by Lowry and Hawkins (1986) which was 7.35 ml per 100g body weight. The values obtained for blood volumes in each tissue were comparable in each group except for the liver and the air-pouch. Induction of the air-pouch increased the liver blood volume both with ($P<0.01$) and without ($P<0.01$) inflammation this is possibly due to increase in porosity of the tissue as a result of the tissue damage and inflammatory stimuli. Vascular porosity in the liver, lung and bone marrow can give higher blood volumes when using ^{125}[I] albumin as the vascular marker (Triplett et al, 1985). The blood volume obtained for the control air-pouch tissue were significantly greater ($P<0.05$) than the normal skin tissue suggesting an increase in blood supply. This is further increased in the inflammatory air-pouch and is significantly greater than the control air-pouch tissue ($P<0.01$) and normal skin tissue ($P< 0.001$). If however these values are compared with the routine skin sample taken from the abdominal region there is no significant difference between the local blood volume in the skin compared to the air-pouch region.

These values were used in subsequent studies to correct the tissue counts for PMNL that remain in the circulation and that have not specifically migrated into or cleared by the tissue involved.

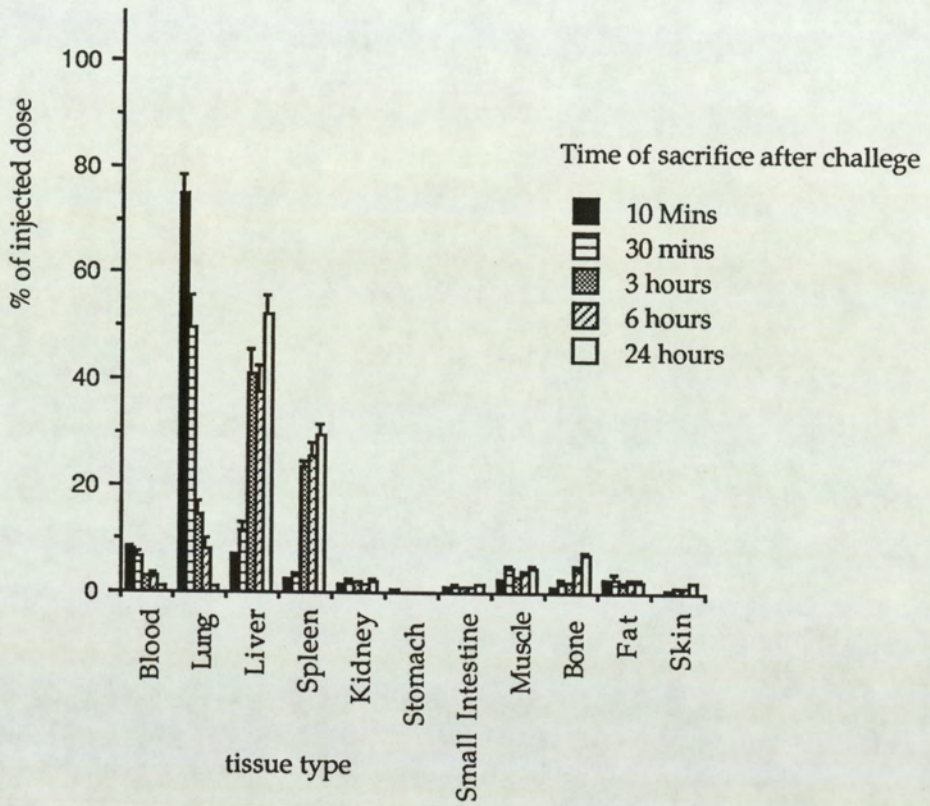
Table 5.01. The blood volume of the male Wistar rat and the respective tissue blood volumes (mls) in: 1. the control rat; 2. the air-pouch rat and 3. the air-pouch with inflammation after injection of 2ml of FMLP. Each value represents the mean ± sem (n=6). * P<0.05, **P<0.01, ***P<0.001, [1] significantly different from control and [2] significantly different from air-pouch with 2ml HBSS.

