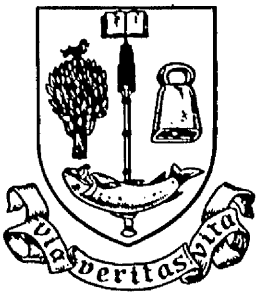


# STUDIES ON CHEMOATTRACTANT-INDUCED POLARISATION AND LOCOMOTION OF HUMAN BLOOD LEUCOCYTES

By

Laila Noor Islam

Department of Bacteriology and Immunology

University of Glasgow. Western Infirmary.

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# List of abbreviations

The following abbreviations have been used in this thesis:

| | |
|---|---|
| AbSRBC | Antibody-coated sheep red blood cells |
| adHSA | Alkali-denatured human serum albumin |
| AHS | Candida spore-activated human serum |
| ANAE | Acid α-napthyl acetate esterase |
| BHK | Baby hamster kidney |
| C5a | Activated fifth component of the complement system |
| C5adA | C5a des Arg |
| C3a | Activated third component of the complement system |
| CMFS-MOPS | $Ca^{2+}$, $Mg^{2+}$ free salt solution containing 10 mM Mops |
| c. | *circa*, about |
| conA | Concanavalin A |
| d | Distance |
| D | Dimension |
| DDGP | Discontinuous density gradient of percoll |
| 2DG | 2-deoxy-D-glucose |
| DIC | Differential interference contrast |
| DMSO | Dimethyl sulphoxide, Grade II |
| EDTA | Ethylene diamine tetra acetic acid |
| EGTA | Ethyleneglycol-bis (β-aminoethyl ether)-N,N'-tetra acetic acid |
| Fc | Crystallisable fragment of immunoglobulins |
| FCS | Foetal calf (bovine) serum |
| Fig. | Figure |
| FITC | Fluorescein isothiocyanate |
| FMLP | N-formyl-L-methionyl-L-leucyl-L-phenylalanine |
| FN | Fibronectin |
| Glut/HBSS-MOPS | 2.5% (v/v) glutaraldehyde in HBSS-MOPS |
| g | Gram/Acceleration of gravity |
| h | Hour(s) |
| HBSS-MOPS | Hanks' balanced salt solution containing 10 mM MOPS |
| HSA | Human serum albumin |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin "G" |
| $LTB_4$ | Leukotriene $B_4$ |
| M | Molar (moles/litre) |
| mg | Milligram |

| | |
|---|---|
| min | Minute(s) |
| ml | Millilitre |
| mM | Millimolar |
| MOPS | 3-(N-morpholino) propanesulphonic acid |
| MNCS | Human peripheral blood mononuclear leucocytes |
| M.wt. | Molecular weight |
| NADH | Nicotinamide adenosine diphosphate (hydrogenated form) |
| n.a | Numerical aperture |
| NADPH | Nicotinamide adenosine diphosphate (phosphorylated hydrogenated form) |
| NAMS | Non-adherent mononuclear leucocytes from human peripheral blood |
| ng | Nanogram |
| NSE | Non-specific esterase |
| O.D. | Optical density |
| OKT | Commercial monoclonal antibodies to human T-cell antigens manufactured by Ortho diagnostics |
| PAF | Platelet activating factor |
| pH | Negative logarithm of hydogen ion concentration |
| P.I. | Polarisation index (length/breadth ratios of leucocytes to express polarisation) |
| PMNS | polymorphonuclear neutrophil leucocytes |
| rms | root mean square |
| RPMI-MOPS | Roswell Park Memorial Institute medium number 1640 containing 20 mM MOPS |
| s | Seconds |
| S.C. | Shape change score |
| S.D. | Standard deviation from the mean |
| S.E.M. | Standard error of the mean |
| SFPS | Serum-free phagocytosis supernatant |
| SIP | Stock isotonic percoll |
| SRBC | Sheep red blood cells |
| temp | Temperature |
| UV | Ultraviolet |
| v/v | Volume per volume |
| w/v | Weight per volume |
| ≥ | Greater than or equal to |
| < | Less than |
| > | Greater than |
| µg | Microgram |
| µl | Microlitre |
| µm | Micrometer |

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# Declaration

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# Summary

Migration of leucocytes into sites of inflammation and to phagocytose damaged cells and invading microorganisms is an immunological reaction of paramount importance. The circulating blood monocytes together with tissue macrophages and blood neutrophils are the cells which carry out this phagocytic function. Studies on different aspects of the locomotion of human blood monocytes are presented in this thesis. The neutrophils and the non-adherent mononuclear cells from human peripheral blood were also used in some experiments.

Studies on monocytes were greatly hampered in the past because of the difficulties in obtaining purified preparations of the cells. Several procedures were tried in the present study, most of which for one reason or another, were unsatisfactory. The BHK microexudate method produced a considerably higher yield of purified populations of monocytes in suspension and was ultimately selected for subsequent studies.

The responses of human blood monocytes to chemotactic factors were studied using an assay of morphological polarisation. Changes in cell morphology from a spherical to a polarised configuration which occurred within a few minutes of adding a chemotactic factor, were scored after incubating the cells in suspension followed by fixation with glutaraldehyde. Polarisation is the initial event in locomotion and can be scored accurately as has been shown in neutrophils. In

contrast to neutrophils where more than 90% cells in a population can respond towards the formylated peptide FMLP, only 60 or so percent of the blood monocytes can do so. The rest of the cells do not bear receptors for FMLP and hence can not respond.

Monocytes showed distinct morphological shapes in different concentrations of FMLP. The cells were most polarised in 5 X $10^{-9}$ M FMLP. Polarisation was quantified by measuring the lengths of monocytes at different doses of FMLP. Due to heterogeneity in the cell size, measurement of lengths to express monocyte polarisation was not accurate. A polarisation index (P.I.) was determined by calculating the length and breadth ratio of each cell which seemed to correlate with the shape change results with different chemotactic factors.

The time course of monocyte polarisation with the optimal dose of different chemotactic factors showed that the cells responded within a short time of exposure. A maximal response was obtained after 10-20 minutes of incubation of the cells with the test substance. Further incubation resulted in loss of polarisation of the cells towards the chemoattractants. This loss of polarisation was studied by incubating the cells with FMLP for different time intervals and then rosetting the monocytes with antibody-coated sheep red cells. The localization of Fc receptors on polarised monocytes (stimulated for an optimum time with FMLP) was at the head region of the cells i.e., the receptors were redistributed at the front as these cells formed head rosettes. On the other hand, many monocytes incubated for a long time in FMLP could

not form Fc rosettes. These cells perhaps internalized their surface receptors.

Incubating monocytes with a combination of different chemotactic factors like FMLP, C5a or $LTB_4$ showed no additive effect, that is to say, no increase over 60% polarised cells in the population. This is similar to the findings of previous workers. In contrast to this, candida spore-activated human serum mixed with FMLP induced over 85% of the monocytes to change shape. This finding suggested that circulating blood monocytes have receptors for different chemotactic factors including unknown factors in serum which are not C5a.

*In vitro* culturing of monocytes showed that the cells increase in size and polarise with the time of culture which can be prevented by adding cycloheximide, an inhibitor of protein synthesis. This observation suggested that cultured cells synthesise proteins to change shape.

A collagen assay was developed for the measurement of the population distribution of neutrophils through the gels in response to an isotropic and a gradient concentration of FMLP. By following the same procedure, invasion of monocytes through collagen gel was studied both in the presence and absence of uniform concentration of FMLP. This is the first report to demonstrate that human monocytes straight from blood can migrate through collagen gels.

Time-lapse cinematography was employed for the visual assays of monocyte locomotion on protein-coated plane surface and through three dimensional matrices of

collagen gels. In the presnce of a uniform concentration of optimal FMLP, monocytes tended to locomote in a relatively straight path with narrow angles of turn. This was true for locomotion on a plane surface as well as through the matrices of collagen gels. When pathogenic yeast *Candida albicans* blastospores in human serum were used as a gradient source, monocytes responded chemotactically and migrated in a nearly straight line path towards the spores and phagocytosed them. This was studied in different conditions.

Thus the behaviour of human blood leucocytes in suspension in response to different chemotactic factors, redistribution of their Fc receptors, leucocyte locomotion on plane surfaces and through gel matrices were studied in the presence of chemotactic factors. It was hoped that these studies would help better understanding of these cells.

# CHAPTER 1

# INTRODUCTION

The ability of blood leucocytes to respond and locomote in response to various stimulants is of vital importance for the defence mechanism of the body. The survey of clinically important immunological phenomena would not be complete without consideration of the function of phagocytic cells. These cells do not carry intrinsic receptors with structural characteristics necessary for antigen recognition nor do they produce antibody, and yet they play an important role in defence of the body against pathogenic microorganisms and in clearance of products of tissue damage and breakdown.

Neutrophil leucocytes and blood monocytes are motile cells and as part of their normal functions, leave the bloodstream and migrate through tissues towards sites of inflammation. This property makes them ideal for studies of locomotion on two-dimensional substrata and through three-dimensional matrices *in vitro*.

A proportion of each type of blood leucocytes is known to respond by changing shape , from spherical to a polarised morphology in response to chemotactic factors present either in a uniform concentration or in a gradient of concentration. Neutrophil leucocytes have been extensively studied in this connection. Lymphocytes are mostly non-responsive towards neutrophil and monocyte stimulating chemoattractants and are less well studied. The polarising responses of monocytes towards different chemotactic factors, their locomotion on plane surfaces, and the locomotion of neutrophils and monocytes through 3-D matrices are the major topic of interest in this work and will be considered in detail in section I-IV.

# SECTION I

## The leucocytes of blood: early studies, origin, function and locomotion

### *Early studies on leucocyte locomotion and inflammation*

The involvement of leucocytes in inflammation was recognized in the nineteenth century. Duntrochet in 1828 (reviewed by Rebuck and Crowley, 1955) observed that leucocytes could locomote from blood vessels into areas of inflammation. During the next 20 years other workers (Waller, 1846 and Addison, 1849) confirmed this by microscopic observation of inflammatory lesions and described the adhesion of leucocytes to blood vessels, their subsequent emigration and amoeboid movement towards the affected area. Forty years later, several workers modified the techniques used to study accumulation of leucocytes in inflammation (Hess, 1887; Leber, 1888 : for a detailed description of the works of these German authors see Wilkinson, 1982a). Among them, Hess (1887) took anthrax bacilli, an inflammatory agent, in a glass chamber and placed it beneath the skin of experimental animals and then observed the migration of leucocytes into the chamber. Leber (1888) introduced capillary tubes containing various substances into the cornea of the rabbit eye. He showed that leucocytes would accumulate in the capillary tube. Leber was uncertain whether this accumulation was due to an attraction of leucocytes towards the substances tested or to immobilization of cells which had reached the tube by random migration. Leber, therefore, slightly changed his experimental

procedure by continuously observing the cells in isolated corneas, excised after the test substances had been injected into it. Based on these experiments, he put forward a theory about inflammation and described chemotaxis as the force which attracted leucocytes into an inflammatory lesion where foreignness of the test substance is the requirement for the initiation of cell migration in a direction.

It was generally believed at that time that leucocytes did not have an active role in immunity against infection and that their accumulation at sites of inflammation was to act simply as scavengers, removing dead cells and organisms. The extensive and revealing investigations of Metchnikoff first focused light upon the importance of phagocytes in resistance to invading microorganisms (see Metchnikoff, 1893, for a review). He made a comparative study of phagocytic cells, starting from single-celled amoebae to human blood leucocytes and concluded that the phagocytes play an important role in the defence of the body against pathogenic microorganisms and in the clearance of products of tissue damage and breakdown. Thus the ability of phagocytes to locomote towards, to ingest and to kill microorganisms by a process involving chemotaxis became widely accepted as an important part of inflammation.

*The white cells*

The white cells or the leucocytes of the blood can be divided into 2 major groups, the mononuclear cells containing round or kidney-shaped nuclei, and the

polymorphonuclear cells containing segmented nuclei. The lymphocytes and monocytes have a single or non-lobed nucleus, and are the mononuclear leucocytes. The mature cells of the myeloid system which circulate in blood are known as granulocytes or polymorphonuclear leucocytes. They are divided into neutrophil, eosinophil and basophil leucocytes on the basis of the affinity of their cytoplasmic granules for Romanovsky stain. The rest of this section will consider monocytes and neutrophils in detail.

*Origin and function of blood monocytes and neutrophils*

(a) Monocytes

These are of variable appearance (8-20 μm in diameter), some of them are larger than other peripheral blood leucocytes. Monocytes possess a large central oval or indented nucleus. Their abundant cytoplasm stains pale blue (with Romanovsky stain) and contains many fine vacuoles. Monocytes are formed in the bone marrow where the earliest recognizable precursor is the monoblast (Goud et al., 1975; Goud and van Furth, 1975). This is the precursor of the promonocyte, which divides and differentiates into monocytes. Monocytes spend only a short time in the marrow and, after circulating for 20-40 h, leave the blood and migrate to various tissues where they mature to become macrophages (van Furth and Cohn, 1968; van Furth et al., 1970, and 1972) and to carry out their principal functions.

Studies in adult animals using bone marrow chimeras or parabiosis (Balner, 1963; Goodman, 1964; Virolainen,

1968) and also kinetic studies with $^{3}H$-thymidine-labelled cells in animals in the normal steady state (van Furth, 1978) have proven that macrophages localized in various tissues or body spaces derive from circulating monocytes. Evidence obtained in liquid cultures of bone marrow exposed to colony-stimulating factor, which shows sequential development from monoblast to macrophages (Metcalf and Moore, 1971; Goud et al., 1975; Goud and van Furth, 1975; van der Meer et al., 1978 and 1979), strongly supports the *in vivo* findings made by kinetic studies.

The extravascular lifespan of monocytes after their transformation into macrophages may be as long as several months or even years. They may assume specific functions in different tissues, e.g., skin, gut, liver etc. Many mononuclear phagocytes are fixed cells located in tissues where they act as traps or filters for material circulating through the tissue. Blood monocytes are phagocytic cells although less efficient at killing ingested microorganisms than the macrophages into which they are destined to develop.

*(b) Neutrophils*

The most common granulocyte is the neutrophil (8-15 μm in diameter), which has a characteristic dense nucleus consisting of 2-5 lobes and a pale cytoplasm. In man its granules stain neither red nor blue but purplish. Neutrophils originate in the bone marrow, ultimately from the pluripotential stem cells (Becker et al., 1963). A number of precursor stages have been defined for

neutrophils, largely on the basis of granule content and morphology (Bainton et al., 1971). Neutrophil precursors do not normally appear in peripheral blood but are present in the marrow. The earliest recognizable precursor is the neutrophil promyelocyte which contains the primary (azurophilic) granules. These cells give rise to myelocytes which have specific or secondary granules. Separate myelocytes of the eosinophil and basophil series can be identified. The myelocytes give rise by cell division to metamyelocytes, non-dividing cells which have an indented or horse-shoe-shaped nucleus and cytoplasm filled with primary and secondary granules. These are very close to the mature neutrophils except that their nuclei are not segmented. Detailed reviews of neutrophil development have been produced by Cline (1975) and Murphy (1976).

The primary granules of the neutrophil contain myeloperoxidase, acid phosphatase and other acid hydrolases. The secondary granules contain the iron-binding protein, lactoferrin, together with lysozyme, a collagenase, a vitamin $B_{12}$-binding protein and neutral proteases.

Mature neutrophils migrate from the bone marrow to the blood stream where they passively circulate up to about 10 h before moving into the tissues. In tissues they perform the phagocytic functions. It has been reported by Cartwright et al. (1964) that blood borne cells segregated into 2 pools. One of them is the pool of freely circulating cells, and the other one is the marginating pool of cells attached to the endothelial

lining of the blood vessels (not included in the blood count). These 2 pools are about equal size and are in dynamic equilibrium. Injury or infection initiates an acute inflammatory response which results in an increase in the size of the marginating pool of neutrophils (for a detailed review, see Ryan and Majno, 1977). It has been estimated that granulocytes spend on average 4-5 days in the tissues before they are destroyed during defensive action or as a result of senescence.

*Common functions of neutrophils and monocytes*

The neutrophil and blood monocytes together with tissue macrophages are commonly known as phagocytes. Their normal function may be divided into 3 phases which are summarised below.

(i) Chemotaxis

This is the process by which phagocytic locomotion is directed by substances formed by bacteria or at the site of inflammation or by substances released from damaged tissues or by complement components.

(ii) Phagocytosis

This is the ingestion of foreign material e.g., bacteria, fungi, etc. and dead or damaged cells of the host's body. Recognition of a foreign particle is aided by opsonisation with immunoglobulin or complement since both neutrophils and monocytes have surface receptors for the Fc fragment of immunoglobulins and for C3 and other complement components. Opsonisation of normal body cells (e.g., red cells or platelets) also makes them liable to

destruction by macrophages. Macrophages also have a role in presenting foreign antigen to the immune system.

(iii) Killing and digestion

This occurs by oxygen dependent and oxygen independent pathways. In the oxygen dependent reactions, superoxide and hydrogen peroxide ($H_2O_2$), are generated from oxygen and NADPH or NADH. In neutrophils, $H_2O_2$ reacts with myeloperoxidase and intracellular halide to kill bacteria; superoxide ($O_2^-$) may also be involved. The non-oxidative microbicidal mechanism involves a fall in pH within the phagocytic vacuoles into which lysosomal enzymes are released. An additional factor, lactoferrin, an iron binding protein present in neutrophil granules is bacteriostatic by depriving bacteria of iron (for previous reviews about oxygen dependent antimicrobial systems, see Klebanoff, 1975; Klebanoff and Hamon, 1975; Klebanoff, 1980).

The locomotion of blood monocytes and neutrophils is of interest in this study and will be described in detail in the next section.

*Locomotion of the leucocytes*

The ability of leucocytes to locomote at random or in a particular direction is determined by environmental influences. Like most other motile metazoan cells, leucocytes move by crawling on surfaces. Locomotion is thus crucially dependent on the nature of the adhesion of the cell with the surface (the substratum) on which it moves. Physiological factors like adhesion to surfaces or deformability play an important role in leucocyte

locomotion. A group of workers (Keller et al., 1977; revised version, 1980) suggested definitions to describe the locomotor reactions of leucocytes and other cells to avoid discrepancies in the use of the various terms.

*Random and directional locomotion of leucocytes*

The locomotor responses of leucocytes in relation to the environment can be of 2 forms. A distinction can be made between 'tactic' reactions which result in directional locomotion towards or away from a gradient source, often with orientation of the body of the organism in relation to that source and with a net flux of organisms in response to the gradient, and kinetic reactions in which the speed or frequency of locomotion of cells and/or the frequency and magnitude of turning (change of direction) of cells or organisms moving at random is determined by substances in the environment. When a chemical substances is stimulating locomotion, the resulting random movement is known as 'chemokinesis' and the directional movement is known as 'chemotaxis'. Frankel and Gunn (1940) distinguished two forms of kinesis as orthokinesis, a reaction by which the speed or frequency of locomotion is determined by the intensity of the stimulus, and klinokinesis, a reaction by which the frequency or amount of turning per unit time is determined by the intensity of the stimulus.

Random locomotion is a complicated phenomenon with a complex definition (Keller et al., 1977 and 1980). It includes two possibilities (Abercrombie, 1965; Gail and Boone, 1970) : (1) the locomotion which is randomly

oriented in relation to the environment and (2) the locomotion which strictly follows the random-walk model (Gail and Boone, 1970) in which if the path of the cell is represented by a series of line segments meeting at angles, the spatial orientation and magnitude of each angle are determined solely by chance. Dunn (1981) considered these two forms of locomotion described above as random locomotion type 1 and type 2, respectively. He pointed out that metazoan cells can never locomote by a pure random Walk : rather than when a cell path is considered over a brief period of time, the path will tend to show **persistence** of direction and the cell will therefore move according to random locomotion type 1. However, over long intervals of time the locomotion of cells does not continue to show persistence but the cells change direction in such a way that the path approximates a random Walk in which mean square displacement is proportional to time (Gail and Boone, 1970). The degree of persistence of direction of locomoting cells can be determined from studies of their turning behaviour in the presence of an isotropic concentration of chemoattractants (described in chapter 6 and 7 for monocyte locomotion). A cell exibiting a pure random movement would make turning angles uniformly distributed between -180° and +180° which is not observed in practice. Zigmond et al. (1981) reported that neutrophils rarely make turns over 90° and have a mean turning angle of about 50°. However, a cell moving in a curved path will not show discrete turns if viewed continuously and choice

of frequency of observation will affect the degree of turn measured (Allan and Wilkinson, 1978).

Anisotropic properties of the cellular environment will result in a deviation from random locomotion (type 1 and type 2) and the cells will move directionally towards the source. Several workers have studied leucocyte locomotion towards gradient sources (McCutcheon, 1946; Harris, 1954; Ramsey 1972a; Zigmond, 1974; Allan and Wilkinson, 1978). In earlier studies of leucocyte chemotaxis, Dixon and McCutcheon (1935) and McCutcheon (1946) introduced a 'chemotropism index' or 'chemotactic ratio' to measure the straightness of the cell path towards the stimulant. They calculated the ratio of the straight line distance from beginning to end of the cell path and the total distance travelled by the cell. Cell locomotion towards the source was scored as +ve and away from the source was -ve. Thus in presence of a stable gradient, if the cells moved accurately towards the gradient source, the chemotactic ratio would be +1.0; and locomotion in a straight path away from the source would give the ratio -1.0. Zigmond (1974) described that leucocytes moving in gradients had chemotactic ratios in between +0.7 and +0.8. Ramsey (1972a) used clumps of *Staphylococcus albus* as a stimulant for the leucocytes and observed that if the bacterial clump towards which cells were moving was moved to a site behind the cells, they responded within the next 40 s by reversing their direction of locomotion towards the new source. Using blastospores of *Candida albicans* in human plasma as gradient source where the spores would continuously

produce chemotactic factors from the surrounding plasma, Allan and Wilkinson (1978) obtained chemotactic ratios up to +0.96 for neutrophils and (Wilkinson and Allan, 1980) +0.89 for monocytes. These workers showed that the mean angle of turn for a population of neutrophils moving towards candida spores was within ±40° of the direction of movement preceeding the turn. Some of the cells in that population were moving directly towards the spores without making any angle of turn. These observation suggested that if a chemotactic factor is present in the environment, changes in its disposition (whether uniform or in a gradient) will affect the direction of leucocyte locomotion. The random and directional locomotion of neutrophils and monocytes is described in chapter 6 and 7. Before considering the locomotion of these cells in detail, the following section will outline methods used in earlier studies, and those frequently used to study leucocyte locomotion.

## SECTION II

### The measurement of leucocyte locomotion

*Early methods for studying leucocyte locomotion*

The first attempt to make direct observations of leucocyte locomotion *in vitro* was by Comandon (1917, an English translation of the work of this author is quoted by Wilkinson, 1982a) who used a thin film of blood spread between a slide and a coverslip and used time-lapse cinematography to record the migration of leucocytes relative to a test object placed under the coverslip.

Using the same technique, Comandon (1919) was able to demonstrate chemotactic locomotion of avain leucocytes *in vitro* where the cells would move directionally towards the erythrocytes of the same species heavily infected with the parasite *Haemamoeba clanilewski*. He described that upon reaching the parasitized red cells, the leucocytes appeared to push it ahead continuously until the red cell membrane burst and released the nucleus and the parasite. Then the leucocytes would follow the previous procedure to burst the cell wall of the parasite and finally ingested the substances of the parasite, the red cell nucleus and the debris. Comandon (1919) subsequently extended his observations to show that starch grains but not carbon particles were chemotactic to human leucocytes.

A quite different attempt was made by Clark and Clark (1920) and Clark et al. (1936) which is based on the direct observation of cell locomotion *in vivo*. They injected different substances including starch granules, croton oil (produces aseptic inflammation) etc. into the transparent tail of a tadpole and observed the subsequent migration of polymorphonuclear leucocytes from the blood vessels and through the tissues towards the injected material. This method and others based on it using spreads of mesentery or transparent rabbit ear-chambers (Sandision, 1928) have been widely and successfully used in studies of the inflammatory response.

Lewis studied the non-chemotactic locomotion of different types of blood leucocytes by time-lapse cinematography analysis. These included studies of

lymphocytes from lymph nodes covered by plasma clots in which the typical locomotor morphology of lymphocytes with a prominent tail and the nucleus placed anteriorly was first demonstrated (Lewis and Webster, 1921). Lymphocytes were migrating vigorously in these studies (between 13-30 μm/min). Ten years later, Lewis (1931 and 1934) described the morphology of locomoting neutrophils, eosinophils and lymphocytes with sequential tracings from time-lapse films to show changes in cell shape during movement. each of these cells moved rapidly in the presence of plasma, neutrophils moved at 19 μm/min (Lewis, 1934).

McCutcheon et al. (1934) and McCutcheon and Dixon (1936) used the slide and coverslip method and studied a lage number of different types of bacteria to show that they would attract human neutrophils. They allowed a clump of bacteria to dry on a microscope slide and then observed the locomotion of neutrophils in plasma towards the clump. By projecting the image of the microscope field on a drawing paper they recorded neutrophil locomotion. Dixon and McCutcheon (1935) tracked the cell path towards the bacteria and determined the "chemotropic ratio" which is a measure of the straightness of path taken by leucocytes towards the bacteria, that could be used to compare the response of different types of leucocytes towards different bacteria. Based on these experiments, McCutcheon and Dixon (1936), showed that different bacteria and yeasts with varying degree of pathogenicity would attract neutrophils to the same degree, the exception being a virulent yeast, *Torula*

*histolytica*, which was significantly less potent in attracting the leucocytes. Lymphocytes however, would not move directionally towards the bacteria (Dixon and McCutcheon, 1935). To test soluble substances against leucocytes. Dixon et al. (1937) used Kaolin (aluminium silicate) to adsorb soluble fractions of bacteria and placed it on a microscope slide as had been done earlier with whole bacteria. Then following the previous procedure they found that a protein-carbohydrate complex from *Staphylococcus hemolyticus* was strongly chemotactic to rabbit peritoneal neutrophils.

*Recent methods for studing leucocyte locomotion*

A number of assay systems are available to study leucocyte locomotion (Wilkinson and Allan, 1978; Wilkinson, 1982a and Wilkinson et al., 1982). The choice of technique depends considerably on what the investigator wants to see and the facilities available. There are two different sorts of assays in use to study leucocyte locomotion. The most widely used assay measures the displacement of a population of cells after a fixed time. Less frequently used are assays in which the behaviour of individual cells is studied (the visual assay).

(a) Micropore filter assays

This method, described by Boyden (1962, commonly Known as the Boyden assay), was originally used to measure the chemotactic migration of neutrophils and has already been used to measure non-chemotactic locomotion

of all blood leucocytes. The assay method utilizes a porous filter paper which separates two compartments from one another, the lower compartment containing the test substance and the upper one containing the cells with or without the test substance (for chemokinetic and chemotactic assays, respectively). The filter has micropores of an appropriate size such that cells are prevented from simply falling through the pores but the activated cells are able to squeeze their way through by active migration towards the stimulus. Filters of pore size 3 μm are generally used for neutrophils and 8 μm is used for monocytes and lymphocytes (lymphocytes, though smaller than neutrophils, have a large and relatively nondeformable nucleus). The most commonly used filter is made of cellulose ester, which is usually 100-150 μm in thickness. The gradient for chemotactic assay is formed across a distance of about 15 cell diameters and the cells have to travel a considerable distance to traverse the filter. Various methods of scoring migration of cells into filters have been used (Boyden, 1962; Keller et al., 1972) but the most widely used is the leading front method described by Zigmond and Hirsch (1973). In this method cells are incubated for a reasonable time interval which allows them to migrate a good distance into the filter but not to reach the lower surface. Then counting the number of cells at different levels within the filter matrix, Zigmond and Hirsch (1973) found that the distribution of cells within the matrix was close to that expected for the random diffusion of particles from a boundary. Also the leading front value determined as the furthest distance travelled by at least two cells in the

same focal plane, correlated well with the distribution of the cell population through the filter. This distance could be easily measured using the calibrated fine-focus control on a microscope. The advantages of this method is that a large sample can be used. This procedure needs a short time and measures the population movement which is generally an end-point assay. As the micropore filter used in this assay is opaque, locomotion of individual cells can not be followed while migrating through it.

(b) The agarose assay

In this assays leucocytes are allowed to locomote underneath an agarose gel (Cutler, 1974; Nelson et al., 1975) and the assay can be used to measure both non-directed and chemotactic locomotion of leucocytes, like the Boyden assay. A layer of agarose gel is allowed to set on a protein-coated glass slide and small wells are cut into the agarose. The centre well is filled with cell suspension which is surrounded by wells containing different chemoattractants, different concentrations of the same attractant, control solution etc. Chemotactic factors placed in nearby wells diffuse through the agarose, and any effect on the cells will be seen as changes in the migration distance towards the attractant and the control solution. The distribution pattern of the cells would be pointed towards the attractant source if the substance tested is chemotactic to the cells. The agarose method, like the filter method, is essentially a method for measuring the locomotion of cell populations

but it is also possible to film cells moving under agarose.

(c) The orientation assay

The chamber used for this assay is described by Zigmond (1977) and is known as an orientation chamber or Zigmond chamber. It is a plastic slide into which two grooves (troughs) separated by a narrow bridge (1 mm diameter) have been cut. Cells are allowed to adhere to a glass coverslip which is then inverted over the slide and secured by the clips. One of the troughs is then filled with a chemotactic factor, the other with a control solution or the two troughs can be filled with different concentrations of the same chemoattractant. If a formyl peptide is used as attractant, the cells (neutrophils or monocytes) gradually orient with their heads up gradient. Within 40 minutes of setting up, over 90% of the neutrophils in locomotor morphology are accurately oriented towards the gradient (Zigmond, 1977). Also neutrophils can detect, by orientation, a concentration difference of the chemotactic factors of as little as 1% between opposite sides of the cells (Zigmond, 1977). This assay measures the morphological orientation of the cells towards the gradient and does not measure the locomotion. However, it can easily be adapted as an assay of chemotactic locomotion by adding serum albumin to the coverslip and filming at 37°C.

(d) Visual assays

Direct microscopic observation of the locomoting leucocyte can provide much information that is very

difficult to obtain with the other assays involving population movement. Details of the behaviour of individual leucocytes and of a small population of cells under various conditions can be studied by the visual assay. A metallic chamber is used for suitable coverslip preparation of the cells to be studied. Details of the procedure are described by Allan and Wilkinson (1978) and will be briefly mentioned in chapter 2 of this study. Time-lapse cinematography is employed to record the locomotion of the cells and the film is subsequently analysed. By tracing cell paths, a number of parameters can be calculated such as cell speed, velocity, turning angles and frequency of turns. The superiority of this method over the others is that these parameters can be measured throughout the filming period. This assay has been used to measure monocyte locomotion through 3-D matrices and on plane substrata (described in chapter 6 and 7, respectively).

(e) Invasion assays

Leucocytes have been cultured with other cell types and have also been allowed to invade monolayers made by other cells. The invasion of chick heart fibroblast aggregates by neutrophils has been studied by Armstrong and Lackie (1975) who reported that these cells can crawl over the surface of fibroblasts and showed a preference for the edge of the latter rather then moving across the central region. Neutrophils were able to invade fibroblast monolayers and showed a preference for underlapping e.g., moving between the monolayer and the

culture flask rather then overlapping. Cramer et al. (1980) grew renal epithelium on the surface of micropore filters and studied the migration of neutrophils through the epithelia and into filters. Lymphocytes also can move vigorously by underlapping reticular cells (Haston, 1979). Russo et al. (1981) followed the movement of neutrophils through the epithelium, basement membrane, and collagenous stroma of human amnion membrane *in vitro*. These properties of leucocytes made them very good candidates for invasion assays *in vitro*.

All the assays described in (a)-(d) have generally been carried out using non-biological substrata such as flat glass or plastic surfaces and filters made of cellulose esters or other unphysiological materials. These are very different from the environment through which leucocytes move *in vivo*. Three-dimensional matrices made from physiological materials are now becoming more popular in leucocyte locomotion studies. In some of the earliest studies, the migration of lymphocytes in serum (fibrin) clots was observed by Lewis (1931). More recently, 3-dimensional collagen gels have been used in studies of a variety of cell types including fibroblasts (Bard and Hay, 1975), lymphocytes (Haston et al., 1982; Schor et al., 1983; Wilkinson, 1985a), neutrophils (Brown, 1982) and monocytes (Brown, 1984). Such gels may be prepared freshly from type 1 collagen from rat tail tendon (Elsdale and Bard, 1972; Schor, 1980). The collagen gel form a fine fibrous 3-D meshwork through which cells can rapidly penetrate provided the collagen concentration is not too high. The gels are transparent

and can be used for both types of assays, that is to say, to study large populations of cells or to study the behaviour of individual cells, since the assays allow both direct observation of moving cells and the measurement of cell distribution after fixation. They provide a better approximation than most of the materials used in other assay methods to environments through which cells have to move in vivo. Collagen gels have been used to study the locomotion of neutrophils and monocytes both in the presence and absence of chemotactic factors (described in chapter 6). In these assays collagen gels have been employed in the same way as the micropore filter assay is used at present, namely to place cells on the upper surface of the gel and then to observe migration and the distribution of the cell population in response to the gradient. Chemotactic factor has also been incorporated into a collagen solution before it formed a gel and then cells with the same dose of the attractant were placed at the upper surface of the gel which allowed a measure of the chemokinetic locomotion of the cells.

## SECTION III

## The morphology of locomoting leucocytes and the biochemistry of the motor apparatus

### *Morphology of leucocytes during locomotion*

Leucocytes move by crawling over surfaces and thus need a suitable substratum for adhesion. The nature of adhesion of the cells with the surface plays an important

role in leucocyte locomotion. A characteristic series of events is seen when a cell locomotes, though, there are considerable variations on the typical sequence of events when individual leucocytes are studied. The first event to be seen is the protrusion of a hyaline membrane or lamellipodium from a rounded leucocyte. This usually forms at one pole of the cell (Lewis, 1934; de Bruyn, 1946; Robineaux, 1964; Ramsey, 1972b; Senda et al., 1975). It is usually a thin and veil-like process which frequently shows ruffling and rapid changes in shape. This protrusion does not contain cytoplasmic organelles, but is rich in actin and actin-binding proteins (Senda, 1977; Oliver et al., 1978). Lamellipodium formation precedes translocation (Zigmond, 1974). The cell body remains rounded at the beginning and as translocation proceeds, the cytoplasmic contents flow forwards into the anterior lamellipodium which continues to protrude forwards. The cell body of many moving cells remain refractile and rounded behind the leading edge. There may be a distinct posterior tail or 'uropod' with retraction fibres.

In neutrophils and monocytes, pronounced tails are commonly seen when the cells are strongly adherent, and cells often become tethered by such tails(see Fig. 61 B). Neutrophils are one of the fastest moving types of metazoan cell. These cells, after stimulation with chemotactic factors, move around 15-20 μm/min on albumin-coated glass surfaces (Allan and Wilkinson, 1978) though McCutcheon (1923) reported speeds up to 30 μm/min

compared to fibroblasts which are much slower, moving at 60 μm/h (Harris, 1973).

The mode of locomotion of human blood monocytes is not significantly different from that described for neutrophils. Monocytes also form a leading lamellipodium and show the same morphological features as neutrophils during locomotion on a less adhesive substratum. A noticeable difference is that monocytes are much more adherent cells than neutrophils and therefore more slowly-moving, but a small proportion of these cells can locomote provided a high concentration of albumin is coating the substratum (see Fig. 61). Albumin reduces adhesion of the cells to the substratum. Glass surfaces coated with a serum albumin concentration of 10 mg/ml allows optimal locomotion of neutrophils. Wilkinson and Allan (1980) studied the speed of unstimulated monocytes on albumin-coated glass and reported that these cells moved at 8-10 μm/min. However, Harris (1961) has reported a slower speed for monocytes.

A minority of lymphocytes taken direct from blood (McCutcheon, 1924; Biberfeld, 1971; O'Neill and Parrott, 1977) or from unstimulated lymph nodes (Russell et al., 1975) are motile but during a period of 4-72 h in culture in serum-containing media (with or without mitogens), the proportion of motile cells increases (Wilkinson, 1986). Formation of a lamellipodium is seen in lymphocytes commencing locomotion. In contrast to neutrophils and monocytes, the nucleus of moving lymphocytes tends to remain anteriorly placed immediately behind the lamellipodium, with cytoplasmic organelles and centriole

behind it. Since the lymphocyte nucleus is round and relatively nondeformable, locomoting cells have been observed to show a prominent tail or uropod which may simply reflect the high nuclear-to-cytoplasmic ratio in lymphocytes.

*The motor apparatus of the leucocytes*

Leucocyte locomotion is accompanied by continuous shape changes with a slow and regulated contractile mechanism propelling the cell forward. There is evidence that this process is mediated by cytoplasmic 'contractile' proteins including actin, myosin, actin-binding protein and others (Stossel, 1978; Hartwig et al., 1980a). These proteins form microfilament networks which are seen to be present in the substratum-adherent cell surface and in the lamellipodium of the moving leucocytes (Reaven and Axline, 1973; Davies and Stossel 1977; Boyles and Bainton 1979; Stendahl et al., 1980). It is not clear how motile force is generated by contraction and relaxation of the microfilament lattice but there is evidence that this force is generated in the anterior lamellipodium. Keller and Bessis (1975) were able to break the stretched and maximally elongated anterior lamellipodium of neutrophils by heating the cells at 46°C. The isolated lamellipodial fragments repaired themselves and continued to show chemotactic movement towards a stimulus. This observation suggested that the organelle- and nucleus-free fragments must contain the apparatus required to maintain a locomotor response.

The broad leading lamellipodium and narrow tail of locomoting neutrophils resembles those of locomoting fibroblasts. The fibroblasts, in general, insert the microfilament bundles (stress fibres) into the leading edge to make points of high adhesion (Abercrombie et al.,1971; Heath and Dunn, 1978) but Couchman and Rees (1979) have shown that rapidly moving fibroblasts have few stress fibres and substratum adhesion sites (focal contacts) compared to slow moving or stationary cells. Neutrophils, which generally translocate at faster rates than fibroblasts, do not have stress fibres and have broad areas of adhesion to the substratum, as shown by interference reflection microscopy, rather than by focal contacts (Armstrong and Lackie, 1975; Keller et al., 1979).

Actin and myosin having ATPase activity were first isolated from horse neutrophils (Senda et al., 1969; Shibata et al., 1972), and are observed to be identical in molecular weights to skeletal muscle actin and myosin. Electron microscopy confirmed that the thick and thin filaments of the isolates were similar to those found in skeletal muscle. Tatsumi et al. (1973) observed that the isolated actin formed helical structures identical to those of skeletal muscle and coprecipitated with the latter in the presence of $Mg^{2+}$ and ATP. Stossel and Pollard (1973) isolated myosin from guinea pig neutrophils and found that it was similar to that of skeletal muscle myosin. Hartwig and Stossel (1975) found an actin-binding protein (Stossel and Hartwig, 1976) present in rabbit alveolar macrophages which was later

found in human neutrophils (Boxer and Stossel, 1976). It was suggested that this protein crosslinked actin filaments reversibly into a gel lattice. Actin-binding protein is associated with the inner surface of the plasma membrane (Boxer et al., 1976). It was suggested that reversible binding of this protein to actin filaments would allow sol-gel transformations and relocation of actin networks without a requirement for depolymerization and repolymerization of the actin molecules themselves. Actin-binding protein conferred rigidity on actin gels by crosslinking actin into a stable lattice (Hartwig et al., 1980 a and b) which was promoted by nanomolar concentrations of $Ca^{2+}$. Yin and Stossel (1979) found another protein to be involved in controlling gel rigidity. This was a $Ca^{2+}$-dependent actin regulator 'gelsolin', which at micromolar concentrations of $Ca^{2+}$ inhibited gelation of actin in the presence of actin-binding protein.

Hartwig et al. (1980b) suggested that the rigidity of actin gels is controlled by the actin, actin-binding protein and gelsolin system. Gels of actin and actin-binding protein purified from neutrophils and macrophages after reconstitution were observed to contract *in vitro* in the presence of purified myosin and $Mg^{2+}$ ATP (Boxer and Stossel, 1976; Stossel and Hartwig, 1976). Stossel and Hartwig (1975) observed that a cofactor protein was necessary for the activation of myosin + $Mg^{2+}$ ATP and gel contraction which they suggested could be a protein kinase that phosphorylates myosin. These observations provide a plausible model for generating contractile

force for cell locomotion when these molecules are appropriately assembled within the cell.

Indirect evidence for the role of actin in the locomotor apparatus is provided by the effects of cytochalasin B which inhibits actin crosslinking and hence inhibits the migration of human and rabbit neutrophils reversibly at doses of 2 to 4 μg/ml (Becker et al., 1972). However , the evidence is circumstantial since cytochalasin B reversibly inhibits transport of D-2-deoxyglucose and glucosamine into leucocytes and leads to suppression of glycolysis (Zigmond and Hirsch, 1972), and at lower concentration can actually stimulate leucocyte locomotion (Ramsey and Harris, 1973).

It seems likely that microfilament networks can be assembled and disassembled to allow contraction and relaxation of different parts of moving cells, and it is important to determine the spatial arrangement of these structures to generate the force required for cell movement. Formation of microfilament networks appears to be stimulated by surface contact. Reaven and Axline (1973) have shown that macrophages and polymorphs which flatten on glass or protein-coated glass form organelle-excluding microfilament networks in the cytoplasm adjacent to the plasma membrane which is in contact with the substratum. Boyles and Bainton (1979) allowed neutrophils to adhere to protein-free glass then detached them from the surface by shearing. They examined the substratum-adherent portion of the cells by high-resolution scanning and transmission electron microscopy. As little as 30 s attachment of the cell to

the substratum left an interlocking 3-D network of globular projections and radiating microfilaments on the surface; the remnants of extending lamellipodia contained a felt of filamentous and fine granular material. However, The strong non-physiological adhesion of neutrophils to clean glass surfaces may account for the fact that the globule-filament complex covered most of the substratum-attached surface of the cells; this may not be the case when neutrophils move optimally on a less adhesive substratum.

The mechanisms by which new adhesions appear at the front of locomoting leucocytes are unknown. Microfilament networks are seen in highest concentration in the leading lamellipodium of locomoting cells. The locomotion and chemotactic responses shown by isolated lamellipodial fragments of neutrophils (Keller and Bessis, 1975) suggests that the local control of microfilament network is the head region of the cells. Both actin and actin-binding protein are present in high quantities in the lamellipodium of neutrophils as has been demonstrated by Senda (1977) using fluorescein-conjugated heavy meromyosin and by Oliver et al. (1978) using anti-actin and a second layer of anti-Ig. Actin is also present in the tail and retraction fibres of locomoting neutrophils (unpublished observation of Dr. W. S. Haston, department of Bacteriology and Immunology, University of Glasgow). Oliver et al. (1978) studied the orientation of rabbit neutrophils in gradients of f-Met-Leu-Phe and observed that high concentrations of actin were present on the side of the cell nearest to the gradient source. This

observation suggested that stimulation by chemotactic factor, as by phagocytosable particles, causes localized formation of microfilament networks.

The motor apparatus of leucocytes based on the observation of several workers is a complex system and further details will not be considered in this study.

## SECTION IV

**Monocyte chemotaxis: factors, receptors, heterogeneity of the population, proportion of responding cells**

The phenomenon of leucocyte chemotaxis whether occuring *in vivo* was controversial for a long time in the early studies. Harris (1954) made a very critical review of the evidence supporting the existence of chemotaxis *in vivo* and concluded that, it was probably of little importance even if it did exist. The difficulty in those days was to design experiments to demonstrate unequivocal chemotaxis in the complex *in vivo* situation. A few years later, Buckley (1963) solved the problem with his *in vivo* chemotactix experiment, described below.

*Chemotaxis in vivo*

Buckley (1963) used a radiofrequency generator to produce very small areas of heat injury in rabbit ear-chambers and then followed the locomotion of granulocytes towards the affected area for several hours. He observed that the cells from the nearby area is moving in a directional fashion to reach the spot which was purely chemotactic locomotion. Upon reaching the lesion, the

cells started to move randomly which indicated that chemotaxis has no role in accumulation of leucocytes at inflammatory foci.

Substances which produce chemotaxis of leucocytes can be divided into 2 classes. Keller and Sorkin (1967) introduced the term 'cytotaxin' to describe chemotactic factors which act directly on cells. They used the term 'cytotaxigen' to describe substances which have no direct capacity to activate the cells and operate indirectly through the activation of serum components, especially of the complement system.

*Chemotaxis in vitro*

Bessis and Burté (1965) carried out an elegant experiment to demonstrate chemotaxis occuring *in vitro* in the absence of plasma factors. They ruptured individual washed blood cells by shining ultra violet or laser microbeam upon them while using a modified microscope and then filmed the response of nearby leucocytes. They found that when a leucocyte or an erythrocyte was killed, leucocytes as far as 500 μm away from the spot would move towards it and the nearest cells were responding within one second. Thus Bessis and Burté (1965) convincingly demonstrated that leucocytes could respond chemotactically to products of cell damage in the absence of plasma or serum factors. This phenomenon of leucocyte chemotaxis towards a product released from injured or dead cells was defined as 'necrotaxis' by Bessis (1974). Living healthy cells may also release chemotactic factors. Leucocytes produce these factors

during phagocytosis which attract other leucocytes. Borel (1970) found that the postgranular fraction of neutrophils obtained after centrifugation of neutrophil homogenates at 9000 g was attracting fresh neutrophils but not macrophages. Some of these cellular chemotactic factors are cytotaxigenic, not capable of attracting leucocytes directly, e.g., lysosomal enzymes which act by splitting complement components.

Activation of complement can be achieved either through the classical pathway (mediated by specific antibody) or through the alternative pathway which can be activated by a variety of substances including polysaccharides, common components of the cell wall of many microorganisms. The cell wall constituent, lipopolysaccharide (endotoxin), of many gram-negative bacteria is an extremely potent activator of the complement system (Keller and Sorkin, 1967; Snyderman et al., 1969). Thus the complement system allows the formation of chemotactic gradients towards a great many different potential pathogens by a common mechanism which leucocytes are able to detect.

*Chemotactic factors for monocytes*

Cells of the mononuclear phagocyte system show chemotactic responses though these responses are less frequently studied and less well understood than those of neutrophils. This is due to the heterogeneity of the mononuclear phagocytes and the technical problems in obtaining these cells in highly purified population in a state suitable to study locomotion. In chapter 3 of this

study is presented an evaluation of several procedures to purify blood monocytes. Heterogeneity of blood monocytes are described later in this section.

Like neutrophils, most of the chemotactic factors for monocytes were investigated using filter assays, it is not clear whether the effect reported is definitely chemotactic or an increased random migration of the monocytes due to recruitment of extra locomotory cells. C5a was the first chemotactic factor described for monocytes and macrophages (Snyderman et al., 1972 and 1975; Dohlman and Goetzl, 1978; Chenoweth et al., 1982). As for neutrophils, C5a induced maximum proportion of human blood monocytes to migrate into filters at $10^{-8}$ M and C5a des Arg at $10^{-7}$ M. Both of these 2 complement peptides as well as C3a and C3a des Arg were used in this study to investigate monocyte polarisation by the shape change assay (see chapter 4).

Mononuclear phagocytes from different sources have responsiveness towards nanomolar concentrations of formyl peptides such as f-Met-Leu-Phe (FMLP) and receptors for these peptides were demonstrated on blood monocytes (Weinberg et al., 1981), and peritoneal (Snyderman and Fudman, 1980) and alveolar (Spilberg et al., 1981) macrophages. Expression of the formyl peptide receptor is related to the state of differentiation of the cell as reported by Pike et al. (1980) who studied a human monocyte line, U937, which responded poorly to FMLP unless cultured with a lymphokine. After culturing for 4 days, the cell synthesized more receptors and increased its chemotactic responsiveness to the peptide. The extent of

monocyte polarity towards different concentrations of FMLP, monocyte locomotion in the presence of FMLP through 3-D matrices and on plane substrata were studied and described in chapter 4, 6 and 7, respectively, of this study.

Mononuclear phagocytes can also respond to lymphocyte derived chemotactic factor (LDCF), released by lymphocytes in chronic inflammation, on exposure to antigen or to polyclonal activators (Ward et al., 1970; Altman et al., 1973). The lymphokine activity is not well characterized after it was reviewed by Altman in 1978.

Bray et al.(1980) described that rat and human neutrophils when exposed to calcium ionophore A23187 released a product of lipoxygenase pathway which caused the aggregation and chemokinesis of fresh neutrophils. This product was identified as leukotriene $B_4$ (5,12-dihydroxy-6,8,10,14 eicosatetraenoic acid) and is active over the concentration range 10 pg to 5 ng/ml (Ford-Hutchinson et al., 1980). Leukotriene $B_4$, prepared from rat neutrophils stimulated with the calcium ionophore A23187, induced chemotaxis as well as chemokinesis of human neutrophils (Ford-Hutchinson et al., 1980). Also it increased dose-dependent chemokinesis of human monocytes and rat macrophages (elicited by sodium caseinate) over the range 30 pg to 10 ng/ml as assessed by an agarose microdroplet technique (Bray et al., 1980). Leukotriene $B_4$ is the most potent chemoattractant for leucocytes, more so than other hydroxy derivatives of lipoxygenase-modified arachidonate (Gallin and Quie, 1978; Goetzl and Sun, 1979).

Denaturation or conformational changes in protein gives them a chemotactic activity not possessed by native forms of the same molecule. Leucocytes probably recognize conformational change in these molecules rather than any specific structural entity. This recognition might reflect a mechanism for clearance of the denatured proteins by the phagocytes. Denatured proteins are not as active as the formyl peptides or C5a towards the leucocytes and activity of these proteins depends upon the degree of denaturation. Wilkinson and Bradley (1981) studied the chemotactic activity of alkali denatured human serum albumin towards blood leucocytes. Removing the haem group from haemoglobin or myoglobin (by cold acid-acetone treatment) results in a loss of the α-helical configuration of the native protein with subsequent unfolding. Wilkinson (1973) observed that the unfolded globin becomes chemotactic for human blood neutrophils. On reconstitution with haemin, the chemotactic activity is progressively lost as the haem to globin ratio is increased. Also a protease inhibitor, $\alpha_2$-macroglobulin, which has no chemotactic activity in its native state, undergoes a conformational change upon binding with proteases. This $\alpha_2$-M-protease complex is chemotactic for human blood monocytes as judged by filter assays (Forrester et al., 1983); this complex is chemokinetic but not chemotactic for neutrophils.

The chemotactic activity of platelet activating factor (PAF, 1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine) towards leucocytes was described by Goetzl et al. (1980). These workers reported the maximum

chemotactic activity of PAF at $10^{-4}$ M when maximum number of neutrophils and mononuclear leucocytes migrated into micropore filters.

Mononuclear phagocyte locomotion has been studied by several workers in relation to tumours. There is evidence to suggest that the growth and proliferation of tumour cells may be inhibited by the activity of macrophages and conversely that growing tumours may inhibit macrophage function. Several workers reported that tumour cells release chemotactic factors for macrophages (Meltzer et al., 1977; Snodgrass et al., 1978; Botazzi et al., 1983) and the role of activated macrophages as killers of tumour cells has been well studied and reviewed by Keller (1980) and Hibbs et al. (1980). However, there are many other reports that suggest that tumours release factors that inhibit locomotion and chemotaxis of macrophages in experimental animals *in vivo* (Snyderman and Pike, 1976a and 1976b; Meltzer and Stevenson, 1977; Normann and Sorkin, 1976 and 1977; Otu et al., 1977). Moreover, it is observed that the locomotion of blood monocytes is depressed in human patients with various tumours including malignant melanoma (Boetcher and Leonard, 1974; Snyderman et al., 1977).

Most of the chemotactic factors described so far are common for both neutrophils and blood monocytes. In the present study, the polarising responses of monocytes were tested against C5a, C5a des Arg, C3a, C3a des Arg, FMLP, leukotriene $B_4$, alkali denatured HSA, PAF, activated human serum (activated by treating with candida spores).