Tissue	(1) Control no air-pouch	(2) Air-pouch +2ml HBSS	(3) Air-pouch +2mlFMLP(10^{-8}M)
Blood (ml/100g)	7.41± 0.76	7.84 ± 0.24	7.48± 0.26
Lung	0.267 ± 0.058	0.310 ± 0.069	0.246 ± 0.037
Liver	0.166 ± 0.041	0.323 ± 0.030**[1.]	0.246 ± 0.037**[1.2.]
Spleen	0.121 ± 0.031	0.125 ± 0.013	0.121 ± 0.023
Kidney	0.321 ± 0.046	0.228 ± 0.044	0.218 ± 0.039
Stomach	0.029 ± 0.009	0.029 ± 0.005	0.032 ± 0.013
Small Intestine	0.036 ± 0.014	0.037 ± 0.009	0.039 ± 0.012
Muscle	0.020 ± 0.025	0.010 ± 0.003	0.011 ± 0.002
Bone	0.067 ± 0.037	0.054 ± 0.008	0.110 ± 0.125
Fat	0.017 ± 0.007	0.012 ± 0.004	0.017 ± 0.007
Skin	0.014 ± 0.003	0.013 ± 0.002	0.022 ± 0.016
Air-pouch	0.011 ± 0.003	0.016 ± 0.004*[1.]	0.022 ± 0.005**[2.] ***[1.]

5.3.3. DISTRIBUTION AND SURVIVAL OF RADIOLABELLED PMNLS AFTER REINFUSION IN TO CONTROL MALE WISTAR RATS .

The PMNLs were successfully labelled with ^{111}In oxine and after washing (section 5.2.5.1) the free ^{111}In was negligable at 1.5 percent of the cell associated radioactivity and the efficiency of labelling was 71.4 percent. The percentage distribution of the reinfused PMNLs is illustrated in figure 5.03. At 10 minutes 74.8 percent of the population was in the lungs with only 8.0 percent free in the circulation. By 30 minutes the lung counts had decreased to 49.9 percent but this was not reflected in a significant mobilisation to the vascular pool which decreased to 6.5 percent of the dose. At 3 hours the lung associated PMNLs had decreased to 14.6 percent but conversely the percentage contained in the liver and spleen had demonstrated an equivalent rise to 41 and 23.5 percent respectively. The blood levels had further decreased to only 2.9 percent. At 6 hours there was not a marked change from the distribution at 3 hours after reinfusion. The lung level decreased to 7.8 percent and the bone marrow demonstrated a small rise too 4.5 percent from 1.8 at 3 hours. The liver and spleen at 24 hours contained greater than 80 percent of the counts (52.4 and 29.7 percent respectively) and the bone marrow increased again to 6.8 percent. The blood however, decreased further containing only 1 percent of the dose at 24 hours. The levels in all other organs were negligible (figure 5.03).

Figure 5.03. The percentage distribution of the reinfused PMNL population at 10 minutes, 30 minutes, 3 hours, 6 hours and 24 hours after I.V injection. Each bar represents the mean ± sem of 4 animals at each time point.



5.3.4. STUDY OF THE INFILTRATION OF REINFUSED PMNLS INTO THE INFLAMMATORY AIR-POUCH.

The infiltration of reinfused PMNLs into the air-pouch rats with and without infammatory stimulus was studied (table 5.02). The PMNLs were successfully labelled with ^{111}In oxine and after washing (section 5.2.5.1) the free ^{111}In was negligible at 2.1 percent of the cell associated radioactivity and the efficiency of labelling was 68.4 percent under the

same conditions as the previous experiment (section 5.3.3). The results (table 5.02) demonstrated that there was no significant difference between tissue associated counts in air-pouch rats with or without injection of FMLP. The air-pouch tissue, which included the counts in the exudate lavaged from the pouch did not demonstrate significant infiltration of ^{111}In PMNLs with FMLP than with just buffer.

Table 5.02. The distribution of indium-111 -labelled donor PMNLs after I.V. administration via the tail vein in the male Wistar rat. Each bar represents the mean ± sem of tissues samples from 4 animals at each time point.