Several workers have observed that mononuclear phagocytes may be attracted into filters by cell specific chemotactic factors that do not attract neutrophils. Borel (1970) described that the subcellular fractions of granulocytes (lysosome fraction) upon incubation with fresh serum could produce a chemotactic stimulus which was specific for neutrophils only. Chemotactic activity for macrophages was only generated by incubation of the lysosome fraction with plasma. Also the 'postgranular' (supernatant) fraction had activity towards neutrophils but not for macrophages. Maroni and Wilkinson (1971) and Maroni et al. (1972) described that spermatozoa activate serum or plasma to produce a factor which is a strong chemoattractant for macrophages but has very little activity for neutrophils. Also the chemotactic activity of lymphokines are specific for macrophages (Ward et al., 1969 and 1970). *Mycobacterium tuberculosis* and its protein fractions activate serum to produce a factor with higher activity for macrophages than for neutrophils (Symon et al., 1972). Thus, although the above reports suggest the possibility of macrophage-specific chemotactic factors, no chemically purified, completely macrophage specific, chemotactic factor has been isolated yet. However, Mundy et al. (1978) found that materials released from resorbing bone are attractant for human blood monocytes but not for neutrophils or lymphocytes. The osteoclasts, which resorb bone *in vivo* are believed to be derived from blood monocytes.

*Monocyte and macrophage plasma membrane receptors*

Macrophages possess on their plasma membrane receptors for the Fc domain of certain IgG subclasses as well as receptors for the C3b component of complement. Using the technique of rosette formation with sensitized erythrocytes, Berken and Benacerraf (1966) first identified a receptor on guinea pig macrophages which is specific for the Fc fragment of guinea pig $IgG_2$ and is resistant to digestion with proteolytic enzymes. Human monocytes and macrophages also have membrane receptors for IgG and C3 (Huber and Fudenberg, 1968; Huber et al., 1969; Huber and Douglas, 1970; Abramson et al., 1970). Later, Melewicz et al. (1981) described the presence of Fc receptors for IgE on human monocytes and rat macrophages.

The function of the C3b receptors varies with the physiological state of the macrophage. C3b receptor on resident mouse peritoneal macrophages mediates binding but not ingestion of complement-coated red cells, while the receptors on thioglycollate broth elicited macrophages mediate ingestion of complement-coated red cells (Bianco et al., 1975). In contrast, Fc receptors on all types of monocytes and macrophages mediate phagocytosis. Although the Fc and C3b receptor system are capable of mediating similar functions, they are not coupled to one another. Signals generated by ligands interacting with the C3b receptor are not transmitted to the Fc receptors, and vice versa (Griffin et al., 1975; Michl et al., 1980). Cultivation of mononuclear phagocytes on surfaces coated with BSA-anti-BSA complex

inhibits Fc receptor mediated phagocytosis but has no effect on particle attachment mediated by Fc or complement receptors (Rabinovitch et al., 1975).

Macrophages have been shown to bind several hormone ligands including insulin (Bar et al., 1976), thyrotropin (Chabaud and Lissitzky, 1977), glucagon (Blecher and Goldstein, 1977), estrogen (Vernon-Roberts, 1969), progesterone (Vernon-Roberts, 1969), cyclic AMP (Vassalli et al., 1976), prostaglandin E1 (Blecher and Goldstein, 1977), parathormone (Minkin et al., 1977), calcitonin (Minkin et al., 1977), histamine (Csaba et al., 1975), serotonin (Csaba et al., 1975) and several others. It is unlikely that any of these hormones share a common receptor, although insulin may compete weakly for receptors specific for somatomedin (Thorsson and Hintz, 1977). In diabetes and obesity, insulin binding to monocytes is decreased because of an increased free insulin concentration and a decreased concentration of insulin receptors (Bar et al., 1976).

Glucocorticoids have anti-inflammatory actions that may be mediated by direct hormonal action on macrophages. Werb et al. (1978a and b) found that monocytes and macrophages contain saturable glucocorticoid receptors, with specificity for cortisol, corticosterone, and related synthetic steroids such as dexamethasone.

During the past decade, considerable progress has been made towards the elucidation of a number of sugar dependent uptake systems (i.e., receptor-mediated pinocytosis). The mononuclear phagocyte system is involved in the clearance mechanism of several

glycoproteins circulating through the tissue. Among them, glycoproteins with terminal mannose (Baynes and Wold, 1976), N-acetyl-glucosamine (Stockert et al., 1976) and various lysosomal glycosidases (e.g., β-glucuronidase) (Stahl et al., 1976) are rapidly cleared from plasma by hepatic kupffer cells. Clearance is strictly dependent upon an intact oligosaccharide unit with terminal mannose, N-acetyl-glucosamine or L-fucose. Alveolar macrophages (Stahl et al., 1978) and hepatic kupffer cells (Schlesinger et al., 1978) possess membrane receptors for mannosyl/fucosyl oligosaccharides. Stahl et al. (1981) found plasma membrane receptors on macrophages which bind and internalize mannose or L-fucose terminated glycoproteins. The physiological role of the mannosyl-fucosyl recognition pathway by the mononuclear phagocytes is unclear. It may function as a salvage pathway for secreted lysosomal enzymes or as a mechanism for the recognition of mannosylated microorganisms like yeasts. Alternatively, it has been suggested by Day et al. (1980) that IgM-antigen immune complex may be taken up via this receptor.

*Redistribution of cell membrane receptors in relation to locomotion*

The movement of cell membranes and the redistribution of membrane components, the process known as patching and capping, have been studied by many workers. It has been known for a long time that membrane movement take place as cells locomote. In fibroblasts, for example, particles are carried backwards from the

leading edge of the cell towards the nucleus (Harris, 1973), or the capping of crosslinked surface molecules occur as cells locomote. Certain surface components of lymphocytes, such as membrane immunoglobulin, Fc receptors and Thy leukaemia antigen cap spontaneously if they are not crosslinked by extrinsic ligands, as the cell assumes a locomotor morphology. These membrane components are later found to be concentrated at the uropod of the lymphocytes (Schreiner et al., 1977; Braun et al., 1978). By using an immunofluorescence technique, Schreiner et al. (1977) and Gabbiani et al. (1977) found myosin, actin and tubulin concentrated under the above mentioned caps produced by membrane components.

Capping has been observed in locomoting neutrophils and lymphocytes. Ryan et al. (1974) found that fluorescein-labelled concanavalin A (con A) is capped at the tail of human neutrophils moving towards a chemotactic factor. By using fluorescein isothiocyanate (FITC) as a monovalent ligand, Ryan et al. (1974) found that neutrophils capped the FITC only when they were in locomotion but did not cap FITC if the cells were not moving. This observation of the authors was in contrast to their findings with con A labelled cells which could form caps at the centre of the cell even when immobilized. Perhaps it is not practical for a cell responding to a chemotactic factor to cap all the receptors to the tail during locomotion and not to retain any of them in its head. Rather, it is more practical for at least a proportion of the receptors to be unoccupied

at the head region of the cell, which would be able to react with new molecules of chemotactic factor.

It has been reported by several workers (Zigmond et al., 1981; Gerisch and Keller, 1981) that the front of a locomoting cell is more responsive to chemotactic factors than the tail, suggesting asymmetry of membrane function in polarised cells. The receptors for FMLP on neutrophils are asymmetric as demonstrated by Sullivan et al. (1984), by scanning electron microscopy using FMLP coupled to haemocyanin. Also Shields and Haston (1985) demonstrated that the C3b and Fc receptors are localized at the leading edge of neutrophils polarised with a dose of FMLP ($10^{-8}$ M) optimal for chemotaxis.

Wilkinson et al. (1980) studied the effect of orientation of human neutrophils in chemotactic gradients on the distribution of Fc and C3b receptors. Neutrophils were allowed to assume a locomotor morphology in gradients of FMLP in an orientation chamber (described by Zigmond, 1977) at 20°C either before or after Fc- (using sheep red cells coated with IgG) or C3b-rosetting (using sheep red cells coated with IgM and complement). Wilkinson et al. (1980) described that if neutrophils were first rosetted, then allowed to orient to FMLP, the rosettes became capped to the tail as the cell assumed locomotor morphology. By employing time-lapse cinematography, the above workers found that the rosette remained stationary relative to the substratum and the cap formed as the front end of the cell moved away from the rosette. This observation suggested that the crosslinked receptors formed relatively immobile patches

of membrane which were actively capped by microfilaments. On the other hand, when neutrophils were polarised in FMLP before rosetting, i.e., Fc and C3b receptors were unoccupied while the cell changed shape, the rosettes were either generally distributed throughout the cell body or formed at the head of the cell. The distribution of the ligand binding receptors (uncrosslinked Fc or C3b) at the microfilament-rich head region of the cell is suggesting that either the receptors are moved to the head of the cell actively or they are functionally more active when present at the head region than elsewhere in the cell body. Walter et al. (1980) also described an asymmetry of Fc receptor distribution on human neutrophils oriented in a chemotactic gradient.

*Macrophages as secretory cells*

Macrophages synthesize and secrete an enormous array of products. The kinds and amounts of products secreted are dependent upon the state of differentiation or activation of the macrophages. Gordon (1980) showed that these cells constitutively secrete lysozyme (Gordon et al., 1974); and can be induced to secrete several neutral proteases including plasminogen activator, elastase and collagenase (Werb and Gordon, 1975). Studies by many investigators have shown that mononuclear phagocytes secrete an incredible variety of biologically active molecules from nucleotidase (Stadecker et al., 1977), arachidonic acid metabolites (Davies et al., 1980), superoxide anion (Johnston et al., 1976), and $H_2O_2$ (Nathan and Root, 1977), to complement components

(Whaley, 1980), procoagulants (Ginkel and Aken, 1980), fibronectin (Johansson et al., 1979; Alitalo et al., 1980), interleukin 1 (Mizel and Farrar,1979) and interferon (Smith and Wagner, 1967).

*Oxygen metabolites in phagocytes from human blood*

Neutrophils has been extensively studied for oxygen metabolism. It has been observed that resting neutrophils consume very little oxygen. During phagocytosis, the oxygen consumption of neutrophils is greatly increased. This is known as the respiratory burst. This increase may be 15 fold or even more, depending on the ingested material (Baldridge and Gerrard, 1933; Sbarra and Karnovsky, 1959 and Weening et al., 1974). The respiration of normal human blood monocytes is less vigorously enhanced during phagocytosis compared to that observed in neutrophils. However, monocytes from neutropenic patients, consume as much oxygen (Biggar et at., 1974), or even more (Baehner and Johnston, 1972), than normal human blood neutrophils. This might be due to a compensatory mechanism.

The importance of the oxygen metabolites (superoxide, $O_2^-$ and hydrogen peroxide, $H_2O_2$) generated by neutrophils during phagocytosis and killing of certain microorganisms is illustrated by the inability of the cells from patients with chronic granulomatous disease (CGD) to generate the products and to kill microorganisms that can not produce $H_2O_2$ themselves. Microorganisms which generate $H_2O_2$ , e.g., streptococci, pneumococci, and lactobacilli, are readily killed by CGD leucocytes,

in contrast to $H_2O_2$-negative mutants of the same organism. By introducing an $H_2O_2$-generating system into the phagosomes of the CGD neutrophils, the defects of the cells could be corrected (Klebanoff, 1980). Monocytes from CGD patients also show impaired killing of non-$H_2O_2$ producing bacteria (Davis et al., 1968; Rodey et al., 1969; Lehrer, 1970 and 1975).

*Heterogeneity of human monocytes*

There is heterogeneity in the sizes and the surface characters of mononuclear phagocytes circulating in the peripheral blood. Norris et al. (1979) observed that blood monocytes can be fractionated into 2 populations by counterflow centrifugation. The major group contains cells with a mean volume of approximately 480 $\mu m^3$ while the minor one contains cells with a mean volume near 400 $\mu m^3$, the latter approximating lymphocytes. In the following year, Arenson et al. (1980) analysed the volume of purified blood monocytes and described that 3 distinct populations of large, intermediate and small cells were present in the circulation. They designated these monocytes as $M_1$, $M_2$ and $M_3$ in order of increasing size, wuth approximate volumes 150, 250 and 480 $\mu m^3$, respectively. Arenson et al. (1980) also found each of these subpopulations of monocytes to be present in the circulation of all 30 individuals tested. They studied the chemotactic response to zymosan-activated human serum by total monocytes, $M_1$ + $M_2$ monocytes together, and $M_3$ monocytes alone by using the agarose assay and found that $M_3$ monocytes were 10-fold more responsive than $M_1$ + $M_2$

monocytes and were significantly more so than total monocytes in all experiments. Their observation suggested that $M_3$ cells are the major subpopulation capable of directional migration.

Böyum (1968) described that monocytes are difficult to separate from lymphocytes by gradient methods because these cells differ only slightly in density. Hardin and Downs (1981) used density marker beads in a re-orienting gradient of percoll and described that lymphocytes have density 1.055-1.058 g/ml and monocytes have density 1.040- 1.045 g/ml which are very close to each other. Also the heterogeneity of monocyte size could make them difficult to separate from lymphocytes as subpopulations of monocytes and lymphocytes with different volumes could overlap in density. Due to the difficulty in obtaining a pure preparation of monocytes with high yield, several workers used an active adhesion step, namely incubating mononuclear cells on protein-coated surfaces, which allowed monocytes to adhere reversibly and then the non-adherent cells, mostly lymphocytes, could be removed by washing the surface. It is described in chapter 3 of this study that about 20% of total blood monocytes are non-adherent. These nonadherent monocytes could be a subpopulation of cells.

Normann and Weiner (1981) reported that there are differences in tumour cytotoxicity between the 2 subsets of human peripheral blood monocytes. They separated the monocyte subsets according to size where the diameter of cells over and below 8.2 μm (diameter) were fractionated by using elutration centrifugation. Normann and Weiner

(1981) found that both populations of monocytes were capable of adherence to plastic and ingested latex or carbon particles. They obtained the large monocytes with greater than 95% purity. These constituted about 70% of the total monocytes in the peripheral blood. Testing the 2 subsets of monocytes for tumoricidal activity, Normann and Weiner (1981) observed that the cytotoxic activity was confined to the subset of small monocytes.

The existence of cells of different size among peripheral blood monocytes could reveal them to be functionally distinct subpopulations. The polarising responses of purified monocytes were studied in detail in the presence of several, pure chemotactic factors (described in chapter 4). A proportion of the cells (about 60%) responded in the presence of the highly active substances. These results correlated with observations of previous workers (Falk and Leonard, 1980 and 1981; Cianciolo and Snyderman, 1981, using mostly different chemotactic factors), suggest that monocyte responsiveness to chemotactic factors could be a property of the subpopulation of cells that can respond to various chemoattractants.

*Proportion of monocytes that respond to attractants*

Blood monocytes are motile cells, and respond towards chemotactic factors. Snyderman et al. (1972) first demonstrated the locomotor responses of monocytes which would migrate into filters towards C5a. Wilkinson and Allan (1980) described that monocytes would migrate directionally towards *Candida albicans* in plasma, as

accurately as neutrophils. The quantitative data of Falk and Leonard (1980) showed that only 20-40% of human blood monocytes are capable of migrating into filters in response to chemoattractants. Using 3 different attractants namely, C5a, a formyl peptide and a lymphocyte-derived chemotactic factor, the same proportion of cells were responding (Falk and Leonard, 1980). These workers also found that cells deactivated to any one attractant, showed responses to other attractants. This suggested that the migrating cells have receptors for all 3 chemotaxins tested.

The capacity of blood monocytes to orient to gradient sources in the orientation chamber (described by Zigmond, 1977) was studied by Wilkinson (1982a). The author reported that about 40% of the monocytes became polarised with good orientation towards the gradient when *Candida albicans* in fresh human serum was placed on the other side of the bridge in the chamber. This observation correlated with that described by Falk and Leonard (1980). Cianciolo and Snyderman (1981) studied the polarisation responses of monocytes in suspension in presence of different chemoattractants: a chemotactic lymphokine, zymosan-activated human serum, and the N-formylated oligopeptides. These authors found that the maximum percentage of monocytes which polarised to any chemotactic factor was about 60%. Also the combination of several chemotactic factors could not increase the percentage of polarised monocytes over 60%. It is not known whether the polarised and unpolarised monocytes represent different stages in monocyte

differentiation or whether they have different fates *in vivo* though several workers have suggested that these are distinct subpopulation of cells (Falk and Leonard, 1980; Cianciolo and Snyderman, 1981).

Pike et al. (1980) observed that human monocyte line U937 showed little capacity to respond by locomotion to FMLP unless the cells were cultured in the presence of a lymphokine. These workers found that the cell line acquired surface receptors during the course of culture and showed chemotactic responses towards FMLP. Leonard and Skeel (1978) isolated a macrophage stimulating protein (MSP) from human serum and later reported that murine resident peritoneal macrophages could not respond to attractants unless MSP was present (Leonard and Skeel, 1980). However, BCG induced peritoneal exudate macrophages did not show enhanced responsiveness to attractants in the presence of MSP.

Wilkinson (1982b) studied the locomotion of mouse macrophages in relation to *Candida albicans* in mouse serum. The author found that the thioglycollate-elicited peritoneal macrophages and a murine macrophage line (J774 B10) responded well to the spores whereas, very few of the resident macrophages showed any response. In a recent study, Wilkinson (1985b) described heterogeneity in locomotor responses of mouse peritoneal macrophages. Very few of the macrophages which were obtained without an eliciting stimulus were motile and only a minority of them showed chemotactic responses towards candida spores. Macrophages which were obtained 4 days after intraperitoneal injection of thioglycollate, an

inflammatory stimulus, were highly motile and showed good chemotaxis towards the spores of candida. On the other hand, macrophages obtained 4 days after injection of *Corynebacterium parvum*, a strong immunopotentiating agent producing a good cell-mediated immune response, were mostly inactive and very few of them showed locomotor or chemotactic responses. There was considerable heterogeneity in size of these macrophages. Many of the immobile cells were as small as the size of blood monocytes, perhaps freshly derived from the circulating cells and recruited into the peritoneal cavity. The loss of typical locomotor characteristics of inflammatory macrophages (developed during immune response against *C. parvum*) could be due to products secreted by lymphocytes, for example, macrophage migration inhibition factor (MIF). These observations suggested that mononuclear phagocytes at different stages of differentiation could have different locomotor capacity which is modulated by unknown factors present in different types of inflammatory lesion.

# SECTION V

## Purpose of the present study

The purpose of the present study was to investigate the locomotor and chemotactic properties of human blood monocytes (also neutrophils in some experiments) using new assays. The first problem for such a study was to obtain pure monocytes. Several procedures are available in the literature. Of them, 6 procedures were tried to establish a suitable one which would give monocytes with high yield and purity. These procedures are described in detail in chapter 2. The method described by Ackerman and Douglas (1978) produced monocytes with more than 96% purity, and was selected for the rest of the study to purify the cells. An evaluation of the procedures followed to purify monocytes is presented by chapter 3.

Monocytes have not previously been studied using the **polarisation assay,** except by Cianciolo and Snyderman (1981). The superiority of the **polarisation or shape change assay** over the commonly used micropore filter assay is that the former is carried out in suspension where the cell-substratum interaction plays no role. On the other hand, adhesion of the cells to the filter surface is unavoidable in the filter assay which can give misleading results.

The polarisation responses of monocytes were studied by the shape change assay using different chemotactic factors. The factors included FMLP, leukotriene $B_4$ ($LTB_4$), candida spore-activated human serum (AHS), activated complement components (C5a, C5adA, C3a, C3adA),

alkali denatured HSA and platelet activating factor, all induced monocyte polarisation in a dose-dependent fashion (described in chapter 4). The maximum percentage of cells polarised (60-65%) towards a defined chemotactic factor did not increase when 2 or more such factors (pure substances) were combined together. In contrast to this, and the observation of previous workers (Falk and Leonard, 1980; Cianciolo and Snyderman, 1981), an additive effect on monocyte polarisation was found when AHS was combined with any chemoattractant like $LTB_4$, C5a or FMLP. With FMLP + AHS a maximum proportion of cells, more than 85%, were polarised after 20 min incubation. Fig. 29 illustrates a population photograph of monocytes polarised in FMLP + AHS.

Monocyte polarisation towards different chemotactic factors was observed to be time-dependent. The cells started to assume a polarised morphology within as little as 30 s of addition of the attractants. The maximum proportion of cells, 35-65%, depending upon the efficacy of the chemotactic factor, were polarised within 10-20 min of addition of the factor, then the proportion of polarised cells started to decrease upon further incubation. This was not reported by earlier workers. Using rosetting assay with antibody-coated sheep red cells, it was observed that Fc receptors capped at the front of the polarised monocytes prestimulated with FMLP for an optimum time (before doing the rosetting assay). On the other hand, a majority of the cells incubated for a long time in FMLP before doing the rosetting assay could not bind to the red cells (see Fig. 32). This

suggests that monocytes lose their surface receptors due to prolonged exposure to the chemotactic factor. This is a new finding.

When monocytes straight from blood were cultured in siliconized glass tubes without an activating factor or mitogen, a majority of the cells, up to 90%, changed shape in RPMI + FCS medium after 72 h (see Fig. 35). The cultured cells were larger in size compared to monocytes direct from blood. Monocytes in culture would synthesize proteins and if inhibited by the addition of cycloheximide, the cells could not assume a locomotor/polarised morphology. This suggests that the cells require proteins to change shape in culture. This has also not been studied by previous workers.

A collagen gel assay was used to study chemoattractant induced migration of human neutrophils. The cells were used in a uniform concentration as well as in presence of a gradient of FMLP and the distribution of cells through the gel was compared. This is the first assay using collagen gels to study neutrophil chemotaxis (Islam et al., 1985). After developing the assay procedure for neutrophils, it was used to study monocyte invasion. This is the first report demonstrating that human monocytes straight from blood can invade collagen gels. This observation is in contrast to that described by Brown (1984) who reported that blood monocytes can not migrate into collagen gels unless cultured for 48 h on a protein-coated surface.

Monocyte invasion through collagen gels was studied in the presence and absence of chemotactic factors. The

cells showed chemokinesis (increased random migration) in the presence of uniform FMLP throughout the gel. Analyses of time-lapse films showed that a wave of circular contraction was developing in the cell body while it was moving through the gel, and the monocyte passed through the constriction which remained fixed with respect to the external environment. Neutrophil and monocyte migration through collagen gels are described in detail in chapter 6 with illustrations.

Chapter 7 of this study describes chemotactic and chemokinetic locomotion of monocytes on plane substrata. The pathogenic yeast *Candida albicans* which can produce an intense, short-range, chemotactic gradient in normal human serum, was used to study monocyte chemotaxis. Cells direct from blood and after culturing for 18 and 72 h were used to study chemotactic locomotion towards the spores. In another study, the locomotion of blood monocytes was studied on different concentration of HSA coated glass coverslips. Also the chemokinetic locomotion of the cells was studied in the presence of fresh human serum, C5a and FMLP. Monocytes made zig-zag tracks when allowed to locomote on plane surfaces (coated with HSA) in the presence of a supraoptimal dose of FMLP whereas in presence of an optimal dose of the peptide, the cells tended to move in relatively straight paths.

Thus by using new assays as well as different existing assay techniques, it was hoped to study certain aspects of chemoattractant-induced polarisation and locomotion of human blood leucocytes.

# CHAPTER 2

# MATERIALS AND METHODS

**Glassware**

Glass universal bottles, cylinders, beakers, test tubes, pipettes and conical flasks were routinely cleaned by soaking them overnight in a 2% v/v solution of stericol (Sterling Industrial, Sheffield, England). They were rinsed thoroughly in running water and sterilized by dry heat.

**Siliconization of glassware**

Glass universal bottles, test tubes and pasteur pipettes were siliconized before using them to handle monocytes. Glassware was cleansed either by dipping in stericol or in chromic acid, washed in running water then dried and allowed to cool before using for siliconization. Repelcote (Hopkin and Williams, Chadwell health, Essex, England), the water-repellent, a 2% solution of dimethyl-dichlorosilane in 1,1,1,trichloro-ethane was used for silicone treatment. All glassware was dipped for 1 min in repelcote and allowed to dry at room temp inside the fume cupboard for about 1 h. There will normally be sufficent water adsorbed on the glassware to hydrolyze repelcote to a highly water-repellent silicone film which is non toxic to cells. After complete evaporation of the solvent the glassware was thoroughly rinsed to remove the small quantity of hydrochloric acid formed during hydrolysis of repelcote followed by a final rinse with distilled water. Then it was dried in a hot oven and sterilized before use.

**Maintenance of *Candida albicans* in culture**

The pathogenic yeast *Candida albicans* was isolated from clinical specimens and maintained in culture in Sabouraud dextrose agar (Oxoid Ltd., Hants., England). It consisted of Mycological peptone (Oxoid L 40 at 10 g/l), Dextrose at 40 g/l and Agar no. 1 (Oxoid L 11) at 15 g/l. The medium was prepared by dissolving 65 g solid media/l of boiling distilled water. The pH of the medium was approximately 5.6. The medium was then sterilized by autoclaving at 121°C for 15 min. When it cooled to about 40°C after autoclaving, aliquots were transferred into plastic petri dishes (Sterilin Ltd., Teddington, England) to form solid agar media. This medium allowed the growth of discrete blastospores about 3 μm in diameter but the mycelial form could not grow as they required the presence of serum protein. Spores were grown for 24 h at 37°C and were stored at 4°C. They were recultured after every 4 weeks of storage (on fresh medium) and thus maintained in culture throughout the study.

**Preparation of standard buffer**

MOPS [3-(N-morpholino) propanesulphonic acid] was used as standard buffer in all experiments. One molar MOPS (Sigma, Poole, Dorset) was prepared by dissolving 20.93 g in 90 ml of distilled water and the pH was adjusted to 7.4 by adding 16 N sodium hydroxide (BDH Chemicals Ltd., Poole, England). Then the final volume of the buffer was made up to 100 ml by adding distilled water. It was sterilized by filtering through 0.45 μm pore sized filters (Microflow 25, Flow Labs. Ltd.,

Rickmansworth, Herts, England) then transferred as aliquots of 10-12 ml into sterile glass universals and was stored at 4°C.

**Preparation of media**

Hanks' balanced salt solution (HBSS), Calcium and magnesium free salt solution, RPMI 1640, L-Glutamine solution (200 mM), Penicillin (5000 units/ml)-streptomycin (5000 µg/ml) and heat inactivated Fetal calf (bovine) serum (FCS) were obtained from Flow Labs. Ltd., Rickmansworth, Herts, England. Ethylene diamine tetraacetic acid (EDTA, disodium salt, M. wt. 372.24) was obtained from BDH Chemicals Ltd., Poole, England. Human serum albumin (HSA) was obtained from Behringwerke AG, Marburg, West Germany.

HBSS was prepared either by diluting a ten-times strength solution with sterile distilled water and adding 1 M MOPS, or by direct use of a single strength medium and 1 M MOPS, to a final dilution of 10 mM MOPS. The pH was adjusted to 7.4 by addition of sterile-filtered 1 N NaOH to the medium. Henceforth, this will be designated as HBSS-MOPS.

Calcium and magnesium free salt solution was always prepared from the single strength medium by adding 1 M MOPS to a final concentration of 10 mM MOPS (pH adjusted to 7.4 with alkali, if necessary) and this medium will be designated as CMFS-MOPS. EDTA was prepared in this medium as a 10 mM solution by dissolving 372.24 mg of EDTA per 100 ml of the medium. This medium (EDTA/CMFS-MOPS) was filter sterilized before use. Different

concentrations of EDTA were prepared by diluting this medium with CMFS-MOPS.

RPMI 1640 medium was always prepared by diluting a ten-times strength solution with sterile distilled water and adding 1 M MOPS to contain 20 mM MOPS, and the pH was adjusted to 7.4 by addition of sterile filtered 1 N NaOH to the medium. This medium will be called RPMI-MOPS. Appropriate volumes of FCS were added to RPMI-MOPS to contain 10 or 20% FCS (v/v) in the medium.

HSA was dissolved either in HBSS-MOPS or in RPMI-MOPS at different concentrations (4 mg/ml-100mg/ml). This will be mentioned in the text where appropriate. These solutions were filter sterilized prior to use.

**Monocyte culture media**

HSA at 10 mg/ml in RPMI-MOPS or 20% FCS in RPMI-MOPS both containing 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin were used as monocyte culture media.

**Preparation of fixative**

Glutaraldehyde (Grade II, 25% aqueous solution, Sigma, Poole, Dorset) was diluted in HBSS-MOPS to give a 2.5% v/v solution, the pH was adjusted to 7.4 by adding 1 N NaOH. This fixative, to be called Glut/HBSS-MOPS, was used to fix cells in most of the experiments.

Formol calcium was used to fix some of the cell preparations for non specific esterase (NSE) stain. It was prepared by dissolving 1% w/v calcium chloride (BDH Chemicals Ltd., Poole, England) in 4% w/v formaldehyde (May

and Baker, Dagenham, England). The pH was adjusted to 6.7 by adding 1 N NaOH.

**Preparation of test reagents**

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) and dimethyl sulphoxide (DMSO, Grade I) were obtained from Sigma, Poole, Dorset. FMLP was prepared as a stock solution of $10^{-2}$ M in DMSO and was stored at -20°C. Fresh peptide solution at different dilutions were made up every day in HBSS-MOPS.

Leukotriene $B_4$ ($LTB_4$), a 5,12 dihydroxy derivative of arachidonic acid, M. wt. 336, was obtained from Miles Labs. Ltd., Slough, England. This compound is highly susceptible to decomposition/isomerisation which is prompted by high temp and low pH values. It was obtained in an amber borosilicate glass ampoule sealed under argon. A stock solution of the compound at $10^{-5}$ M was prepared in cold HBSS-MOPS by breaking the ampoule on the day of the experiment. Different dilutions were made up in the same medium. All solutions of $LTB_4$ were kept cool (at 4°C) until use. Occasionally HSA (5 mg/ml) in HBSS-MOPS was used to prepare different dilutions of $LTB_4$ from the $10^{-5}$ M stock solution and were stored at -20°C up to 2 weeks.

**Fresh human serum**

Blood samples from healthy volunteers were taken into sterile glass universals and left undisturbed for 40 min at room temp for clotting. Then the clot was detached from the sides of the universal with a wooden applicator/

orange stick (Henleys medical supplies Ltd., London) and was incubated at 37°C for 1 h when it retracted to release maximum fluid. This straw coloured fluid (serum) was collected in a sterile conical base plastic tube (Sterilin Ltd., Teddington, England) and centrifuged at 500 g for 5 min at room temp to remove remaining red cells. The clear supernatant was transferred into small glass universals as aliquots and stored at -70°C until use. There were 6 serum donors, but only one was used for each experiment.

**Candida spore-activated human serum**

*Candida albicans* blastospores are potent sources for the continuous generation of chemotactic factors from normal human serum. These factors may be generated from the classical (antibody mediated) pathway of complement activation as most normal human sera contain some level of anti-candida antibody. Also the polysaccharides present in the cell wall of the spores are known to activate serum complement by the alternative pathway which will produce chemotactic factors even in the absence of antibody in the serum. *C. albicans* can not produce potent chemotactic factors in the absence of serum. In all experiments to be described, the serum was used from a healthy individual with anti-candida IgG antibody at 16 µg/ml (estimations by courtesy of Dr. C. McSharry, department of Bacteriology and Immunology, University of Glasgow).

Viable *C. albicans* blastospores, from a 24 h, 37°C culture were suspended in HBSS-MOPS and washed at 800 g for 5 min. Then the medium was removed and the spores

were resuspended in fresh human serum at 5-6 X $10^6$/ml and incubated for 1 h at 37°C. After that the spores were removed from the serum sample by two sequential spins at 800 g X 5 min. This candida spore activated (untreated) human serum (AHS) was stored at -20°C until use. Also aliquots of untreated AHS was heated at 56°C for 30 min. This heat inactivated form of AHS, designated as 'InAHS' was stored at -20°C until use.

**Serum-free candida spore phagocytosis supernatant**

About 2 X $10^6$ monocytes in 1 ml of HSA (20 mg/ml) in HBSS-MOPS in a siliconised glass tube were mixed with a suspension of living *C. albicans* (previously washed once with HBSS-MOPS) at 1.2-1.5 X $10^7$ spores in 1 ml of HSA. The mixture was incubated at 37°C for 1 h. Candida spores would not produce any chemotactic stimulus to the monocytes in the absence of fresh human serum. As a result monocytes could not detect the presence of candida in the medium. But very few of them could phagocytose 1 or 2 spores which came in contact with the monocytes (observation from time-lapse film). The basis of this experiment was to see if monocytes released any metabolite or factor(s) during phagocytosis (in the absence of serum) which might be able to induce shape change or activate fresh monocytes.

At the end of the incubation period, monocytes and candida spores were removed from the suspension by spinning twice at 600 g X 10 minutes. This serum free

phagocytosis supernatant (SFPS) was stored at -20°C until use.

Platelet activating factor (PAF) was obtained from Nova Biochem, Switzerland. It is 1-O-Hexadecyl-2-O-acetyl-Sn-glycero-3 phosphorylcholine-$2H_2O$ with chemical formula $C_{26}H_{54}NO_7P.2H_2O$. A stock solution was prepared at $10^{-5}$ M in HBSS-MOPS and kept at 4°C. Different dilutions were prepared in the same medium prior to use.

**Complement derived peptides**

Purified complement peptides C5a, C5a des Arg, C3a and C3a des Arg were isolated from hog and were kindly provided by Dr. B. Damerau, University of Göttingen, West Germany (Damerau et al., 1980). The stock solutions and different dilutions were prepared in HSA (5 mg/ml) in HBSS-MOPS and were stored at -20°C until use.

**Alkali denatured human serum albumin**

Different batches of denatured HSA were prepared by Prof. P. C. Wilkinson as described previously (Wilkinson and Bradley, 1981). Briefly, a solution of HSA at 2 mg/ml was prepared in distilled water and brought to pH 12 by the addition of 16 N sodium hydroxide. The solution was left overnight at 20°C and then brought to pH 7.4 by adding concentrated HCl (May and Baker Ltd., Dagenham, England). Denaturation was confirmed by the presence of a peak at 287 nm in the difference spectrum against native HSA (performed with a spectrophotometer, SP 1800, Pye Unicam Ltd., Cambridge). This alkali denatured HSA (adHSA) was dialysed against excess of HBSS-MOPS (medium

changed thrice). The adHSA solution was concentrated up to a desired level by passing through Amicon filters (PM 10 membranes, Amicon Co., Mass., Lexington, U.S.A.) and was stored at -20°C until use.

**Isolation of neutrophil leucocytes**

Human blood was collected from normal donors by venepuncture and was mixed with preservative-free heparin (Evans Medical Ltd., Speke, Liverpool) at 10 units/ml of blood in sterile plastic universals (Sterilin Ltd., Teddington, England). One part of dextran (M. wt. 110,000, "Dextraven 110" a 6% solution of dextran in 0.9% sodium chloride, obtained from Fisons Ltd., Loughborough) was added to 4 parts of heparinized blood and was left for 40 min at room temp. This treatment caused erythrocytes to form rouleaux, accelerating their sedimentation. The leucocyte-rich supernatant was layered onto lymphocyte separation medium (Ficoll-Hypaque, Sp. gravity 1.077 g/ml at 20°C (first described by Böyum, 1968), which was obtained from Flow Labs. Ltd., Rickmansworth, Herts, England) in the ratio of 3 ml separation mixture : 7 ml of supernatant and centrifuged at 400 g for 30 min. All centrifugations were carried out at 20°C and sterile conical base plastic centrifuge tubes (Sterilin Ltd., Teddington, England) were used throughout the procedure. The pellet was transferred to a fresh tube and washed once with HBSS-MOPS for 5 min at 400 g. Some red blood cells contained in the pellet were lysed by resuspending it in 0.5 ml of cold sterile distilled water. This was allowed to stand for 30 s and

then transferred to another tube followed by washing with excess of medium (HBSS-MOPS) for the same time and speed as mentioned before. The cells were washed once again which removed most of the red cell debris. They were finally transferred to a new tube after resuspending in HBSS-MOPS and were counted in an improved Neubauer haemocytometer. The yield was 1.5-2.3 X $10^6$ cells/ml of blood. The cell suspension consisted of >95% polymorphonuclear neutrophil leucocytes (PMNS) the remainder being mostly eosinophil and basophil leucocytes (as confirmed by Giemsa stain). This procedure gave a population of >94% round neutrophils as examined by phase- contrast microscopy with a X40 objective. The neutrophils (resuspended at $10^6$ cells/ml) were always used within 1 h of preparation.

**Checking cell viability**

A 0.2% solution of trypan blue (BDH Chemicals Ltd., Poole, England) was prepared in normal saline and was filtered to remove any undissolved dye. One hundred microlitres of cell suspension (at $10^6$ cells/ml) was mixed well with 100 µl of the dye solution and left for 1 min at room temp. One drop of this mixture was taken on a microscope slide, mounted with a coverslip, examined under X20 phase objective of an inverted microscope (Nikon, MSD). Viable cells excluded the dye solution and were refractile whereas dead cells took up the dye and were stained blue when examined under the microscope. Neutrophils isolated by the above mentioned procedure always contained >97% viable cells.

**Isolation of mononuclear cells**

*(a) Isolation by centrifugation of diluted blood*

Human peripheral blood was collected and mixed with heparin as mentioned earlier (under "Isolation of neutrophil leucocytes"). Heparinized blood was mixed with HBSS-MOPS in the ratio of 2:3 at room temp. About 16-17 ml of this diluted blood was layered over 12 ml of lymphocyte separation medium in a sterile conical bottomed centrifuge tube (Nunclon, Denmark) and was spun at 440 g for 30 min at 20°C. Mononuclear cells collected from the interface between the diluted plasma and separation medium, were transferred to conical-base plastic centrifuge tubes (as used to isolate PMNS) and washed with excess of CMFS-MOPS medium at 4°C for 15 min at 440 g. The pellets after resuspension in small volumes of CMFS-MOPS were pooled together into a fresh tube, filled up with the cold medium and washed for 10 min at 440 g. The cell pellet was finally transferred to another new tube and washed once more. Thus changing tubes after every wash minimized the platelet contamination of the mononuclear cells (MNCS). The cells were resuspended at 1.5-9.0 X $10^6$ cells/ml in the appropriate medium according to the subsequent procedure followed to separate them into monocytes and lymphocytes. Cell viability, as mentioned for PMNS, judged by trypan blue exclusion exceeded 97%. The yield of MNCS was variable and was 0.5-2.0 X $10^6$ cells/ml of blood used.

*(b) Isolation by centrifugation of whole blood*

Heparinized blood (collected as mentioned before) was layered directly over lymphocyte separation medium in the ratio of 3 ml separation medium : 7 ml of blood in a sterile, conical base, plastic tube and was centrifuged at 440 g for 30 min at 20°C. MNCS were collected from the interface between the plasma and the separation medium and were washed thrice with CMFS-MOPS medium (at 4°C) as mentioned before. Spinning whole blood always gave some red cell contamination of the mononuclear cell fraction. It was observed that red cells which are non-adherent, were washed away with the lymphocytes and did not remain in the monocyte fraction after the purification procedure. This isolation procedure also gave >97% viable mononuclear cells finally resuspended at the prescribed concentration in the required medium necessary for their separation. The yield of MNCS in this procedure was similar to that mentioned above. The purification procedure of MNCS was always started as soon as the cells had been isolated.

**Procedure followed to purify monocytes in suspension**

Several procedures were followed to purify monocytes in suspension. Of them, procedure (a) and (b) utilized a density centrifugation step to separate monocytes, procedures (c), (d), (e) and (f) utilized an active adhesion step to purify monocytes from lymphocytes which are non-adherent. These procedures are described below.

*(a) Discontinuous density gradient of percoll*

Percoll, colloidal silica coated by polyvinyl pyrrolidone, density 1.130 g/ml at 20°C, osmolality 12 mOs/Kg $H_2O$, was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Stock isotonic percoll solution (SIP) was prepared by using 9 parts of percoll, 0.9 parts of X10 HBSS and 0.1 part of 1 M MOPS, added together to give a pH value of 7.4. SIP was diluted by using HBSS-MOPS medium to prepare different solutions of percoll with different densities (1.04-1.08 g/ ml) at 21°C from the regression equation described by Ulmer and Flad (1979).

Density (g/ml) = (% SIP X 0.001186) + 1.0041

Three different densities of percoll, at 2 ml of each, were carefully layered in a conical base plastic tube so that the densities were in ascending order from top to the bottom of the tube and the interface between different densities was distinct. Mononuclear cells, 1 X $10^7$ in 2 ml of HBSS-MOPS medium were carefully layered over the top layer of percoll in the tube and were centrifuged at 500 g for 20 min at room temp. Then the cells were collected in separate tubes from different interfaces and were washed once with EDTA at 2.5 mM in CMFS-MOPS at 4°C for 15 min at 440 g. They were washed twice more with 10% FCS in RPMI-MOPS for 10 min each, followed by resuspension in fresh medium. Mononuclear cells were not separated well in this procedure. For further experiments SIP was diluted according to Fluks (1981) to prepare different densities (1.05-1.08 g/ml at 22°C) by using the formula below :

Density (g/ml) = (% SIP X 0.001151) + 1.0096

Two to 3 different densities of percoll were layered in a tube in ascending order of densities (as described before). MNCS were suspended in HBSS-MOPS and 1.0-1.8 X $10^7$ cells in 2 ml medium were placed on the top layer of percoll in the gradient tube, were centrifuged, washed and finally resuspended as described before.

*(b) Nycodenz-monocyte separation medium*

The procedure described by Böyum (1983) was followed to purify monocytes where cells would be separated by density centrifugation on a non-ionic iodinated gradient medium. The separation medium containing nycodenz [5-(N-2, 3-dihydroxypropylacetamido) -2,4,6-tri-iodo-N,N-bis (2,3-dihydroxypropyl) isophthalamide with density 1.068 g/ml at 20°C and osmolality 335 mOsm] which was obtained from Nyegaard and Co., Oslo, Norway.

Human peripheral blood was mixed with disodium EDTA at 1.4 mg/ml instead of using heparin as an anti-coagulant. Dextran was mixed with the EDTA-blood at 1:4 and the plasma layer containing the leucocytes was removed when the erythrocytes had settled after 40 min. A 5 ml aliquot of plasma was layered over 5 ml of nycodenz-monocyte separation medium in a conical base plastic tube and was centrifuged for 15 min at 600 g. There was no distinct band at the interface after centrifugation and cells were collected from 3-4 mm above the interface between the plasma and the separation fluid up to slightly more than half the volume of the latter. These cells were washed thrice, using CMFS-MOPS containing 5 mM EDTA for the first wash at 440 g for 15 min and RPMI-MOPS

+ 10% FCS for the last two washes for 10 min each. Cells were resuspended in RPMI-MOPS containing 10% FCS.

*(c) Gelatin-plasma-coated flasks*

A 2% solution of gelatin (BDH Chemicals Ltd., Poole, England) was prepared in distilled water at the boiling point. When the solution cooled to about 40°C, aliquots of 5 ml were poured into several Nunclon flasks (surface area 25 $cm^2$) and were incubated for 2 h at 37°C. Then gelatin was removed from the flasks by pasteur pipettes and the flasks were allowed to dry. Gelatin-treated flasks were stored at 37°C in a dry incubator before use. They were used within one week of preparation. The use of gelatin-plasma coated flasks for the separation of mononuclear cells into lymphocytes and monocytes was described by Freundlich and Avdalovic (1983).

About 10 ml of 50% autologous plasma (diluted by HBSS-MOPS) was introduced into each gelatin coated flask and was incubated for 1 h at 37°C. Then plasma was removed, the flask was washed twice with CMFS-MOPS, and MNCS up to 1 X $10^7$ in 5 ml of RPMI-MOPS + 20% FCS was introduced into each flask followed by incubation for 40 min at 37°C. Then non-adherent cells were decanted and the flask was washed 3-4 times with prewarmed RPMI-MOPS + 20% FCS medium. After that a 6 ml 1:1 mixture of EDTA at 10 mM in CMFS-MOPS with RPMI-MOPS + 20% FCS was added to the flask and incubated for 20 min at 37°C. Finally the detached monocytes were collected, washed thrice with RPMI-MOPS + 10% FCS for 10 min each at 440g and were resuspended in the fresh medium.

*(d) Gelatin-FCS-coated flasks*

Gelatin-treated flasks were incubated with 5 ml of heat inactivated FCS for 1 h at 37°C. The rest of the procedure was the same as described for gelatin-plasma-coated flasks.

(e) *FCS-coated flasks*

The use of FCS-coated flasks for the purification of blood monocytes was first described by Kumagai et al. (1979). The procedure was slightly modified for the present study.

About 5 ml of heat inactivated neat FCS was introduced into each tissue culture flask (surface area 25 $cm^2$, obtained from Nunclon, Denmark) and incubated overnight at 4°C. Then FCS was removed and the flask was washed thrice with HBSS-MOPS. MNCS up to 1 X $10^7$ in 5 ml of RPMI-MOPS + 10% FCS was added into each flask and was incubated for 1 h at 37°C. After that the non-adherent cells were decanted and the flask was washed 3-4 times with RPMI-MOPS + 10% FCS prewarmed at 37°C. Then the flask was incubated for 20 min at 37°C with 6 ml EDTA at 4 mM in CMFS-MOPS containing 5% FCS [Kumagai et al. (1979) used cold RPMI + 10% FCS medium for washing off non-adherent cells and used 0.2% EDTA in 5% FCS for the detachment of adherent cells while incubating the flask at 4°C]. It detached some of the monocytes from the flask. The cells were collected and the flask was washed 3-4 times with the same medium to recover the detached cells. Monocytes were washed thrice with RPMI-MOPS + 10%

FCS as described before and were resuspended in the fresh medium.

*(f) BHK microexudate-coated flasks*

Ackerman and Douglas (1978) first reported the use of BHK microexudate-coated flasks for the purification of monocytes. Preparation of microexudate-coated flasks are described later in this chapter. Mononuclear cells, up to 1 X $10^7$ in 5 ml of RPMI-MOPS + 10% FCS medium were introduced into each small falcon flask or 1.6-4.0 X $10^7$ cells in 10-12 ml of RPMI-MOPS + 10% FCS medium were introduced into a large flask coated with BHK microexudate. The flask with the cell suspension was incubated immediately for 1 h at 37°C which allowed monocytes to attach to the microexudate-coated surface by an active adhesion process. Non-adherent cells were then decanted and the flask was rinsed with 3 to 4 changes of RPMI-MOPS + 10% FCS medium at 37°C leaving behind the adherent monocytes. A detachment medium was prepared by mixing EDTA at 10 mM in CMFS-MOPS and RPMI-MOPS + 10% FCS at 1:2 so that EDTA concentration was not more than 3.3 mM in the medium. A 5 ml aliquot of this medium (prewarmed at 37°C) was introduced into a small flask, or 8-10 ml medium was added to a large flask, and incubation was continued for 40 min at 37°C in a shaker at 160 revolutions/min for maximum detachment of monocytes. After that the cells were decanted from the flask and the flask was washed with 3-4 changes of the detachment medium. The cells were collected in a siliconised tube (6 X 5/8") and were washed thrice with RPMI-MOPS + 10% FCS

at 440 g X 10 min, each. After the final wash, the supernatant was removed by inverting the tube on a piece of Kleenex medical wipe (for maximum removal of the medium) for few seconds followed by resuspending the monocytes, usually in HSA/ HBSS-MOPS. In some experiments the detached monocytes were washed with RPMI alone or RPMI containing HSA at 4 mg/ml.

The non-adherent cells were regularly used for non specific esterase activity (described later) which determined the proportion of non-adherent monocytes washed away with the lymphocyte fraction . In a few cases the non-adherent cells were washed twice with RPMI-MOPS at 440 g X 10 min each, and were used for shape change assays.

**Preparation of non specific esterase (NSE) stain**

Mononuclear phagocytes contain non specific esterase enzyme. It is one of the most reliable markers for the identification of these cells. When α-napthyl butyrate or acetate is used as substrate, almost all monocytes and macrophages are positive, although the intensity may differ with species, developmental stage, culture conditions and functional state. In the present study α- napthyl acetate was used as substrate of the NSE enzyme.

The procedure described by Mueller et al., (1975) was followed to prepare reagents for acid α-napthyl acetate esterase (ANAE) stain. A 0.067 M solution of $KH_2PO_4$ (M. wt. 136.09, BDH Chemicals Ltd., Poole, England) was prepared by dissolving 9.08 g/l of the

solution (which will be called solution A). Similarly, a 0.067 M solution of $Na_2HPO_4,2H_2O$ (M. wt. 177.99, BDH Chemicals Ltd., Poole, England) was prepared by dissolving 11.9 g/l of the solution (which will be called solution B). A buffer of pH 5.0 was prepared by mixing 98.5 ml of solution A to 1.5 ml of solution B. One gram pararosaniline (Raymond A. Lamb, London) was dissolved in 20 ml distilled water and 5 ml conc. HCl (May and Baker Ltd., Dagenham, England). After gentle warming, cooling and filtration, the solution was stored in the dark at 4°C. A 4% solution of $NaNO_2$ (BDH Chemicals Ltd., Poole, England) was freshly prepared every day in 5 ml distilled water. An equal volume of this solution was mixed with the pararosaniline solution (usually, 1.5 ml to 1.5 ml) and shaken for a few seconds until the colour became amber to give hexazotized pararosaniline.

ANAE stain consisted of 40 ml 0f 0.067 M phosphate buffer, pH 5.0, 2.4 ml 0f hexazotized pararosaniline and 10 mg of α-napthyl acetate (Sigma, Poole, Dorset) in 0.4 ml acetone (BDH Chemicals Ltd., Poole, England). The solution was adjusted to pH 5.8 using 2 N NaOH. The ANAE incubation medium was always prepared just before use.

**Preparation of smears for ANAE stain**

*(a) Cell fixation*

Mueller et al., (1975) used cytocentrifuge preparations of cells, dried at room temp. for 30 min before fixing with formol calcium at 4°C for 10 min.

It was observed that morphological detail of the cells was lost considerably (particularly for monocytes) if they were allowed to dry in the smear before fixation. For this reason, preparations of mononuclear cells were fixed immediately after the final wash of the isolation procedure. Monocytes and lymphocytes were fixed after washing followed by the separation step. Formol calcium or Glut/HBSS-MOPS was used to fix the cells in suspension for 10 min at 4°C. The fixed cells were washed twice with 0.9% w/v NaCl (BDH Chemicals Ltd., Poole, England) at 440 g X 10 min at 20°C followed by resuspension in 4-5 drops of saline. These cell suspensions could be stored at 4°C for 24 h without apparent loss of the ANAE activity.

*(b) Preparation of smears*

Fixed cell suspensions were used to prepare smears on microscope slides by placing 1-2 drops on each slide. These were allowed to dry at room temp and then incubated immediately in the ANAE stain.

*(c) Staining time*

Smears were incubated at 4°C for different time intervals (1-10 h). After that the slides were rinsed 3-4 times in tap water followed by counterstaining either by using 0.1% w/v toluidine blue (Raymond A. Lamb, London) or 0.012% washed methyl green (BDH Chemicals Ltd., Poole, England), both solutions were applied for 20 s. After counterstaining the slides were rinsed in tap water, air dried, mounted by DPX and a coverslip, and examined at

1,000 times magnification using bright field objective. At least 600 cells were counted from two identical slides to determine the percentage composition of lymphocytes, monocytes and neutrophils in different cell fractions. Neutrophils did not take the ANAE stain but their nuclei were stained with methyl green.

Mononuclear cells with the morphological features of monocytes were diffusely stained with the reddish brown reaction product within 2 h of incubation and their number was constant after prolonged incubation (see Fig. 1 A and B). T-lymphocytes stained with ANAE showed 1-2 (maximum of 4) discrete reddish brown spots of the esterase enzyme in their cytoplasm (see Fig. 1 A and B). Incubation up to 6 h increased the number of T-lymphocytes (up to 4%) in the cell population. B-lymphocytes could not give the positive reaction due to lack of the NSE enzyme in their cytoplasm. However, incubation over 6 h developed light yellow colours in all the lymphocyte cytoplasm (smudges from the reaction product). So an incubation period of 2 h was considered to be the standard time because monocytes could be distinguished from the lymphocytes after that interval.

Counterstaining with methyl green had the advantage over toluidine blue that it stained the cell nucleus, which helped to confirm their identity. This was useful particularly for distinguishing monocytes with poor staining from lymphocytes with 3 or more granules of reaction product or from esterase negative B-lymphocytes.