Tissue	Air-pouch without FMLP	Air-pouch with FMLP
Blood	3.77 ± 1.57	4.50 ± 1.6
Lung	6.70 ± 1.4	5.17 ± 1.37
Liver	66.50 ± 9.1	63.07 ± 6.97
Spleen	6.69 ± 1.60	8.76 ± 4.16
Kidney	1.57 ± 0.33	1.78 ± 0.43
Stomach	0.08 ± 0.06	0.07 ± 0.03
Small Intestine	0.88 ± 0.62	0.88 ± 0.38
Muscle	3.19 ± 3.26	2.42 ±0.69
Bone	3.15 ± 1.14	2.92 ± 1.23
Skin	0.45 ± 0.37	0.79 ± 0.18
Air-pouch	0.37 ± 0.16	0.63 ± 0.77
% Recovery of dose	93.51 ± 7.08	91.93 ± 8.5

5.4. DISCUSSION

The results of this chapter culminated in the development of a potential model for the study of PMNL infiltration of an inflammatory lesion. The air-pouch model could be manipulated to facilitate the study of a wide variety of inflammatory conditions which have been reviewed in section 5.1.3. as well as for study of *in vivo* chemotaxis.

The infiltration of inflammatory cells were easy to quantify by conducting cell counts after lavage of the exudate. Using FMLP (2ml 10^{-8}M FMLP) as the irritant in the air-pouch PMNLs infiltrated reaching a peak aproximately 6 hours after challenge where greater than 5×10^7 PMNLs responded to the stimulus. The total number of PMNLs in the circulation of a rat of approximately 175g is approximately 8×10^7 (calculated from figures from Farris & Griffiths, 1949) (table 5.03). This figure includes both the marginated and freely circulating pool of cells which each constitute 50% of the total pool (Boggs, 1974). The response to the FMLP challenge was therefore substantial and involved recruitment of more than 50 percent to the air-pouch.

Table 5.03. White cell population of the rat (Farris & Griffiths, 1949)

Cell Type	Number of cells
White Blood Cells (Nº/ml)	9×10^6
neutrophils (PMNLS)(Nº/1.3 x 10^7wbc/ml)	3.15×10^6
Lymphocytes (Nº/1.3 x 10^7wbc/ml)	5.4×10^6
Monocytes (Nº/1.3 x 10^7wbc/ml)	1.8×10^5
Basophils (Nº/1.3 x 10^7wbc/ml)	0.9×10^4
Eosinophils (Nº/1.3 x 10^7wbc/ml)	1.8×10^5

The development of an *in vivo* assay of chemotaxis in this investigation relied on the viability of the PMNL and the ability to function with normal pathophysiology after reinfusion. The results of section 5.3.3. illustrated that the PMNLs isolated by the method detailed in section 2.2 and labelled with indium-111-oxine do not survive in the circulation. The maximum blood level being observed at just 10 minutes (8.0 percent) and decreasing subsequent to this time point. The majority of the PMNLs (74.8 percent) were located in the lung at the early times (10 and 30 minutes). This is characteristic of the pulmonary sequestration of PMNLs observed following intravenous infusion of chemotactic agents and intravenous activation of complement (Harlan, 1985) also following reinfusion of radiolabelled cells in previous studies (Peters et al, 1980). This characteristic may not represent a specific homing of cells to the pulmonary microcirculation, but may instead reflect the fact that the

lung is the first capillary bed encountered after intravenous administration of mediators and cells. The lung sequestration could be the result of aggregation in the microcirculation and retarded transit through the capillary beds or margination in the post-capillary venules, the site of the first major decrease in vessel wall shear stress. The observation that the lung accumulation of PMNLs is a characteristic of infusion of PMNL activator molecules and also labelled cells indicates that in the latter case the PMNLs reinfused may be preactivated so aggregation or sticking in the post-capillary venules were both possible alternatives. Following the period of lung sequestration the PMNL population became redistributed to the liver and spleen which by 24 hours contained greater than 80 percent of the counts. These organs contain the greatest proportion of macrophages in the MPS and the PMNL distribution suggests clearance of the reinfused cells from the circulation. The conclusion of this study was that the PMNL population despite exhibiting normal physiological properties *in vitro* were not surviving *in vivo.* From the results in section 5.3.4. there was no significant accumulation of reinfused PMNLs into inflammatory air-pouches which suggests that they do not behave with normal pathophysiology *in vivo* . This could be accounted for by the fact that the cells used had already been elicited when the rat peritoneum was challenged with sodium caseinate. Stimulation with chemoattractants has been demonstrated to cause expression of adhesion receptor molecules on the cell surface (Bainton et al. 1987) which promote PMNL endothelial interactions and clearance from the circulation. The washing, purification and erythrocyte lysis have also been shown to decrease PMNL survival when the cells have been labelled with a variety of isotope complexes and reinfused (Hardeman, 1980). Finally the actual cell labelling procedures demonstrate marked differences in PMNL kinetics,