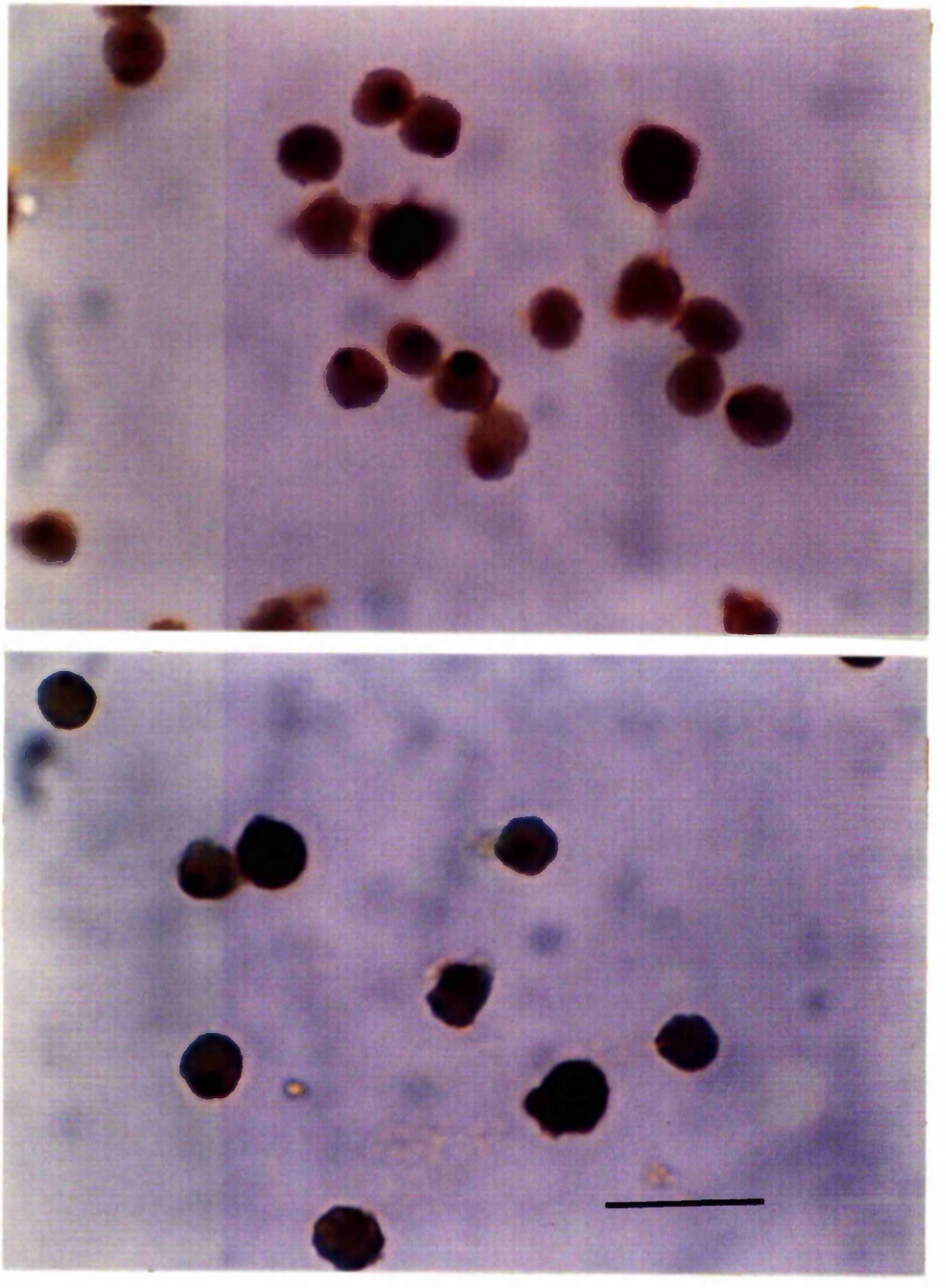



Fig. 1 Acid α-napthyl acetate esterase (ANAE) stain on mononuclear cells from human peripheral blood. The fixed cells were stained for 2 h in the ANAE incubation medium and then counterstained for 20 s in 0.012% washed methyl green. T-lymphocytes stained with ANAE show discrete reddish brown stain, whereas monocytes are diffusely stained (2 cells in both photographs, A and B). Normal human peripheral blood mononuclear cells usually contain 16±9% monocytes. Bar = 20 μm.

**ANAE stain of monocytes after phagocytosis of latex beads**

About 8 X $10^5$ monocytes were suspended in 200 µl of heat inactivated FCS in a siliconized glass tube. Latex beads (an aqueous suspension of polystyrene particles, 10% w/v, average diameter 1.091 µ, a minimum of 5 X $10^{10}$ particles/ml) were obtained from Sigma, Poole, Dorset. Beads were suspended at 5-8 X $10^7$ particles/ml in HBSS-MOPS. About 200 µl of this suspension was mixed with the monocytes and incubated for 1 h at 37°C. Then the cells were fixed with Glut/HBSS-MOPS for 10 min at room temp followed by washing twice with sterile saline (500 g X 10 min, each). Fixed cell suspensions were taken onto 4 slides, 2 were wet mounted and examined under the phase-contrast objective at X40 and X100. Cells containing 3 or more latex particles were scored as phagocytic. At least 400 cells were counted from two identical slides to determine the percent phagocytic monocytes. The remaining 2 slides were air dried and incubated in the ANAE stain for esterase activity on phagocytosed monocytes. Slides were air dried, mounted and examined as described before (see Fig. 2 A).

In some experiments monocytes suspended in neat FCS were allowed to adhere to clean glass coverslips (22 X 22 mm, the procedure to be described in "monocyte adhesion assay") by incubating for 30 min at 37°C. Then latex beads were added to them on the coverslips and the incubation was continued up to 1 h. After fixing and washing the cells to remove excess latex particles, they were stained with Leishman stain (0.15%, w/v in absolute

methanol) for 5 min. Then the coverslips were washed with saline and wet mounted. The cells were examined under X100 phase objective when monocytes appeared with blue stain in their kidney-shaped nucleus and a pale blue staining in the cytoplasm containing phagocytosed latex beads (see Fig. 2 B).

**Culture and maintenance of BHK cells**

Spontaneous baby hamster kidney cell line, BHK 21, clone 13 was obtained from the Cell Biology department, University of Glasgow (courtesy of Dr. J. M. Lackie).

*(a) Growth media for BHK cells*

BHK cells were grown either in Glasgow modification of Eagle's medium (GMEM) or in Ham's F-10 medium. Both media (Flow Labs. Ltd., Rickmansworth, Herts, England) were prepared by diluting a X10 strength solution with sterile distilled water and supplemented with 20 mM MOPS, 10% FCS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml), pH adjusted to 7.4. Sometimes the BHK cell culture was found contaminated either by *Enterobacter aerogenes* or by mucoid lactose fermenting coliforms (culture checked in the department of Bacteriology and Immunology, University of Glasgow). Both type of bacterial contaminants were sensitive to gentamicin and cefotaxime. Contaminated BHK cultures were always thrown out. Fresh cells were grown in presence of gentamicin (obtained as 80 mg/ml solution from Roussel Labs. Ltd., England) and fungizone (obtained as 250 µg/ml

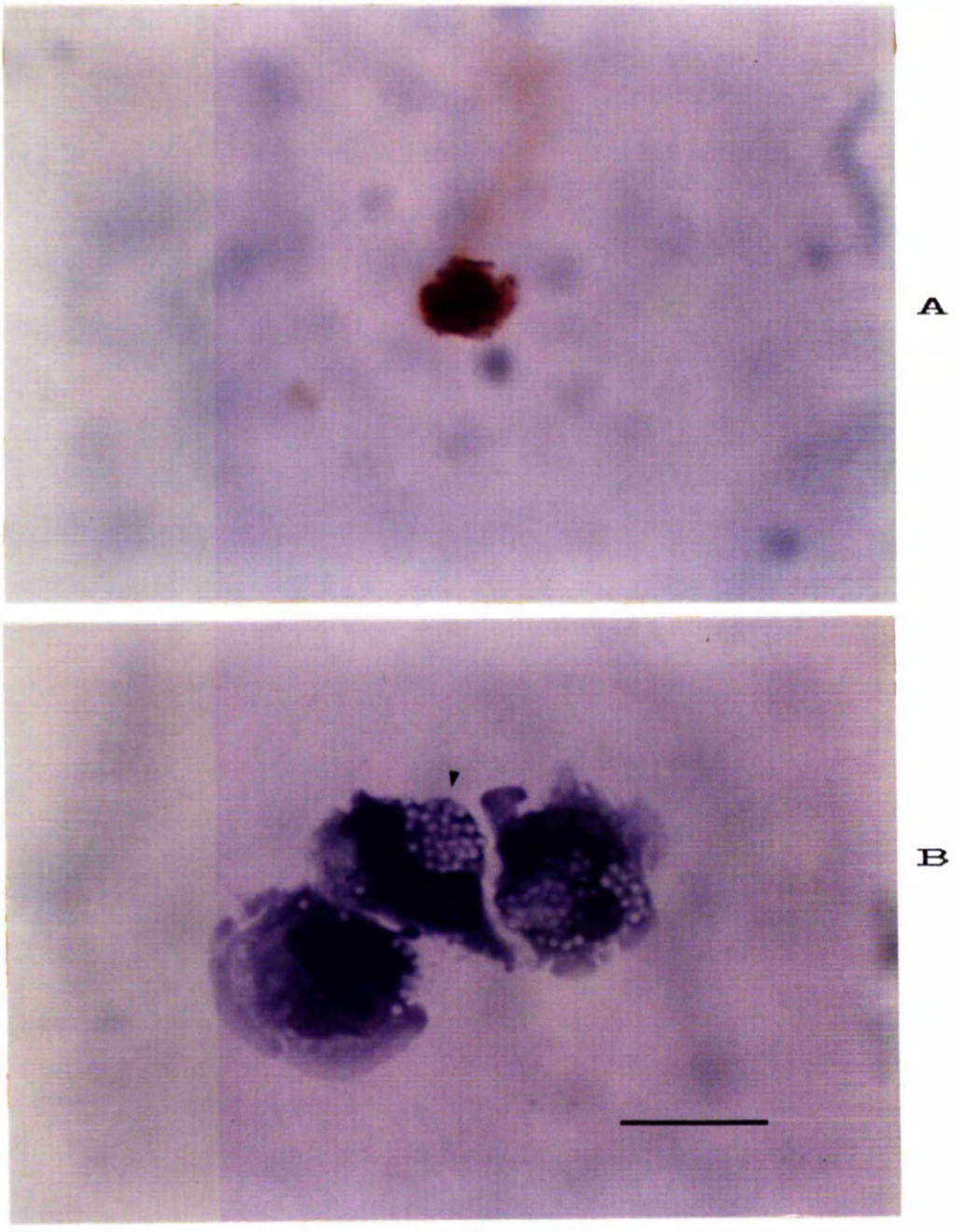



Fig. 2 Identification of cells which phagocytosed latex beads as monocytes. A: Monocyte stained with ANAE after latex phagocytosis. Beads are seen inside the cell. B: Monocytes stained with Leishman stain after latex phagocytosis. Beads are mostly in the cytoplasm of the cell. About 85% of the peripheral blood monocytes phagocytosed the latex beads. Bar = 20 μm.

solution from Gibco, Paisley, Scotland), both used at a low concentration in the BHK growth medium (2.0 μg/ml and 2.5 μg/ml, respectively).

*(b) Typical plating of BHK cells*

BHK cells grow like fibroblasts in culture *in vitro*. When they make contact with one another, ruffling of the leading lamellipodium of the contacting cell ceases at the point of contact. This process of contact inhibition (Abercrombie, 1967) contributed to the cells forming an evenly dispersed monolayer on the surface of the tissue culture flask. When this monolayer formed the culture was called confluent and the cells needed to be transferred to fresh surfaces to keep them growing. BHK cells released some extracellular microexudate on the culture flask which covered the total surface when the cells were at confluence.

Tissue culture flasks (25 $cm^2$ and 75 $cm^2$ surface area, Falcon, Division of Becton Dickinson U.K. Ltd., Oxford) were used to grow BHK cells. It was observed that passage of 1 X $10^6$ cells in 8 ml medium to a 25 $cm^2$ flask (small flask) yielded a confluent culture in 48 h and 1/2 X $10^6$ cells took 72 h to be confluent. Similarly, 2 X $10^6$ cells in 20 ml medium yielded a confluent culture in a 75 $cm^2$ flask (large flask) in 48 h and 1 X $10^6$ cells took 72 h. About 4-5 X $10^6$ cells could be obtained from a small flask and 8-10 X $10^6$ cells could be obtained from a large flask when the cultures were at confluence.

*(c) Detachment of BHK cells from confluent culture*

EDTA (10mM) in CMFS-MOPS, pH 7.4 or 0.2% trypsin (1,000 units/mg, Sigma, Poole, Dorset) in HBSS-MOPS could be used to detach BHK cells from the confluent culture. As trypsin would hydrolyse the microexudate from the culture flask (on which monocytes were to be incubated for active adhesion) EDTA was routinely used to detach BHK cells from the confluent culture.

The culture medium was decanted from the flask and 10 ml EDTA solution was introduced into a large flask (5 ml EDTA solution was used for a small flask) and incubated for 10 min at 37°C. The cell monolayer detached from the flask, leaving behind the extracellular microexudate (Poste et al., 1973). Then the cells were decanted and the flask was shaken vigorously with 5-10 ml of fresh EDTA solution (the volume used depended on the size of flask used) to remove all BHK cells.

*(d) Culture of detached BHK cells*

BHK cells were washed twice (using the growth medium) immediately after detachment, to remove EDTA (spinning for 5 min at 400 g). Finally the cells were resuspended very well to make a single cell suspension in the culture medium. They were counted, viability checked by trypan blue dye exclusion (described earlier for PMNS) and were allowed to grow in the new flasks if the viability exceeded 90%. EDTA detached cells yielded a confluent culture (according to the typical plating cell density) in exactly the same way as trypsin detached cells did.

*(e) Freezing down of BHK cells*

BHK cells were harvested from the culture flask during the log phase (i.e., one day after passage), were washed twice with the growth medium and finally resuspended at 5-6 X $10^6$ cells/ml in the freezing medium (pH 7.4) containing 20% FCS, 10% DMSO and 70% growth medium at 0°C. About 1 ml of this cell suspension was transferred to each sterile storage ampoule (Flow Labs. Ltd., Rickmansworth, Herts, England) at 0°C. The ampoules were placed in a polystyrene box and were cooled at a rate of 1°C/min (approximately) to -70°C, with compensation for heat of crystallization followed by transferring them to the vapour phase of a liquid nitrogen container at -196°C.

*(f) Thawing and re-growing*

The frozen BHK cell sample was thawed by gently shaking the ampoule in a water bath at 37°C. As soon as the sample had melted, the outside of the ampoule was washed with 70% v/v ethanol and the cell suspension was slowly mixed with about 20 ml of the growth medium at 20°C. The cells were washed once and viability checked after resuspending in the growth medium. For re-growing, the cell suspension was mixed with the BHK conditioned medium (described below) at a proportion of 25:75 and allowed to grow at a density 2-3 times higher than the usual cell density in a continuously growing culture. Because 20-30% of the thawed cells died during the first 24 h in culture. The medium was decanted from

the flask after the first 24 h when most of the dead cells and debris were removed. Then fresh growth medium was added to the flask. Thawed cells had a slower growth rate and took 6-10 days to yield a confluent culture.

*(g) Conditioned medium*

Conditioned medium was the decanted medium from a healthy confluent culture which was spun down to exclude dead cells and debris. This was stored at -20°C until use. It consisted of several metabolites synthesised by the cells while growing. Supplementation of this medium to the thawed cells helped them to survive until their synthetic apparatus could work.

**Storage of microexudate-coated flasks**

All BHK cells were detached and decanted from the confluent cultures by using EDTA (described before) which left the microexudate on the culture flask. The flask was washed 3-4 times with HBSS-MOPS to remove remaining cells and EDTA. Finally, an excess of HBSS-MOPS was added into the flask to cover the microexudate. The flask was either used on the same day or was stored at 4°C. The stored flasks were always used within 2 weeks of preparation.

**Isolation of collagen**

Type I collagen was prepared from rat tail tendons by slight modification of the methods of Elsdale and Bard (1972) and Schor (1980). Tails were collected from rats weighing 230-250 g. They were soaked in 70% ethanol for 5

min before removing the skin. Tendons were stripped and dipped in HBSS-MOPS containing 50 units/ml penicillin and 50 µg/ml streptomycin for 5 min. The medium was removed and the tendons were soaked in 3% v/v acetic acid (BDH Chemicals Ltd., Poole, England) in a sterile conical flask and left overnight at 4°C. Then the content of the flask was thoroughly shaken for 1 h with a magnetic stirrer and undissolved materials were removed by centrifugation at 800 g for 30 min. The almost clear tropocollagen solution was mixed with 20% w/v sodium chloride (at 1:2) to precipitate the collagen. Then it was centrifuged at 600 g for 40 min when collagen precipitates accumulated at the top of the centrifuge tube and undissolved materials were removed as a pellet. The collagen precipitates were collected by using a spatula and were washed once with distilled water. They were dissolved again in 3% v/v acetic acid (keeping in mind to make the collagen solution quite concentrated) and were centrifuged twice at 800 g X 20 min, each time rejecting a portion of the solution at the bottom of the centrifuge tube. This treatment completely removed small undissolved materials from the collagen solution with an increase of optical quality. Then the collagen solution was exhaustively dialysed against distilled water adjusted to pH 4.0 with HCl. The concentration of collagen in the solution was determined by comparing the optical density value at 234 nm (using matched cuvettes in a Pye Unicam spectrophotometer, SP 1800) to a standard graph prepared with freeze dried samples of the same

collagen preparation. Aliquots of the collagen solution were then transferred into sterile plastic universals and stored at -20°C until use.

**Preparation of collagen gels and assay of phagocyte locomotion**

Three dimensional collagen matrices were prepared as described by Shields et al. (1984) by adjusting the osmolarity and pH of the stock collagen solution (at 5 mg/ml) to physiological levels. Briefly, 1 ml of 10X HBSS, 100 µl of 1 M MOPS (pH 7.4), distilled water, FMLP in HBSS-MOPS and 0.142 M NaOH (added if necessary to adjust the pH) were rapidly mixed to the stock collagen to give a 10 ml solution of collagen at 1.5 mg/ml (collagen A) with FMLP added at any desired concentration. Another solution of collagen was prepared at 1 mg/ml (collagen B) without FMLP. Once these solutions were prepared the collagen formed a gel within a few minutes.

Gels for chemokinetic experiments with neutrophils were prepared by rapidly pouring a 0.5 ml portion of collagen A (containing FMLP at a uniform concentration between $10^{-9}$ M and $10^{-6}$ M) into the wells of a tissue culture multi-well plate (Flow Labs. Ltd., Rickmansworth, Herts, England) with 24 flat-bottomed wells (1.7 X 1.6 cm). Provided the collagen stock solution was used within 2-3 months of preparation, it gelled within 5-10 min at room temp. The gels were allowed to set for 1 h before use. Then they were overlaid with neutrophils in

suspension containing the same concentration of FMLP as was present in the gel, so that the FMLP concentration was constant throughout ( Fig. 3 A ). For control experiments, FMLP was absent from both collagen and the cell suspension. Neutrophils, suspended in HBSS-MOPS (±FMLP) were used at $10^6$ cells/well in 1 ml.

Gels for chemokinetic assays with monocytes were prepared by rapidly pouring a 0.5 ml portion of collagen A containing 5 X $10^{-9}$ M FMLP into the wells of a multi-well tissue culture dish. The concentration of FMLP incorporated into the gels was the optimal dose that induced the maximum number of monocytes to assume locomotor morphology, as will be described later. After formation of a firm gel they were overlaid with monocytes in suspension in the same concentration of FMLP as was present in the gel, so that FMLP concentration was constant throughout. For control experiments, FMLP was absent from both collagen and cell suspension. Monocytes were suspended in HBSS-MOPS (± FMLP), HSA (20 mg/ml) in HBSS-MOPS (± FMLP) and HSA (20 mg/ml) in HBSS-MOPS + (FMLP + AHS, both at optimum dose) and used at $10^6$ cells/well in 1 ml. The optimum dose for (FMLP + AHS) was determined by the shape change assay, as will be described later.

Gels for chemotactic assays were prepared as follows: a 0.4 ml portion of collagen A containing FMLP at a chosen uniform concentration between $10^{-10}$ M and $10^{-5}$ M was poured as above and allowed to set. This was to serve as a gradient source. One hour later a 0.3 ml

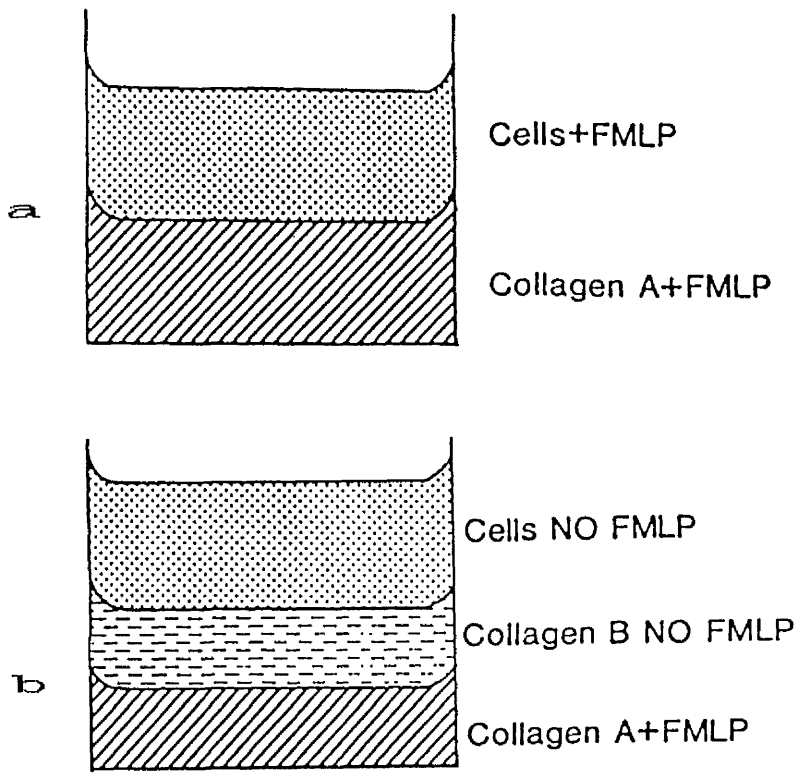



Fig. 3 Schematic diagrams of the population distribution assay. a: The assay for neutrophil/monocyte invasion of collagen gels in an isotropic concentration of FMLP. b: The assay for neutrophil invasion of collagen (1.0 mg/ml) in a gradient of FMLP. Hatched areas indicate collagen gels containing FMLP which then diffuse through the upper layer (broken lined area) of collagen B (containing no added FMLP) to form a gradient. The dotted areas indicate liquid media containing cells with or without FMLP. For control experiments, FMLP was absent from both collagen gels and the cell suspension.

portion of collagen B (without FMLP) was quickly and carefully layered over the first gel and allowed to set. This was to form a layer through which the FMLP could diffuse to form a gradient (Fig. 3 B). A suspension of neutrophils at $10^6$ cells in 1 ml of HBSS-MOPS was added to the top of the collagen B layer 20-60 min later. In control experiments, the same procedure was followed except that collagen A lacked FMLP. The height of the combined layers A and B was approximately 3.5 mm and the gels were optically clear enough to allow good visualization of cells deep within the gel using the fine focusing adjustment of an inverted phase-contrast microscope (Nikon, MSD). The tissue culture dishes were immediately incubated at 37°C for cells to invade the gel.

Neutrophils and monocytes were allowed to invade the gels for various times. The gels were then fixed by addition of 1 ml Glut/HBSS-MOPS for 30 min. Then the upper surface of the gel was washed 5 times with HBSS-MOPS to remove non-invaded cells. The following measures of cell invasion were used.

(i) A leading front measure, i.e., the distance that the leading 2 cells in the same focal plane in a microscopic field had migrated (Zigmond and Hirsch, 1973). Four randomly chosen fields in each of 6 identical wells were counted for neutrophils. For monocytes, 10 randomly chosen fields were counted from each of 2 identical wells.

(ii) The vertical distribution of the neutrophil and monocyte populations through the gel was determined by

counting the number of cells in each of a series of planes starting 40 μm below the gel surface and moving down at 40 μm intervals until there were no more cells to be counted. The number of cells in 100 squares of a 10 X 10 graticulated eyepiece was counted in each plane at X200 magnification. Ten to 16 readings were taken from 4 identical wells for neutrophils and from 2 identical wells for monocytes for the average cell number (N) at a particular distance (d, μm) from the upper gel surface.

(iii) To determine the absolute number of cells inside the gels, the number of neutrophils (for chemokinesis assays only) and monocytes recovered by washing the gel surface 5 times with HBSS-MOPS was counted in a haemocytometer. Since the number of cells initially added to the gel surface was known, the number invading the gels could be indirectly calculated.

In some of the experiments, collagenase (127 units/mg, Worthington, Millipore Ltd., U.K.) at 2 mg/ml in HBSS-MOPS was allowed to digest the gels from the upper portion so that some of the invaded cells were detached. This was done after washing the gels (at the end of incubation) with HBSS-MOPS at 37°C before fixation. One ml of collagenase was added to each well at 37°C with gentle shaking of the dish from time to time so that the digestion of gels in the well was uniform. Collagenase was quickly removed after 5 min and the remaining gels were fixed with 1 ml of Glut/HBSS-MOPS which at the same time stopped the collagenase reaction. In control experiments it was observed that 5 min

incubation could digest gels up to 85 μm (range 70-100 μm) below the surface. After fixation, the gels were washed several times to recover the detached cells. These cells were counted as before. Thus it was possible to determine the approximate number of cells remaining which had invaded the gel to about 100μm below the surface.

**Mathematical analysis of final cell distribution**

The mathematical analysis of final neutrophil distribution was carried out by Dr. I. C. McKay, Department of Bacteriology and Immunology. University of Glasgow.

*(a) Simple diffusion model*

If cells start from the top surface of the gel and move into it by random migration, then their distribution at the end of the experiment should correspond to one half of a normal distribution curve, and could be represented by the equation below.

$$N = \frac{2a}{s} e^{-d^2/2s^2}$$

where N is the number of cells in focus at a depth d in the gel, a is a constant proportional to the number of cells in the gel and S is root-mean-square displacement attributable to random migration.

*(b) Diffusion with drift model*

If cells start from the top surface of the gel and move into it partly by random migration and partly by a

superimposed directed movement of uniform velocity v normal to the surface, then their distribution at the end of the experiment should be given approximately by the equation (personal communication of Dr. G. A. P. Wyllie, Department of National Philosophy , University of Glasgow).

$$N = \frac{a}{s} \left[ e^{-(d-vt)^2/2s^2} + e^{-(d+vt)^2/2s^2} \right]$$

whhere N, a, s, and d are as defined above, and vt is the distance of drift (i.e., drift velocity v, multiplied by time t).

Each of these equations was made to fit the observed cell distributions for each microscopic field by adjusting the values of a, s and v by means of a trial and error computer programme, until the value of chi-squared, which measures the discrepancy between observed and theoretical cell number, was as small as possible. The optimum values of s and v, as judged by this criterion, were taken as measures of random migration and directional migration, respectively. These mathematical analyses were carried out for final neutrophil distributions only.

**Preparation of collagen-coated coverslips**

Circular glass coverslips (diameter 16 mm) were immersed in a hot solution of stericol (5% v/v) for 30 min. They were then washed thoroughly in running tap water and finally rinsed with distilled water followed by

blot drying with soft tissues. The procedure described by Brown (1982) was followed to prepare collagen-coated coverslips. Each clean coverslip was immersed in 1 ml solution of collagen at 100 μg/ml in 1% v/v acetic acid for 10 min. Then the collagen was precipitated onto the coverslip by salting out with 0.5 ml of 20% w/v sodium chloride. After that the coverslips were incubated at 37°C for 1 h followed by washing with HBSS-MOPS to remove acetic acid and NaCl.

**Monocyte adhesion assay**

Earlier workers reported that human monocytes (Douglas, 1976 ; Bumol and Douglas, 1977) and mouse macrophages (Rabinovitch and DeStefano, 1973a and b) undergo cytoplasmic spreading following attachment to a glass surface . Adhesion to different substrata was determined by placing 4 X $10^5$ monocytes in 0.4 ml of HSA at 20 mg/ml in HBSS-MOPS on clean glass and collagen-coated glass coverslips (both 16 mm in diameter) placed at the bottom of the wells in multi-well plates. In some experiments, FMLP at 5 X $10^{-9}$ M was added to the cell suspension before adding to clean glass coverslips. The coverslips with cells on were incubated at 37°C for 30 min, the cells were fixed with Glut/HBSS-MOPS for 10 min then washed 10 times through an air/HBSS-MOPS interface to detach non-adherent cells. Then the cells on the coverslips were stained with Giemsa (BDH Chemicals Ltd., Poole, England, 1% w/v solution prepared in 50% glycerol and 50% methanol) for 5 min, washed in tap water and wet

mounted. The number of cells in 5 fields of a X40 phase objective in a Vickers microscope were then counted on each coverslip and the mean ± standard error of the mean of several replicates was calculated. Adhesion to clean glass was taken as 100%. In all experimental controls, 90-95% of the total cells adhered to clean glass.

**Monocyte shape change assay**

The basis of this experiment was to measure the polarising responses of monocytes in suspension in the presence of uniform concentrations of chemotactic factors. Several substances were tested (either alone or in combination with others) which have at least some effect on neutrophil/monocyte locomotion as reported in the literature.

Monocytes suspended in HSA at 5 mg/ml in HBSS-MOPS tended to form small aggregates when incubated for 20-30 min at 37°C. As single cells only could be counted and their outlines drawn, monocytes were routinely suspended in 20 mg/ml HSA in HBSS-MOPS for the polarisation assays. At this HSA concentration aggregation was less of a problem. Monocytes were suspended at $10^6$ cells/ml in a siliconized glass tube. Aliquots of 200 µl of the cell suspension were taken into several siliconized tubes to which the appropriate factor was added. Then the cells were incubated for various times at 37°C. After that they were fixed by Glut/HBSS-MOPS for 10 min. The fixed cells were washed twice in HBSS-MOPS at 450 g X 10 min each, followed by resuspension in a few drops of the medium.

**Scoring of monocytes in the shape change assay**

The fixed cell suspension was examined by phase-contrast optics using a Vickers microscope with X40 objective. Any cell deviating from a spherical outline was scored as polarised and polarisation was expressed as a percentage of the total cells counted. Monocytes with spherical outlines but containing a few filamentous projections were counted as round cells. When a proportion of the cell surface was ruffled with a constriction separating the ruffle from the spherical part of the membrane then the cell was scored as a non-round, i.e., polarised cell. A total of 300-400 cells were counted from each preparation.

For all the monocyte preparations used in the shape change assays, a side by side non specific esterase staining was carried out to determine the percent ANAE positive cells in the initial cell suspension. The percentage of monocytes that were polarised in the shape change assay was calculated by the formula below.

% monocytes polarised = (% total cells polarised / % ANAE positive cells in the monocyte preparation) X 100

**Monocyte morphology in suspension**

After scoring monocytes in the shape change assay some of the preparations were used to draw cell morphology. The image of 100 randomly chosen monocytes from each of these preparations was projected onto paper and the outline traced using a camera lucida drawing tube fitted to a wild microscope (X100 objective, n.a. 1.3). The longest axis of each cell was measured accurately to

give the length and the perpendicular distance at the mid-point of the length was measured to give the breadth of each cell. The ratio of length/breadth was calculated to give a "polarisation index" (P.I.) of each monocyte. Monocyte lengths and their P.I. values were compared between different doses of chemoattractant stimulated populations.

**Measurement of the polarisation index of neutrophils**

Neutrophils at 5 X $10^5$ cells in 1 ml of HBSS-MOPS were incubated alone and in the presence of FMLP at $10^{-8}$ M in HBSS-MOPS for 30 min at 37°C. Then the cells were fixed, washed, and the percentage of polarised cells counted in different preparations, as described for monocytes.

The outline of 100 randomly chosen neutrophils from each preparation were drawn, length and breadth ratios determined, and their P.I. values were compared to those of monocytes at their optimum polarisation with FMLP.

**Monocyte shape change in culture**

Monocytes at $10^6$ cells in 1.5 ml were cultured in two different culture media, i.e., (a) 20% FCS in RPMI and (b) 10 mg/ml HSA in RPMI, both containing penicillin-streptomycin and glutamine. The cells were cultured in siliconized glass tubes (4 X 1/2") up to 3 days at 37°C. An aliquot of cells was removed from the culture at a particular time and, without being allowed to cool were fixed in suspension at 37°C with Glut/HBSS-MOPS. The fixed cells were washed twice with HBSS-MOPS and the

percent polarised cells was determined as described before. In every case a small aliquot of cell preparation was withdrawn from the culture tube at 20 min after setting up. After fixation at 37°C and 2 washes, microscopic examination of this was used as a control (short-term) for the shape change in culture experiments. In one occasion, lymphocyte activating monoclonal antibody, anti-T3 (OKT3, obtained from Ortho Diagnostics, Raritan, NJ) was added to the cells at 5 ng/$10^6$ cells and was used in a short-term and in a culture experiment.

For the determination of cell viability, the cultured monocytes were examined under a phase objective and also by their ability to exclude trypan blue dye. In all experiments, the proportion of polarised cells was estimated from the total of viable cells. The outlines of monocytes were drawn in short-term and in culture experiments and their lengths as well as P.I. values were compared as described before.

Monocyte culture supernatant (MCS, collected after culturing the cells for 3 days) was added to freshly prepared cells and incubated for 20 min at 37°C. Then they were fixed and the polarised cells were counted. In some experiments $10^{-5}$ M cycloheximide (3[2(3,5-di methyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide, M. wt. 281.3, obtained from Sigma, Poole, Dorset ) was added to the monocytes and cultured thereafter for 24 h and the supernatant was collected. Also the same dose of cycloheximide was added to the supernatant obtained from a 24 h monocyte culture (with no cycloheximide added to the cells during culture). The polarising responses of

freshly prepared monocytes were tested against these supernatants.

**Visual assays of monocyte locomotion**

***(a) Chemotaxis towards Candida albicans*** *blastospores in human serum.*

The filming chamber used for visual assays of monocyte locomotion was a rectangular aluminium slide (76 X 26 X 1.5 mm) with a hole of 20 mm diameter drilled out of the centre. The details of assays with these chambers were described previously by Allan and Wilkinson (1978). The metallic slide was washed in stericol and rinsed thoroughly before use. Square coverslips (22 X 22 mm) were also washed in stericol, rinsed thoroughly in running water, finally rinsed with distilled water and then blot dried with soft tissues. A clean coverslip ringed with silicone vacuum grease (Beckman Inst., Palo Alto, Calif., U.S.A.) was attached to the hole on the filming chamber to form a well with a volume of 0.5 ml.

Living *C. albicans* blastospores, from a 24 h, 37°C culture were suspended in HBSS-MOPS and washed at 600 g for 5 min. They were opsonised by resuspending in human serum followed by incubation for 30 min at 37°C. Then the spores were washed once with HBSS-MOPS and finally resuspended in the same medium at 3.0-3.5 X $10^6$ spores/ml. In some of the experiments unopsonised candida spores were used for chemotactic assays.

A sample of 100 µl of human monocytes at $10^6$ cells/ml in HSA/HBSS-MOPS was taken on to the coverslip at the bottom of the well in the filming chamber. It was

placed in a moist atmosphere for 5 min at room temp which allowed the cells to settle on and adhere to the coverslip. The medium was carefully removed from the well leaving the cells on the coverslip. Then the well was filled immediately with 0.6 ml of a mixture prepared with candida suspension (100 µl), normal human serum and HSA/HBSS-MOPS (added in variable proportion to make 500µl). A clean square coverslip (22 X 22 mm) was ringed with silicone vacuum grease and placed on to the open well. The coverslip was pressed gently on the side of the well to set it, taking care to exclude air bubbles from the chamber while excess medium was wiped off with soft tissues. The filming chamber was immediately placed on a Nikon phase-contrast, inverted microscope (model MSD) stage where the temp was maintained at 37°C using a warm air curtain. Thus, at the onset of the assay, monocytes were present on the bottom coverslip and *C. albicans* spores were in suspension. During the next 5-10 min the preparation reached 37°C and the cells completed the transient adaptation changes (Zigmond and Sullivan, 1979) and most of the spores settled on to the lower coverslip around the adherent monocytes. The filming chamber used for this assay provided good optical quality to visualize the behaviour of either a population of cells or a single cell (using better optics) and prevented evaporation of medium.

Each spore or group of 2 or 3 spores acted as a very small source continuously generating chemotactic factors from the surrounding serum. As described earlier, chemotactic factors may be produced from the classical

(antibody mediated) pathway of complement activation or from the alternative pathway as the polysaccharides present in the cell wall of spores are known to activate complement. As mentioned before, the serum used for the visual assays contained anti-candida IgG antibody at 16 μg/ml. The response of monocytes to the appearance of spores around them could often be followed from the moment a spore appeared in the field to the time of phagocytosis. This assay therefore allowed the visual observation of the initial reaction of the mononuclear phagocytes to a gradient of chemotactic factors, their migration towards the source (in this case, spores) and the ultimate phagocytosis of the spore.

To record monocyte chemotaxis towards candida spores, filming was started 10 min after setting up the chamber with a Bolex H 16 reflex 16 mm camera and Nikon cine autotimer (CFMA) attached to the microscope. A suitable field was filmed (with an area 280 X 400 μm) near the middle of the well to minimize the effects of convection currents. Filming was performed at 1 frame/ 4 s, using a X10 objective and X10 eyepiece and Kodak plus-X reversal film 7276. Each sequence was filmed for 45-50 min. These cine films were developed commercially (Brent Labs. Ltd., London).

In some experiments, monocytes kept in culture for 18 h and 3 days (in 20% FCS / RPMI-MOPS containing penicillin-streptomycin and glutamine) were used for candida phagocytosis. The culture medium was removed by centrifugation and the cells were suspended in HSA at 10

mg/ml in HBSS-MOPS. The rest of the procedure was as described before.

*(b) Locomotion on two-dimensional substrata*

The setting up of the filming chamber for this assay was as described before. Monocytes were suspended in HSA/ HBSS-MOPS. The list below describes the substances used for time-lapse filming on two-dimensional substrata.

(i) HSA (5, 20, 50 and 100 mg/ml)

(ii) FMLP at $10^{-7}$ M and 5 X $10^{-9}$ M, in HSA (5, 20, 50 and 100 mg/ml)

(iii) C5a at $10^{-8}$ M in HSA (20 mg/ml)

(iv) Fresh human serum with and without 5 X $10^{-9}$ M FMLP

The concentrations 5 X $10^{-9}$ M for FMLP and $10^{-8}$ M for C5a were the respective optimum doses as determined by the shape change assay.

The filming chamber was filled with the cell suspension to contain 2.5-3.0 X $10^{5}$ monocytes. This concentration was chosen to minimize cell-cell collisions, which make analysis difficult. The chamber was sealed with an upper coverslip and was placed on the warmed (37°C) stage of an inverted Nikon microscope. Filming started 10 min after setting up and extended over a period of 1 h.

In some experiments, Fc rosetted monocytes were used for time-lapse filming. The procedure for the rosetting assay will be described later. Monocytes in 20 mg/ml HSA were allowed to adhere to clean glass coverslips by incubating for 20 min at 37°C. Then these cells were incubated with antibody-coated sheep red cells to form Fc

rosettes. After that the coverslips were washed several times to remove unbound red cells. These coverslips were used as bottom coverslips in the matallic filming chamber. HSA at 100 mg/ml in HBSS-MOPS + 5 X $10^{-9}$ M FMLP was used to fill the chamber. The rest of the procedure was as described before except that cells were filmed at 20 and 30°C.

*(c) Locomotion in three-dimensional matrices*

About 0.4 ml of collagen A (preparation collagen A was described earlier) containing FMLP at 5 X $10^{-9}$ M was poured into the wells of several aluminium filming chambers and allowed to set. Then a 200 µl suspension of monocytes at $10^{6}$ cells/ml in HSA at 20 mg/ml in HBSS-MOPS containing 5 X $10^{-9}$ M FMLP was carefully layered over the gels in the filming chamber and allowed to settle for 5 min at room temp. Thus the concentration of FMLP in the gels and in the cell suspension was uniform. Then the chambers were sealed with an upper coverslip and incubated at 37°C for various times (45 min to 2½ h), thus allowing the cells to migrate into the gels before filming started. At the end of incubation, each chamber was placed on the warm stage (37°C) of an inverted microscope (Nikon, MSD) and a field near the centre of the well was chosen. The microscope was focused so that the behaviour of monocytes within the collagen gel could be observed using X20 phase-contrast optics. Locomotion of these monocytes, usually 40-120 µm below the gel surface was then filmed by time-lapse cinematography

using a lapse interval of 1 frame per 4 s. Each sequence was filmed for approximately 1 h.

**Analysis of monocyte locomotion in visual assays**

Films were projected onto drawing paper using a stop-action 16 mm cine projector. The position of each monocyte was marked at the first frame. After each subsequent 10 frames (40 s) the cell centre was marked in chemotaxis sequences and after each 20 frames (80 s) the cell centre was marked for chemokinesis sequences. These mid-points were joined to give a cell track. The positions of *C. albicans* spores and points of phagocytosis were also recorded. Since the distance between each recorded step represented the distance covered in a fixed time and the dimensions of the field were known, measurement of the steps could be expressed directly as speeds in μm/min. The cell tracks were analysed for velocity and persistence of individual cells and for the population. This otherwise time-consuming procedure was made possible and greatly simplified by using a graphic digitizing tablet (Summagraphics Ltd., Bit Pad One) linked to a BBC computer, with a programme written by Dr. J. M. Lackie, (Department of Cell Biology, University of Glasgow) based on the mathematical calculation done by Dr. G. A. Dunn (MRC Cell Biophysics Unit, London). Details of this type of analysis (Wilkinson et al., 1984) and the programme used are published elsewhere (Lackie and Burns, 1983; Lackie and Wilkinson, 1984). This programme expressed S as the rms speed of the cells (μm/min), P as a measure of the

directional persistence time (min) and R as the rate of diffusion of the cells ($\mu m^2$/min).

The angle of turn between succeeding steps was measured from the cell tracks. To avoid inaccuracy in estimating the mid-points of the translocating monocytes, angles below 10° were not considered as discrete turns. It should be mentioned here that moving cells, in general, gradually change direction rather than making sudden turns; also a cell moving in a curved path will not show any discrete angle of turn if observed continuously but the choice of frequency of observation will affect the degree of turn measured. However, if the frequency of observation is constant between different population of cells, their turning behaviour could be compared.

The term "chemotropism index" described by Dixon and McCutcheon (1935) was adopted and hereafter referred to as "chemotactic ratio" was used for quantitative expression of chemotaxis. It was obtained by dividing the straight line distance from the point at which a cell started locomotion up to the end of its path (i.e., the point of phagocytosis) by the total distance travelled by the cell between the two points. The original definition describes positive and negative chemotaxis on a scale where +1.0 is for locomotion in a straight line towards the gradient source, and -1.0 is for locomotion in a straight line away from the source.

For locomotion on 3-dimensional collagen matrices, the position of each monocyte was marked at the first frame and after each subsequent 20 frames (80 s) the cell

centre was marked. The mid-points were joined to give a track which then allowed the cell speed and angle of turn to be calculated as described above. Frame by frame analysis of the time-lapse film indicated the morphological changes involved in monocyte migration through the gel matrix..

**Studies of Fc receptor distribution on monocytes using antibody-coated sheep red cells**

The localization of several ligand binding receptors, for example, C3b, FMLP and Fc were studied in neutrophils by different workers (Sullivan et al., 1984; Shields and Haston; 1985). In this study the distribution of Fc receptors on polarised monocyte membranes is demonstrated by using antibody-coated sheep red cells.

*(a) Determination of subagglutinating dose of antibody*

To obtain good Fc rosettes, it is essential to use a subagglutinating dose of antibody, so the agglutinating dose of the antibody was determined first. Rabbit anti-sheep red blood cell antibody, (hemolysin), prepared by Difco Labs., Detroit, Mich., U.S.A., was obtained by courtesy of Dr. D. A. R. Simmons, Department of Bacteriology and Immunology, University of Glasgow. Sheep red blood cells (SRBC, obtained in Alsever's solution, Flow Labs. Ltd., Rickmansworth, Herts, England) were washed thrice with HBSS-MOPS for 5 min each, at 350 g. Then the packed cells were resuspended to give a 1% v/v solution in HBSS-MOPS. The antibody was diluted with HBSS-MOPS to give different dilutions as shown in the

table below. One hundred microlitres of each dilution were mixed with 100 µl of the red cell suspension in different wells of a 96 well round-bottomed microtitre plate (Sterilin Ltd., Teddington, England) and incubated at 37°C for 1 h. Agglutination was detected by the sedimentation of red cells at the bottom of the wells. Agglutination was present in the first 3 wells containing higher concentrations of antibody. In the last 5 wells most of the red cells were in suspension with a tiny pellet at the bottom of the wells, were identical with the control wells (because of undisturbed incubation). The well containing 1/160 dilution showed some agglutination of red cells but the antibody concentration was not enough to link up all the red cells. As a result a lot of red cells were in suspension. There was no red cell

| Dilution of anti-sheep red cell antibody | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **1/40** | **1/80** | **1/120** | **1/160** | **1/200** | **1/250** | **1/300** | **1/350** | **1/400** | **1/500** |
| **++++** | **+++** | **++** | **±** | **-** | **-** | **-** | **-** | **-** | **-** |

+ve sign indicates agglutination

-ve sign indicates no agglutination

agglutination in the well containing 1/200 dilution. So this dilution was considered to be the highest subagglutinating dose of the antibody used, and was used to coat sheep red cells for Fc receptor studies.

*(b) Fc receptor distribution on polarised monocytes*

Monocytes were suspended in HSA (5-20 mg/ml) in HBSS-MOPS at 5 X $10^5$ cells/ml. About 400 µl of the cell suspension with or without FMLP at 5 X $10^{-9}$ M was placed on clean glass coverslips (22 X 22 mm) and incubated for various times (20-60 min) at 37°C. Sheep red blood cells, washed thrice with HBSS-MOPS, resuspended at 1% v/v in HBSS-MOPS containing 1/200 dilution of antibody, were incubated for 30 min at 37°C. Then the red cells were washed thrice with HBSS-MOPS to remove excess antibody. The antibody-coated red cells were finally resuspended at 1% v/v with HBSS-MOPS at 4°C.

Monocytes on coverslips were washed once with HBSS-MOPS at 4°C after the end of incubation. Then they were covered by the antibody-coated cold red cell suspension and were incubated for 1 h at 4°C for Fc rosetting. After that monocytes were fixed with Glut/HBSS-MOPS for 10 min at room temp. Then the fixative and excess red cells (unbound) were washed off the coverslips with HBSS-MOPS. The coverslips were finally washed through an air/medium interface to remove the unbound cells and then wet mounted. The preparations were examined under a X40 phase objective in a Vickers microscope. Three or more red cells attached to a monocyte was scored as Fc rosetted. The total percentage of monocytes forming Fc rosettes and

the distribution of rosettes in polarised and round cells were determined by counting at least 300 cells from each preparation.

In some experiments, monocytes were incubated for 10 min at 37°C with antibody-coated red cells to form Fc rosettes. The proportion of cells forming Fc rosettes were scored as described before.

**Photomicrography**

Still photographs were taken using an Olympus microscope equipped with phase-contrast and differential interference contrast (Nomarski) optics with X40 and X100 objectives and a Nikon inverted microscope (model MSD) fitted with X20 phase-contrast optics. Photomicrographs were taken on Kodak Panatomic X and Kodak Ektachrome 35 mm films with a Nikon microflex camera (model AFBM). Panatomic X films were developed through HC 110 and Unifix and were printed on Kodagraph projection (P84) paper, and Kodabrome II RC paper using Dectol and Unifix. All photographic reagents and papers were obtained from Kodak Ltd., Manchester, U.K. Coloured films (Ektachrome) were developed and printed commercially.

# CHAPTER 3

# EVALUATION OF METHODS FOR PURIFICATION OF HUMAN MONOCYTES IN SUSPENSION

The participation of mononuclear phagocytes in immunological reactions, the modulation of other cell functions, the elimination of damaged cells and invading microorganisms are an area of active investigation. However, the exact role of these cells is not well understood, partly because purified, functionally intact monocyte populations are difficult to obtain. Thus, finding a suitable method for monocyte purification is important.

At the beginning of this investigation, a choice had to be made between the different available methods for purifying monocytes from blood. This chapter describes the procedures that were tried. Most were for one reason or another unsatisfactory, and the method of Ackerman and Douglas (1978) using BHK microexudate-coated plastic was eventually chosen for the rest of the study, as detailed below.

**(a) Discontinuous density gradients of percoll**

Several workers used percoll gradients to separate human peripheral blood mononuclear cells (MNCS). Hardin and Downs (1981) used density marker beads in the reorienting gradients of percoll and reported the density of monocytes to be 1.040-1.045 g/ml, lymphocytes 1.055-1.058 g/ml and platelets 1.034 g/ml. Based on this idea, different densities of percoll (1.04, 1.05 and 1.08 g/ml) were prepared according to the regression equation described by Ulmer and Flad (1979). The equation is

$$\text{Density (g/ml)} = (\%\ \text{SIP} \times 0.001186) + 1.0041$$

The percoll solutions were carefully layered in ascending order of densities from top to bottom in a conical base plastic centrifuge tube and MNCS (1-2 X $10^7$) suspended in 2 ml of HBSS-MOPS were placed at the top of the gradient and the tube was centrifuged. Then the cells were collected from different interfaces and washed thrice. It was observed that a lot of cells were stuck to the tube used for percoll density centrifugation. After staining the cells for acid α-napthyl acetate esterase (ANAE) stain, it was observed that 30-50% cells isolated from the top 2 interfaces were monocytes. Similar results were obtained in 3 experiments with these densities of percoll. The maximum purity of monocytes obtained in these experiments was 55% and the total recovery of cells in each band combined together was about 30-40% of the total cells applied to the gradient.

Another approach was made to prepare different densities of percoll according to the formula below, described by Fluks (1981)

$$\text{Density (g/ml)} = (\%\ \text{SIP} \times 0.001151) + 1.0096$$

Different densities of percoll (1.05-1.08 g/ml) were prepared and used in different combinations. In 2 experiments, percoll densities of 1.06, 1.07 and 1.08 g/ml were used in ascending order from top to the bottom of the tube and MNCS were placed at the top. After centrifugation and subsequent washing of cells, it was observed that about 50% of the total cells applied were recovered in different fractions. The purity of monocytes (isolated from each percoll interface) varied from 30-

40%. That is, monocytes were present in all the interfaces.

In 2 other experiments, 4 different densities of percoll (1.05-1.08 g/ml) were layered in one tube as described before and MNCS were centrifuged after placing at the top of the gradient. Fractions were collected from every interface in between (i) the medium and percoll at 1.05 g/ml, (ii) percoll at 1.05-1.06 g/ml, (iii) percoll at 1.06-1.07 g/ml and (iv) percoll at 1,07-1.08 g/ml. There was no cell in (i); (ii) consisted of MNCS, mostly non-separated; about 72% cells recovered in (iii) were monocytes and 90% cells recovered in (iv) were lymphocytes. Unfortunately, 20-30% of the total cells applied on the gradient were recovered from all the fractions.

For the last 2 experiments in each set, 2 densities of percoll (i) 1.06 and 1.07 g/ml were used in one centrifuge tube and (ii) 1.07 and 1.08 g/ml were used in another tube. Equal numbers of MNCS were placed in both the tubes and they were centrifuged under identical conditions. The result suggested that the maximum separation of MNCS occurred at the interface between 1.06 and 1.07 g/ml of percoll in tube (i) where 50-60% of the total cells were monocytes. In tube (ii) the percoll interface fraction consisted of about 35% monocytes. Again, the total cells recovered from these tubes was poor. Cells were lost during several washing steps and a lot of them adhered to the tube used for percoll density centrifugation. Considering the poor separation and low yield, the discontinuous density gradient centrifugation

of mononuclear cells over percoll was not suitable to purify monocytes.

**(b) Use of Nycodenz-Monocyte separation medium**

The commercially available medium Nycodenz-Monocyte was used to purify MNCS into lymphocytes and monocytes. The principle of this procedure was described by Böyum (1983) who mentioned that increasing osmolarity caused cells to expel water and shrink while their densities increased; then lymphocytes, which perhaps overlap in density with monocytes, were more sensitive to an increase of osmolarity than the monocytes. As a result lymphocytes would move through the density barrier that was present initially and move to the bottom of the tube during centrifugation while monocytes would remain at the upper portion of the interface (Böyum, 1983).

Though Böyum (1983) reported that this procedure can purify monocytes up to 95-98%, it was not possible to obtain more than 50% pure monocytes in 3 experiments. The reason for this could be partly due to an estimate of the fraction to be collected after density centrifugation of the leucocyte-rich plasma over the Nycodenz-Monocyte medium. The fraction was collected from 3-4 mm above the interface between the plasma and the separation fluid up to slightly more than half the volume of the latter, as described by Böyum (1983). It was observed that the majority of cells present in the separation fluid were lymphocytes (about 50%) with up to 20% polymorphs. Even fractions collected only from the interface did not consist of more than 65% monocytes. Again, monocytes

purified by this procedure were completely surrounded by platelets during centrifugation over the separation fluid. It was not possible to remove them by washing the cells with 5 mM EDTA. The adherence of platelets to the monocytes made them less useful for the shape change and other proposed study.

**(c) Use of gelatin-plasma-coated flasks**

Recently, Bevilacqua et al. (1981), while studying fibronectin (FN) receptors on monocytes, demonstrated that human monocytes reversibly adhere to FN coated gelatin treated surfaces via a $Mg^{2+}$ dependent mechanism. The authors showed that human plasma could function as the source of FN and they suggested that this process might be used for the purification of monocytes. Based on this idea, Freundlich and Avdalovic (1983) described this procedure by using autologous plasma on gelatin-coated surfaces for the separation of mononuclear cells. Details of this procedure was described in chapter 2.

Due to practical limitations of obtaining 10-12 ml of platelet free plasma (neat) e.g., to draw about 30 ml of blood, it was not possible to use autologous plasma over 50% concentration (collected from 20-22 ml of blood).A lot of cells were observed to be adherent to the gelatin-plasma-coated surface after incubating the MNCS for 40 min and subsequent washing of the non-adherent cells. Only a fraction of these cells (up to 45%) were detached after incubating the flask with 10 mM EDTA in CMFS-MOPS/20% FCS in RPMI-MOPS (mixed at 1:1). The results from 3 experiments suggested that a maximum of

60% cells purified by this procedure were monocytes. These unsatisfactory results of low yield and purity made this procedure less useful for further experiments.

**(d) Use of gelatin-FCS-coated flasks**

This procedure was similar to procedure (c) described above except that neat FCS was used as the source of FN instead of using 50% autologous plasma to coat the gelatin treated flasks. It was considered that if there were patches of bare surfaces present in procedure (c), not coated with FN due to use of 50% plasma, those surfaces would be covered by FN present in the undiluted FCS. It was hoped that these surfaces might help better detachment of monocytes after using EDTA. Hence MNCS were incubated on gelatin-FCS coated flasks as described before. After washing off the non-adherent cells, detachment of the adherent cells with EDTA evolved similar problems as described in (c). Also the purity of the monocyte preparation was significanty lower (about 50%) in 2 similar experiments.

**(e) Using FCS-coated flasks**

The procedure described by Kumagai et al. (1979) was followed to purify monocytes from mononuclear cell preparation. Details of this procedure were as described earlier. After EDTA treatment of the adherent cells on FCS-coated flasks, it was observed that almost 50% of the total adherent cells remained stuck to the flask. Staining the recovered cells for ANAE suggested that 70-75% of the total cells purified by this procedure were

monocytes. Using FCS from 2 different batches to coat the plastic surface resulted in similar detachment of adherent cells. That is about half of the total adherent cells came off the flask after treating them with EDTA. As the yield was poor, this procedure was not convenient for working with small volumes of blood (20-22 ml).

**(f) Use of BHK microexudate-coated flasks**

The procedure described by Ackerman and Douglas (1978) was the most efficient one to separate monocytes from lymphocytes. The method followed was described in detail in the previous chapter. Some features of the cells purified by this procedure are presented below.

*(i) Purity of the monocyte fraction*

The results from more than 60 experiments suggested that monocytes purified by procedure (f) were more than 96% (S.D. = 2.7) pure as they were diffusely stained with the ANAE stain. The rest of the cells present in the monocyte fraction were probably esterase negative B-lymphocytes. In some experiments the purity of the monocyte fraction exceeded over 99%. Fig. 4 A, B and 5 B illustrates populations of monocytes positive for esterase.

*(ii) Viability and yield*

Over 97% of the monocytes purified by procedure (f) were viable as they excluded trypan blue. About 66% (S.D. = 13.3, n = 61) of the total monocytes present in the MNCS were purified by this procedure. A small

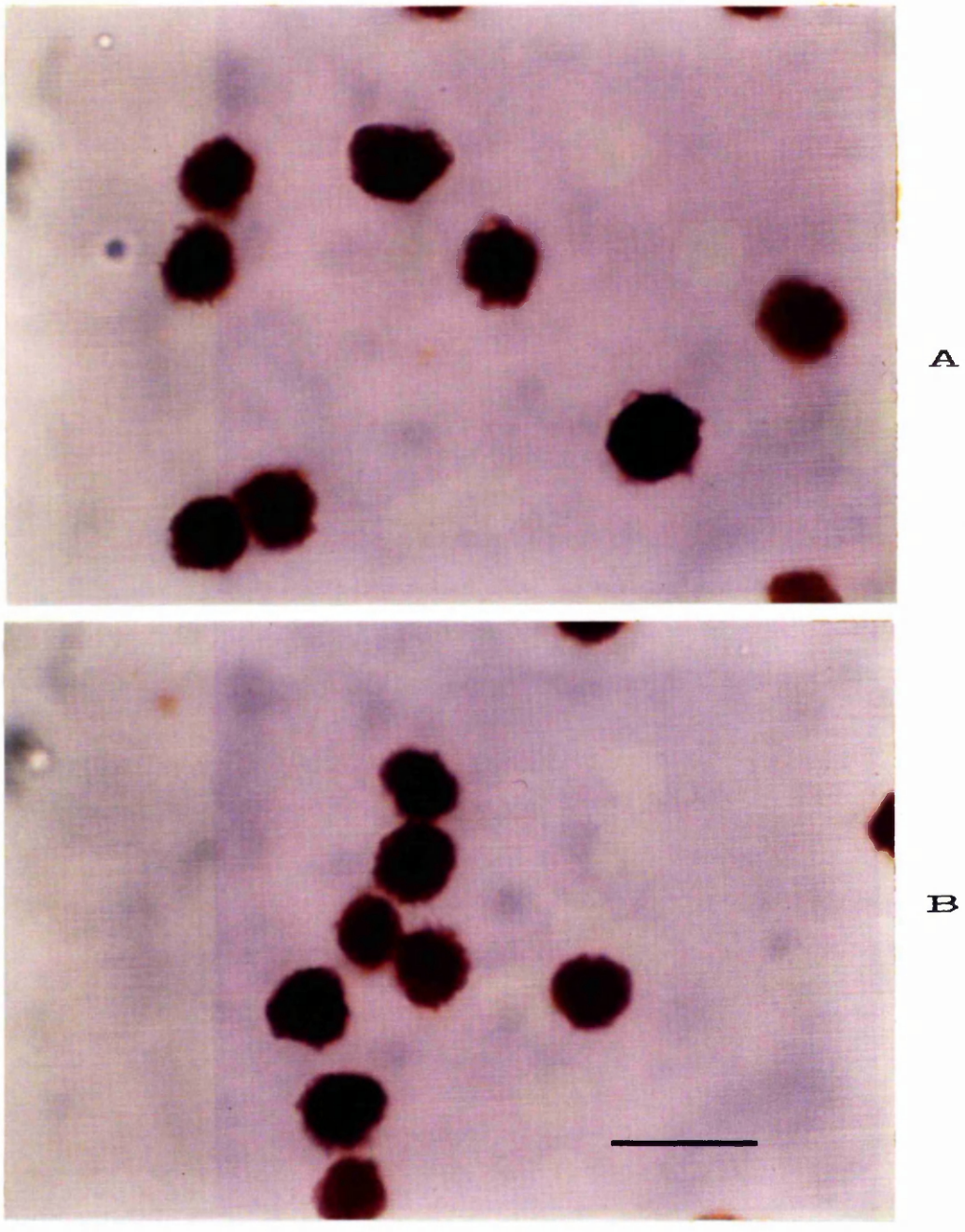



Fig. 4 Acid α-napthyl acetate esterase (ANAE) staining on monocytes purified on a BHK microexudate-coated surface. The purified monocyte preparations consisted of more than 96% ANAE positive cells. All the cells seen in A and B are monocytes (cf. Fig. 1 and 5 for ANAE stain on T-lymphocytes). Note the heterogeneity of monocyte size. Bar = 20 μm.

proportion of the adherent cells were not detached from the microexudate-coated flask after EDTA treatment. The proportion of these cells varied from 1% to a maximum estimate of 15% of the total blood monocytes. The rest of the cells, not possible to purify, were non-adherent. These cells were removed with the lymphocyte fraction, The presence of 3-8% monocytes in the lymphocyte fraction was confirmed by the ANAE stain.

*(iii) Morphology of the monocytes*

About 85% of the monocytes purified by procedure (f) had spherical morphology (cf. Fig. 6 A). The rest of the cells had polarised morphology even if they were unstimulated. It was considered that this small proportion of cells with polarised morphology was due to FCS (10%, v/v) present in RPMI-MOPS medium used to wash the cells after EDTA detachment of the monocytes. Hence serum free medium was used to wash the cells in some experiments. It was observed that monocytes were forming small aggregates during several centrifugation steps of washing at room temp. Moreover, the same proportion of cells were observed to be polarised in both cases when another fraction of cells detached from the same surface was washed with the RPMI-MOPS medium containing 10% FCS. In some experiments, the detached monocytes were washed with RPMI-MOPS containing HSA at 4 mg/ml. It was observed that almost the same proportion of cells were polarised after the final wash compared to the fraction of cells washed by the usual procedure. These results suggested that the small proportion of polarised cells present in

the unstimulated population of monocytes could be due to adhesion of the cells to the microexudate-coated surface. However, for routine washing of the detached cells, RPMI-MOPS containing 10% FCS was used throughout the study.

It was described in the Introduction that heterogeneity of human blood monocytes was reported by several workers. Differences in size were noticed among the monocytes present in the mononuclear cells and also in the monocyte fraction purified on a BHK microexudate-coated surface. That is monocytes of different sizes could be purified by this procedure. Fig. 5 illustrates the ANAE staining of monocytes of different sizes present in (A) the mononuclear cells and in (B) the purified monocyte fraction.

*(iv) Surface and cytoplasmic characteristics of isolated human monocytes*

About 95% monocytes purified on the BHK microexudate-coated surface were able to adhere to glass coverslips at 37°C during 30 min incubation. The spread cells were easily identified by their characteristic cytoplasmic veils with homogeneous and non-refractile cell body under phase optics (cf. Fig. 61 A).

Monocytes purified by procedure (f) changed shape in suspension with variable proportions in response to different chemoattractants. Up to 85% of the cells were polarised after incubating with a combination of 2 or more chemoattractants (cf. Fig. 29 and Table 5).

A proportion of the purified monocytes could invade three-dimensional collagen matrices and locomote on a

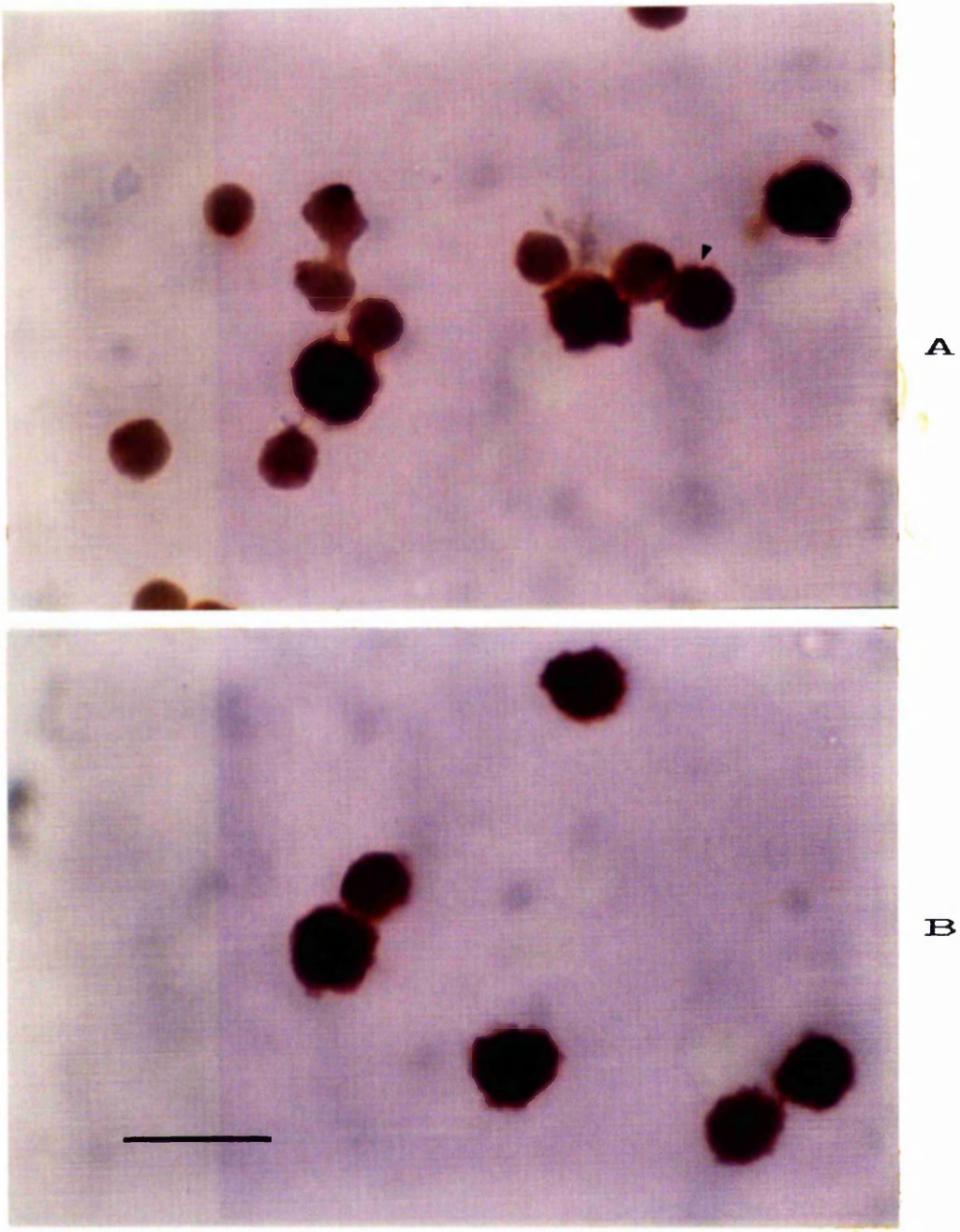



Fig. 5 ANAE staining of monocytes of different sizes. A: staining on mononuclear cells before separating the monocytes. Note the monocyte marked with an arrow is of the same size as the nearby lymphocyte and is much smaller than other monocytes seen in the photograph. B: Monocytes of different size stained with ANAE after purification on a BHK microexudate-coated surface. Bar = 20 μm.

protein coated plane substratum (described in chapter 6 and 7).

Over 90% Of the purified monocytes formed Fc rosettes with antibody coated-sheep red cells (cf. Fig. 32 A and Table 7a).

Also 85-90% of the cells ingested latex particles when incubated under appropriate conditions (described in chapter 2). Fig. 2 (see page 78a) shows photographs of monocytes after ingesting latex beads followed by staining the cells with (A) ANAE and (B) Leishman stain. More interestingly the same proportion of monocytes (86-90%) were able to phagocytose *Candida albicans* blastospores in the presence of human serum (cf. Table 19).

These observations clearly indicated that monocyte populations purified on the BHK microexudate-coated surface were functionally intact.

In summary, several procedures (a) to (f) were followed to separate monocytes from the lymphocytes. Most of them, namely procedure (a) to (e) were not suitable because of poor yield and poor purity of the cells. Using BHK microexudate-coated surfaces for active adhesion of monocytes (procedure f) yielded a highly purified fraction of monocytes with an average yield of 2/3rd of the total monocytes present in the peripheral blood. A small proportion of the blood monocytes are not adherent and could not be purified by this method. Monocytes purified by this procedure were morphologically and functionally intact.

# CHAPTER 4

# MONOCYTE SHAPE CHANGE IN SUSPENSION IN UNIFORM CONCENTRATIONS OF CHEMOTACTIC FACTORS AND THE LOCALIZATION OF Fc RECEPTORS ON POLARISED CELLS

Monocytes were suspended at $10^6$ cells/ml in HSA (20 mg/ml)/HBSS-MOPS for the polarisation assays. Using the mononuclear cell preparation techniques described in the Materials and Methods and following the purification procedure reported by Ackerman and Douglas (1978) it was possible to obtain populations of monocytes with 84.0% (S.D. = 3.54, n = 56) spherical cells. When these cells were incubated in siliconized glass tubes without an attractant they remained spherical. In isotropic solutions of chemotactic factors, some cells responded within 1 min and became non-spherical with a morphology which showed early polarisation. Fig. 6 represents photomicrographs of monocytes incubated (A) without an attractant, (B) and (C) with an attractant (in this case FMLP). It is clear from Fig. 6 that cells were spherical after incubating for 20 min at 37°C without an attractant (A), a proportion of them changed shape in the presence of $10^{-7}$ M FMLP (B) and in 5 X $10^{-9}$ M FMLP (C) when incubated for the same length of time.

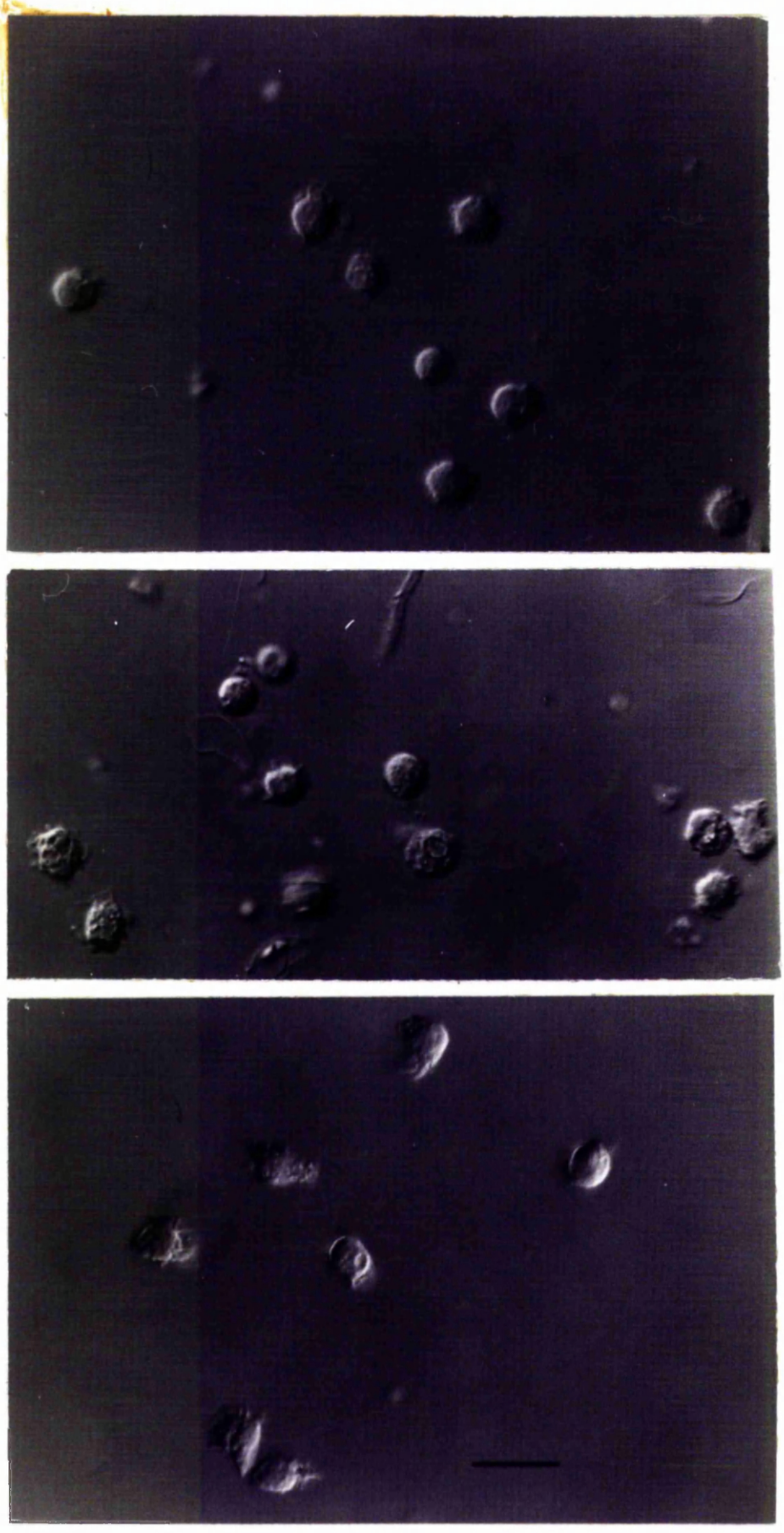
This section will consider the measurement of polarisation in detail using a single factor, FMLP. The responses to other chemotactic factors will be considered in section II and III. The localization of Fc receptors on polarised monocytes will be described in section IV.

## SECTION I

### Dose response for monocyte polarisation in FMLP

The polarisation responses of monocytes were tested using a wide range of FMLP concentrations ($10^{-12}$ M to

Fig. 6 A-C. Differential interference contrast micrographs of monocytes incubated with and without a uniform concentration of FMLP. The cells were fixed in suspension with 2.5% glutaraldehyde after incubation for 20 min at 37°C in HSA (20 mg/ml) in HBSS-MOPS ± FMLP. A: With no FMLP; B: $10^{-7}$ M FMLP in HSA (20 mg/ml); C: 5 X $10^{-9}$ M FMLP in HSA (20 mg/ml). Bar = 20 μm.



A

B

C

$10^{-6}$ M). The cells responded in presence of these FMLP doses but there were differences in both concentration and time of incubation in their responsiveness. Similar dose response curves were obtained in 7 experiments (presented in Fig. 7) in which monocytes were incubated for 20 min at 37°C. The maximum percentage of polarised cells, 65.73 % (S.D. = 5.67), was obtained with 5 X $10^{-9}$ M FMLP.

Monocytes in high concentrations of FMLP ($10^{-6}$ M or $10^{-7}$ M) were not well polarised. The few cells which responded had an irregular outline with cytoplasmic projections at many points on the cell surface. A portion of the cell membrane was ruffled which was sometimes separated from the spherical part of the membrane by a constriction (Fig. 6 B), with 5 X $10^{-9}$ M FMLP. Though there were some poorly responding cells, most of the polarised cells had an elongated morphology (Fig. 6 C).

**Time course of polarisation**

To assess the effects of time of incubation on the polarisation of monocytes, 5 X $10^{-9}$ M FMLP was added to cell suspensions that had been equilibrated at 37°C and were incubated at varying times thereafter from 30 s up to 2 h. Then the cells were fixed and the percentage of polarised monocytes were calculated. The results from 9 experiments are shown in Fig. 8. It was observed that in some cells membrane ruffling occurred within as little as 30 s of exposure to the peptide and by 2 min more than 50% cells in the populations were responding. Incubation up to 10 min resulted in a recognizable leading edge with

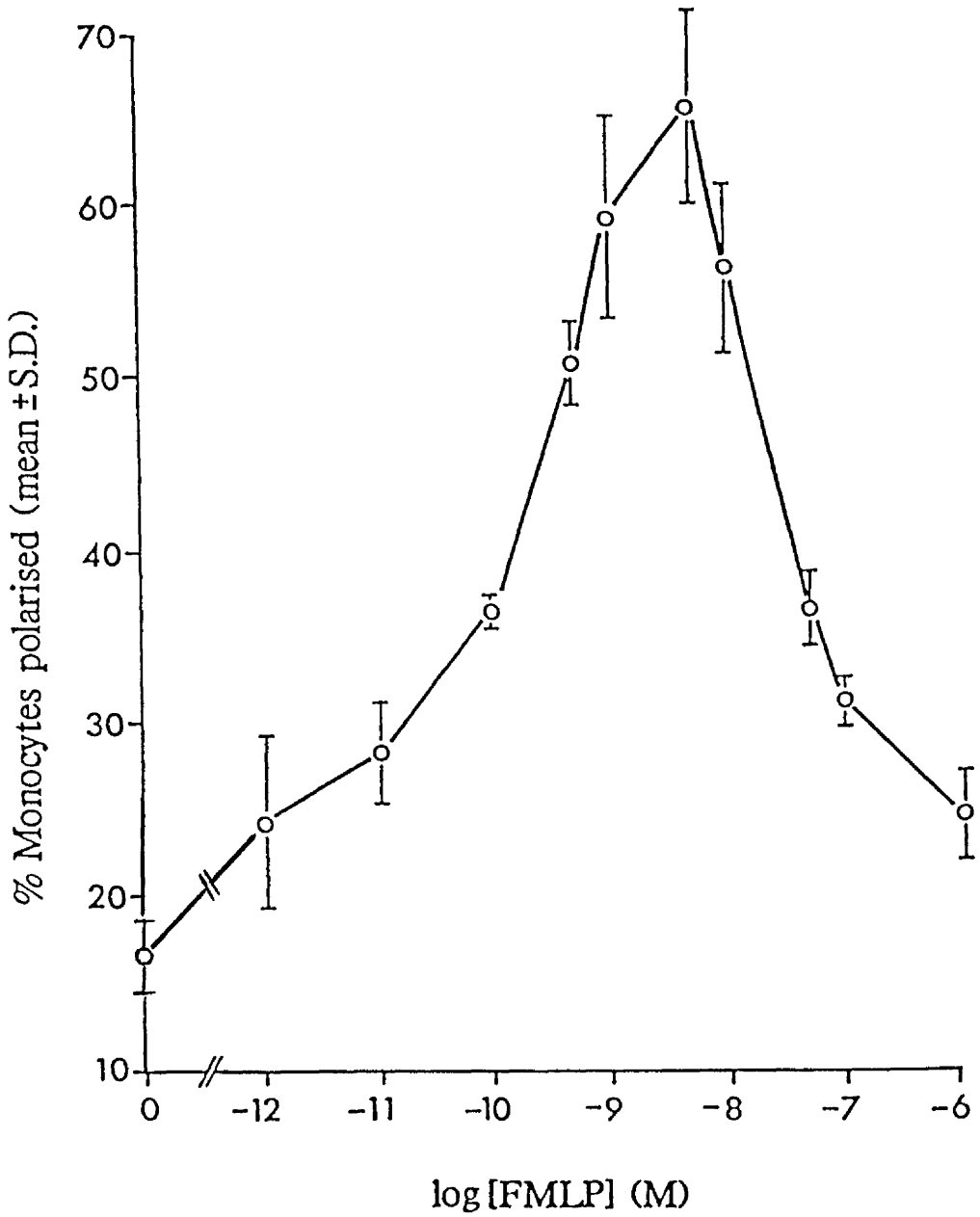



Fig. 7 Dose-response for monocyte polarisation in FMLP. The cells were incubated in suspension with different doses of FMLP for 20 min at 37°C and then were fixed with 2.5% glutaraldehyde. The maximum proportion of monocytes was polarised at 5 X $10^{-9}$ M FMLP. The point on the ordinate represents the proportion of monocytes polarised in the unstimulated population. The graph summarises the results of 7 experiments, each using 10 concentrations of FMLP and monocytes from one of 4 human donors. All points on the graph are therefore comparable, being based on the same 7 experiments and the same 4 donors.

constriction rings noticeable in the cell body of some monocytes. Huge lamellipodia as well as blebs were also seen in some monocytes. These effects were at maximum after 20 min, when 67.55% (S.D. = 5.5) monocytes were polarised. The rest of the cells (30-40%) had a spherical morphology, and did not respond to FMLP.

It is evident from Fig. 8 that about 55% (or more) monocytes in the populations remained polarised during the course of incubation from 5 min up to 30 min followed by a loss of polarisation. Only 40.78% (S.D. = 4.89) monocytes were polarised after 60 min incubation with 5 X $10^{-9}$ M FMLP. Many cells were rounded up after 2 h of incubation with FMLP and only 35% monocytes in the populations were polarised. Incubating monocytes in HSA alone up to 2 h increased the percentage of polarised cells up to 1.5% over the values obtained after 20 min incubation (not shown in Fig. 8). These observations indicated that monocytes could respond to FMLP within a short time of exposure by showing rapid shape changes, which were dose- and time-dependent, and the response started to fall after a certain period of incubation.

The loss of monocyte polarity with time of incubation is interesting. It is not known how the responding cells are rounded up again due to prolonged exposure to the chemotactic factor (FMLP in this case). The loss of responsiveness could be due to loss of cell surface receptors for FMLP or due to internalization of the peptide receptors. Further related experiments are described at the end of this chapter (Section IV).

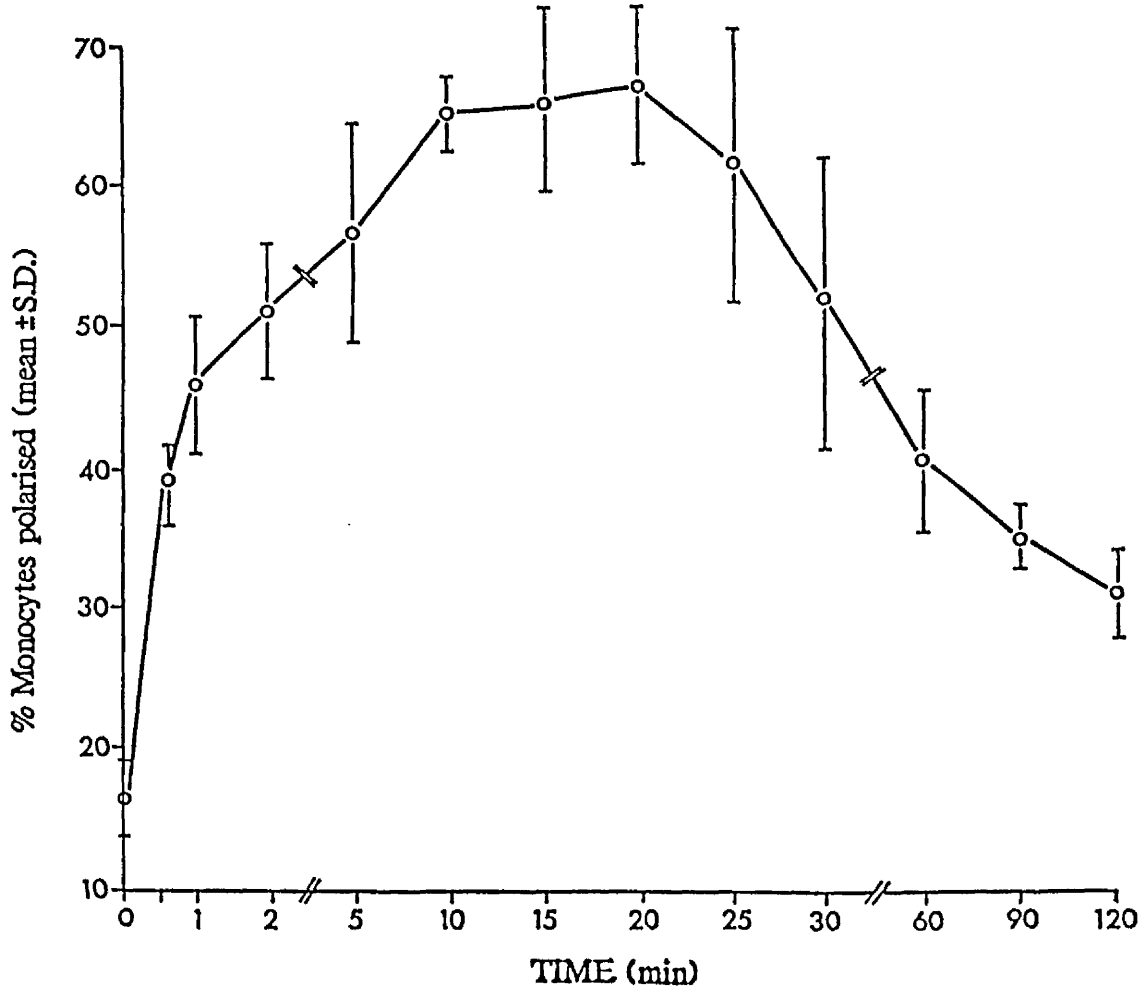



Fig. 8 Time course of monocyte polarisation in 5 X $10^{-9}$ M FMLP. The cells responded within 30 s of exposure to the peptide and by 2 min about 50% of the total cells were responding. This percentage of polarised cells increased to a maximum of 67.55% after 20 min. Then the proportion of polarised cells decreased due to prolonged incubation in FMLP.

**Monocyte morphology in FMLP**

The difference in cell morphology at different uniform concentrations of FMLP ($10^{-12}$ M up to $10^{-6}$ M) was quantified by measuring accurately the longest axis of the monocytes after incubating them with the peptides followed by fixing the cells in suspension. Table 1 contains a summary of the measurements of mean cell lengths in different concentrations of FMLP from 3 experiments. It is clear that monocytes have maximum lengths at 5 X $10^{-9}$ M FMLP, the concentration that induced maximum shape change. Though suboptimal concentrations like $10^{-11}$ M or $10^{-10}$ M FMLP gave a lower percentage of polarised cells compared to the optimal dose, the mean cell lengths in these doses were comparable to that of the optimal dose. It was observed that some monocytes were well polarised in $10^{-11}$ M or $10^{-10}$ M FMLP in exactly the same way as they were with 5 X $10^{-9}$ M FMLP. The reason for this is not known. Fig. 9 represents the distribution of monocyte lengths in supraoptimal, optimal and suboptimal concentrations of uniform FMLP and in the controls from several experiments.

**Sources of inaccuracy in measuring monocyte polarisation**

It is clear from Fig. 6 B and C that when a cell is polarised it has one axis longer than the other. Two factors were considered to give inaccurate results in comparing monocyte lengths to quantify cell polarity in different doses of FMLP. These are described below.

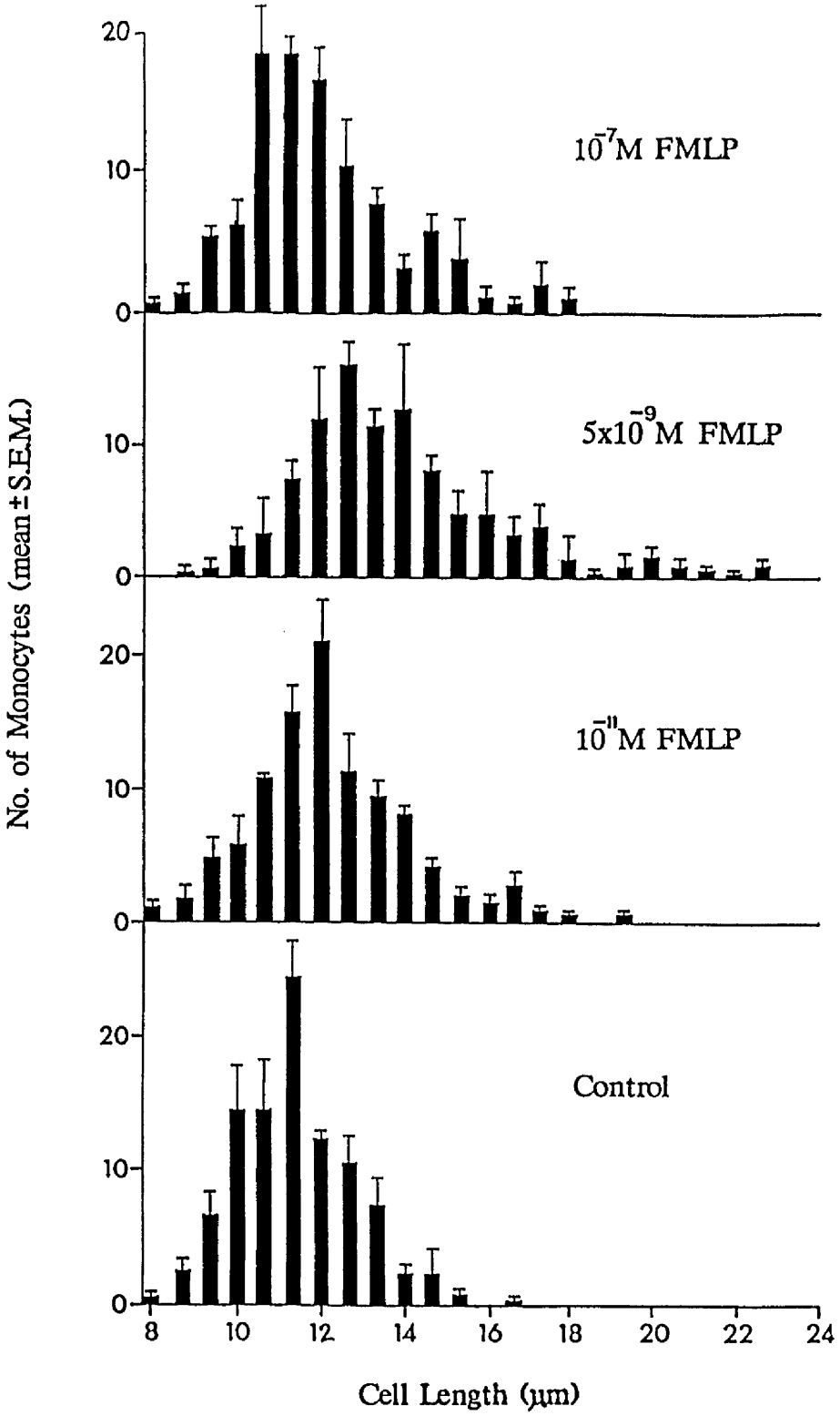



Fig. 9 Distribution of monocyte lengths in uniform concentrations of FMLP. The cells were incubated for 20 min with different doses of FMLP. Then they were fixed in suspension and their lengths were measured. Monocytes had maximum lengths at 5 X $10^{-9}$ M FMLP, when the cells were most polarised. A summary of the mean of 3 experiments is shown in Table 1.

Table 1. Effect of uniform concentrations of FMLP on mean cell length of 100 randomly chosen cells

| Concentration of FMLP | Mean cell length ± S.D. (μm) |
|---|---|
| 0 | 11.20 ± 0.27 |
| $10^{-6}$ M | 11.15 ± 0.17 |
| $10^{-7}$ M | 11.62 ± 0.27 |
| 5 X $10^{-8}$ M | 11.67 ± 0.97 |
| $10^{-8}$ M | 12.70 ± 0.67 |
| 5 X $10^{-9}$ M | 13.28 ± 0.49 |
| $10^{-9}$ M | 12.85 ± 0.81 |
| 5 X $10^{-10}$ M | 12.98 ± 0.31 |
| $10^{-10}$ M | 12.58 ± 0.36 |
| $10^{-11}$ M | 12.33 ± 0.01 |
| $10^{-12}$ M | 11.79 ± 0.32 |

*(a) Size difference*

Variations in size were always observed in populations containing over 95% monocytes. The identity of these cells was confirmed by their characteristic ANAE staining (see Fig. 4 A, B and Fig. 5 B). There were big round cells, small round cells as well as big and small polarised cells in the FMLP stimulated populations. These heterogeneity of cell size made it difficult to compare cell lengths as a measure of polarity in different populations.

*(b) Polarised unstimulated monocytes*

There were some polarised cells in the unstimulated (control) populations of monocytes in every experiment. The control cells in Fig. 9 consisted of 14.49% (S.D. = 4.97%) polarised cells. These cells might account for the long tail on the control histogram.

In Fig. 9 is shown that the number of cells with smaller lengths (8-10 μm) were reduced in all the FMLP stimulated monocyte populations. There were very few cells with longer axes when monocytes were incubated with supraoptimal ($10^{-7}$ M) and suboptimal ($10^{-11}$ M) doses of FMLP. The number of cells with longer axes were at maximum in 5 X $10^{-9}$ M FMLP. Also cell lengths were maximally spread (over 22 μm) at this dose.

The distribution pattern shown in Fig. 9 can not give any information about the polarised cells in different populations. This is because measuring polarisation of monocytes of different sizes by measuring their lengths is inaccurate. A direct attempt to solve

this problem was taken by measuring accurately the breadth of each monocyte as the perpendicular distance at the mid-point of the longest axis (length). Then the length/breadth ratio was calculated to give the "polarisation index" of each monocyte. A cell having a polarisation index (P.I.) of 1.0 is an unstimulated or round cell with equal length and breadth.

Tracing the outlines of several hundred cells (using a cameralucida drawing tube as described in the Materials and Methods) from different control populations of monocytes followed by determination of the length/breadth ratio of each cell it was observed that P.I. value up to 1.30 never represented a polarised cell. When monocytes were incubated with FMLP ($10^{-12}$ M up to $10^{-6}$ M) there were some poorly responding cells as well as some triangularly polarised cells, both types having almost equal length and breadth. Fig. 10 shows the photomicrographs of some monocytes in 5 X $10^{-9}$ M FMLP where cells were polarised without distinct head and tail.

In practical terms it was difficult to show convincing polarisation in cells with P.I. values below 1.3. When length/breadth ratio was <1.35 it was difficult to be sure whether a cell was polarised or irregular in shape for some other reason. At P.I. $\geq$ 1.35 the measurement was more reliable. Therefore, a P.I. value of 1.35 was considered as the lower limit for a polarised monocyte irrespective of the population from which the cell was selected i. e., whether the cell was stimulated by FMLP or not. This classification of the P.I. values of

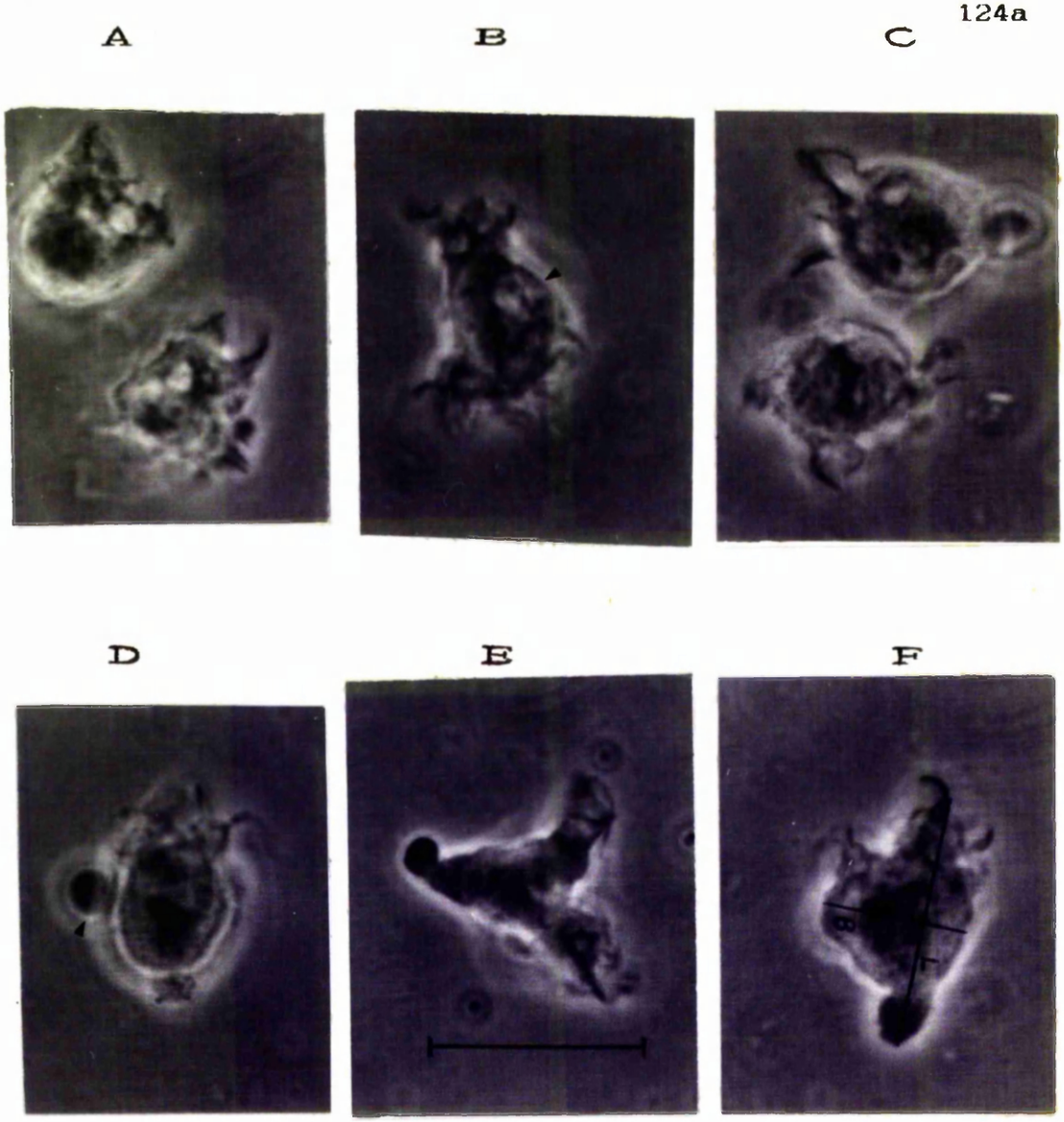



Fig. 10 A-F. Phase-contrast photographs of some poorly responding monocytes fixed in suspension after incubating for 20 min at 37°C in 5 X $10^{-9}$ M FMLP in HSA (20 mg/ml) in HBSS-MOPS. A, B and C: Ruffling of the membrane occurred in almost all over the cell surface. Spherical bleb was seen in a few cells (arrowed in B and D) and a knob-like tail was seen infrequently in the poorly responding cells (see C, E and F). F: schematic presentation for the measurement of the length (L)/breadth (B) ratio of monocytes to give the polarisation index, P.I. Bar = 20 μm.

polarised and unpolarised cells seemed to correlate with the outline drawings of about 95% monocytes in any population.

Fig. 11 compares the two assays so far described, i.e., the percent monocytes polarised in the shape change assay and the P.I. values $\geq 1.35$ of the same population of cells from 3 similar dose response experiments with FMLP. The two dose response curves were almost superimposable. This indicated that the new definition of polarised cells by the P.I. determination was correlating to the shape change values obtained in the polarisation assay.

Table 2 contains a summary of the measurements of mean P.I. of monocytes and their P.I. group distributions (described below) over a wide concentration range of FMLP from several experiments. Although monocytes responded towards different FMLP doses, qualitative differences in cell morphology were observed at different peptide concentrations, with an optimal dose (in terms of cell polarity) around the dissociation constant (Kd) of the receptor for FMLP ($10^{-9}$ M to $10^{-8}$ M). Monocytes, therefore, have maximum P.I. values at $5 \times 10^{-9}$ M FMLP.

The polarisation index, defined as a criterion to determine the extent of cell polarity, was different for different monocytes. The value increased as the cell was more polarised or elongated and a lower value was obtained for a poorly responding cell. These values were divided into 7 sub-groups so that the P.I. ratio distribution at different concentrations of FMLP could be compared with each other as well as with the control.

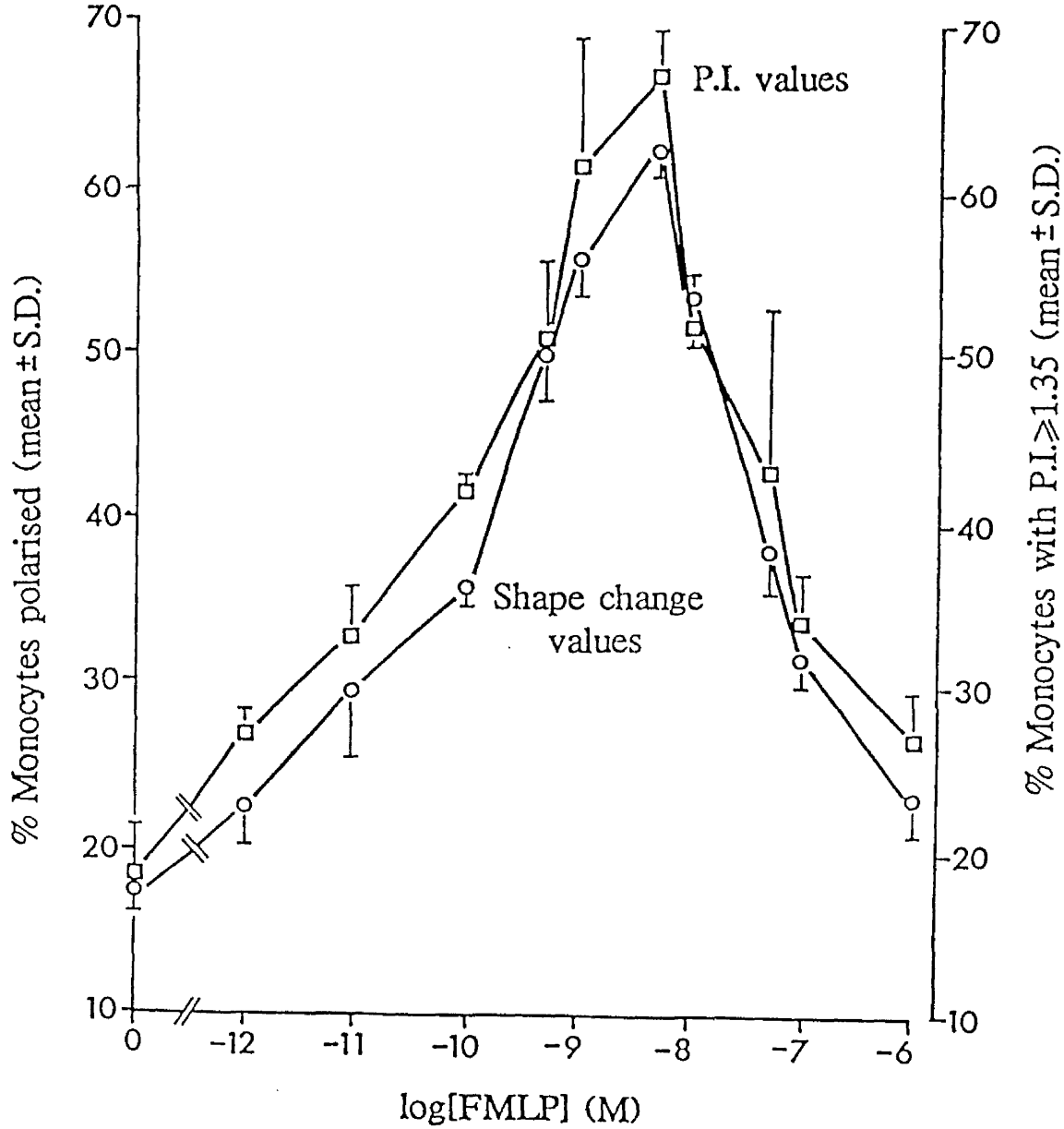



Fig. 11 Comparison of the shape change values and the measurement of the polarisation index (P.I.) values for the dose-response of monocyte polarisation in FMLP. The cells were incubated in suspension for 20 min in different doses of FMLP and then fixed for both the assays. Any deviation of the cell morphology from the spherical outline was scored as polarised or shape changed and expressed as the percentage of total cell counted. The length and breadth ratio (polarisation index, P.I. value) of each monocyte was calculated and then the proportion of cells with P.I. values ≥1.35 was determined. Measurement of P.I. value was more accurate to describe polarity of monocytes with variable size. The assays were almost superimposable.