survival and focal accumulation at inflammatory sites (Thakur & McKenney, 1980; McAfee et al, 1980). The problem with viability testing for the purpose of PMNL activity is that the structural integrity of the cell membrane can be proven and the functionality in terms of phagocytosis and chemotaxis can be determined but this does not demonstrate the capacity of the cells to circulate with normal disappearance kinetics. This was a very important question and in retrospect could have been answered sooner. With the consequence of demonstrating cell infiltration in the air-pouch model. The progress of this study involved the use of the same cell isolation technique throughout because of the need for large numbers of cells for the *in vitro* investigations. The use of PMNL isolation techniques from whole blood were ruled out for the reason of cell yield (Schmitt *et al* , 1986) but this constraint does not apply in the *in vivo* study as very few cells would be required. The evidence presented in diagnostic imaging studies (Williamson *et al*, 1986; Reba & Chandeysson, 1980; Thakur & McKenney, 1980; McAfee *et al* , 1980; Wiblen et al, 1979) have demonstrated circulating half-lives of between 5 to 7 hours and imaging of inflammatory loci with indium 111 labelled PMNLs. Indium 111 has become the agent of choice and though development of a complex (Indium 111 troponolone (Burke *et al* , 1982; Peters *et al* , 1980) the cells can be labelled in plasma and extensive washing and pelleting of the cells is not required. Isolation and labelling of the cells therefore needs to be conducted with the minimum of trauma for the cells and in the minimum time (Thakur & McKenney, 1980). The next step in the progress of this project would be the development of a technique to isolate and reinject a PMNL population which exert normal pathophysiology. Then to investigate whether these cells will carry the model particles to the inflammatory air-pouch and determine the targeting potential *in vivo*.

CHAPTER 6

CHAPTER 6. CONCLUSIONS

The possibilities in the field of drug targeting are wide reaching and as a consequence many and varied approaches have been investigated and exploited, some of which are progressing slowly towards clinical utility. The rationale behind the inception of this project was the inherent targeting capacity of the PMNL. PMNLs are the first cells to respond in the vast majority of inflammatory conditions and as such they may be used to carry drugs to these sites. The target condition may be as simple as an abscess, or as complex as the joints involved in progressive rheumatic disease. Before these cells could be used in the clinical situation many questions had to be answered. This study investigated some of the fundamental issues essential for development of such a concept.

A reproducible method was established for the isolatation of a large numbers of viable PMNLs. These cells were then used in the development of reliable assays to study phagocytosis and chemotaxis *in vitro* . The assays enabled PMNL migration to be measured following phagocytic loading with polystyrene latex beads. The PMNL populations studied avidly phagocytosed the 1.1μm PSL beads and the loading of the cells increased in proportion to the incubation concentration. The chemotaxis and random migration of PMNLs after differential loading at concentration of 5, 10, 20 and 30 beads per cell demonstrated no significant diffence from control (table 4.02). The leucotactic index reflected the distribution of the PMNL population in the filter. However, when the bead distribution was expressed the results suggested that there was a disproportionately large number of beads in the least responsive

PMNLs in the blindwell chamber assay. Approximately 80% of the PMNL population were able to migrate uninhibited by phagocytosis and these cells contained 50% of the beads. If these PMNLs could exhibit normal pathophysiology *in vivo* there is the potential to deliver 50% of the dose to inflammatory lesions.

A rat air-pouch model was then developed to demonstrate the focal accumulation of these cells *in vivo* . Using ^{125}I albumin, as an intravascular marker, the tissue blood volume were determined. These values were used in the study of survival indium-111 labelled PMNLs after reinfusion; and to demonstrate focal accumulation of the cells in the inflammatory air-pouch. The PMNLs isolated and labelled in this study demonstrated prolonged lung sequestration then clearance of the radioactivity in the liver and spleen. As the cells did not survive in the circulation the focal accumulation of radiolabelled PMNLs in the airpouch, was not demonstrated in these preliminary studies.