Table 2. Effect of different uniform concentrations of FMLP on the polarisation distribution and the mean P.I. of the monocytes

| Conc. of FMLP | No. of cells (percent) values as indicated below in different groups with polarisation index (mean ± S.E.M of 4 experiments) | | | | | | | P.I. of the population |
|---|---|---|---|---|---|---|---|---|
| | 1.00–1.34 | 1.35–1.65 | 1.66–1.96 | 1.97–2.27 | 2.28–2.58 | 2.59–2.89 | 2.90–over 3.0 | (mean ± S.E.M.) |
| 0 | 82.5 ± 2.5 | 16.0 ± 1.0 | 1.0 ± 1.0 | 0.5 ± 0.5 | 0 | 0 | 0 | 1.25 ± 0.01 |
| $10^{-6}$ M | 73.0 ± 2.0 | 24.5 ± 0.5 | 1.5 ± 1.5 | 0.5 ± 0.5 | 0.5 ± 0.5 | 0 | 0 | 1.26 ± 0.01 |
| $10^{-7}$ M | 66.0 ± 2.0 | 28.0 ± 2.0 | 4.5 ± 0.5 | 1.5 ± 0.5 | 0 | 0 | 0 | 1.31 ± 0.01 |
| 5 X $10^{-8}$ M | 57.0 ± 7.0 | 34.0 ± 4.0 | 7.0 ± 2.0 | 1.5 ± 0.5 | 0.5 ± 0.5 | 0 | 0 | 1.35 ± 0.07 |
| $10^{-8}$ M | 48.0 ± 2.0 | 35.5 ± 2.5 | 9.5 ± 1.5 | 5.5 ± 1.5 | 1.0 ± 1.0 | 0.5 ± 0.5 | 0 | 1.42 ± 0.04 |
| 5 X $10^{-9}$ M | 33.0 ± 2.0 | 35.0 ± 2.0 | 17.0 ± 1.0 | 9.0 ± 2.0 | 3.0 ± 2.0 | 1.5 ± 0.5 | 1.5 ± 1.5 | 1.57 ± 0.02 |
| $10^{-9}$ M | 38.5 ± 5.5 | 28.0 ± 4.0 | 20.0 ± 3.0 | 10.5 ± 2.5 | 1.5 ± 0.5 | 0 | 1.5 ± 0.5 | 1.54 ± 0.04 |
| 5 X $10^{-10}$ M | 49.5 ± 3.5 | 24.5 ± 0.5 | 18.0 ± 4.0 | 4.5 ± 0.5 | 2.5 ± 0.5 | 0.5 ± 0.5 | 0.5 ± 0.5 | 1.46 ± 0.03 |
| $10^{-10}$ M | 58.5 ± 0.5 | 19.0 ± 4.0 | 12.0 ± 2.0 | 7.5 ± 1.5 | 1.5 ± 0.5 | 0.5 ± 0.5 | 1.0 | 1.44 ± 0.06 |
| $10^{-11}$ M | 67.0 ± 2.0 | 20.0 ± 5.0 | 9.5 ± 4.5 | 2.0 ± 1.0 | 1.0 ± 1.0 | 0.5 ± 0.5 | 0 | 1.36 ± 0.07 |
| $10^{-12}$ M | 72.0 ± 2.0 | 10.0 ± 1.0 | 13.0 ± 1.0 | 4.0 | 1.0 | 0 | 0 | 1.33 ± 0.05 |

Fig. 12 shows the P.I. group distribution of monocytes in controls, supraoptimal, optimal and suboptimal concentrations of FMLP from several experiments. Each set of histograms represents the P.I. of 100 cells. In $10^{-7}$ M FMLP about 68% cells were unpolarised with P.I. values below 1.35. Most of the polarised cells in this population had P.I. values between 1.35 and 1.65 with very few cells having higher polarisation indices. In 5 X $10^{-9}$ M FMLP about 33% cells were unpolarised with P.I. values in between 1.0 and 1.34. The rest of the cells were polarised as divided into different P.I. sub-groups. With $10^{-11}$ M FMLP polarised cells fell in a wide range of P.I. groups though they were few in number. It is clear that monocytes were most polarised in 5 X $10^{-9}$ M FMLP compared to the other doses. Cells that were polarised in the medium (HSA alone) were mostly in the first polarised group with P.I. value 1.35-1.65, indicating that the extent of polarity was not high without any stimulant.

**Comparison of the shape change values for the time course of monocyte polarisation in FMLP and the P.I. values of the cells**

To assess the effect of time of incubation upon the polarisation indices of monocytes, 5 X $10^{-9}$ M FMLP was added to the cell suspensions that had been equilibrated to 37°C and the incubation was continued for different time intervals up to 40 min. Then the cells were fixed, the percentage of polarised cells were determined and the outlines of 100 cells were drawn from each preparation

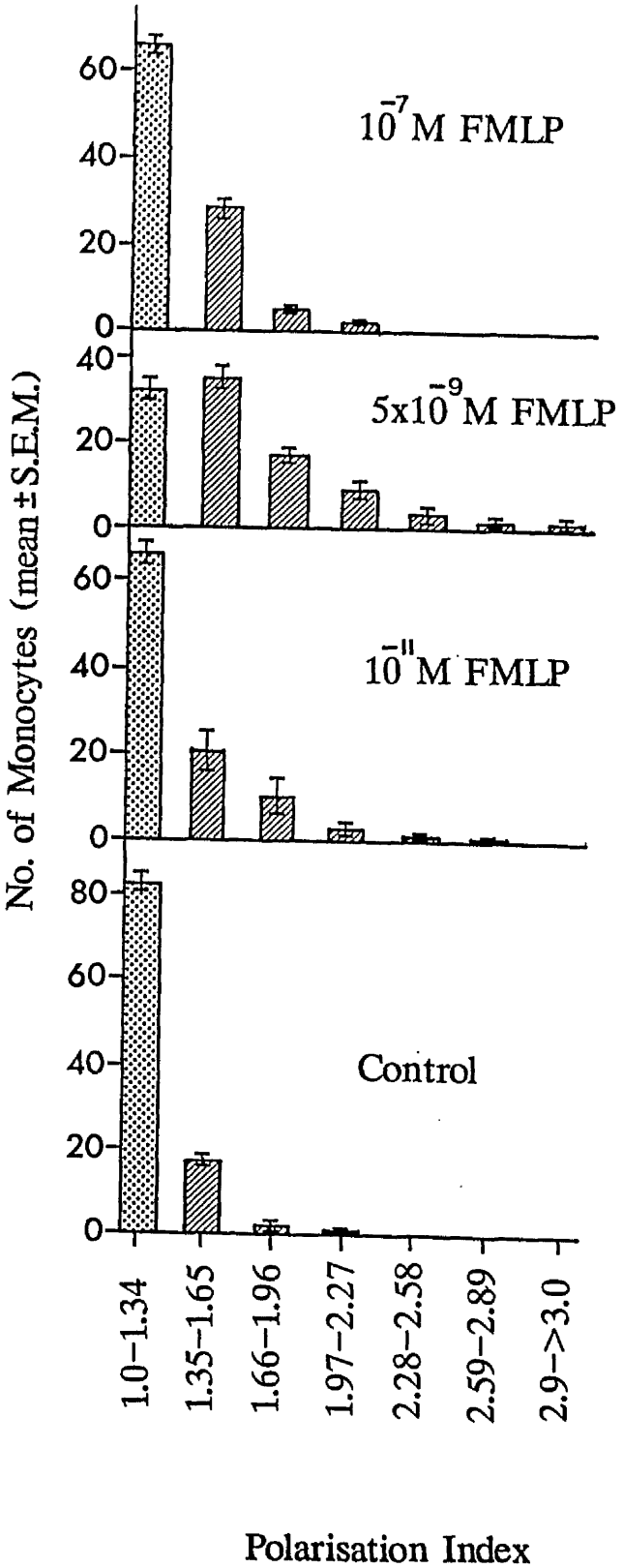



Fig. 12 Distribution of polarisation index (P.I., calculated as length/breadth) ratio of monocytes at different concentrations of FMLP. Cells with P.I. values 1.0-1.34 were not polarised. Fewer monocytes with higher P.I. values were present at $10^{-7}$ M and $10^{-11}$ M FMLP and in the control. The cells were more elongated at 5 X $10^{-9}$ M FMLP. A summary of 3 experiments is given in Table 2.

followed by accurately measuring their length and breadth. The length/breadth (L/B) ratio was calculated to determine the polarisation index of each cell which was then divided into different P.I. groups.

Fig. 13 shows the percent monocytes polarised in the shape change assay and the time course for P.I. values in optimum FMLP. At 20, 30 and 40 min 0f incubation the number of monocytes polarised by the P.I. determination was lower than that obtained from the shape change score though the results were not significantly different. The number of polarised monocytes in the control did not increase significantly from the marginal level (when cells were just at 37°C) after incubation for 20 or 40 min in either assays.

The distribution of the polarisation indices of monocytes (from 5 X $10^{-9}$ M FMLP time course) are illustrated by Fig. 14. It is noticeable that a short time exposure of monocytes to FMLP resulted in a rapid increase of polarised cells during the first 2 to 10 min. The maximum response of the population was observed after 20 min when nearly the maximum number of polarised monocytes were obtained in each P.I. group. Then the response started to fall after incubation for 30 and 40 min when cells in the unpolarised group started to increase with the disappearance of polarised cells in different higher P.I. groups.

It was interesting to see the overall effect of the time of incubation upon the mean P.I. of monocytes with and without FMLP. The results from 3 separate experiments are shown in Fig. 15. The polarisation indices of control

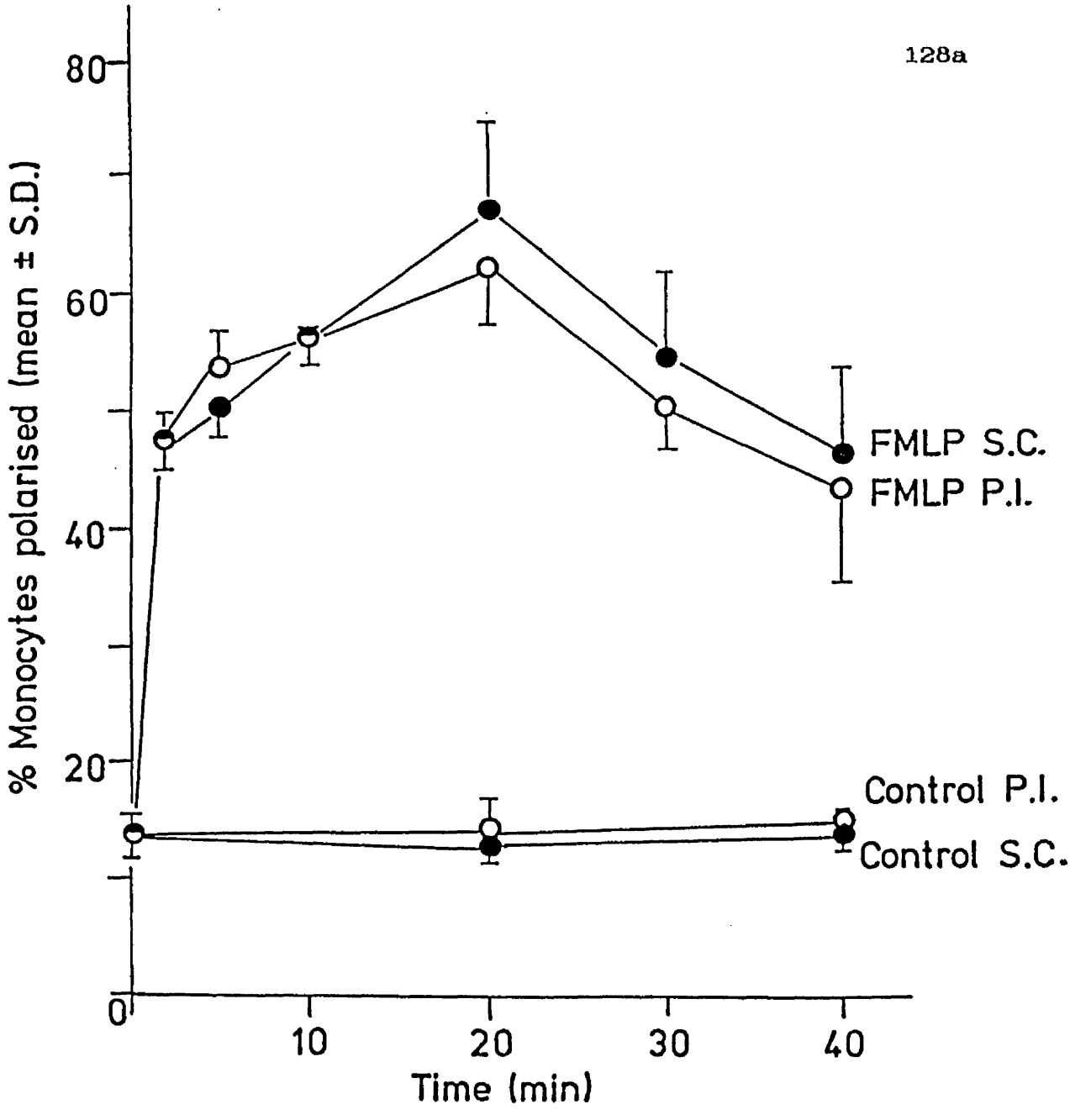



Fig. 13 Comparison of the time course of monocyte shape change (S.C.) and polarisation index (P.I.) determination in 5 X $10^{-9}$ M FMLP. The S.C. and P.I. values of control cells were almost the same during 40 min incubation at 37°C but the values increased as soon as the cells were exposed to FMLP. The S.C. and P.I. values of monocytes were at maximum after 20 min and then the proportion of polarised cells decreased with the time of incubation in FMLP. Thus the 2 assays correlated very well in showing loss of monocyte polarisation due to prolonged incubation in FMLP.

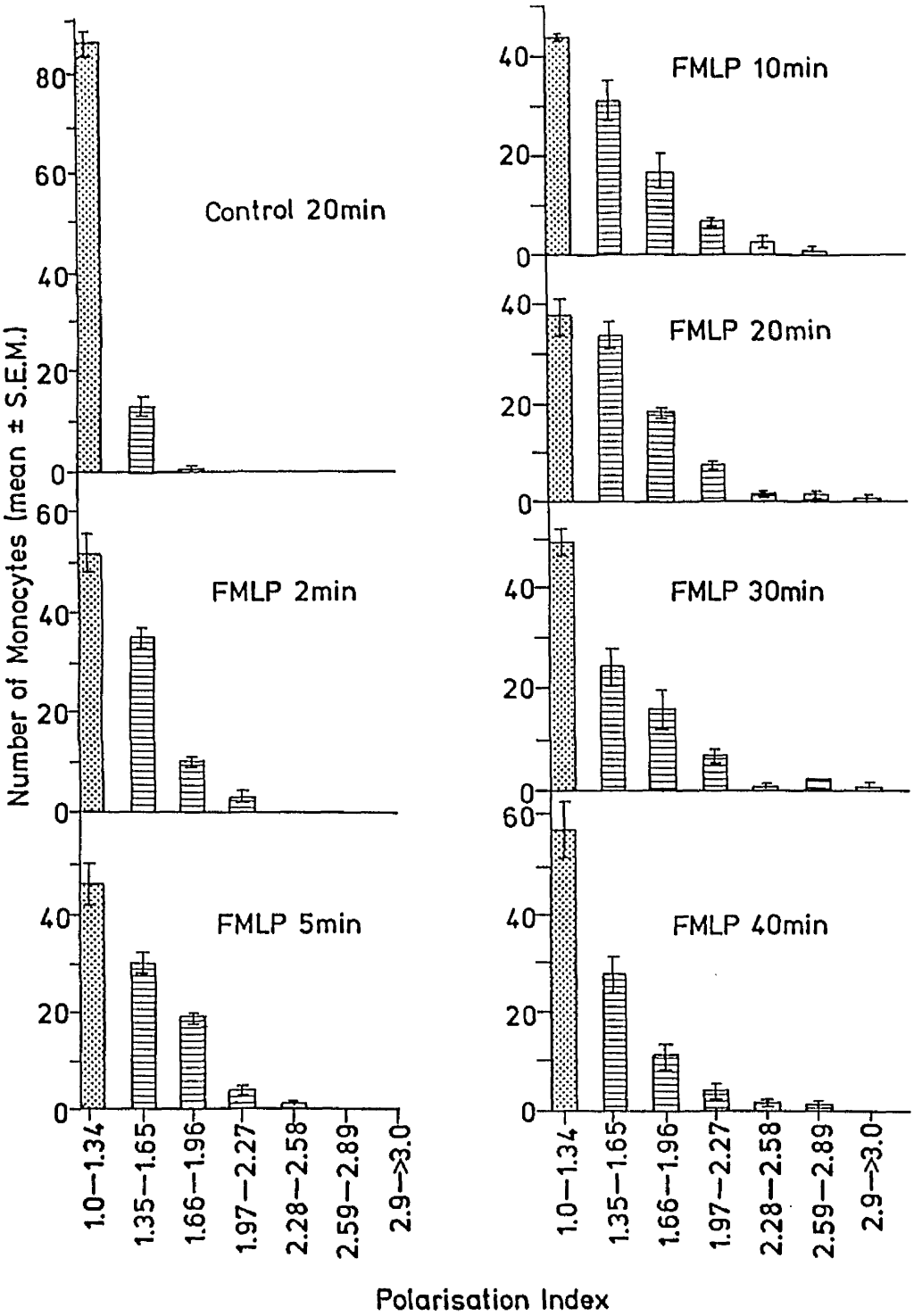



Fig. 14 Distribution of the polarisation index values of monocytes at different times of incubation in 5 X $10^{-9}$ M FMLP. The number of cells with higher P.I. values in FMLP increased during 20 min of incubation. Prolonged incubation reduced the extent of monocyte polarity as the number of cells in higher P.I. group decreased at 30 and 40 min incubation in FMLP. Monocytes with P.I. value 1.0-1.34 were not polarised.

monocytes were almost identical when incubated for 20 and 40 min at 37°C (on right of Fig. 15)

In isotropic environments of optimum FMLP, monocytes displayed rapid morphological changes with increase in lengths during the course of incubation which were maintained for a considerable period (20 min). Cells were more polarised and longer when incubated for 20 min with 5 X $10^{-9}$ M FMLP.

Though the mean P.I. of the monocyte population was 1.51 ± 0.06 at optimum incubation with the optimum dose of FMLP (Fig. 15) it is evident that about 40% cells in the population did not respond towards FMLP. This will reduce the mean P.I. of the polarised cells. There was a good number of polarised cells in the population with P.I. over 1.5, even up to 3.0 (or over 3.0) when the distribution of the P.I. ratios were considered (Fig. 12 and 14).

**Determination of the polarisation indices of neutrophils**

After determination of the P.I. ratios of monocytes to measure polarity, it was interesting to see whether length/breadth ratio ⩾1.35, as considered for a polarised monocyte, was consistent for other cell type. Neutrophils are the most extensively studied leucocytes from human blood. The polarisation and locomotion of these cells were studied by several workers. Also these cells respond toward FMLP. Hence neutrophils were selected to compare polarity with monocytes when both of them were stimulated with FMLP.

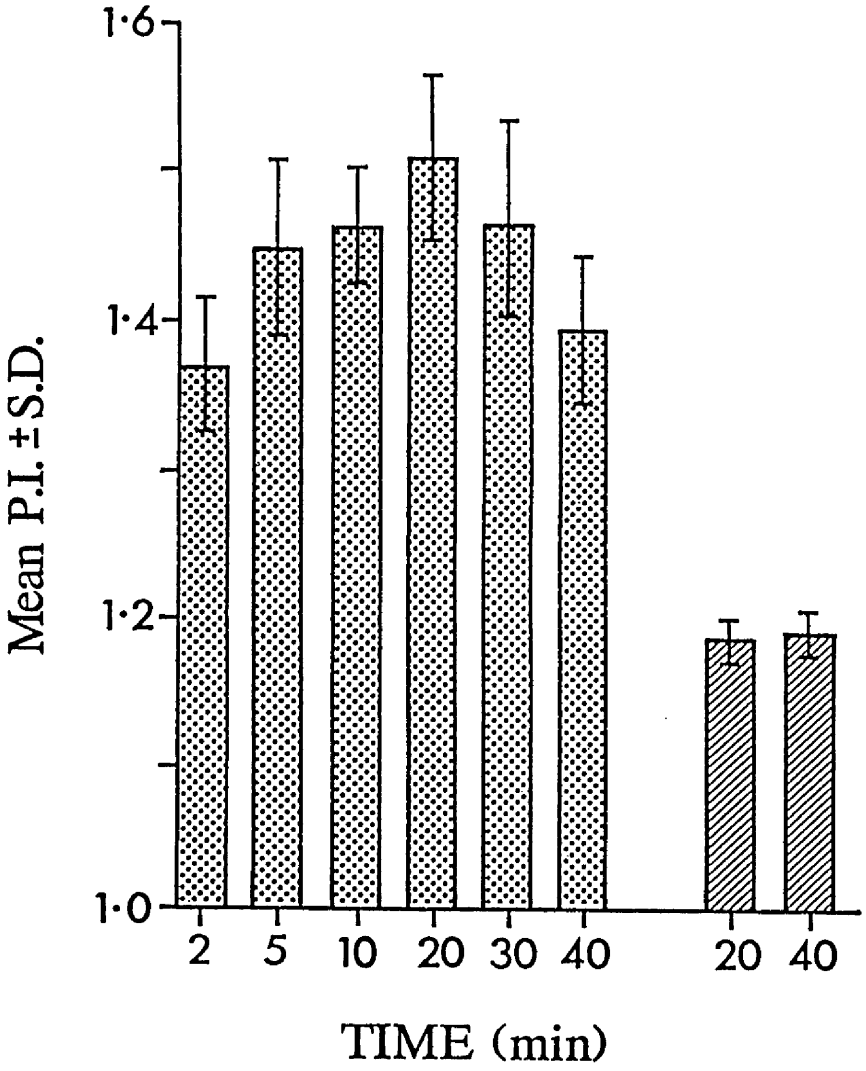



Fig. 15 Time course of the mean polarisation index (P.I.) values of monocytes in .5 X $10^{-9}$ M FMLP ( ▩ ) and in the control ( ▨ ). The mean P.I. values of the cells in FMLP increased with time of incubation and was maximum at 20 min, after which the values decreased with time of exposure. The mean P.I. value of the control population of cells was the same at 20 and 40 min of incubation. Each column represents the mean of 3 experiments.

Neutrophils incubated in suspension in uniform concentration of FMLP or purified hog C5a showed rapid shape changes, which were dose- and time-dependent (Shields and Haston, 1985; Haston and Shields, 1985). Although neutrophils can respond over a wide range of FMLP doses (2.5 X $10^{-10}$ M to $10^{-6}$ M), cells were consistently polarised with anterior and posterior regions at $10^{-8}$ M FMLP. It is reported by the above workers that these cells have maximum lengths when incubated for 30 min at 37°C in an isotropic environment of $10^{-8}$ M FMLP.

Neutrophils in suspension (HBSS-MOPS) were incubated for 30 min at 37°C with $10^{-8}$ M FMLP and as controls. The cells were fixed and the percent polarised cells were counted in each preparation (round and polarised cells were assessed as described earlier for monocytes). By drawing the images of 100 randomly chosen neutrophils from each preparation, followed by measuring the P.I. (length/breadth ratios, as described for monocytes) from 3 similar experiments, it was observed that 93.33% (S.D. = 1.53) cells were polarised with FMLP in the shape change assay compared to 91.33% (S.D. = 2.31) polarised cells obtained by the P.I. measurements (L/B ratios $\geq$1.35). Only 5.5% (S.D. = 0.71) neutrophils in the control showed polarised morphology as scored by the shape change assay, and 6.0% (S.D. = 1.41) polarised cells were counted by the P.I. measurements. These results showed that the P.I. ratio, which was initially introduced to define a polarised monocyte was also applicable to designate a polarised neutrophil.

The P.I. of neutrophils were divided into several sub-groups and their distribution pattern in comparison with monocytes is presented in Fig. 16. The results from 3 experiments (Fig. 16) suggested that in a population of neutrophils where more than 90% cells were polarised in FMLP, many cells were in each polarised P.I. group (with values ≥1.35) compared to the population of monocytes where at least 35% cells were not responding. The majority of the polarised monocytes had low P.I. ratios i.e., between 1.35 and 1.65. In the highest P.I. group with value 2.9 - over 3.0, there was only one monocyte compared to 8 neutrophils with a higher extent of polarity. Fig. 17 shows population photographs of neutrophils and monocytes polarised in FMLP.

Table 3 contains a summary of the mean P.I. of neutrophil and monocyte populations in their respective optimum dose of FMLP at optimum incubation and of the control populations.

Thus neutrophils when incubated with an optimum dose of FMLP for an optimum period of incubation, displayed a polarised morphology such that the average length was twice the breadth of the polarised neutrophils. With monocytes, in presence of the respective optimum dose and incubation in FMLP, the average length was 1.77 times the breadth of the polarised monocytes. Fig. 18 shows high power photomicrographs of neutrophils and monocytes polarised in FMLP.

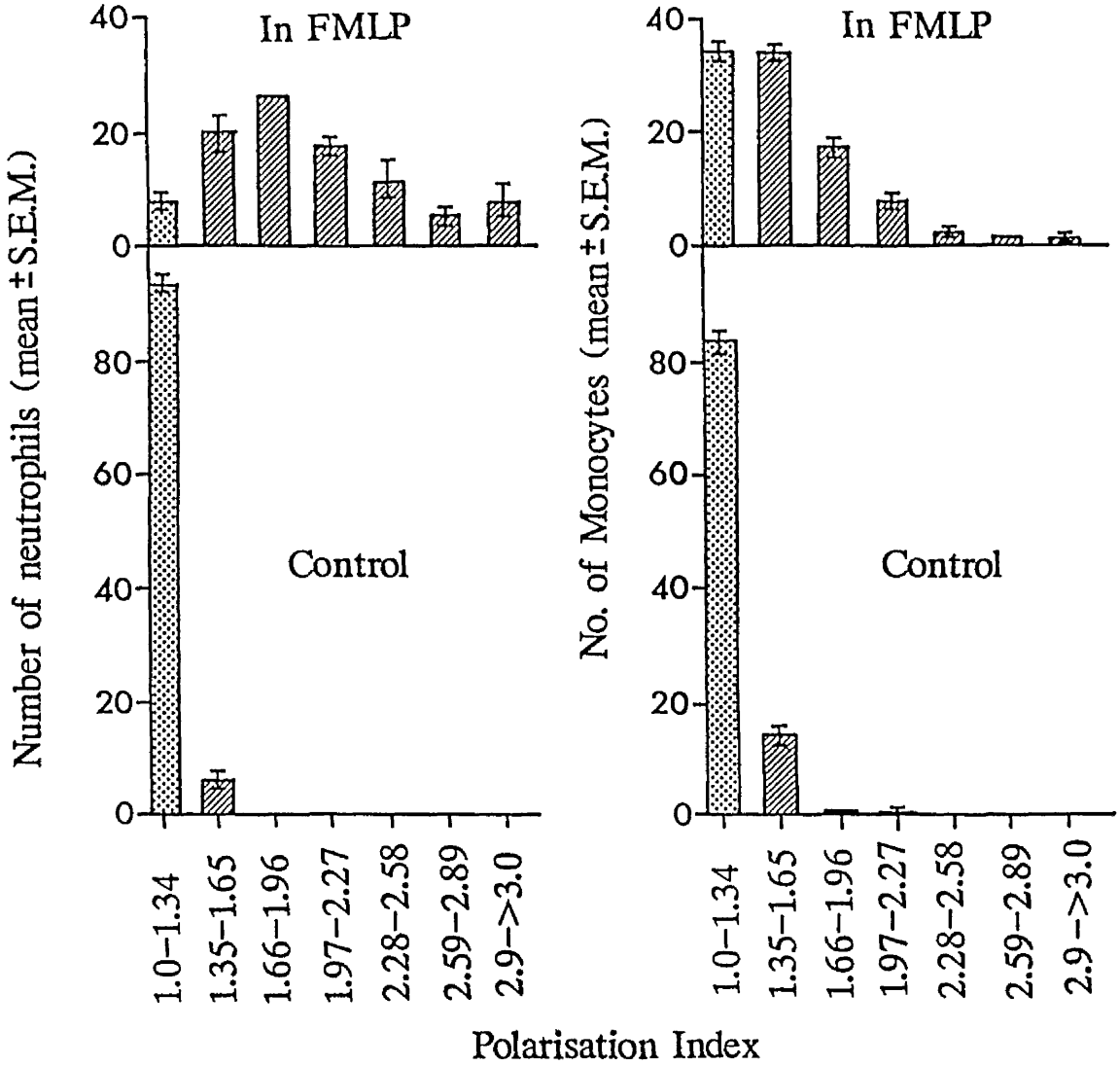



Fig. 16 Comparison of the polarisation index (P.I.) values of neutrophils and monocytes both stimulated with the respective optimum dose of FMLP. More than 90% neutrophils were responding in $10^{-8}$ M FMLP and were more polarised compared to about 65% monocytes responding in 5 X $10^{-9}$ M FMLP. Cells with P.I. values 1.0-1.34 were considered to be unpolarised and 1.35 to onwards were considered to be polarised.

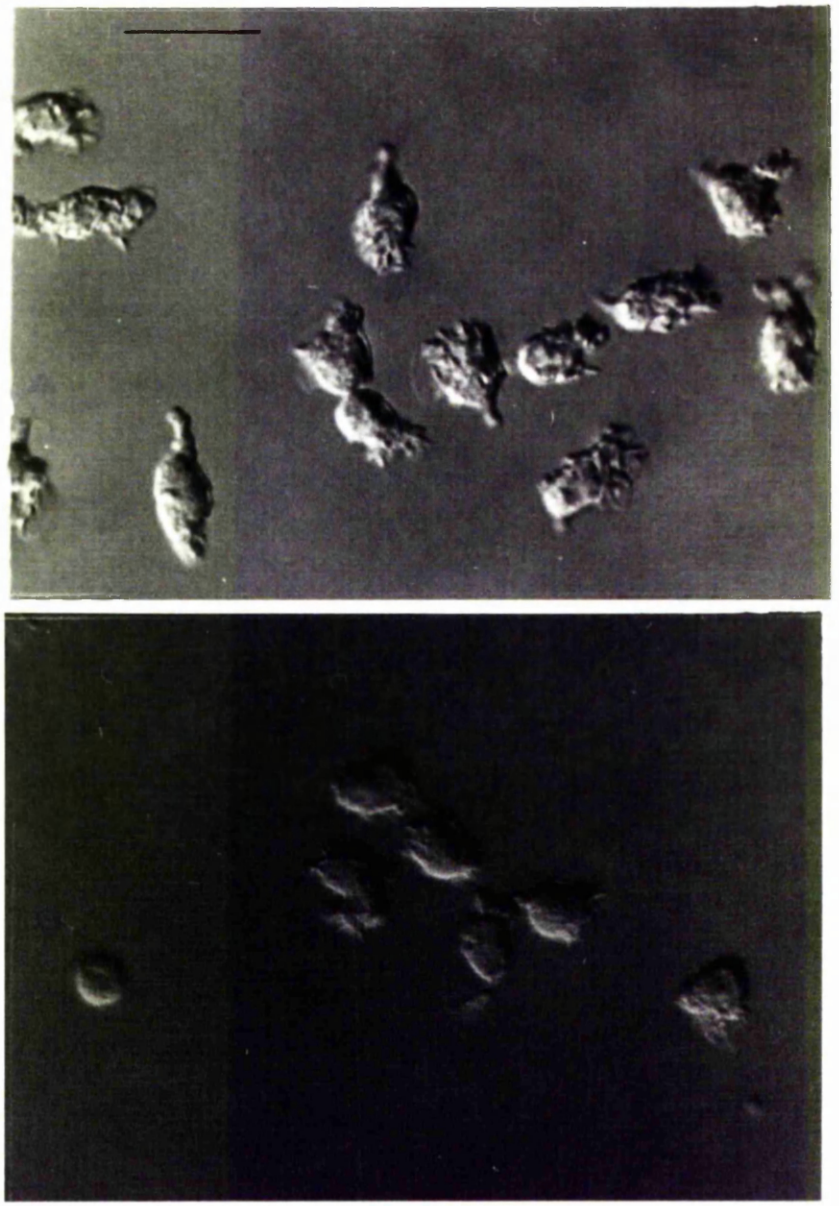



Fig. 17 Differential interference contrast photomicrographs of populations of human neutrophils and monocytes polarised in FMLP. A: Neutrophils fixed in suspension after incubating for 30 min at 37°C in $10^{-8}$ M FMLP; B: Monocytes fixed in suspension after incubating for 20 min in 5 X $10^{-9}$ M FMLP. The dose of FMLP and the time of incubation was optimum for the respective cells. Neutrophils were more polarised in FMLP than the monocytes. Bar, 20 μm.

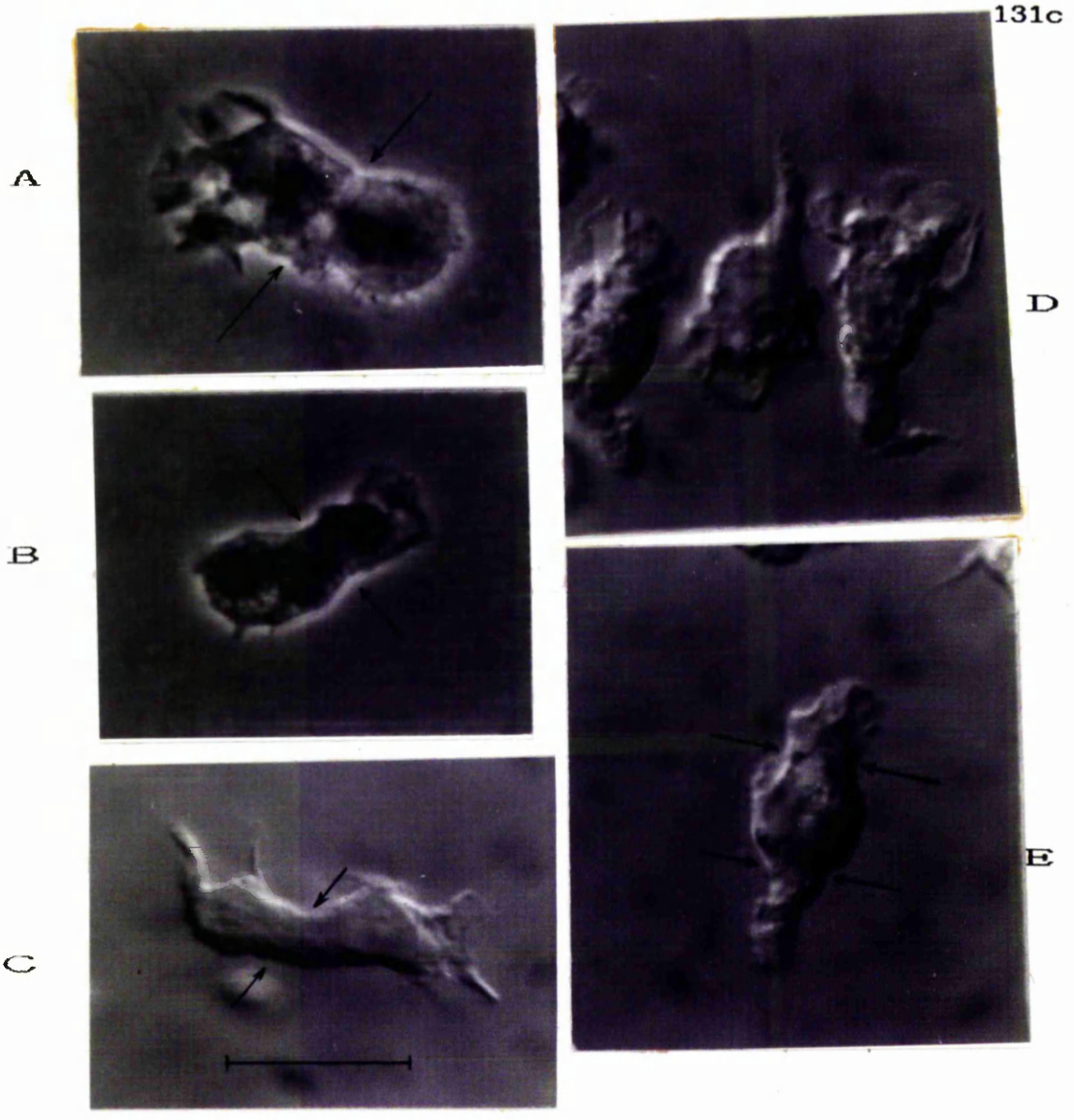



Fig. 18 High power photomicrographs of neutrophils and monocytes polarised in FMLP. A and B: Phase-contrast photographs of monocytes incubated in suspension for 20 min with 5 X $10^{-9}$ M FMLP; C: monocyte under similar conditions as A and B except under differential interference contrast (DIC) optics; D and E: DIC photographs of neutrophils incubated in suspension for 30 min with $10^{-8}$ M FMLP. Arrows indicate constriction ring(s) in the cell body. Bar, 20 μm.

Table 3. Effect of optimum concentration of FMLP on mean polarisation index of neutrophils and monocytes in suspension

| Cells | Medium | FMLP Conc. (M) | incubation at 37°C (min) | P.I. of the population (mean ± S.D.) | P.I. of the polarised cells (mean ± S.D.) |
|---|---|---|---|---|---|
| Neutrophils | HBSS-MOPS | 0 | 30 | 1.14 ± 0.03 | 1.45 ± 0.05 |
| " | " | $10^{-8}$ M | 30 | 1.99 ± 0.10 | 2.03 ± 0.13 |
| Monocytes | 20 mg/ml HSA in HBSS-MOPS | 0 | 20 | 1.18 ± 0.05 | 1.48 ± 0.06 |
| " | " | $5 \times 10^{-9}$ M | 20 | 1.58 ± 0.06 | 1.77 ± 0.03 |

In summary, studies of monocyte polarisation in FMLP suggested that not all monocytes responded to FMLP. The cells responded within a short time of exposure by showing rapid shape changes, which were dose- and time-dependent. The maximum percentage of monocytes polarised (only 60% or so) was with 5nM FMLP. The cells maintained their polarised morphology for a considerable period followed by the loss of polarity with time. Measurement of P.I. was better than cell length because of size differences. An average length of a polarised monocyte was 1.77 times the breadth of the cell.

Neutrophils responded better than monocytes towards FMLP. Almost all neutrophils responded in presence of this peptide. The responding cells were more polarised than monocytes and their average lengths were twice the breadth.

SECTION II

**Effect of various chemoattractants on monocyte polarisation**

Several substances were tested to see their ability to induce monocyte shape change in suspension. The results with different substances are described below.

*(a) Leukotriene $B_4$* ($LTB_4$)

Monocytes were incubated with different concentrations of leukotriene $B_4$ ($10^{-12}$ M to 5 X $10^{-6}$ M) for various time intervals. The results from 6 similar dose-response experiments for the percentage of monocytes that were polarised are shown in Fig. 19. The maximum percentage of monocytes polarised, 50.84% (S.D. = 5.51) to 50.40% (S.D. = 7.10), was obtained in a range between $10^{-9}$ M and $10^{-8}$ M $LTB_4$ after 10 min incubation at 37°C. As the polarised cell lengths were at maximum with $10^{-9}$ M $LTB_4$, it was considered as the optimum dose. The time course of monocyte polarisation in $10^{-9}$ M $LTB_4$ was followed from 30 s up to 30 min at 37°C. The results from 4 experiments are shown in Fig. 20 which shows that upon the addition of $LTB_4$ to the cell suspensions, monocytes responded within 30 s of incubation. The response was maximum after 10 min when 50.60% (S.D. = 5.34) cells in the population were polarised. The cells were not as elongated as they were with 5 X $10^{-9}$ M FMLP after 20 min incubation (see Fig. 31, described later). Also the response started to drop upon further incubation, as had been seen with FMLP.

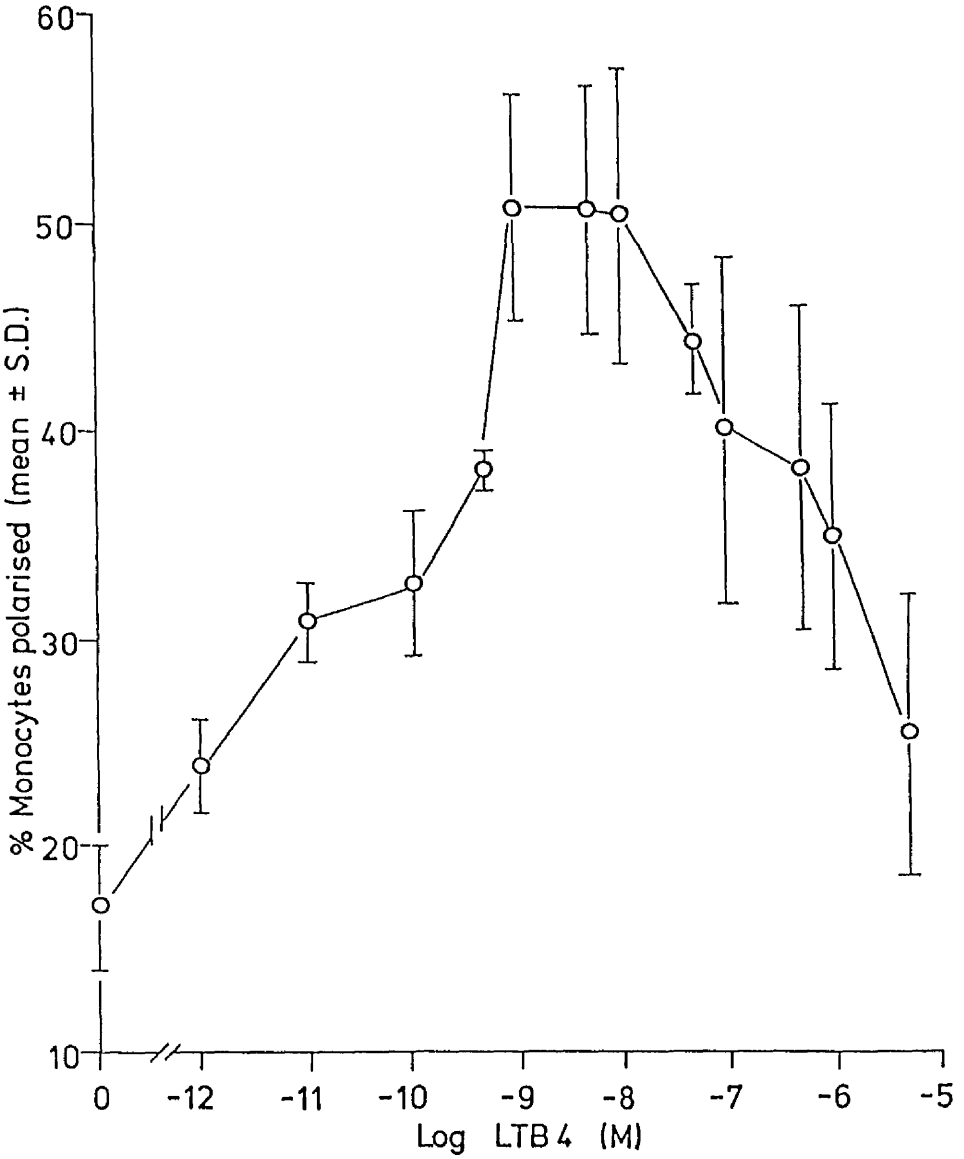



Fig. 19 Dose-response of monocyte polarisation in leukotriene $B_4$ ($LTB_4$). About 50% of the cells were polarised in a range between $10^{-9}$ M and $10^{-8}$ M $LTB_4$ after 10 min incubation. Monocytes were more polarised in $10^{-9}$ M $LTB_4$ than in $10^{-8}$ M $LTB_4$. Hence $10^{-9}$ M $LTB_4$ was considered as the optimum dose.

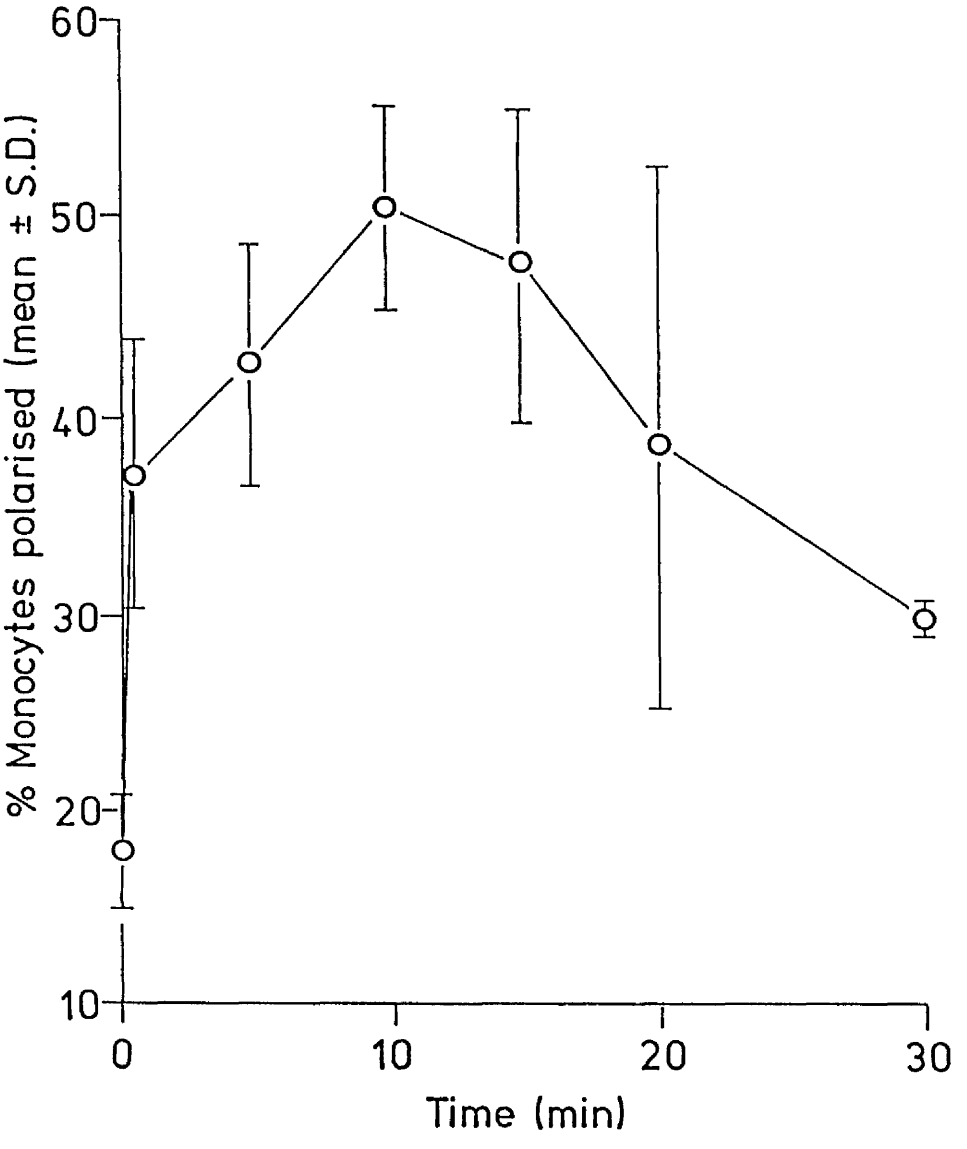



Fig. 20 Time course of monocyte polarisation in $10^{-9}$ $LTB_4$. The cells responded within 30 s of addition of $LTB_4$ and the maximum percentage, about 50% cells in the population, changed shape at 10 min incubation. Further incubation reduced the proportion of polarised cells, as observed earlier with FMLP (cf. Fig. 8).Each point represents the mean of 4 experiments.

*(b) Activated human serum (AHS)*

Monocytes upon incubation with fresh human serum (untreated) changed shape up to 32.10% (S.D.=3.75) when incubated for 10-20 min at 37°C. This response was obtained by using 10% human serum in the medium and the percent monocytes polarised did not increase by increasing the concentration of serum up to 100%. When fresh, unactivated serum was preheated to 56°C for 30 min, it lost the ability to induce monocyte shape change.

Fresh serum activated by incubating for 1 h at 37°C with *Candida albicans* blastospores followed by collecting the cell free supernatant. When AHS was added to monocytes in suspension, the cells showed rapid shape changes and assumed a polarised configuration. Different doses of AHS (1%-40%) were tested against monocytes and the response was highly reproducible as presented in Fig. 21 which represents 5 experiments. The effect was dose-dependent and as little as 1% AHS induced monocyte polarisation with the peak response at 10% when 61.51% (S.D. = 6.80) cells were polarised. Increasing the concentration of AHS up to 40% gave variable results with high standard deviations and the average response appeared to drop.

The time-course of monocyte response to 10% AHS was followed up to 60 min at 37°C. The results from 7 experiments are presented in Fig. 22 which shows that more than 40% monocytes were polarised within 30 s of exposure to the activated serum. The response increased sharply with the time of incubation and by 2 min about 50% cells were polarised. The extent of monocyte polarity

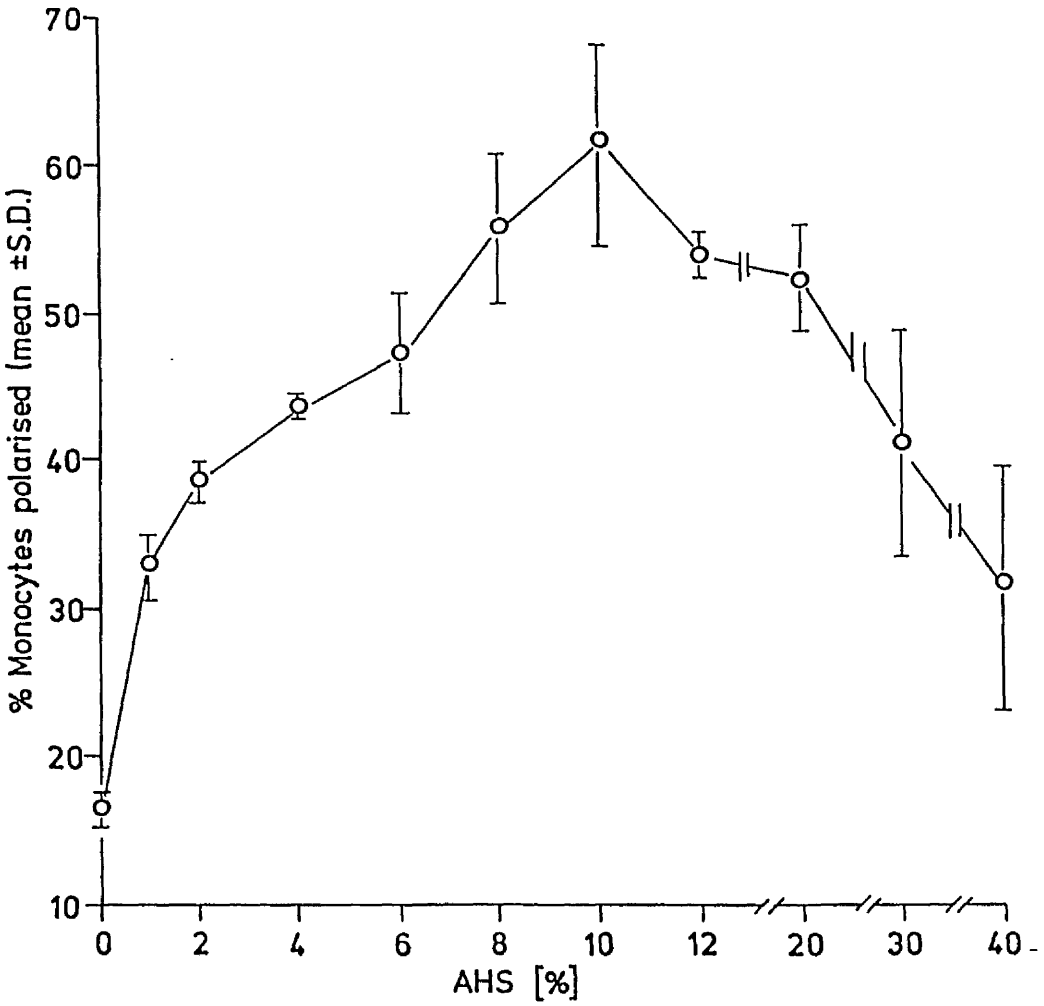



Fig. 21 Dose-response for monocyte polarisation in *Candida albicans* blastospore-activated human serum (AHS). The peak response was observed at 10% AHS when 61.5% monocytes changed shape during an interval of 10 min incubation. The polarised cells were not as elongated as they were in 5 X $10^{-9}$ M FMLP (see Fig. 31).

in AHS was similar to that observed in $LTB_4$ and the effects were similar to those obtained with 5 X $10^{-9}$ M FMLP. Cytoplasmic projections or veils and blebs were noticeable in several cells and constriction rings appeared in the cell body of some elongated monocytes. The response was maximum at 10 min when 63.28% (S.D. = 5.72) monocytes were polarised. Further incubation resulted in a loss of response and about 45% cells were polarised at 20 min. Incubation up to 60 min gave only about 35% polarised cells in the populations.

When AHS was subjected to heat treatment at 56°C for 30 min its capacity to induce monocyte shape change was unaltered. The dose response curve and the time course of the 'heat inactivated form of AHS' was almost superimposable upon the graphs of monocyte polarisation with untreated AHS. However, when fresh serum was heat inactivated at 56°C for 30 min before incubating with candida spores, it had no subsequent activity on monocyte polarisation.