It is clear from these investigations that PMNLs will migrate after phagocytosis of PSL beads *in vitro* whether they will do the same *in vivo* still has to be answered.

CHAPTER 7

CHAPTER 7. PROPOSALS FOR FURTHER INVESTIGATION

In vitro studies with neutrophils bear little resemblance to the complex situation encountered *in vivo* . Consequently the first question that has to be asked is: will neutrophils migrate *in vivo* and carry beads to a site of inflammation?

To answer this question a technique has to be employed to isolate and reinfuse PMNLs to demonstrate normal survival kinetics *in vivo*. Evidence that transfused PMNLs have been successfully used in conditions of profound neutropenia (Boggs, 1974; Klastersky, 1979) and focal accumulation of reinfused PMNLs in inflammation (Thakur and McKenney, 1980; McAfee, 1980) means that this is a realistic goal. The future of the project would then depend on whether PMNL could be loaded with beads and still exhibit their normal pathophysiology *in vivo* .

If targeting of beads with PMNLs to an inflammatory site is demonstrated the potential for further investigation is immense. There are two possible approaches to neutrophil targeting. In the first case the drug-carriers would:

1. Be injected into the circulation where they would specifically bind and be internalised by PMNLs.
2. The cells would then circulate normally with the payload of drug and eventually encounter the inflammatory stimulus required to stimulate the cells.
3. The loaded PMNLs would marginate in the disease location and then migrate from the vessel through the endothelial lining into the tissue.

4. In the tissue they demonstrate their normal pathophysiological effect during which time the release of the drug from the carrier would be facilitated and its therapeutic effect exerted at the target site.

This rationale involves targeting to the PMNL in the circulation and exposure of the beads to cells of the MPS where non-specific clearance would be expected. Stabilisation of the bead surface with poloxamers (Davis and Illum; 1986) has been shown to prevent MPS clearance. To achieve specific interaction and phagocytosis by the PMNL the beads would have to recognise the cells. This may be possible by incorporation of neutrophil specific ligand like the family of glycoproteins Gp165/95 (including Mac-1 and CR3) (Buyon et al, 1988) or the monoclonal antibody 31D8 (Selligman et al, 1985) into the poloxamer surface layer; but this has to be investigated.

The second possibility involves isolation, loading and reinfusion of PMNL, the approach already adopted in this study. This alternative relies solely on the inflammation targeting ability of the PMNL which has been proven in previous studies (McAfee, Subramanian and Gagne, 1980, Thakur and McKenney, 1980, Rayuda, 1983). The targeting potential however, could be enhanced by better understanding of the heterogeneity of these cells.

The question of PMNL heterogeneity with respect to their phagocytic loading is important. The observation that 80% of PMNLs readily express the Fc receptor and also demonstrate superior adhesiveness, aggregation and chemotaxis (Klempner & Gallin, 1978) correlates well with the finding in this study, when approximately 80% of the PMNLs migrated in the blindwell chamber assay. In addition findings that abscess PMNLs are

enriched to 95% (Gallin, 1984) with cells expressing Fc receptors suggests preferential mobilisation of these cells in response to the inflammatory stimulus. This is strong evidence for differential purification or preferential phagocytic loading of the active sub-population to enhance the targeting potential *in vivo* . By density gradient centrifugation Pember et al (1983) demonstrated PMNLs can be separated into two groups - those with high density and those with intermediate density. It may therefore be possible to isolate the sub-population that migrate in the blindwell chamber assay and reevaluate the bead targeting potential compared to the whole population.

Disease targets should be established early in the next phase of work so the carrier-drug complex can be investigated and an answer found to the question: how is the carrier going to protect the PMNL from the drug until release of its payload at the target site? As only one size of solid bead was looked at in this study it is important to discover the optimal loading capacity of the PMNLs with different sizes and composition of beads. The question might be raised that the effect show in this study was characteristic of PSL beads; therefore will more complex beads and interaction with specific ligands have a detrimental effect on PMNL migration.

The findings from this study need to be substanciated by demonstation of PMNL carrying beads to an inflammatory site in vivo. Then the many areas of potential study that have been touched in this section could be fomulated into a coordiated programme of research in which a multidiscipinary team would be needed.

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