*(c) Platelet activating factor (PAF)*

Monocytes in suspension were incubated with a wide range of PAF concentrations ($10^{-9}$ M - $10^{-4}$ M). The response was very poor and there was almost no effect with high doses ($10^{-6}$ M - $10^{-4}$ M) of PAF. Very few monocytes, up to 30%, responded in a range between $10^{-8}$ M and $10^{-7}$ M PAF when incubated for 20 min at 37°C. The results from 5 experiments are presented in Fig. 23 which shows that low doses of PAF can induce monocyte shape change only up to 10-15 % higher than the background

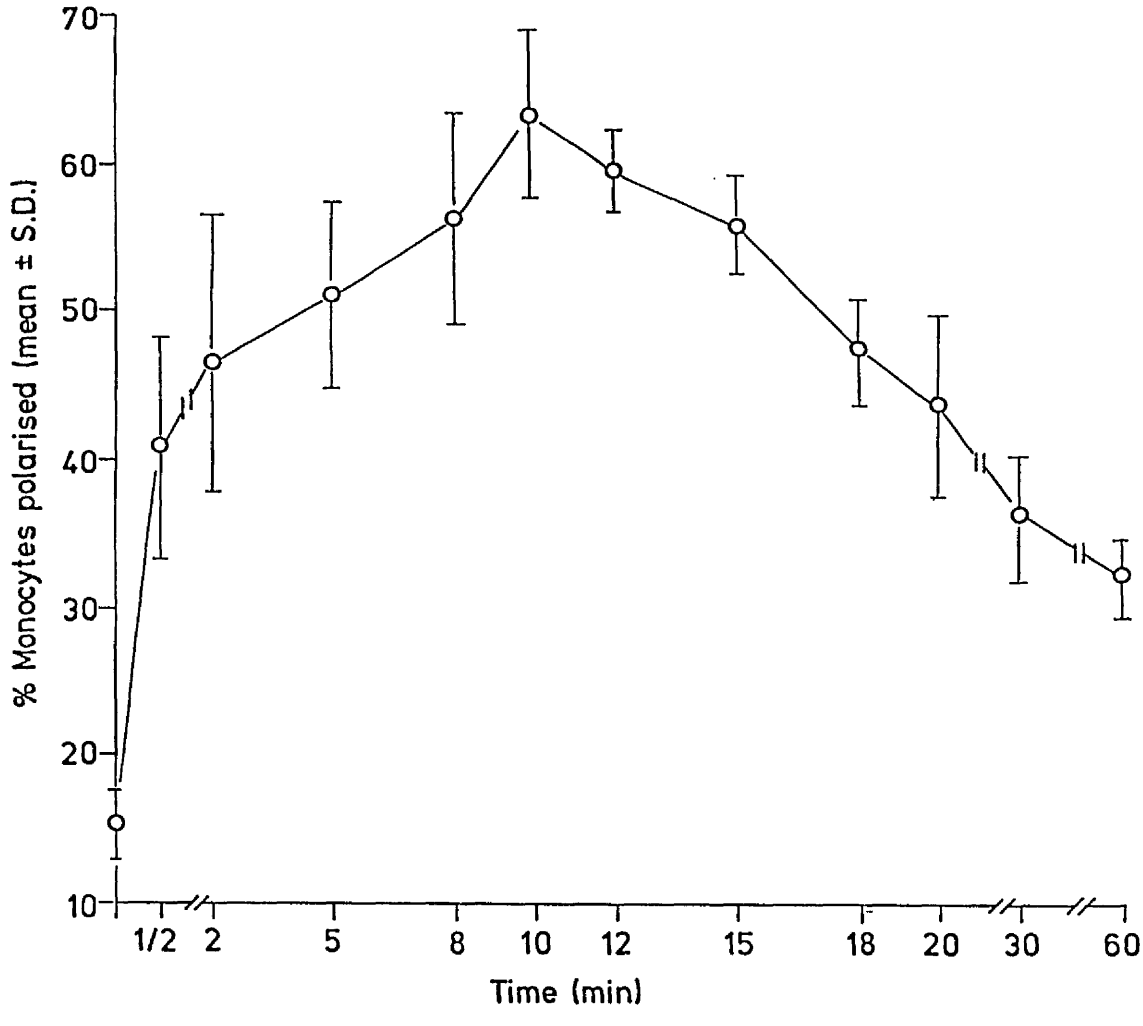



Fig. 22 Time course of monocyte polarisation in 10% AHS. The cells rapidly changed shape in the presence of AHS and more than 40% of them were polarised within 1/2 min of exposure to the activated serum. About 63% of the total monocytes changed shape at 10 min of incubation in AHS. Further incubation resulted in loss of polarisation and only about 35% cells were responding after 60 min of incubation.

level. Also the extent of cell polarity was poor. There was no appreciable change of the percentage of monocytes polarised when the cells were incubated with $10^{-7}$ M PAF for 10 min and 30 min at 37°C.

*(d) Alkali-denatured HSA (adHSA)*

Monocytes were incubated with different batches of adHSA. There was considerable variation between the batches of adHSA used and the results were inconsistent. Only one batch caused over 60% monocyte polarisation, whereas with the others only 25-35% cells were polarised. The variation presumbly reflected the degree of denaturation which could not be easily controlled. The maximum percentage of monocytes were polarised by incubating the cells for 10 min with adHSA. Incubation for 20 min and 30 min reduced monocyte responsiveness towards the most active adHSA by 10-15% respectively. The results with 4 batches of adHSA after 10 min incubation are shown in Table 4.

*(e) Serum-free phagocytosis supernatant (SFPS)*

The serum-free phagocytosis supernatant (SFPS) was obtained by incubating monocytes in HSA alone (without serum) with *Candida albicans* blastospores followed by collecting the cell free supernatant (described in detail in the Materials and Methods). Monocytes were incubated with neat SFPS for 10, 20 and 30 min at 37°C. In 5 experiments, only 36.0% (S.D.= 10.32) cells were polarised after 10 min incubation. There was no significant change of percent monocytes polarised after

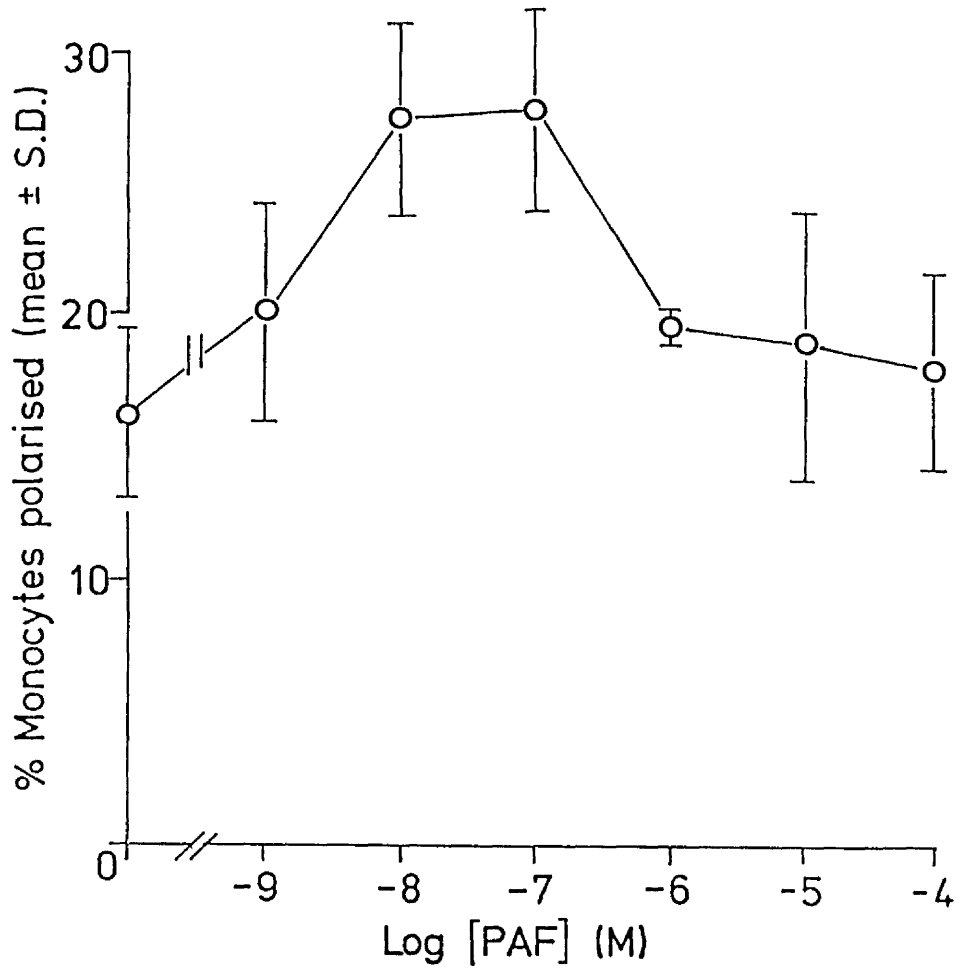



Fig. 23 Dose-response for monocyte polarisation in platelet activating factor (PAF). The cells responded poorly in a range between $10^{-9}$ M and $10^{-7}$ M PAF and a maximum of 28% cells changed shape at this range. Higher doses of PAF ($10^{-5}$ M or $10^{-4}$ M) had almost no effect on monocyte polarisation.

Table 4. Effect of alkali-denatured HSA on monocyte polarisation

| Batch of adHSA | Concentration (mg/ml) | % monocytes polarised (mean ± S.D.) |
|---|---|---|
| 1 | 0.75 | 15.0 ± 1.5 |
| | 1.50 | 20.0 ± 3.1 |
| | 3.00 | 26.5 ± 2.5 |
| 2 | 0.75 | 32.0 ± 1.8 |
| | 1.50 | 35.0 ± 4.2 |
| | 3.00 | 34.5 ± 2.2 |
| 3 | 0.70 | 18.0 ± 0.6 |
| | 1.40 | 32.0 ± 1.9 |
| | 2.80 | 30.0 ± 1.6 |
| 4 | 0.85 | 28.5 ± 9.2 |
| | 1.70 | 64.5 ± 16.3 |
| | 3.40 | 54.0 ± 4.8 |
| Cells in native HSA | 20.00 | 13.7 ± 2.1 |

20 and 30 min incubation. Also 20% and 50% SFPS had almost the same effect as 100% SFPS towards monocyte polarisation.

In summary, with all the chemoattractants tested so far, AHS induced up to 60% monocytes to change shape. It (10%, v/v AHS) was as effective as 5 X $10^{-9}$ M FMLP. $LTB_4$ was less potent in monocyte polarisation and it was maximally effective at nM concentrations. Monocyte responsiveness towards the optimum dose of AHS and $LTB_4$ started to decrease after 10 min of incubation, the same trend of the time course of cell polarisation was observed as obtained with FMLP. PAF was a poor chemoattractant judged by monocyte polarisation. Out of 4 batches of adHSA tested, only one batch induced up to 60% monocytes to change shape at a concentration of 1.7 mg/ml.

**Monocyte shape change in presence of complement peptides**

The polarising responses of monocytes were tested in the presence of several complement peptides. The results are described below .

*a) C5a*

Monocytes in suspension were incubated with uniform concentrations of purified hog C5a. The cells responded in the presence of these doses by showing rapid shape changes which were dose- and time- dependent. The results from 3 experiments are presented in Fig. 24 which shows that the peak response was in a range between 5 X $10^{-9}$ M

and 5 X $10^{-8}$ M C5a. Monocytes were consistently polarised with more elongated morphology in the presence of $10^{-8}$ M C5a. Hence this was considered as the optimum dose since 56.42% (S.D. = 4.20) cells were polarised. Due to small available quantities of the substance it was not possible to use high doses of C5a ($10^{-6}$ M and $10^{-5}$ M) in more than one experiment.

Though the percent monocytes polarised was lower in $10^{-8}$ M C5a than in 5 X $10^{-9}$ M FMLP, the extent of cell polarity was comparable to the latter (see Fig. 31). Monocytes were incubated with $10^{-8}$ M C5a for various time intervals. Fig. 25 shows the results from 5 experiments for the percentage of monocytes that polarised at different incubation periods. The cells responded very quickly towards the optimum dose of C5a and 35% of them changed shape within 30 s of exposure to the complement peptide. At 1 min of incubation there was considerable variation in the percent monocytes polarised in different experiments and almost all C5a responding cells 48.32% (S.D. = 4.62) showed polarised morphology. Incubation up to 10 min induced a maximum of 54.62% (S.D. = 3.75) monocytes to change shape (a small increase above the 1 min result) which was followed by rapid loss of response by the cells due to further incubation. It was interesting to see that almost all the cells lost their polarised morphology after 40 min.

*(b) C5a des Arg*

A wide range of C5a des Arg (C5adA) concentrations ($10^{-12}$ M - $10^{-6}$ M) was tested for monocyte shape change

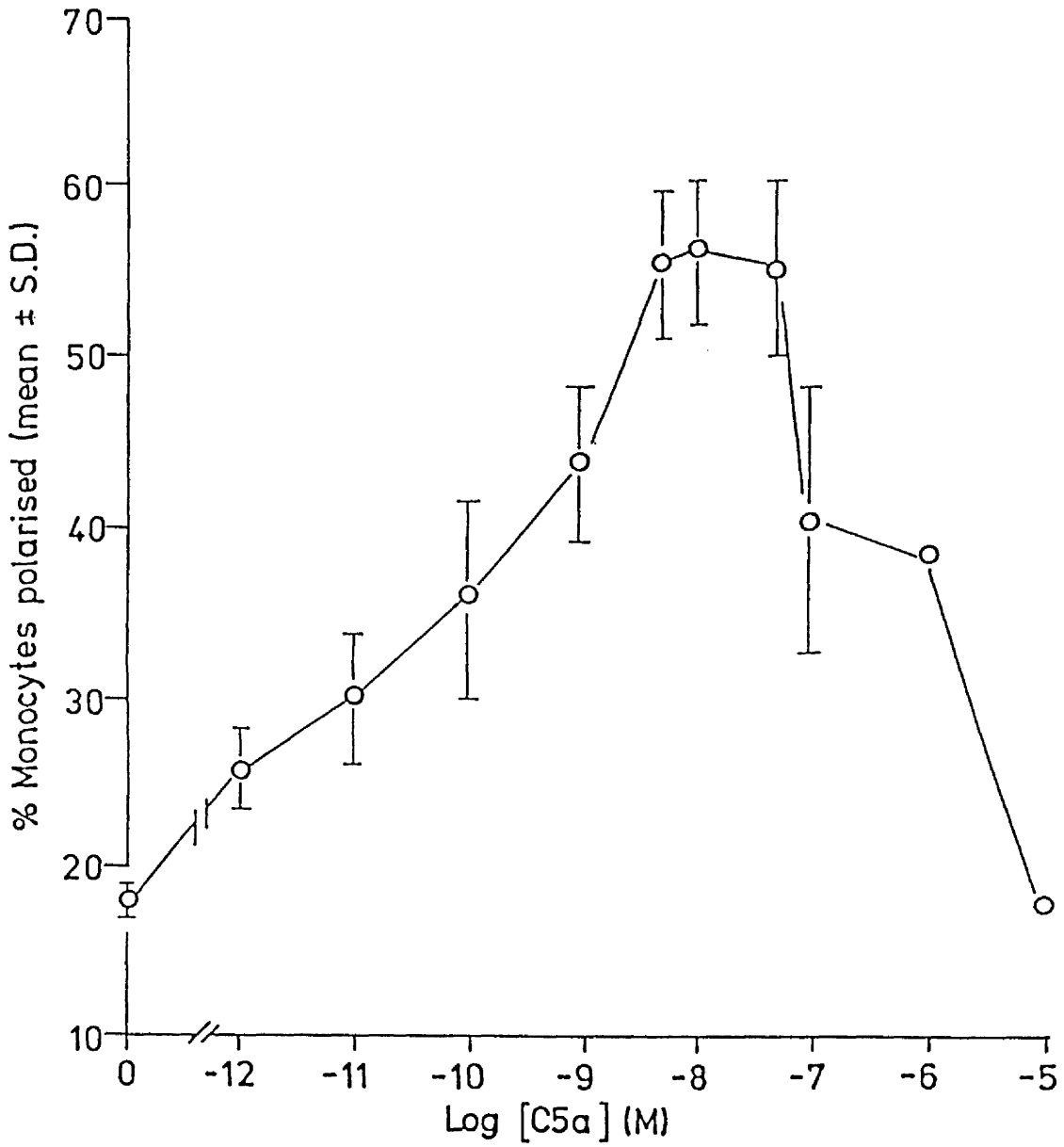



Fig. 24 Dose-response for monocyte polarisation in C5a. The cells were incubated for 10 min with different doses of C5a and then fixed in suspension and counted. Monocytes were maximally polarised at $10^{-8}$ M C5a when the highest proportion of 56.4% cells responded. The dose $10^{-6}$ M and $10^{-5}$ M were not used more than once due to shortage of the sample.

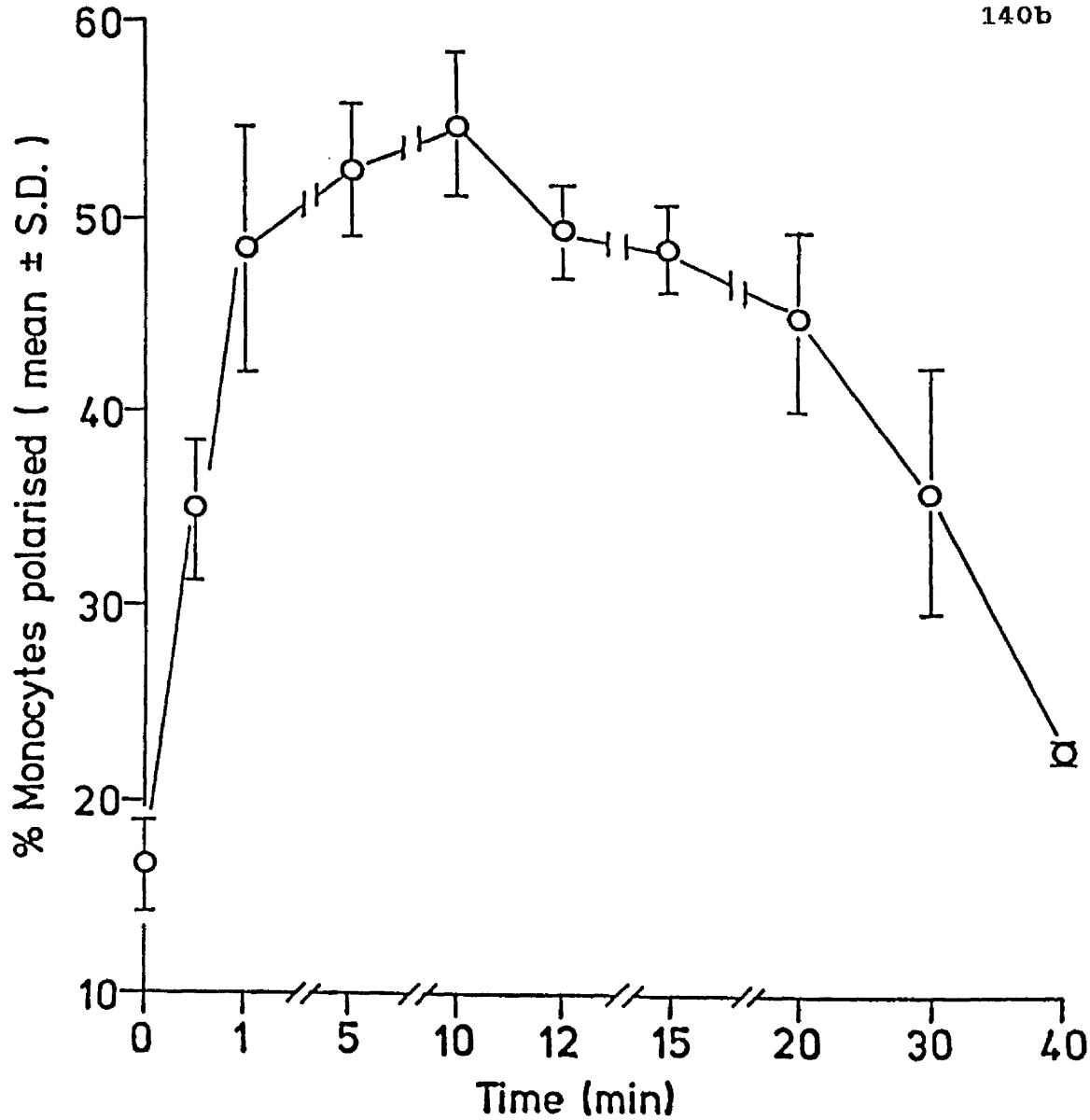



Fig. 25 Time course of monocyte polarisation in $10^{-8}$ M C5a. Almost all the cells responding towards the complement peptide changed shape within 1 min of incubation with the complement peptide. Then the polarised cells increased in length during incubation up to 10 min, when a maximum percentage of 55% cells were responding. Like loss of monocyte polarisation towards FMLP, $LTB_4$ and AHS, loss of polarity was observed due to prolonged incubation and almost all C5a responding cells lost their polarised morphology after 40 min incubation.

in suspension. The results from 4 experiments are presented in Fig. 26 which shows that low doses of C5adA ($10^{-12}$ M - $10^{-9}$ M) had similar effects to C5a at $10^{-12}$ M to $10^{-9}$ M, respectively, towards monocyte polarisation. The maximum percentage of monocytes (50.86%, S.D. = 4.89) changed shape at $10^{-7}$ M C5adA . This dose is an order of magnitude higher than the maximal effective dose for C5a. Due to lack of the substance it was not possible to use $10^{-6}$ M C5adA in more than one experiment. Fig. 26 represents the dose response curve after 10 min incubation at 37°C. A similar trend of the graph for the time course of monocyte polarisation was expected with $10^{-7}$ M C5adA as obtained with $10^{-8}$ M C5a, so the time course was not done.

*(c) C3a*

The percent monocytes polarised with different doses of C3a from 4 experiments is presented in Fig. 27. C3a was active at a high dose and the most effective concentration was in between $10^{-6}$ M and $10^{-5}$ M. Due to shortage of the complement peptide it was not possible to use 3 X $10^{-6}$ M and $10^{-5}$ M C3a in more than one experiment. The maximum percentage of monocytes, 64.90%, was polarised with 3 X $10^{-6}$ M C3a after 10 min incubation at 37°C.

*(d) C3a des Arg*

Monocytes were less responsive to C3a des Arg (C3adA) than to any other complement peptide tested. The results from 4 experiments suggested (Fig. 28) that the

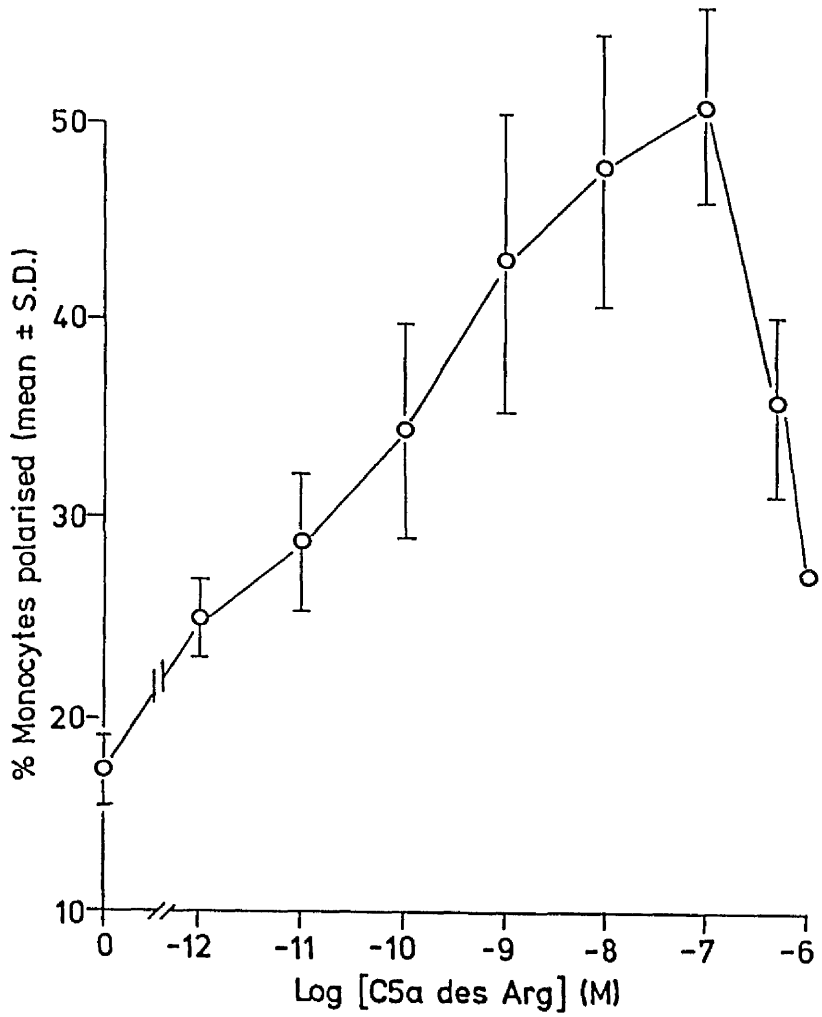



Fig. 26 Dose-response for monocyte polarisation in C5a des Arg. A dose of $10^{-7}$ M C5a des Arg induced the maximum percentage of monocyte polarisation. This dose was one order of magnitude higher than that for C5a.

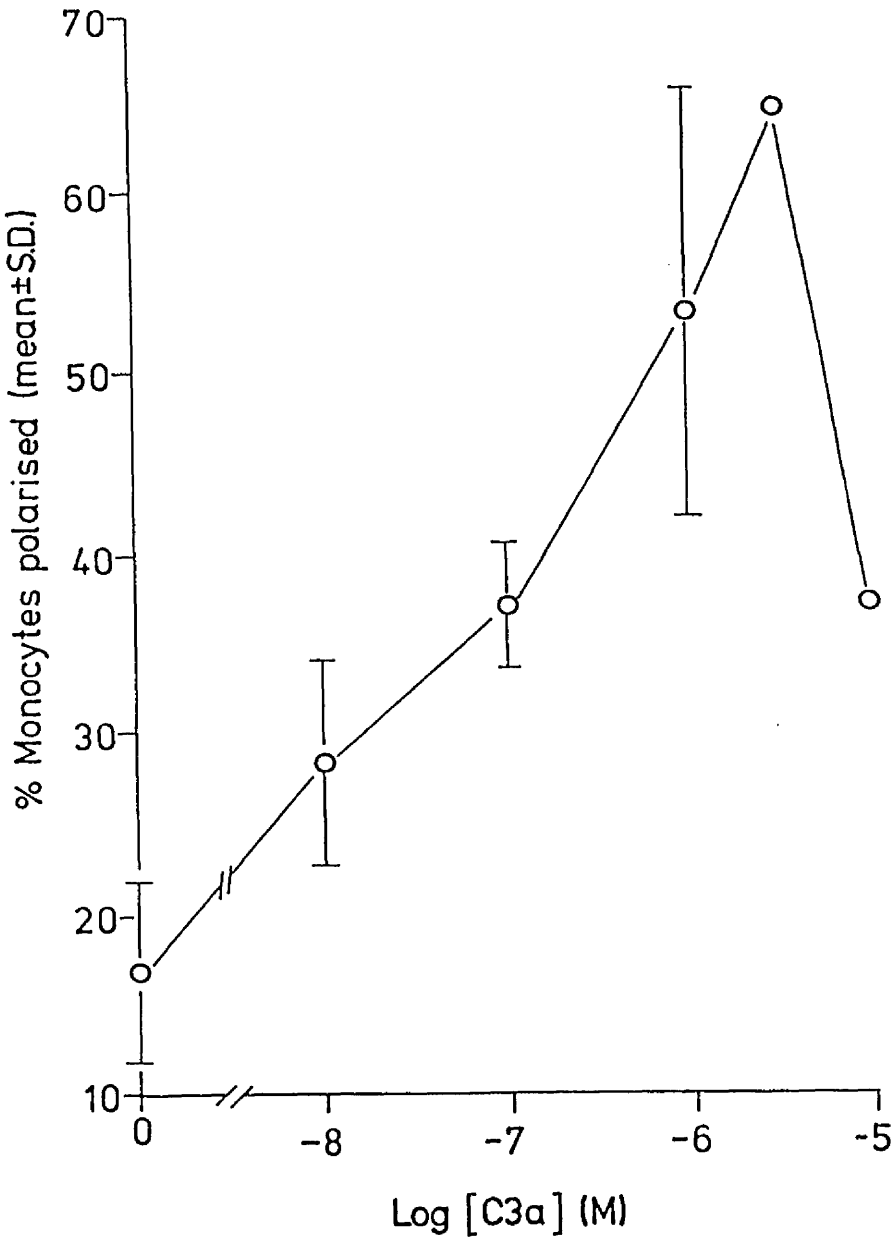



Fig. 27 Dose-response for monocyte polarisation in C3a. The maximum proportion of cells, about 64%, were polarised at 3 X $10^{-6}$ M C3a after 10 min incubation. Due to shortage of the available C3a, the dose 3 X $10^{-6}$ M and $10^{-5}$ M were not used more than once.

maximum response was in between $10^{-7}$ M and 5 X $10^{-6}$ M C3adA when about 40% monocytes changed shape after 10 min incubation at 37°C.

In summary, of all the complement peptides tested towards monocyte polarisation, C3adA was least active. Due to small available quantities of purified C3a the maximum effective dose was not clear. However, it was around $10^{-6}$ M C3a. The potency of the complement peptides towards monocyte polarisation and the order of maximum effective doses are given below .

Potency: C5a or C5adA or C3a > C3adA

Dose: C5a < C5adA < C3a or C3adA

SECTION III

**Combined effect of different chemoattractants on monocyte polarisation**

With no single chemoattractant reported in the earlier sections was it possible to get over 65% polarised monocytes. This is similar to the results of earlier workers (Falk and Leonard, 1980 and 1981 ; Cianciolo and Snyderman, 1981) who suggested that only 60 or so percent of monocytes responded towards chemoattractants because only these cells had chemotactic receptors and the rest lacked them. This hypothesis can be tested by studying the effect of combined chemotactic factors on monocyte polarisation.

To test whether the cell population that was polarised by one chemoattractant could be induced to

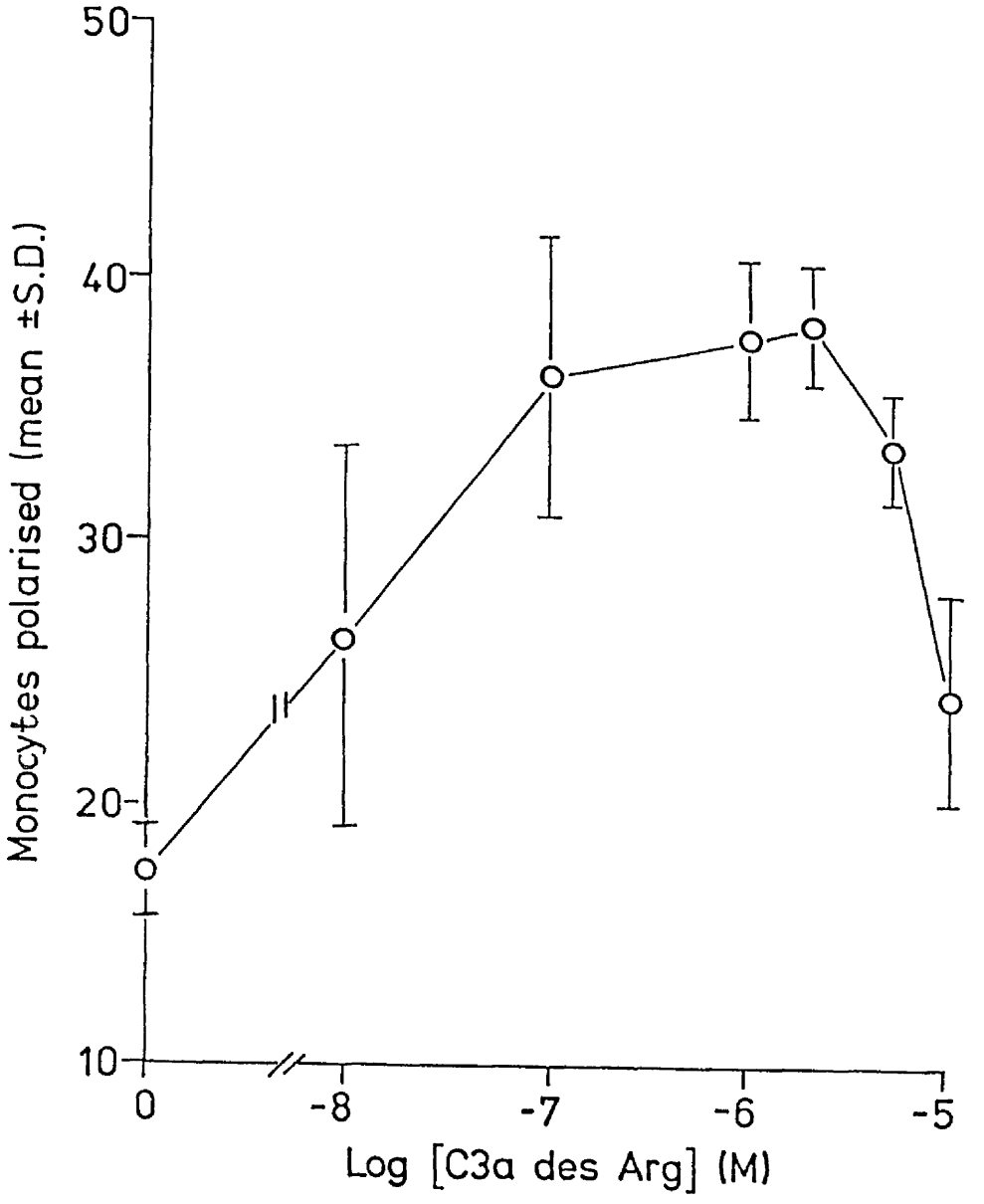



Fig. 28 Dose-response for monocyte polarisation in C3a des Arg. The maximum proportion of cells, about 40%, changed shape in a range between $10^{-7}$ M and 5 X $10^{-6}$ M C3a des Arg. Monocytes responding to C3a des Arg were not well polarised.

polarise further in the presence of additional chemoattractants, monocytes were incubated with the respective optimum dose of more than one chemoattractant for 10, 20 and 30 min at 37°C. When FMLP was combined with other substance(s), the optimum incubation period was 20 min. With other chemoattractants (except FMLP) the optimum incubation period was 10 min.

The results from several experiments are summarised in Table 5 which shows that when **pure substances** were combined together the percentage of polarised cells did not exceed the number obtainable with the maximally effective concentration of any chemoattractant tested alone. That is to say that the combination of 2 or 3 of these chemoattractants did not have any additive effect in increasing the percentage of polarised cells in the population. When 5 X $10^{-9}$ M FMLP was added to the optimum dose of any one of C5a, $LTB_4$, C3a or (C3a + C5a), approximately the same maximal percentage (57.29-69.48%) of monocytes was always polarised as with FMLP alone.

When fresh human serum was combined with either C5a, FMLP or (C3a + C5a), there was no additive effect on monocyte polarisation. $LTB_4$ in combination with human serum had an additive effect (63.37 ± 22.60%) though there was considerable variation in the results from 3 experiments.

In contrast to the above findings and the findings of earlier workers, when FMLP was combined with $LTB_4$ + serum, 80.01%, (S.D. = 11.77) monocytes were polarised. Most interestingly, AHS always induced further

**Table 5. Combined effect of various chemoattractants on monocyte polarisation**

| Test substances | Concentration (respectively) | % monocytes polarised (mean ± S.D.) |
|---|---|---|
| C5a + FMLP | $10^{-8}$ M, 5 X $10^{-9}$ M | 57.29 ± 5.69 |
| FMLP + SFPS | 5 X $10^{-9}$ M, 50% | 55.67 ± 3.28 |
| C5a + SFPS | $10^{-8}$ M, 50% | 59.80 ± 2.15 |
| C5a + $LTB_4$ | $10^{-8}$ M, $10^{-9}$ M | 49.50 ± 5.82 |
| $LTB_4$ + SFPS | $10^{-9}$ M, 50% | 57.70 ± 1.52 |
| C5a + AHS | $10^{-8}$ M, 10% | 71.54 ± 7.00 |
| $LTB_4$ + AHS | $10^{-9}$ M, 10% | 75.35 ± 9.70 |
| ($LTB_4$ + C5a + | ($10^{-9}$ M, $10^{-8}$ M, | |
| FMLP + AHS) | 5 X $10^{-9}$ M, 10%) | 85.40 ± 2.81 |
| FMLP +AHS | 5 X $10^{-9}$ M, 10% | 85.89 ± 7.28 |
| FMLP + $LTB_4$ | 5 X $10^{-9}$ M, $10^{-9}$ M | 68.13 ± 9.02 |
| Serum + C5a | 10%, $10^{-8}$ M | 52.67 ± 3.76 |
| (C5a + FMLP + | ($10^{-8}$ M, 5 X $10^{-9}$ M, | |
| Serum) | 10%) | 69.70 ± 0.78 |
| Serum + FMLP | 10%, 5 X $10^{-9}$ M | 66.02 ± 5.89 |
| C3a + C5a | $10^{-6}$ M, $10^{-8}$ M | 58.16 ± 8.66 |
| (C3a + C5a + | ($10^{-6}$ M, $10^{-8}$ M, | |
| Serum) | 10%) | 51.78 ± 1.81 |
| (C3a + C5a + | ($10^{-6}$ M, $10^{-8}$ M, | |
| FMLP) | 5 X $10^{-9}$ M) | 69.48 ± 5.63 |
| (FMLP + $LTB_4$ + | (5 X $10^{-9}$ M, $10^{-9}$ M, | |
| Serum) | 10%) | 80.01 ± 11.77 |
| $LTB_4$ + Serum | $10^{-9}$ M, 10% | 63.37 ± 22.60 |
| (FMLP + $LTB_4$ + | (5 X $10^{-9}$ M, $10^{-9}$ M, | |
| AHS) | 10%) | 87.62 ± 4.38 |
| C3a + FMLP | $10^{-6}$ M, 5 X $10^{-9}$ M | 58.15 ± 2.95 |

polarisation of monocytes when combined with any chemoattractant, $LTB_4$, C5a or FMLP. With FMLP + AHS the effect was maximum and 85.89 ± 7.28% monocytes in the populations were polarised after 20 min incubation at 37°C. Fig. 29 shows a population photograph of monocytes polarised in FMLP + AHS.

This suggested that the earlier proposal that 40% of blood monocytes lack chemotactic receptors cannot be true. Almost all monocytes responded to mixtures of FMLP with activated serum. This also suggested that the chemotactic properties of activated serum cannot be ascribed to C5a alone. However, the nature of other factors and their receptors is not known.

**Effect of various chemoattractants on the polarisation indices of monocytes**

The polarisation indices of monocytes were calculated in the presence of the optimum dose of AHS, C5a and $LTB_4$ (each after 10 min at 37°C) and the percent monocytes with P.I. values ≥1.35 were determined. Also the P.I. values in the presence of FMLP + AHS were determined in experiments in which over 85% monocytes in the populations changed shape after 20 min incubation at 37°C. These P.I. values were compared to the corresponding shape change scores (from 4 experiments with each chemoattractant and the combination) and the results are illustrated by Fig. 30 which also contains histograms for $5 \times 10^{-9}$ M FMLP and the control (both after 20 min at 37°C). It was observed that the P.I. value ≥1.35, determined to designate a polarised cell

Fig. 29 Differential interference contrast photomicrograph of monocytes responding to a mixture of 5 X $10^{-9}$ M FMLP and AHS (10%, v/v). The cells in suspension were incubated for 20 min at 37°C in presence of the chemotactic factors and then fixed with 2.5% glutaraldehyde. About 85% of the monocytes changed shape in presence of the 2 chemotactic factors, combined together. Bar, 20 μm.

(particularly in FMLP), was also applicable to different chemoattractants and also the combination of FMLP + AHS with different maximal percentage of monocytes polarised.

The P.I. group distributions with the above attractants are presented by Fig. 31 which illustrates the different extent of monocyte polarity in the presence of different substances. $LTB_4$ was the least potent chemoattractant with very few cells in the higher P.I. groups. AHS was comparable to FMLP in terms of maximal percentage of monocytes polarised but there were no cells in the highest and second highest P.I. groups (compared to FMLP). C5a was less effective than FMLP in inducing monocyte shape change but the P.I. distribution pattern was similar to that of FMLP. This indicated that polarised monocytes were as elongated in C5a as they were in FMLP. With FMLP + AHS when ⩾85% cells changed shape, there were more cells in all the polarised P.I. groups (compared to other chemoattractants) with the maximum number of cells in the P.I. group 1.35-1.65. This reflected the fact that many of monocytes wre not well polarised.

The mean P.I. of the monocyte population in presence of the above attractants (from 4 experiments) is summarised in Table 6.

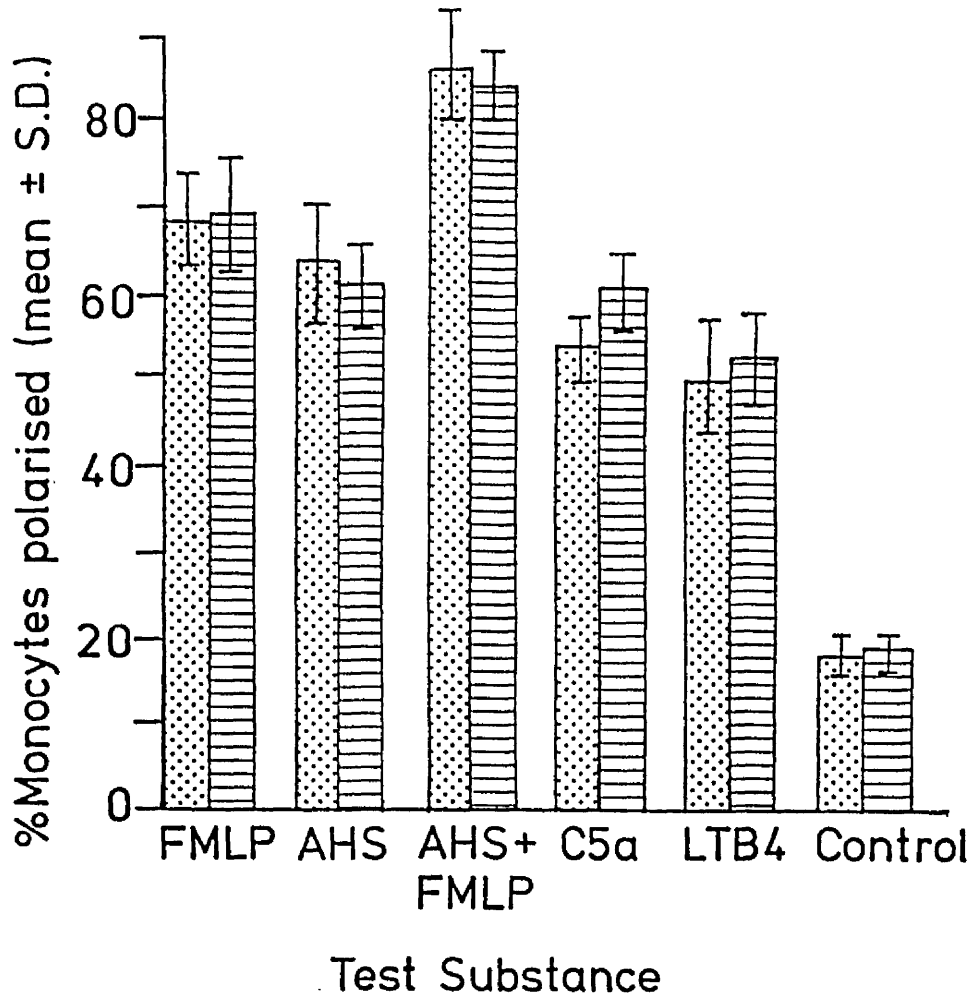



Fig. 30 Comparison of the shape change ( ⊠ ) and the polarisation index (P.I.) values ( ▤ ) of monocytes stimulated with the optimum dose of different chemotactic factors. P.I. values of 1.35 onwards were considered to designate monocytes polarised in 5 X $10^{-9}$ M FMLP and seemed to correlate with the proportion of cells polarised in AHS (at 10%), $10^{-8}$ M C5a, $10^{-9}$ M $LTB_4$ and AHS at 10% and FMLP at 5 X $10^{-9}$ M, combined together. The cells were incubated for 10 min with C5a, $LTB_4$, AHS and for 20 min with FMLP, AHS + FMLP and in the control and then fixed in suspension and examined.

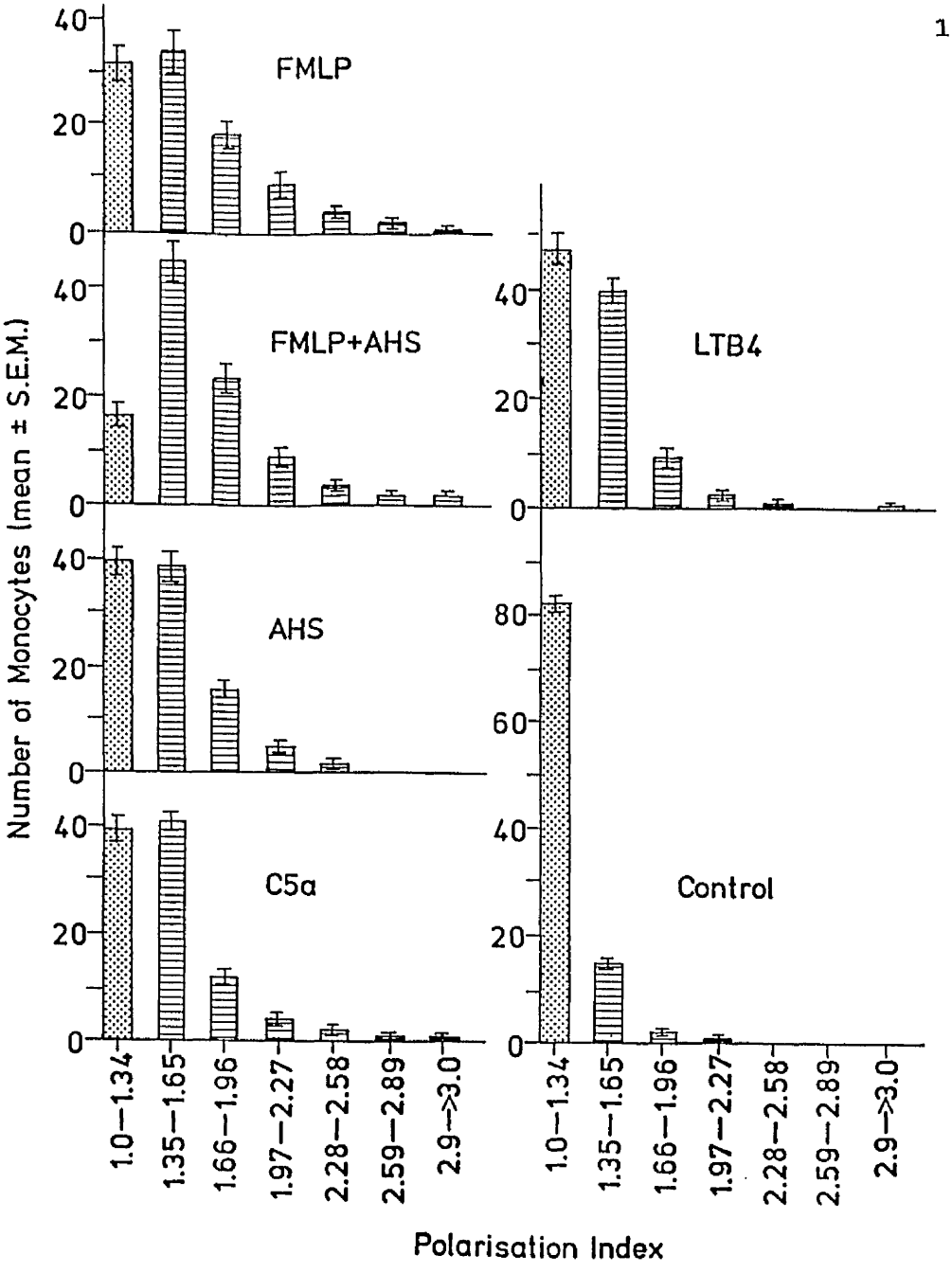



Fig. 31 The distribution of the polarisation index (P.I.) values of monocytes in different chemotactic factors. The dose used and the time of incubation was optimal for the respective chemotactic factor (see Fig. 30). The extent of monocyte polarisation was higher in FMLP and C5a compared to that in $LTB_4$ and AHS as there were very few or no cells with higher P.I. values in $LTB_4$ and AHS. About 85% of the monocytes were polarised in FMLP + AHS where the majority of the cells had P.I. value 1.35-1.65, indicating that the cells were not highly polarised.

Table 6. Effect of various chemoattractants on mean polarisation indices (P.I.) of monocytes in suspension

| Test substances | P.I. of the population (mean ± S.D.) | P.I. of polarised monocytes (mean ± S.D.) |
|---|---|---|
| FMLP | 1.58 ± 0.06 | 1.77 ± 0.03 |
| AHS | 1.46 ± 0.03 | 1.63 ± 0.03 |
| FMLP + AHS | 1.63 ± 0.01 | 1.73 ± 0.02 |
| C5a | 1.48 ± 0.06 | 1.67 ± 0.08 |
| $LTB_4$ | 1.39 ± 0.05 | 1.57 ± 0.06 |
| Control | 1.25 ± 0.04 | 1.43 ± 0.04 |

SECTION IV

**Studies on Fc receptor distribution on monocytes stimulated with optimum concentration of FMLP**

It was observed in the earlier sections that monocytes incubated in the presence of any chemoattractant like FMLP, $LTB_4$, C5a, AHS or even an effective batch of adHSA responded in a time-dependent fashion so that a maximum percentage of cells was polarised after an optimum period (10 or 20 min). After incubating monocytes with these chemoattractants beyond the optimum period, a proportion of cells which were responding earlier lost their polarised morphology and were rounded up again. It was interesting to study the distribution of receptors on the cell membrane after incubation for different time intervals in the presence of a chemoattractant, to see whether monocytes lose their surface receptors when incubated for a long time.

The localisation of Fc and C3b receptors has been reported to be at the front of the neutrophils pre-incubated with an optimum dose of FMLP for an optimum period (Shields and Haston, 1985). Also Sullivan et. al. (1984) demonstrated that the receptors for FMLP on neutrophils are asymmetric. In T-lymphocytes it was observed that the surface marker Thy 1-2 is at the front of the cells fixed while motile (Haston and Shields, 1984).

Based on the findings of earlier workers (using different cell types) it was expected that monocytes stimulated by an optimum dose of FMLP would have an

elongated morphology and that different ligand-binding receptors, including Fc, move towards the front of the polarised cells. FMLP receptors are more difficult to work with compared to Fc or C3b receptors. Hence the localisation of Fc receptors on monocytes was studied in presence of 5 X $10^{-9}$ M FMLP.

Fc receptors on the membranes of polarised and unpolarised monocytes were studied using antibody-coated sheep red blood cells (AbSRBC). The concentration of serum albumin present in the medium used to suspend monocytes and the temperature at which the cells were incubated with AbSRBC seemed to play key roles in the rosetting assays. Their importance is described below.

*(a) Role of HSA in the medium*

Adhesion to the substratum was important for Fc rosetting by monocytes. Cells suspended in HSA at 5 mg/ml in HBSS-MOPS were allowed to adhere to clean glass coverslips for various time intervals at 37°C. It was observed that with prolonged incubation (60 min at 37°C) adhesion was strong which resulted in poor rosette formation when these monocytes were incubated later with AbSRBC. Increasing the concentration of HSA in the cell suspension seemed to reduce strong adhesion to the coverslips during 1 h incubation at 37°C. At the same time a lot of monocytes could not adhere enough during a 20 min incubation. Considering these difficulties 20 mg/ml HSA was found suitable to suspend monocytes for Fc receptor studies.

*(b) Effect of temperature on Fc rosetting*

Monocytes on coverslips covered with AbSRBC were incubated for various time intervals at 0°C (on ice), 4°C, 20°C and 37°C. It was observed that rosetting took a long time (1 h or more) when incubated at 4°C and at 0°C, respectively. Rosetting as well as phagocytosis was favoured with increasing temperature. Incubation at 20°C for 30 min allowed more than 80% monocytes to form Fc rosettes but a lot of red cells were phagocytosed. Monocytes incubated with AbSRBC at 37°C took only 8-10 min for rosette formation but red cells were quickly phagocytosed by the monocytes. Hence they were incubated at 4°C for 1 h to form Fc rosettes since internalization of red cells was at a minimum. Again, prolonged incubation in the cold was not suitable for FMLP stimulated cells to maintain their polarised morphology.

**Studies on monocyte Fc receptors**

Monocytes were allowed to adhere to clean glass coverslips for 20, 40 and 60 min at 37°C in the presence and absence of 5 X $10^{-9}$ M FMLP in 20 mg/ml HSA. Then the cells were incubated for 1 h at 4°C with AbSRBC to form Fc rosettes. The results from 3 experiments are summarised in Table 7a and 7b.

It is clear from Table 7a that when monocytes were allowed to adhere to clean glass coverslips in the presence of HSA alone, there was no effect of the time of incubation (i.e., 20, 40 and 60 min at 37°C) upon the total percentage of monocytes that could form Fc rosettes

Table 7a. Effect of the time of incubation in 5 X $10^{-9}$ M FMLP on the formation of Fc rosettes by stimulated and unstimulated monocytes (rosetted at 4°C for 1 h)

| Monocyte incubation medium (20 mg/ml HSA) | Time of incubation at 37°C (min) | % monocytes rosetted (mean ± S.E.M.) | % polarised monocytes rosetted (mean ± S.E.M.) |
|---|---|---|---|
| HSA alone | 20 | 91.00 ± 2.89 | 9.10 ± 3.03 |
| FMLP in HSA | 20 | 91.30 ± 2.59 | 29.10 ± 1.06 |
| HSA alone | 40 | 93.67 ± 1.33 | 10.67 ± 2.43 |
| FMLP in HSA | 40 | 86.70 ± 6.36 | 22.83 ± 4.47 |
| HSA alone | 60 | 86.77 ± 3.96 | 8.02 ± 2.77 |
| FMLP in HSA | 60 | 55.00 ± 3.00 | 6.00 ± 1.52 |

**Table 7b. Effect of the time Of incubation in 5 X $10^{-9}$ M FMLP on the localization of Fc rosettes by stimulated and unstimulated monocytes (rosetted) at 4°C for 1 h)**

| Monocyte incubation medium (20 mg/ ml HSA) | Time of incubation at 37°C (min) | Position of Fc rosettes in round cells (mean ± S.E.M.)% | | polarised cells (mean ± S.E.M.)% | |
|---|---|---|---|---|---|
| | | general | capped | general | head |
| HSA alone | 20 | 82.9±3.7 | 17.1±3.7 | 77.9±2.1 | 22.1±2.1 |
| FMLP in HSA | 20 | 71.9±7.3 | 28.0±7.4 | 62.5±4.1 | 37.5±4.1 |
| HSA alone | 40 | 77.8±8.3 | 22.2±8.3 | 75.2±7.8 | 24.8±7.8 |
| FMLP in HSA | 40 | 79.6±3.9 | 20.4±3.9 | 65.9±7.2 | 34.1±7.2 |
| HSA alone | 60 | 80.1±3.4 | 19.9±3.3 | 85.5±2.4 | 14.5±2.4 |
| FMLP in HSA | 60 | 81.7±1.7 | 18.3±1.7 | 87.8±1.1 | 12.2±1.1 |

(over 90%). When the cells were incubated with 5 X $10^{-9}$ M FMLP for different time intervals (particularly at 60 min) the total percentage of monocytes that could form Fc rosettes with the AbSRBC was greatly reduced (55.0%, S.E.M. = 3.0) compared to the corresponding controls (86.77%, S.E.M. = 3.96).

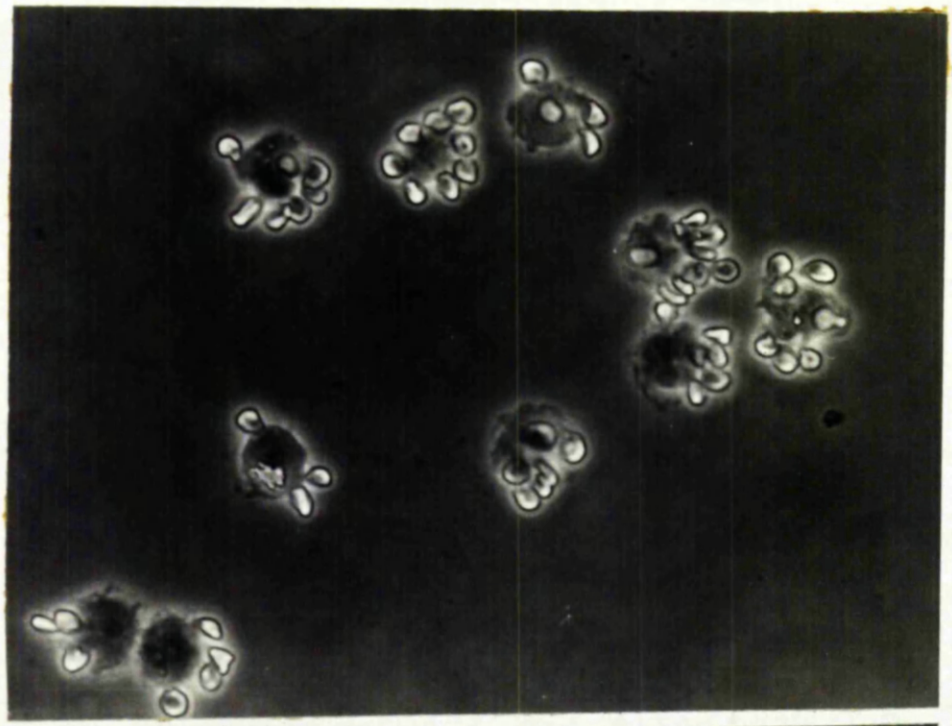
Fig. 32 A, B, C and D shows photomicrographs of Fc rosetted monocytes in (A) HSA after 60 min, (B) and (C) FMLP after 20 min and (D) FMLP after 60 min. It is clear from Fig. 32 D that a lot of monocytes could not bind with antibody-coated sheep red cells due to prolonged incubation in FMLP (60 min) before doing the rosetting assay. Fig. 33 shows one head rosetted monocyte in FMLP after 20 min (under high power) which illustrates that Fc receptors are at the front of the cell when stimulated for an optimum time.

In another set of experiments monocytes on coverslips (in 5 mg/ml HSA) were rosetted at 37°C by incubating with AbSRBC for 10 min (Table 8). It was observed that the total percentage of Fc rosetted cells was reduced when they were preincubated for a longer period in FMLP. Though a lot of red cells were phagocytosed by the monocytes at 37°C, these experiments also supported the results shown in Table 7a. The results are shown in Table 8.

These observations suggested that in the presence of an attractant, monocytes lost their surface receptors (in this case Fc, perhaps internalised) due to prolonged exposure and lost their responsiveness towards that attractant upon further incubation.

It was described earlier that monocytes pre-stimulated with FMLP at 37°C were incubated at 4°C with AbSRBC to form Fc rosettes. Incubation for 1h in the cold reduced both the proportion of cells polarised and the extent of polarity in those that were polarised. Since all the monocytes previously incubated either with or without FMLP for various time intervals were later incubated at 4°C with AbSRBC for rosette formation, the proportion of polarised monocytes rosetted in each group could be compared. Pre-incubation of monocytes for 20, 40 and 60 min in HSA alone had no effect on the proportion of polarised cells that could form Fc rosettes. On the other hand, pre-incubation for different time intervals in FMLP gave different proportions of polarised monocytes that could form Fc rosettes. The results presented in Table 7a (column 4), showing a decrease in the number of polarised monocytes rosetted with the time of incubation in FMLP, are consistent with the earlier observation of loss of polarity due to prolonged incubation of monocytes in suspension with FMLP. However, Table 7b shows that a high proportion of the cells could form head rosettes when pre-incubated for 20 min in FMLP before doing the rosetting assay. The proportion of head rosetted monocytes decreased greatly when the cells were pre-incubated for 60 min in FMLP before doing the rosetting assay.

A



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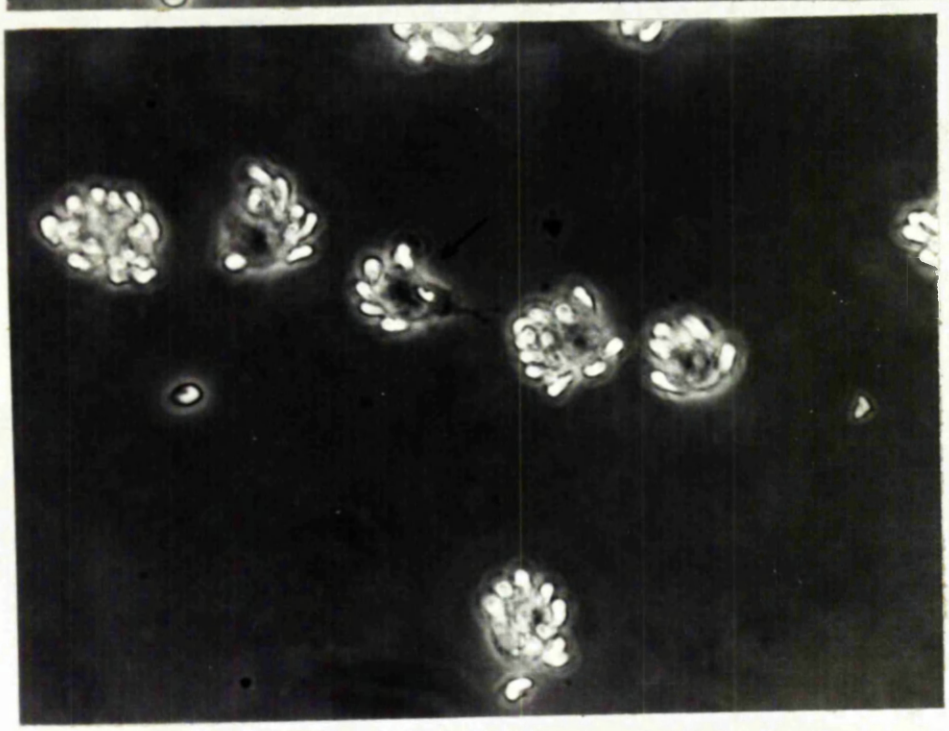
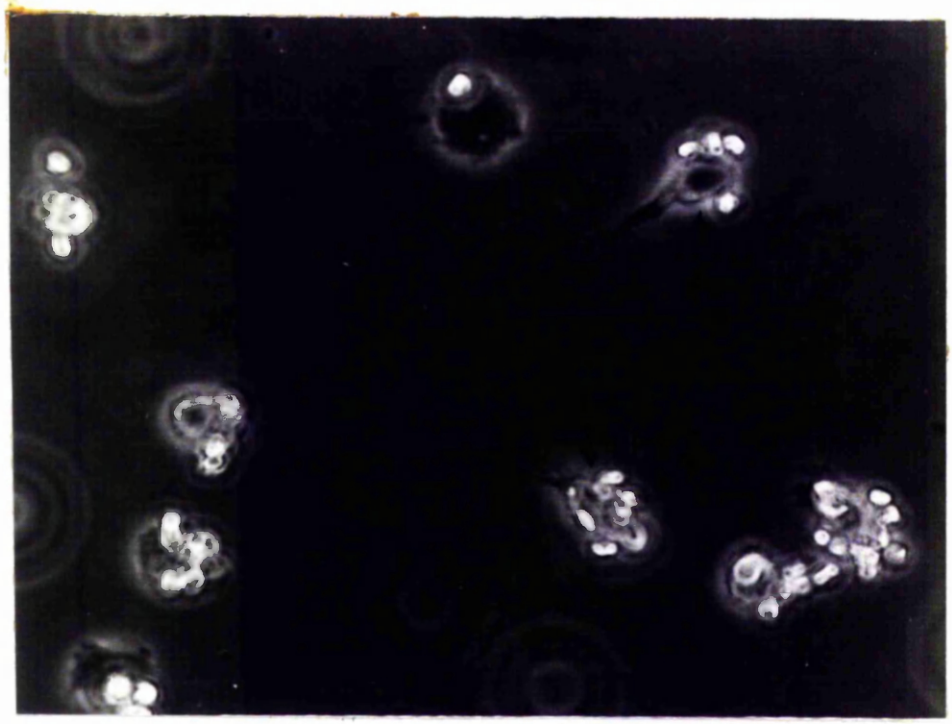
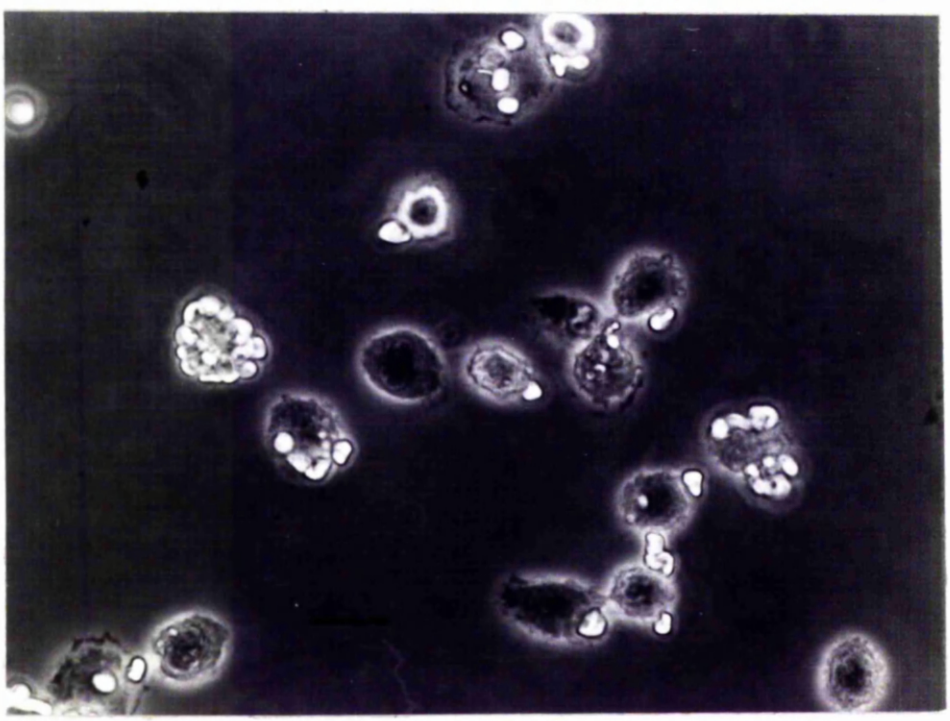


Fig. 32 A-D. Phase-contrast photomicrographs of monocytes rosetted with antibody-coated sheep red cells (Fc receptor). A: Monocytes incubated for 60 min at 37°C in HSA (20 mg/ml) before the rosetting assay. B and C: Fc rosettes in monocytes preincubated for 20 min at 37°C in 5 X $10^{-9}$ M FMLP/20 mg $ml^{-1}$ HSA. Note monocytes with locomotor morphology capped the red cells at the head region (shown with arrows).



C



D

D: Lack of Fc rosette formation by monocytes preincubated for 60 min at 37°C in 5 X $10^{-9}$ M FMLP/ 20 mg $ml^{-1}$ HSA. Experiments presented in this thesis describes that monocytes internalise their surface receptors due to prolonged incubation in chemotactic factor. Bar, 20 µm.

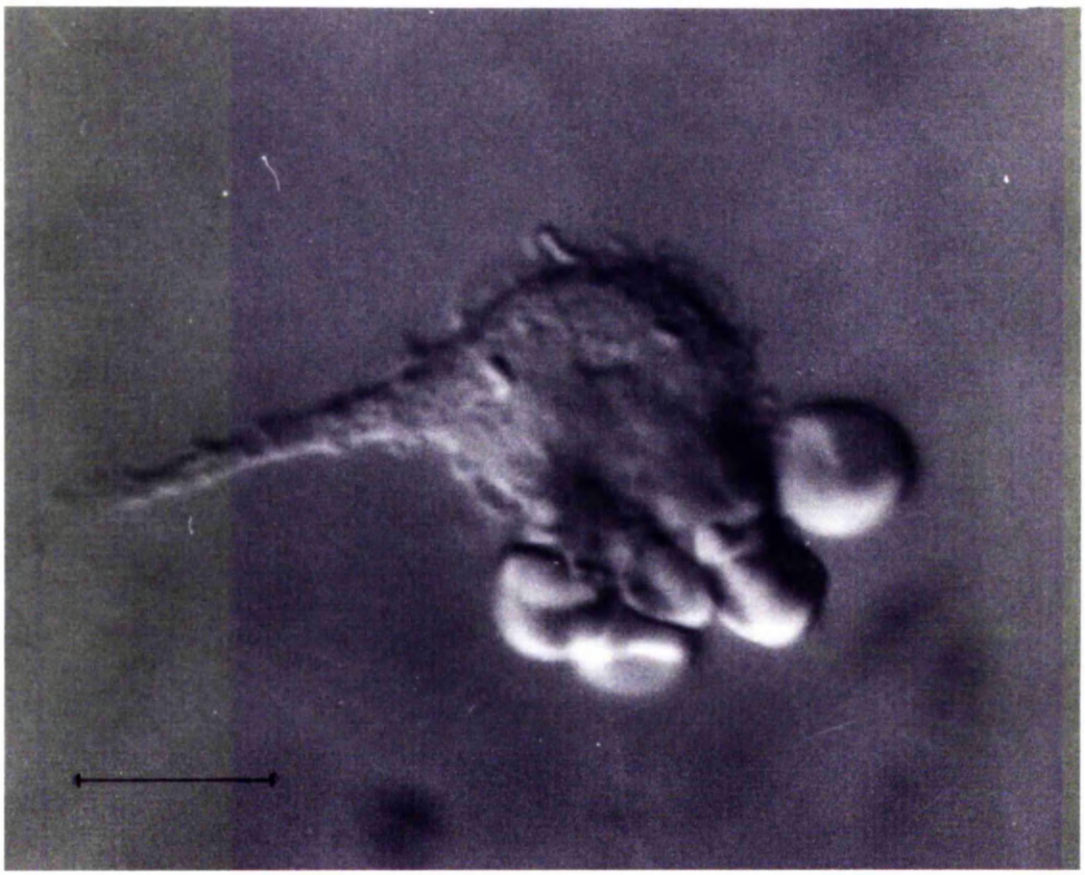


Fig. 33 High power differential interference contrast photomicrograph of the distribution of ligand-bound Fc receptors on a polarised monocyte. The monocyte was incubated for 20 min at 37°C in 5 X $10^{-9}$ M FMLP in HSA (20 mg/ml) before rosetting with red cells. Formation of head rosettes in cells with locomotor morphology indicates that the receptors are localised at the front of the cell. Bar = 10 μm.

Table 8. Effect of the time of incubation in 5 X $10^{-9}$ M FMLP on the formation of Fc rosettes by stimulated and unstimulated monocytes (rosetted at 37°C for 10 min)

| Monocyte incubation medium (5 mg/ml HSA) | Time of incubation at 37°C (min) | Monocytes rosetted (% of total cells) |
|---|---|---|
| HSA alone | 20 | 87.0 |
| FMLP in HSA | 20 | 79.5 |
| HSA alone | 40 | 81.5 |
| FMLP in HSA | 40 | 81.0 |
| HSA alone | 60 | 79.0 |
| FMLP in HSA | 60 | 61.5 |

In summary, if monocytes were preincubated on coverslips (up to 60 min at 37°C in the presence of HSA at 20 mg/ ml) up to 90% or so of them could form Fc rosettes with antibody-coated sheep red blood cells (AbSRBC). In contrast to this, when cells were incubated in the presence of an attractant, like FMLP, for a long period (60 min, while the optimum incubation period is 20 min), the maximum percentage of cells that could form Fc rosettes was greatly reduced. A lot of cells did not have Fc receptors to form rosettes with AbSRBC. These observations clearly indicated that the cells had lost their Fc receptors due to prolonged incubation in FMLP. Also the monocytes might have lost their FMLP receptors (which are technically impossible to study). If the cells did lose their chemotactic receptors, this would clearly explain why they rounded up when incubated for longer periods in the presence of different chemoattractants. Hence the loss of monocyte polarisation with time using the various substances tested in earlier sections could be explained.

# CHAPTER 5

# MONOCYTE SHAPE CHANGE IN CULTURE

The polarising responses of monocytes in the presence of various chemoattractants were described in the previous chapter. The cells were incubated for 30 s up to a maximum interval of 2 h with those substances. This chapter describes the results when monocytes were cultured in 2 different media for various time intervals up to 3 days. No chemoattractant or mitogen was added to the cells to induce polarisation.

**Studies of shape change in short-term and during culture**

A very low proportion of unstimulated monocytes direct from blood showed typical polarised morphology in RPMI-FCS or RPMI-HSA at 20 min incubation. It should be recalled here that 16% ( S.D. = 3.1, n = 56 ) of cells showed polarised morphology when they were just purified from blood using a BHK microexudate-coated surface. The proportion of polarised cells increased dramatically when the cells were cultured for long intervals in either medium with a maximum effect in RPMI-FCS. The results from 3 experiments are shown in Fig. 34 which shows that about 60% monocytes in both culture conditions showed polarised morphology at 18 h incubation. Then the rate of polarisation increased slowly until in RPMI-HSA 65-75% cells had changed shape at 24 h. This percentage did not increase upon further incubation up to 72 h. At this time cell viability was falling rapidly in RPMI-HSA. In RPMI-FCS the proportion of polarised cells increased with the time of culture and about 90% cells showed polarised morphology after 72 h. Also cell viability was significantly higher in this medium than in RPMI-HSA.

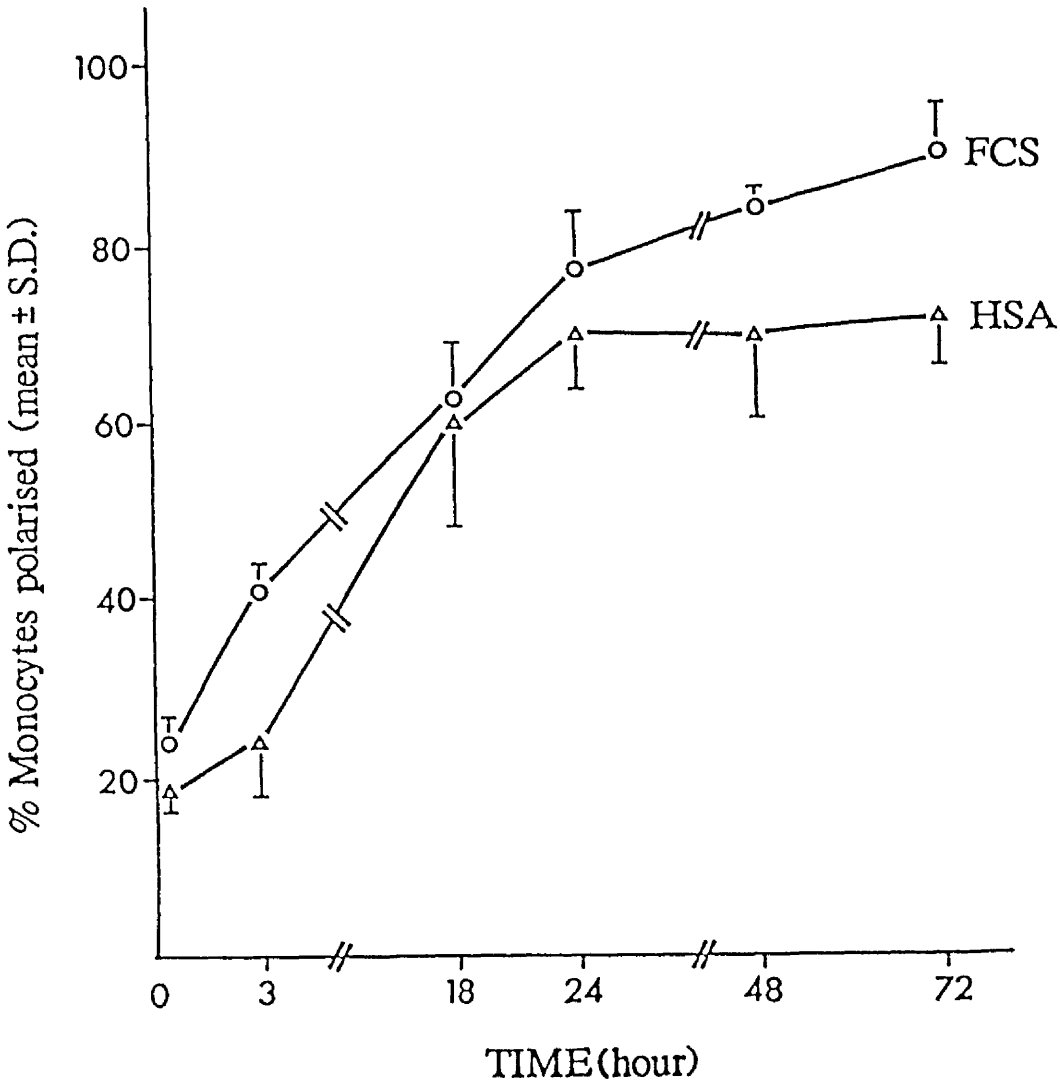



Fig. 34 Monocyte shape change in different culture conditions. The cells were cultured in siliconized glass tubes in 20% FCS in RPMI and in 10mg/ml HSA in RPMI. A starting proportion of 15-25% cells changed shape after 20 min of setting up the experiment and then the proportion of polarised cells increased with the time of culture. The maximum percentage of monocytes, about 90%, changed shape in FCS and also the cell viability was better in the serum (FCS) containing medium than in HSA.

This suggests that serum was necessary for cell culture over long intervals. Monocyte viability results in 2 culture conditions are presented by Table 9. Fig. 35 illustrates the differential interference contrast appearance of monocytes (A) after short-term assay in RPMI-FCS, (B) cultured for 72 h in the same medium, (C) and (D) elongated morphology of cells cultured for 72 h in RPMI-HSA and (E) the appearance of the non-adherent population (mainly lymphocytes) after culturing for 72 h in RPMI-FCS (described later).

**Effect of monocyte culture supernatant on freshly isolated cells**

After obtaining a high proportion of cells that changed shape in culture it was expected that there might be certain metabolite(s) secreted in the culture supernatant which could induce shape change of fresh monocytes. Hence freshly isolated cells were suspended in the supernatants collected from monocyte cultures (after 72 h) and were incubated for 20 min at 37°C. The control cells suspended in freshly prepared media were incubated for the same length of time. The results are presented in Table 10 which shows that the culture supernatant from RPMI-HSA had no effect on fresh cells. There was considerable variation in the percentage of monocytes that changed shape with 3 batches of RPMI-FCS culture supernatants and the average effect was more than the background response. This observation suggested that there were some cell released factor(s) or metabolite(s)

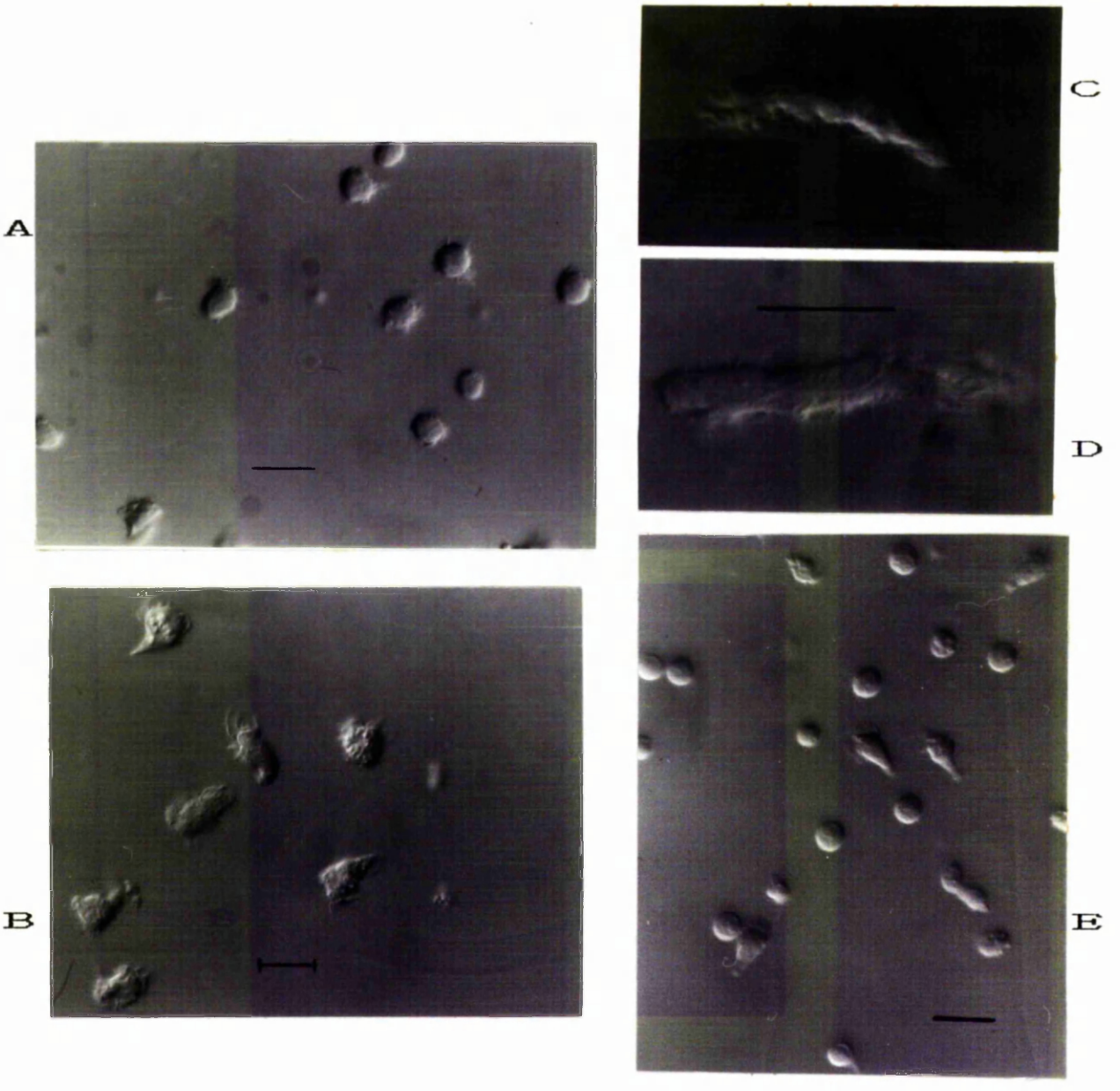



Fig. 35 A-E. Differential interference contrast photographs of monocytes and non-adherent mononuclear cells under different culture conditions. A: Monocytes after 20 min in FCS (20%. v/v) in RPMI showing non-motile cells; B: after culture for 72 h in the same medium showing highly polarised cells; C and D: elongated morphology of monocytes cultured for 72 h in RPMI-HSA (10 mg/ml); E: non-adherent mononuclear cells after culture for 72 h in RPMI-FCS (20%, v/v) showing many polarised cells. Bar = 20 µm.

**Table 9. Monocyte viability in different culture media**

| Medium | % viable cells at different time of culture | | | |
|---|---|---|---|---|
| | 18 h | 24 h | 48 h | 72 h |
| RPMI-FCS (20%, v/v) | 92 | 88 | 82 | 77 |
| RPMI-HSA (10 mg/ml) | 85 | 81 | 70 | 58 |

**Table 10. Effect of monocyte culture supernatant on freshly isolated cells**

| Medium used to suspend cells | % polarised monocytes (mean ± S.D.) |
|---|---|
| Fresh medium : | |
| RPMI-FCS | 14.75 ± 3.95 |
| RPMI-HSA | 13.67 ± 4.04 |
| Culture supernatants (collected at 72 h) : | |
| RPMI-FCS | 27.14 ± 14.05 |
| RPMI-HSA | 15.33 ± 02.31 |

present in the culture supernatant (containing FCS) which induced shape change of fresh cells. However, the nature of the active substance is not known.

**Addition of OKT3 to monocytes**

To see if the lymphocyte activating monoclonal antibody, anti-T3 (OKT3), had any effect on monocyte shape change in short-term assays, OKT3 was added to the cells at 5 ng/$10^6$ cells and was incubated for 20 min at 37°C. The monocytes did not respond towards OKT3.

It had recently been reported by Wilkinson (1986) that anti-T3 (OKT3) induced lymphocyte shape change after 24 h in culture. It was hoped that the supernatant from OKT3-cultured lymphocytes might induce shape change of fresh monocytes in short-term assays. Hence mononuclear cells and the non-adherent (monocyte depleted) lymphocytes (separated by incubating mononuclear cells on BHK microexudate-coated surface) suspended in RPMI-FCS at 2 X $10^6$ cells/ml containing 10 ng/ml OKT3 were cultured for 24 and 48 h, respectively. Then the culture supernatants were collected and added to freshly isolated monocytes. The results suggested that mononuclear cell and monocyte depleted lymphocyte culture supernatants (both containing OKT3) had no effect on monocyte shape change in short-term assays ( after 20 min).

**Effect of cycloheximide in monocyte culture**

It was interesting to explore what metabolic process was occurring in the monocyte population when about 90% cells changed shape during culture for long periods. To

see whether protein synthesis was necessary, the protein synthesis inhibitor, cycloheximide ($10^{-5}$ M), was incorporated with monocytes suspended in RPMI-FCS and the cells were then cultured for 24 h. It was observed that only 15% cells had polarised morphology after this time which was no higher than the baseline response of uncultured monocytes. That is, shape change did not occur in culture with cycloheximide. It was mentioned earlier that culturing cells for 24 h resulted in over 70% monocyte shape change (with no cycloheximide). This observation suggested that monocytes synthesise proteins necessary for the locomotor response in culture. Similar results were obtained with lymphocytes in cycloheximide-treated cultures (Wilkinson, 1986).

The supernatants collected from monocytes cultured for 24 h both in the presence and absence of cycloheximide were tested on freshly obtained cells. These cells showed no response towards the cycloheximide containing supernatant. In contrast, there was about 26% shape change of the monocytes towards the cultured supernatant without cycloheximide. This percentage response was about 10% more than the background count of polarised cells. When cycloheximide ($10^{-5}$ M) was added to fresh cells suspended in the ideal culture supernatant (where cells were cultured in RPMI-FCS without cycloheximide), the same percentage of cells ($\simeq$26%) responded again. This indicated that cycloheximide did not interfere with cell polarisation in the short-term assay.

Incubating monocytes direct from blood in 5 X $10^{-9}$ M FMLP with and without cycloheximide resulted in about 64% shape change in both cases (monocyte responsiveness towards FMLP was described in the previous chapter). This suggested that protein synthesis was not necessary for stimulation of polarisation in cells that already possess polarisation capacity and which have cell-surface receptors for chemotactic factors but is necessary for the induction of polarity as they synthesise more receptors in culture (Pike et al., 1980).

**Experiments with the non-adherent mononuclear cell population from BHK microexudate-coated surfaces**

It was reported earlier that about 20% of the blood monocytes were non-adherent to the BHK microexudate-coated surface used to separate them from lymphocytes. Hence the lymphocyte preparation always contained some monocytes (mostly non-adherent cells). The identity of the cells was confirmed by ANAE stain (described in chapter 2). It was of interest to see whether the non-adherent monocytes could respond towards chemoattractants and also change shape in culture.

The non-adherent mononuclear cells (NAMS) suspended in HSA (at 20 mg/ml) in HBSS-MOPS were incubated with candida spore-activated human serum (AHS) for 10 min at 37°C (experiments with AHS were described in the previous chapter). Only 12% cells changed shape towards AHS. This suggested that it could be the monocytes and a proportion of lymphocytes which responded towards the AHS. Staining

the NAMS preparation for ANAE suggested that it contained 8% monocytes.

The NAMS were used for short-term assays and also cultured in 2 media as described for monocytes. The results presented in Fig. 36 show that there was very little shape change of NAMS in the short-term assay. Only 7% cells in RPMI-HSA had polarised morphology at 20 min which increased up to 13% at 18 h, 15% at 24 h and did not increase further with time of culture. In RPMI-FCS, about 12% of the NAMS changed shape at 20 min which increased up to 21% at 18 h then very slowly increased up to 26% at 48 h followed by sharp increase during the next 24 h when about 40% cells were polarised at 72 h. It had been reported recently that a very small proportion of lymphocytes could change shape when cultured in HSA and about 30% cells changed shape when cultured in 20% FCS, both for 72 h (Wilkinson, 1986). These observations seemed to correlate with the results obtained from NAMS culture experiments. Hence the small percentage of shape change occurring in the culture experiments with RPMI-HSA and the further shape change, up to 40% in RPMI-FCS (considering 30% of the lymphocytes could change shape), could be ascribed to the small proportion of non-adherent monocytes present in the NAMS. Fig. 35 E illustrates a Nomarski photomicrograph for the shape change of non-adherent mononuclear cells (NAMS) cultured for 72 h in RPMI-FCS.

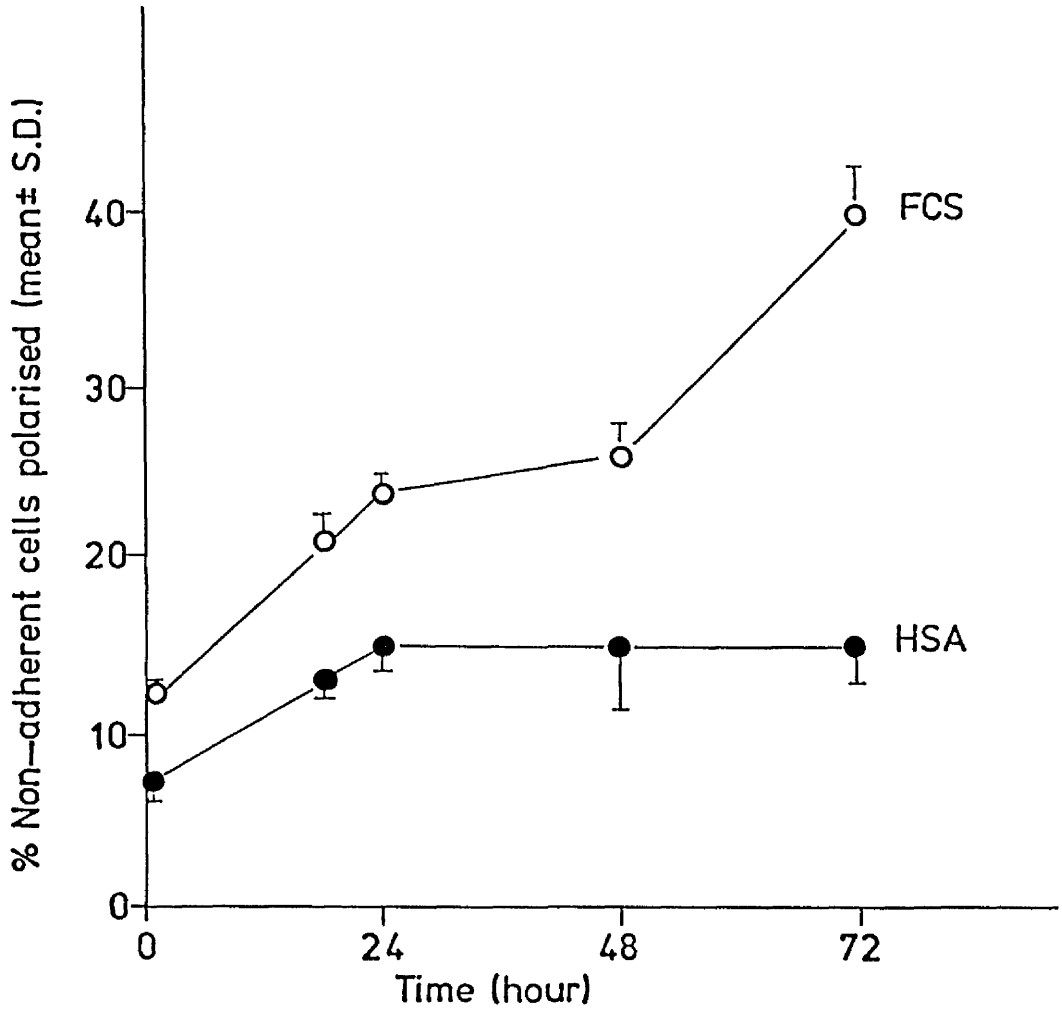



Fig. 36 Shape change of non-adherent mononuclear cells (NAMS) during culture in 20% FCS in RPMI and in 10 mg/ml HSA in RPMI. The proportion of polarised NAMS in HSA reached a maximum level of 15% at 24 h and did not increase further with time of culture. On the other hand up to 40% of NAMS changed shape in FCS containing medium. Up to 30% of human lymphocytes would change shape during culture under similar conditions (Wilkinson, 1986). Lymphocytes were the majority of cells in the NAMS preparation. The data suggested that a proportion of the non-adherent monocytes changed shape in culture to give values over 30%.

**Morphology of monocytes in culture**

The morphology of monocytes in the presence and absence of different chemoattractants was described in detail in the previous chapter. The present chapter considers monocyte shape change when the cells were cultured for a long time. It appeared (p160-2) that the cells probably synthesise proteins while in culture. It was interesting to see that there were a lot of cells in both the culture media having much more elongated morphology than those observed in the presence of chemoattractants. Hence morphology of the cells straight from blood, suspended in RPMI-FCS, at short-term assay and after culturing for 3 days were compared by measuring their lengths. The results presented by Fig. 37 shows that the cells were highly polarised and elongated while they were in culture. The mean cell length in the short-term assay was 10.8 μm (S.D. = 0.93) and after 72 h in culture was 18.1 μm (S.D. = 1.81) (cf. Fig. 9 and Table 1 in chapter 4). Calculating the length/breadth ratios to give the polarisation index (P.I.) of these cells, it was observed that the mean P.I. for cells after the short-term assay was 1.26 (S.D. = 0.03) and for cells after culturing 3 days was 1.79 (S.D. = 0.07)

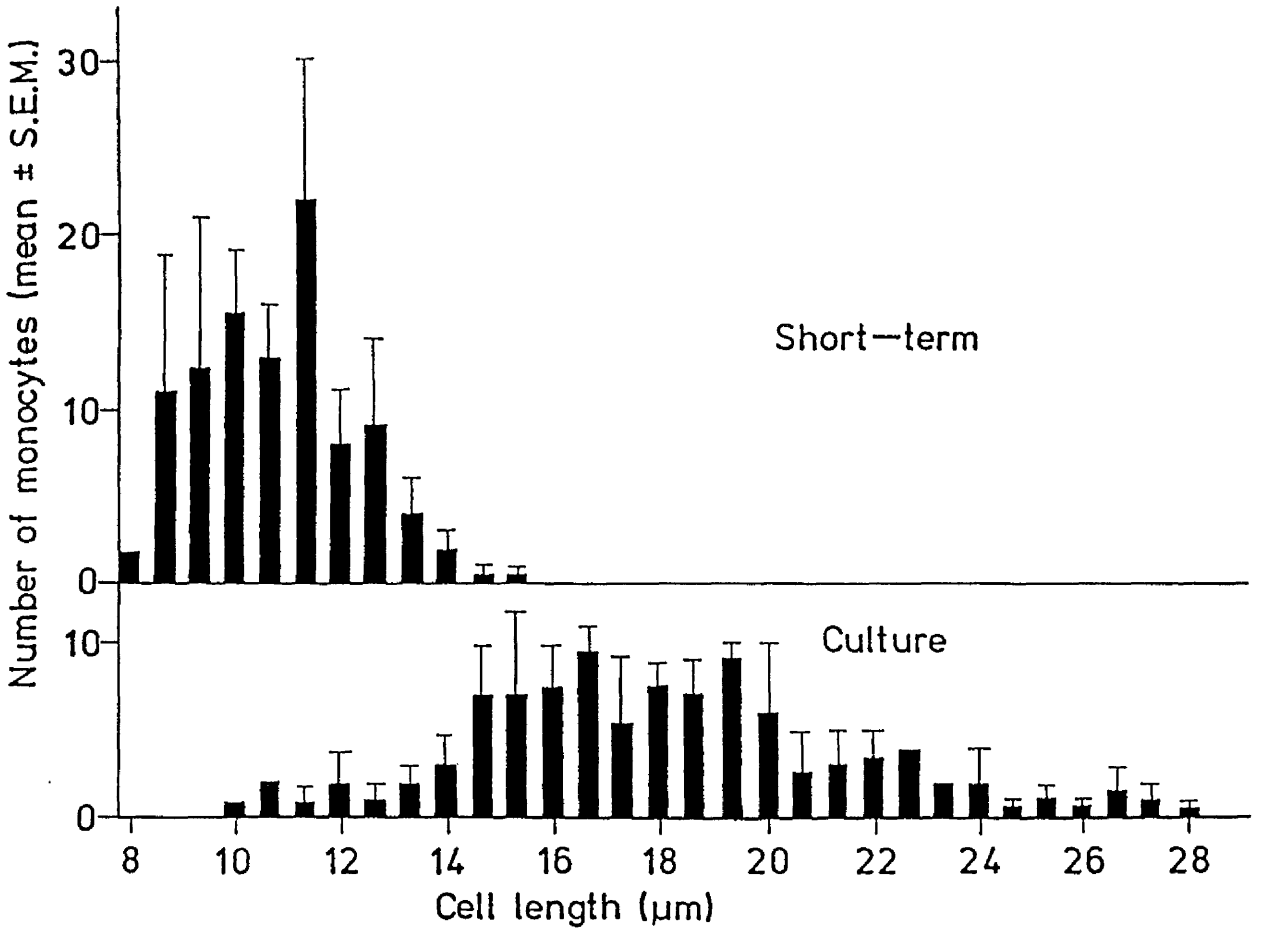



Fig. 37 Distribution of monocyte lengths in RPMI-FCS (20%) in the short-term (20 min) and in culture (72 h). The cells were bigger in size and were more elongated during culture than they were in FMLP (cf. Fig. 9) and their average length/breadth ratio was 1.79.

In summary, a very low proportion of unstimulated monocytes direct from blood showed polarised morphology in RPMI-HSA and RPMI-FCS (20%, v/v), and this proportion increased with time of culture. About 90% monocytes changed shape when cultured for 72 h in RPMI-FCS. At this time up to 70% cells were polarised in RPMI-HSA. Monocyte viability was higher in the presence of serum (FCS) than in its absence. The RPMI-FCS culture supernatant induced shape change of fresh cells, while RPMI-HSA was inactive. Lymphocyte activating monoclonal antibody, OKT3, or supernatants obtained by culturing the lymphocytes or mononuclear cells in the presence of OKT3 added to freshly isolated monocytes could not induce shape change. Monocytes in culture synthesise proteins which were responsible for induction of polarity during culture. The small proportion of blood monocytes which could not adhere to protein-coated surfaces would also change shape if cultured in RPMI-FCS for long periods. Monocytes were larger in size and were much more elogated when they were cultured compared to those obtained direct from blood.

# CHAPTER 6

# CHEMOATTRACTANT INDUCED LOCOMOTION OF PHAGOCYTES THROUGH GELS

Most of the studies published up till now using 3-dimensional gels of collagen have been studies of non-chemokinetic and non-chemotactic locomotion. In this chapter the chemokinetic and chemotactic locomotion of human neutrophil leucocytes were studied using collagen gels. The first and second section will consider experiments with neutrophils since the basic conditions for the assay were established using these cells. Later, in the third section, the chemokinetic locomotion of human blood monocytes will be described.

## SECTION I

### Chemokinesis of neutrophils in collagen gels

The dose response curve for the leading front measure of neutrophils invading collagen gels in the presence of different uniform concentrations of FMLP after incubating for 2 h at 37°C is shown in Fig. 38. Similar dose-response curves were obtained in 4 similar experiments. The optimum concentration of FMLP in this assay was $10^{-8}$ M. In the absence of FMLP the leading front value was low which is shown in the figure by the point on Y-axis.

### Time course of invasion

In Fig. 39 is shown the time course of invasion of collagen gels by neutrophils in the presence and absence of an isotropic concentration of $10^{-8}$ M FMLP. The figure represent two experiments in each set where experiments

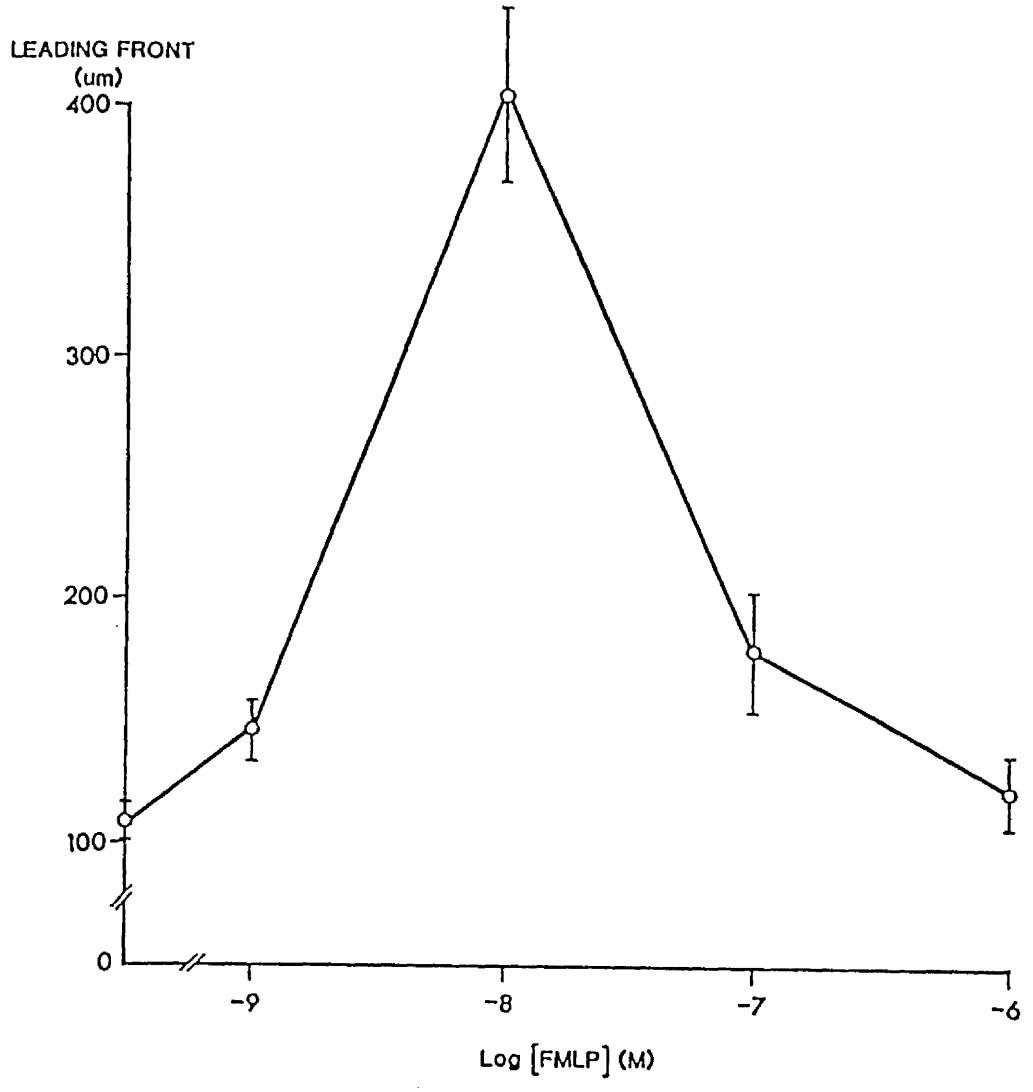



Fig. 38 Dose-response curve for the leading front of neutrophils migrating into collagen gels (1.5 mg/ml) in different concentrations of FMLP. Leading front value at 2 h ± S.D.

FMLP (i) and control (i) were carried out with the same preparation of neutrophils. Similarly, experiments FMLP (ii) and control (ii) were done with the same batch of neutrophils.

It is shown in Fig. 39 that the leading front measure increased with time but the relationship was not linear. In experiments with FMLP, neutrophils rapidly migrated into the gel and they had maximum population velocity during the first hour of incubation. Then the velocity decreased while the cells were incubated for 4-5 h. The leading front values were variable in the controls and the cells were moving much more slowly. At the end of a 2 h incubation period, control cells invaded 90-180 μm into the gel, while cells in the presence of $10^{-8}$ M FMLP migrated 320-450 μm. For further experiments an incubation time of 2 h was chosen. If longer times were chosen, it was found that the leading front values began to diverge both in FMLP and in the controls (Fig. 39). If shorter times were chosen, neutrophil penetration of the gel was insufficient to allow accurate plotting of cell numbers at different levels. Table 11 contains a summary of 4 experiments for the migrating velocity of neutrophils at the leading front at different times in the presence and absence of uniform concentration of $10^{-8}$ M FMLP in the gel. Fig. 40 A and (B, C) illustrate population photographs of neutrophils within collagen gels in the presence of uniform concentration of $10^{-8}$ M FMLP under phase-contrast and differential interference contrast optics, respectively.

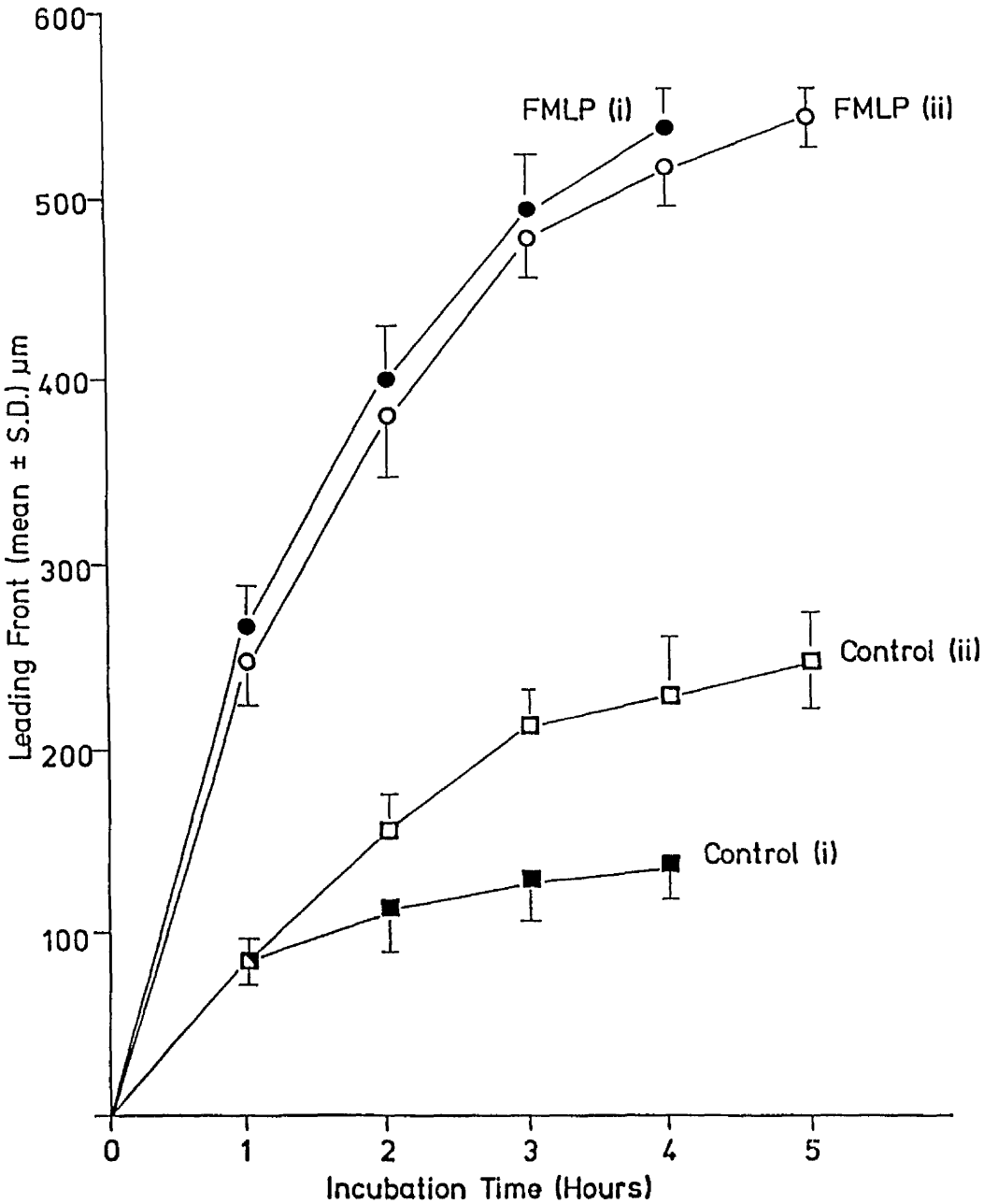


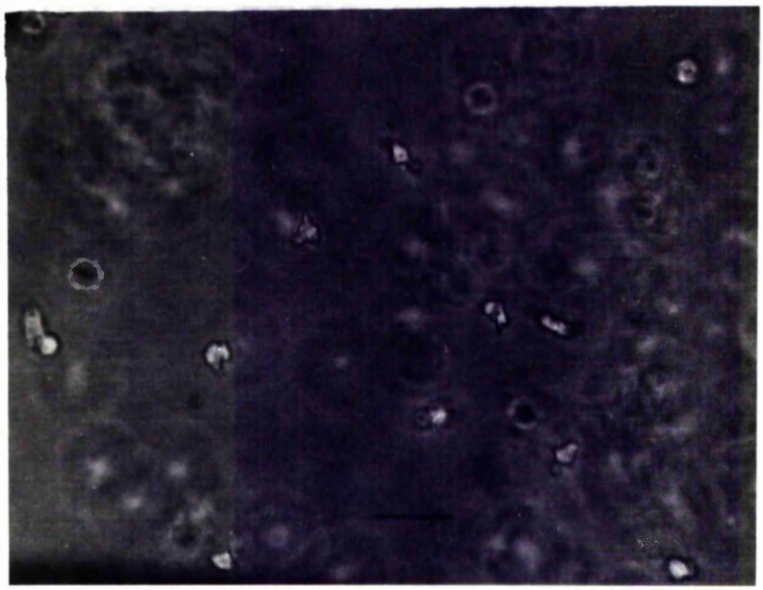

Fig. 39 Time course of invasion by neutrophils of collagen gels (1.5 mg/ml) in the presence and absence of $10^{-8}$ M FMLP (throughout). The figure illustrates the leading front assay for 2 separate set of experiments [FMLP (i), control (i) and FMLP (ii), control (ii)] with the gels. The leading front values with FMLP were similar in experiments (i) and (ii) during 4 h of incubation but the values were different for control (i) and (ii). This perhaps reflects the physical properties of the gel.

**Proportion of cells invading gels**

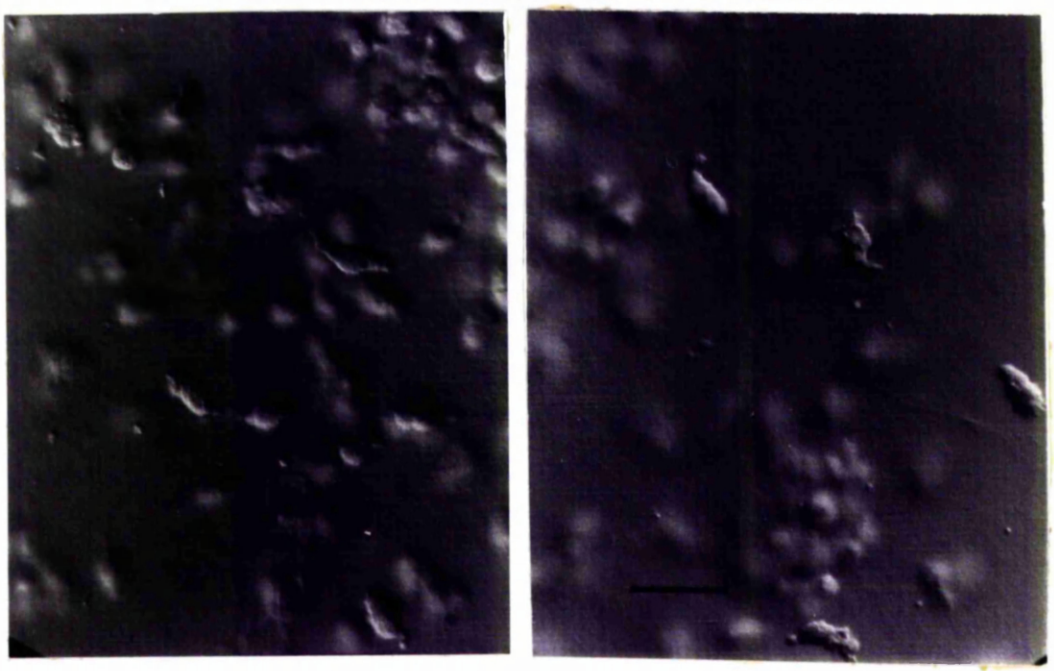
To obtain information on the absolute number of neutrophils inside the gel, the number of cells recovered by washing the gel surface 5 times with HBSS-MOPS was counted in a haemocytometer from 3 identical wells after a particular time of incubation. Since the total number of cells initially placed at the top of the collagen matrix was known, the number associated with the gel could be indirectly calculated. It should be mentioned here that almost all the cells applied on the gel were found to be attached to the surface within 1 h of incubation. It was not possible to remove these cells by washing even before fixing the gel. It might be considered that these cells were anchored to the gel by pushing their cellular processes through the 3-D network of the gel. More direct experiments for the absolute number of cells inside gels will be described later.

**Morphology of the cells in gels**

Neutrophils inside collagen gels assumed rapid configurational changes both in the presence and absence of $10^{-8}$ M FMLP. Cells which were anchored to the gel surface had a spherical morphology in the controls, whereas neutrophils attached to a gel surface in an isotropic concentration of $10^{-8}$ M FMLP had variable morphologies at different times of incubation. The results from 4 experiments are summarised in Table 11.



A



B C

Fig. 40 Neutrophils within the matrix of collagen gels (1.5 mg/ml) containing an isotropic concentration of $10^{-8}$ M FMLP. A: Phase-contrast photograph; B and C: differential interference contrast photographs of neutrophils after 2 h incubation at 37°C. The plane of focus of photographs A and B are showing neutrophils 120 µm from the gel surface; C: 200 µm from the gel surface. Bar : 50 µm.

Table 11. Effects of time of incubation on invasion of collagen gels by neutrophils in the presence and absence of uniform concentration of $10^{-8}$ M FMLP

| Time of incubation (hours) | Velocity (μm/min) of cells at the leading front | | Morphology of cells at the gel surface in FMLP (% polarised cells) |
|---|---|---|---|
| | in control | in FMLP | |
| 1 | 1.00 - 1.83 | 3.83 - 5.00 | 90 - 95 |
| 2 | 0.75 - 1.50 | 2.67 - 3.75 | 70 - 80 |
| 3 | 0.50 - 1.39 | 2.50 - 3.00 | 50 - 55 |
| 4 | 0.44 - 1.12 | 2.04 - 2.37 | 40 - 47 |
| 5 | 0.40 - 0.97 | 1.73 - 1.90 | 25 - 30 |

Fig. 41 is a phase-contrast photograph of neutrophils on a 3-dimensional gel surface. Most of the cells showed extensively polarised morphology when the photograph was taken 30 min after putting cells on the gel. Fig. 42 A, and B represent high power phase-contrast photographs of neutrophils deep within a gel containing FMLP. The cells had broad lamellipodia (l) at the anterior, several constrictions (c) in the cell body and a noticeable tail (t) at the posterior.

**Neutrophil distribution within collagen gels**

The distribution of cells through collagen gels was plotted as mean cell number against distance from 40 μm below the top surface of the gel in the presence and absence of a uniform concentration of $10^{-8}$ M FMLP and is shown in Fig. 43. Similar results were obtained in 4 experiments. The distribution of cells moving randomly into a gel would be identical to that expected for a simple diffusion (Zigmond and Hirsch, 1973); thus, if the logarithm of the cell number were plotted against the square of the distance migrated, a linear plot would be expected. The data of Fig. 43 were therefore transformed in this way and are shown in Fig. 44. This shows linear plots both for cells entering a gel in the absence of an attractant and for cells responding to a uniform concentration of FMLP (it also shows a plot for a chemotaxis assay, described below). These plots are suggestive of random locomotion in the absence of an attractant and of chemokinesis (increased random locomotion either due to an increase in cell speed or to recruitment of more

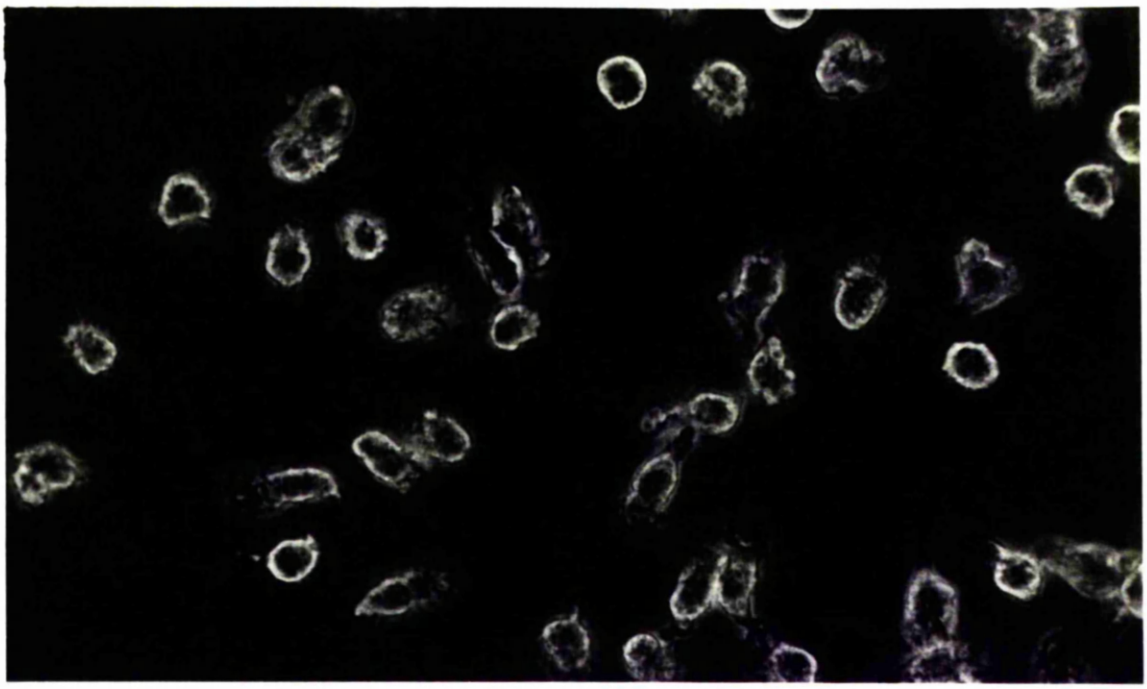


Fig. 41 Phase-contrast appearance of neutrophils on the surface of collagen gels (1.5 mg/ml) containing an isotropic concentration of $10^{-8}$ M FMLP. The photograph was taken after incubating the cells for 30 min at 37°C. Note neutrophils are highly polarised on the gel surface. Bar: 50 μm.

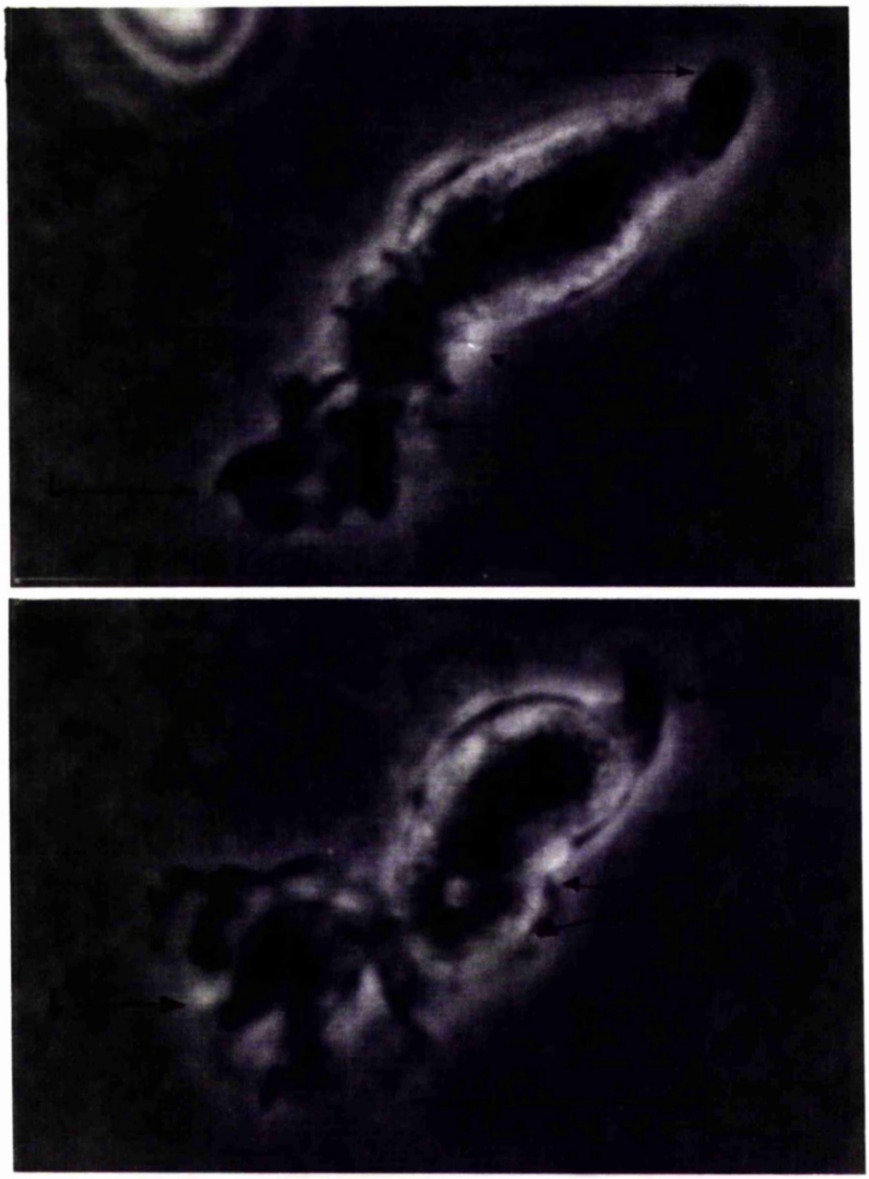


Fig. 42 High power phase-contrast photographs of neutrophils within collagen gels (1.5 mg/ml) containing a uniform concentration of $10^{-8}$ M FMLP. Neutrophils had broad lamellipodia (l) at the anterior, a knob-like tail (t) at the posterior and several constrictions (c) in the cell body. Photographs were taken 90 min after incubating the gels at 37°C with neutrophils. Bar = 20 μm.

cells or to both) in the presence of FMLP. In fact, the number of cells entering the gel was greater in the presence of FMLP than in its absence (Fig. 43).

A more direct assessment was made by counting the total number of cells in a series of microscopic fields starting from the top of the gel followed by moving down at each 40 μm interval until there were no more cells to be counted. As almost all neutrophils initially added to the gel surface were found to be associated with the gel during 2 h of incubation, the number which actually penetrated the gel (at least 40 μm below the surface) could be calculated. The results from 4 experiments suggested that 80% (range 74-84%) and 32% (range 28-35%) of the total cells layered on the gel surface were within the gel matrix in the presence and absence of uniform concentration of $10^{-8}$ M FMLP, respectively. This observation is also suggestive of chemokinetic locomotion of neutrophils into collagen gels in the presence of FMLP.

By using collagenase to digest 70-100 μm depth of gels from the upper surface of the wells (after 2 h of incubation with cells on) it was shown that only 3-8% of the total cells initially added to the control wells were within the remaining gel. Similar digestion of gels from wells where neutrophils had migrated in the presence of uniform concentration of $10^{-8}$ M FMLP (for 2 h) showed that more than 50% of the total cells initially added to the wells were within the remaining gel. However, the distribution of neutrophils within gels does not allow a direct measure of cell speed, though FMLP has been

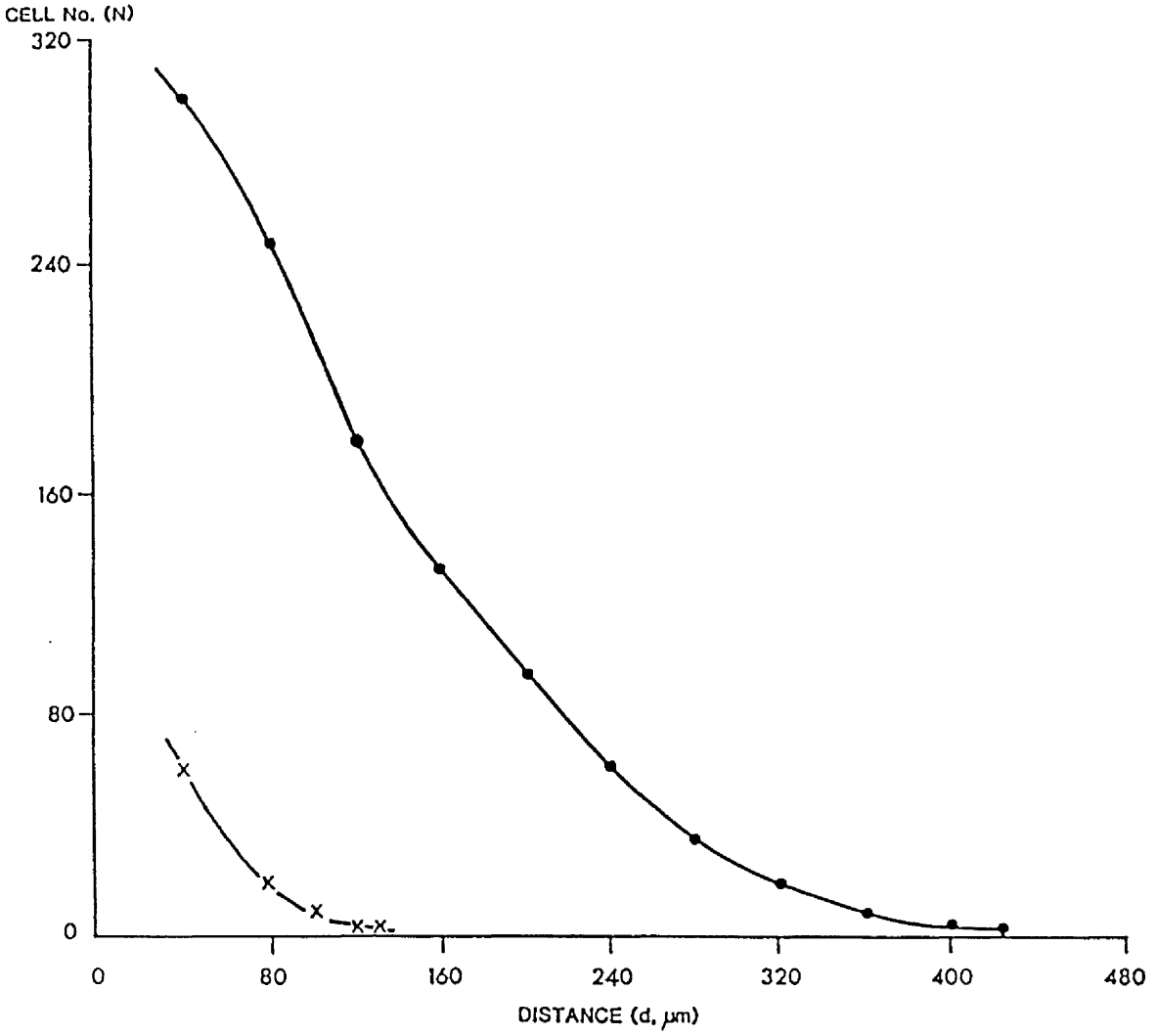



Fig. 43 Distribution of neutrophils through collagen gels (1.5 mg/ml) after 2 h of incubation in the presence of an isotropic concentration of $10^{-8}$ M FMLP ( ● ) and in the absence of FMLP ( x ) after 2 h.

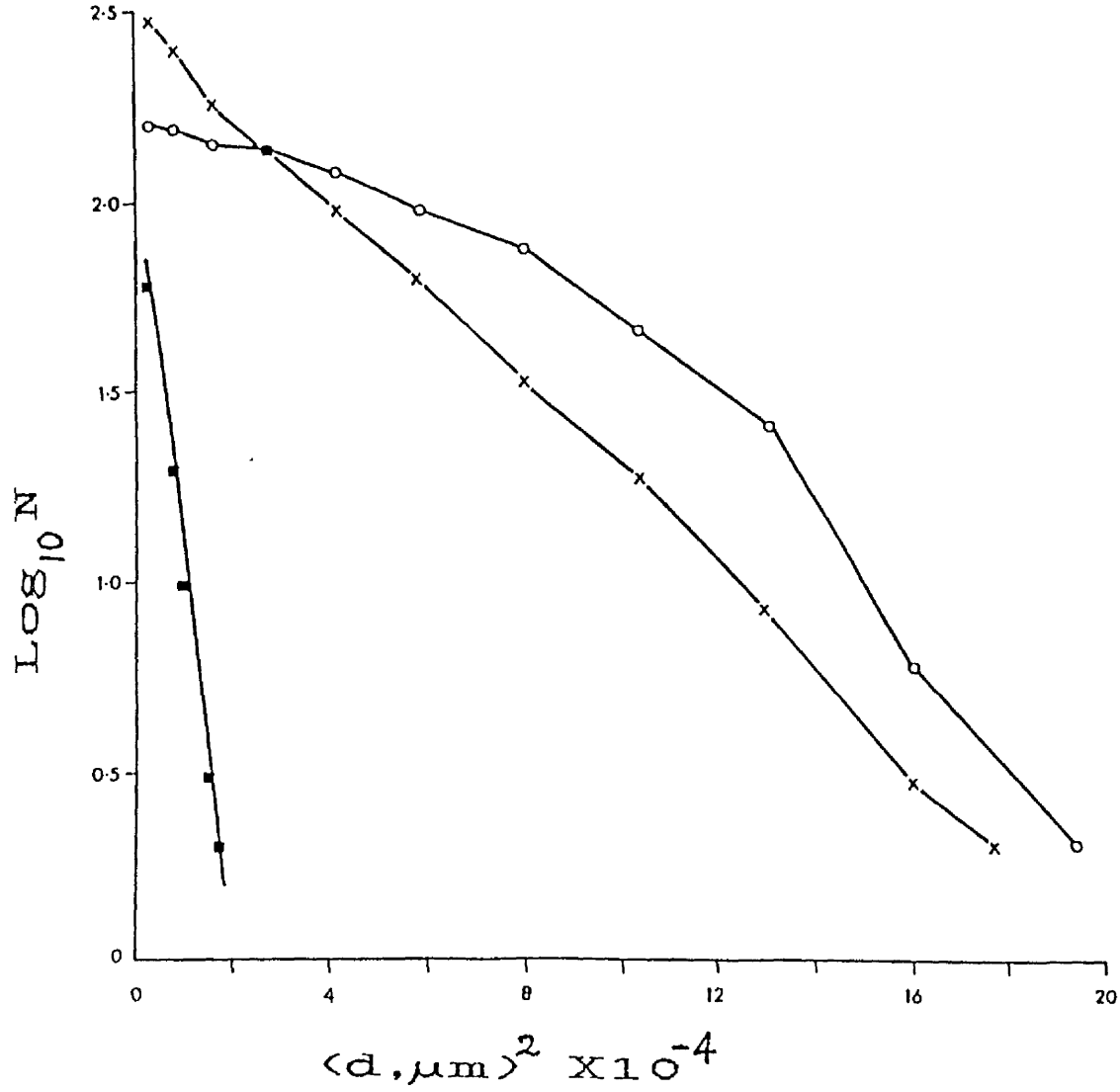



Fig. 44 Transformed plot of data from Fig. 43 and Fig. 46. Logarithm of cell numbers ($log_{10}N$) against $d^2$. No FMLP ( ■ ) and isotropic FMLP ( x ) from Fig. 43; FMLP gradient ( o ) from Fig. 46.

shown in other studies to increase the velocity of neutrophils as well as recruitment of extra locomotory cells (Shields and Haston, 1985).

SECTION II

**Migration of neutrophils into collagen gels in response to gradients of FMLP**

In Fig. 45 is shown the dose-response curve for the leading-front measure for cells migrating in gradients originating from sources of FMLP at different concentrations and shows that $10^{-8}$ M FMLP was the optimal concentration in this assay. Similar results were obtained from 9 experiments

**Cell distribution in gels**

If neutrophils exposed to gradients of FMLP respond by directional locomotion towards the peptide, it will be expected that the distribution of cells through the gel will no longer be that expected for a simple diffusion, but that there will be a flux of cells into the gel. If this is the case, the plot of log number of cells against the square of the distance migrated will no longer be linear. The effectiveness of the chemotactic response will depend on the amplitude of the gradient and, under the assay conditions described, this will depend on how long the FMLP, present at uniform concentration in collagen A (collagen at 1.5 mg/ml containing FMLP, in this case $10^{-8}$ M, described in chapter 2) is allowed to diffuse through collagen B (collagen at 1.0 mg/ml without

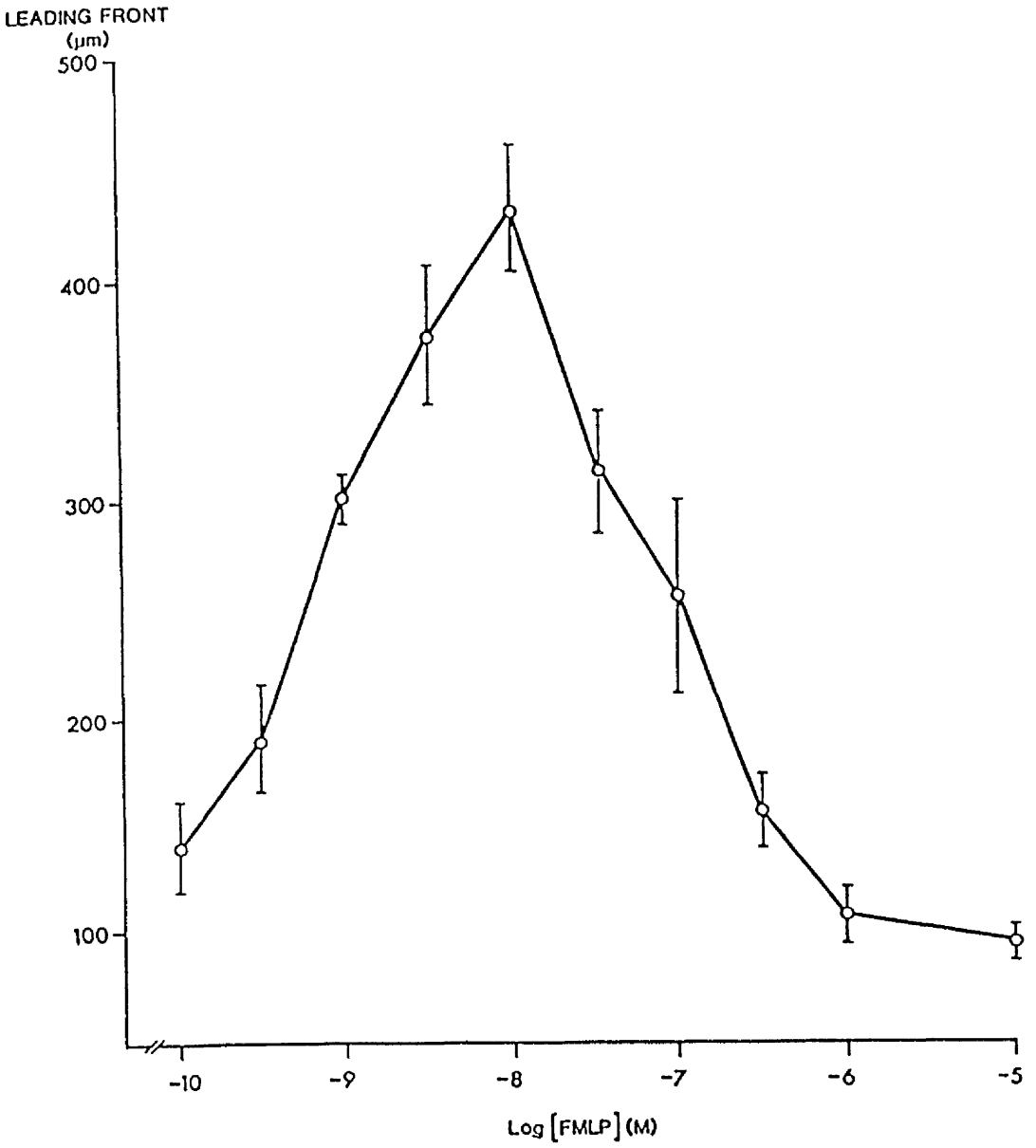



Fig. 45 Dose-response curve for the leading front of neutrophils migrating through collagen gels (1.0 mg/ml) towards gradient sources of FMLP at different concentrations. Leading front values were measured after 2 h (±S.D.).

FMLP) before cells are placed on the upper surface of the latter. Therefore, preliminary experiments were performed to determine the optimal time for gradient diffusion that was consistent with formation of a firm gel, and at the same time allowed good detection of the gradient by cells. A good chemotactic response was judged in this assay on the basis of the non-linearity of the plot of log cell number against $d^2$. It was found that a twenty minute lapse for gradient diffusion before layering cells onto the upper gel was the minimum consistent with the practical limitation of having a firm gel to place the cells on. In practice, 30 min was adopted as the optimum time for FMLP diffusion through the upper gel and the cells were allowed to migrate into the gel for 120 min. The distribution of cells through the gel in response to a gradient of FMLP ($10^{-8}$ M at source) is shown in Fig. 46 (cf. Fig. 43). This is a non-linear transformed plot and the same data transformed to show log cell number against $d^2$ is shown in Fig. 44. This plot is clearly non-linear. Similar distribution patterns of neutrophils and their corresponding non-linear transformed plots were obtained in 6 other experiments.

Analysis of these distributions by curve-fitting as described in the methods gave cell numbers entering the gel, displacement due to random migration and velocity of directional migration for each microscopic field studied. These estimates are summarised in Table 12. These mathematical analyses of the distribution curves were made by Dr. I. C. McKay, Department of Bacteriology and Immunology, University of Glasgow.

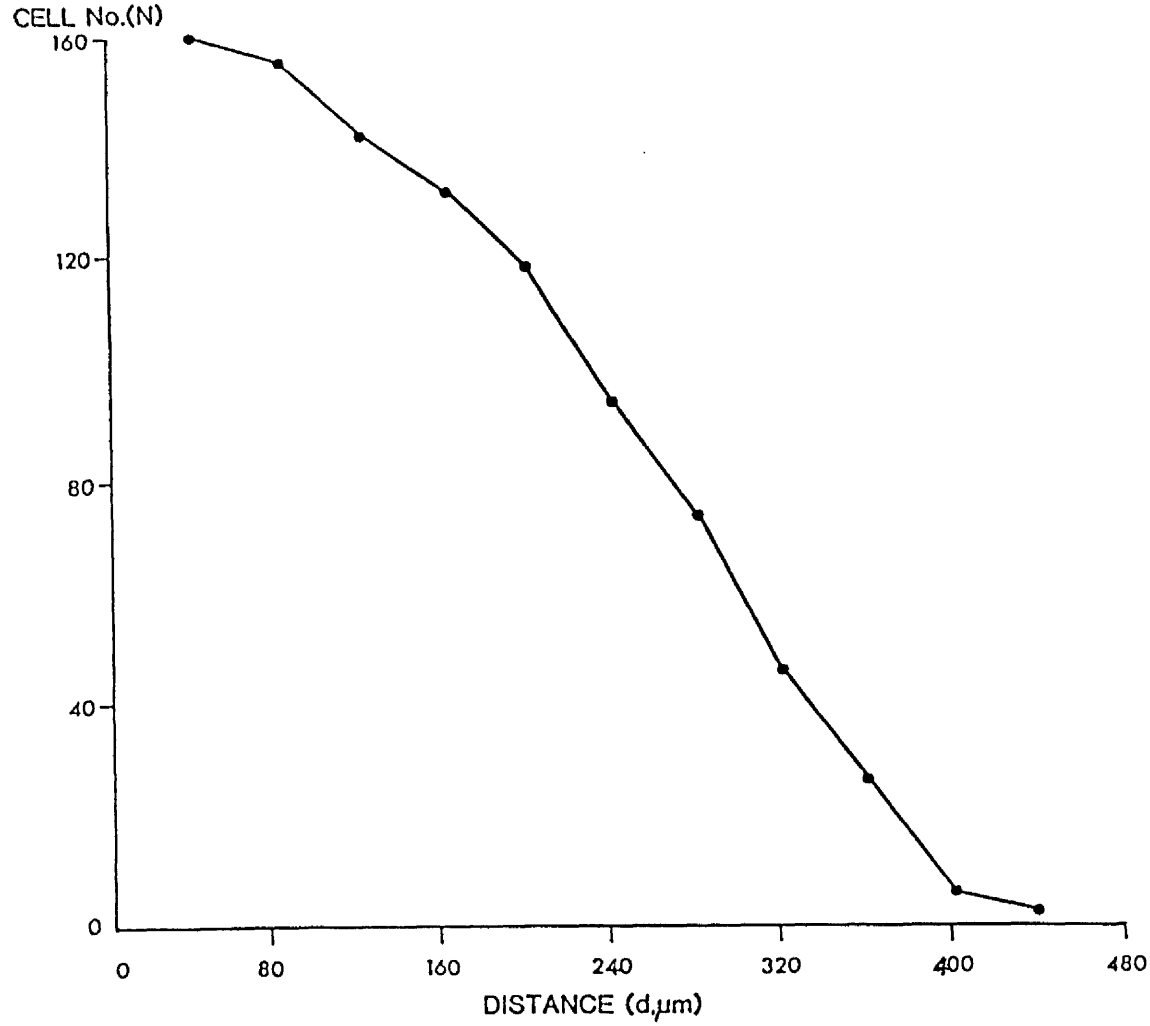
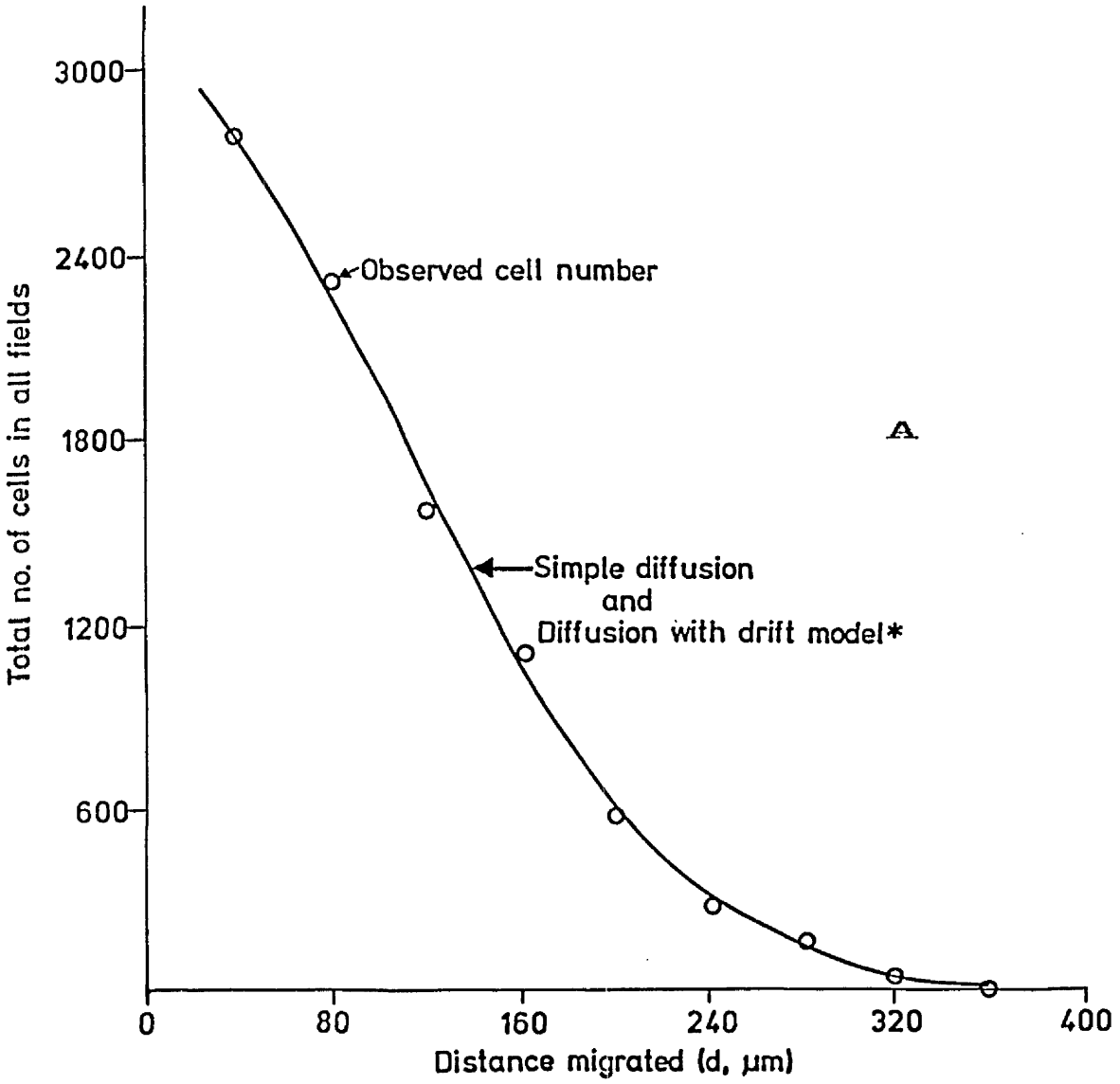



Fig. 46 Distribution of neutrophils through a collagen gel after 2 h incubation in the presence of a gradient of FMLP ($10^{-8}$ M at source) set up 30 min before adding the cells.

As examples of the comparison between the fitted theoretical curves and the observed cell numbers, the two graphs presented in Fig. 47 A and B show the results obtained in experiment (a) with a uniform concentration of $10^{-8}$ M FMLP and (1) with an FMLP gradient, respectively. The total number of cells obtained by the simple diffusion model and the diffusion with drift model was the same for uniform FMLP (as there was no gradient), the 2 graphs for the 2 models were superimposed and the observed cell number has been shown by encircled points on Fig. 47 A. For experiment (1) with gradient concentration of FMLP, the observed cell number, shown by encircled points on Fig. 47 B, seemed to fit the diffusion with drift model, better than the simple diffusion model.

The displacements attributable to random migration in Table 12 show no evidence of being influenced by the concentration gradient of FMLP, nor do the numbers of cells entering the gels. The second last column of the table is of interest as an indicator of whether the last 4 experiments (with uniform FMLP concentration) showed less evidence of directional drift than the first 7 experiments (with an FMLP concentration gradient). In the presence of a gradient the mean of the mean estimate of directional migration was 44.2 ± 7.0 μm/h, whereas in the absence of a gradient the mean was 19.49 ± 9.3 μm/h. Comparison of the two sets of numbers in the velocity column gives P = 0.06 by student's 2-tailed t-test or P = 0.04 by Wilcoxon's rank sum test.


3000
2400
1800
1200
600
Total no. of cells in all fields
Observed cell number
A
Simple diffusion
and
Diffusion with drift model*
0
80
160
240
320
400
Distance migrated (d, μm)

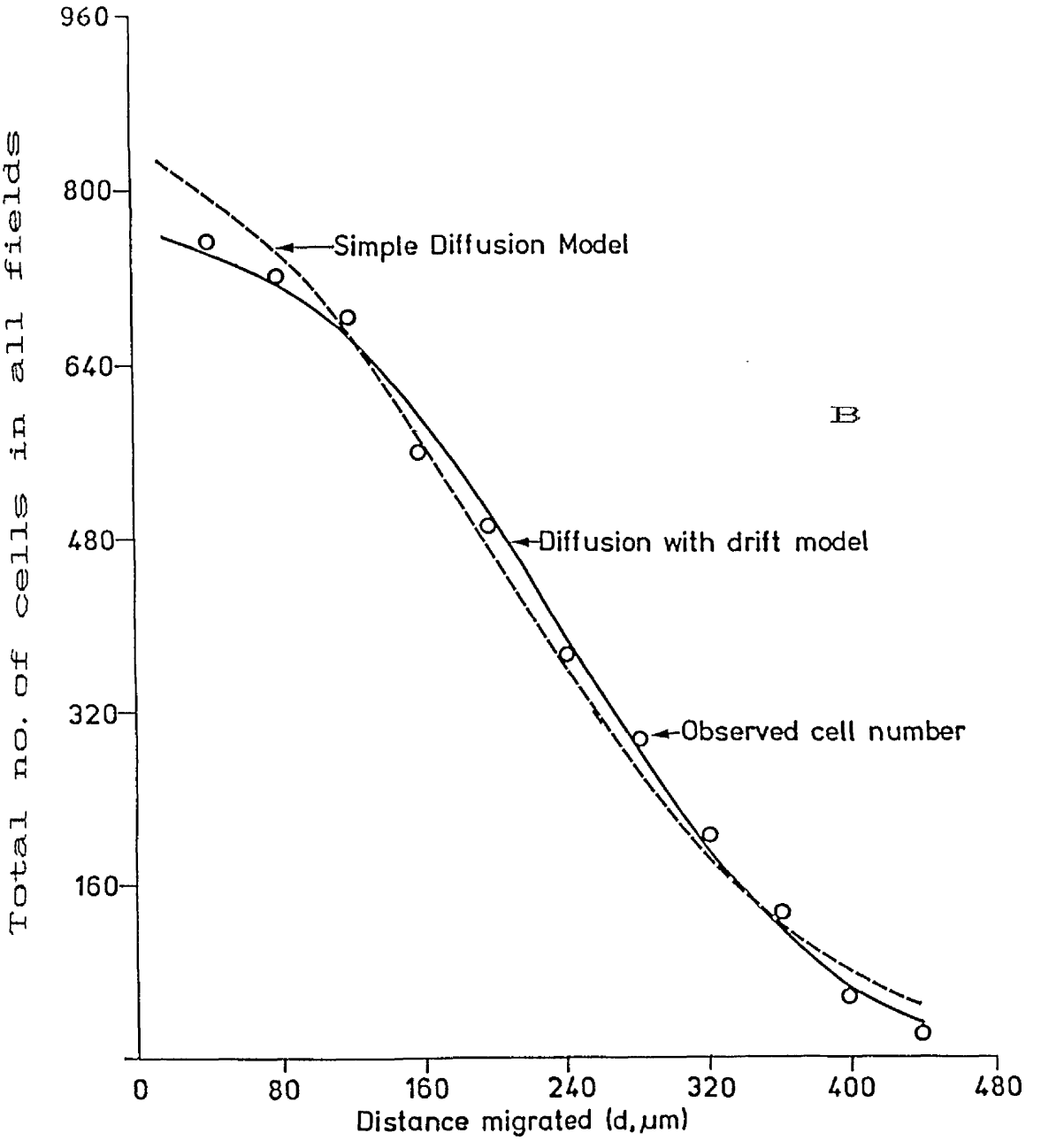



Fig. 47 A-B. Comparison between the fitted theoretical curves and the observed cell numbers. A: Shows the results obtained in experiment (a) with a uniform concentration of $10^{-8}$ M FMLP; B: Shows the results obtained in experiment (i) with an FMLP gradient (see Table 12). The simple diffusion model and the diffusion with drift model coincided almost exactly (*) in A since there was no gradient, the observed cell number ( o ) satisfied both the theoretical models. In B the observed cell number ( o ) fitted the diffusion with drift model better than the simple diffusion model.

Table 12. Summary of migration parameters estimated from final distributions of cells that started on the top surface of gels and migrated either in a vertical concentration gradient of FMLP or in a uniform FMLP concentration

| | Experiment No. | No. of fields observed | a (proportional to number of cells entering the gel) mean of all fields ± S.E.M. | s/μm Root-mean-square displacement attributable to random migration mean of all fields± S.E.M. | v/μm $hr^{-1}$ Estimated velocity of directional migration mean of all fields± S.E.M. |
|---|---|---|---|---|---|
| with an FMLP concentration gradient (0 to $10^{-8}$ M) | 1 | 10 | 7498 ± 269 | 134 ± 5 | 59.4 ± 3.9 |
| | 2 | 10 | 9418 ± 897 | 118 ± 4 | 52.4 ± 5.6 |
| | 3 | 10 | 17123 ± 410 | 125 ± 5 | 36.1 ± 6.5 |
| | 4 | 10 | 16595 ± 643 | 122 ± 2 | 65.9 ± 0.6 |
| | 5 | 10 | 13223 ± 464 | 120 ± 4 | 22.6 ± 6.4 |
| | 6 | 16 | 9733 ± 329 | 100 ± 5 | 54.7 ± 2.2 |
| | 7 | 12 | 10143 ± 286 | 112 ± 3 | 18.3 ± 6.4 |
| with a uniform concentration of FMLP ($10^{-8}$ M) | a | 10 | 16440 ± 578 | 102 ± 4 | 15.8 ± 6.5 |
| | b | 10 | 19668 ± 966 | 126 ± 4 | 12.9 ± 6.2 |
| | c | 10 | 10361 ± 396 | 96 ± 2 | 2.8 ± 2.4 |
| | d | 10 | 19683 ± 777 | 109 ± 3 | 45.9 ± 2.1 |

The statistical significance of the experiments with uniform and gradient concentrations of FMLP through gel is rather marginal, possibly on account of the anomalous result of experiment d.

In summary, neutrophils migrate into collagen gels both in the presence and absence of FMLP. The cells showed increased random migration when a uniform concentration of $10^{-8}$ M FMLP was present both in the cell suspension and in the gel. More cells penetrated into the gel and the leading front value was higher i.e., the cells migrated further deep within gels when $10^{-8}$ M FMLP was throughout. By definition this locomotion was chemokinetic. About 80% of the total neutrophils placed on the top surface migrated into the gel matrix in response to uniform concentration of $10^{-8}$ M FMLP and 28-35% of the control neutrophils migrated into the gel during 2 h incubation.

In another set of experiments, different doses of FMLP were incorporated into collagen solutions before it formed a gel. Then solution of collagen (without FMLP) was poured on it to form a second layer of gel through which FMLP could diffuse to form a gradient. Neutrophils were placed on the upper surface of the gel. The cells responded with a maximum leading front value when $10^{-8}$ M FMLP was present in a gradient. The distribution of the cell population in response to the gradient did not appear to be one half of a normal distribution curve. Also the transformed plot of log cell number against $(\text{distance})^2$ was not linear which would be expected for a

simple diffusion of cells through the gel. There was a flux of neutrophils into the gel in response to the gradient of FMLP which was clearer in some experiments than in the others (Table 12). As a whole the statistical significance was marginal for the 7 experiments with a gradient of FMLP through the gels as a convincing evidence for neutrophil chemotaxis.

## SECTION III

### Invasion of collagen gels by monocytes in the presence and absence of uniform FMLP

There is one recent report available in the literature (Brown, 1984) which suggests that human monocytes straight from blood do not migrate through collagen gels. However, if the adherent mononuclear cells were cultured on a protein-coated plane surface for 48 h, then the monocyte population which became detached would move into collagen gels. This hypothesis can be tested by putting monocytes just after purification from blood on collagen gels and then incubating the gels with cells on. It was observed that the cells migrate into collagen gels like other blood leucocytes.

### Chemokinesis of monocytes in collagen gels

Invasion of collagen gels by monocytes was studied in the presence and absence of uniform concentration of $5 \times 10^{-9}$ M FMLP. This dose of FMLP was observed to induce maximum shape change of the monocytes (60% or so as described in the polarisation assays). The time course of

invasion of the collagen matrices by monocytes, using the leading front method, is shown in Fig. 48. The leading front measure increased with time but the relationship was not linear. The values were higher in presence of FMLP but the rate of migration of the monocytes at the leading front was much slower than that of the neutrophils in FMLP (cf. Fig. 39). After an incubation of 4 h monocytes in the presence of FMLP migrated 180-240 μm within the gel, while control cells migrated 115-140 μm deep into the gel. It was observed that cells suspended in HBSS-MOPS tended to form small aggregates at the gel surface which made fewer cells available to invade gels during the course of incubation. For further experiments, monocytes were suspended in HSA (20 mg/ml) in HBSS-MOPS.

To see if suspending cells in HSA had an effect on their migration into the gel, monocytes in HSA in the presence and absence of uniform concentration of 5 X $10^{-9}$ M FMLP were allowed to invade collagen gels for various time intervals. Similar results were obtained in 3 similar experiments, one of which is presented in Fig. 49. This shows that the leading front values increased slightly (cf. Fig. 48) in both cases when cells were suspended in HSA. After 4 h of incubation the cells invaded 220-280 μm and 125-200 μm deep inside gels in the presence and absence of uniform concentration of 5 X $10^{-9}$ M FMLP, respectively. When the cells were allowed to invade the gels during overnight (16 h) incubation, the leading front values reached up to 500-650 μm in presence of FMLP and the control cells migrated up to 200-260 μm. At the same time viability of the monocytes had fallen to

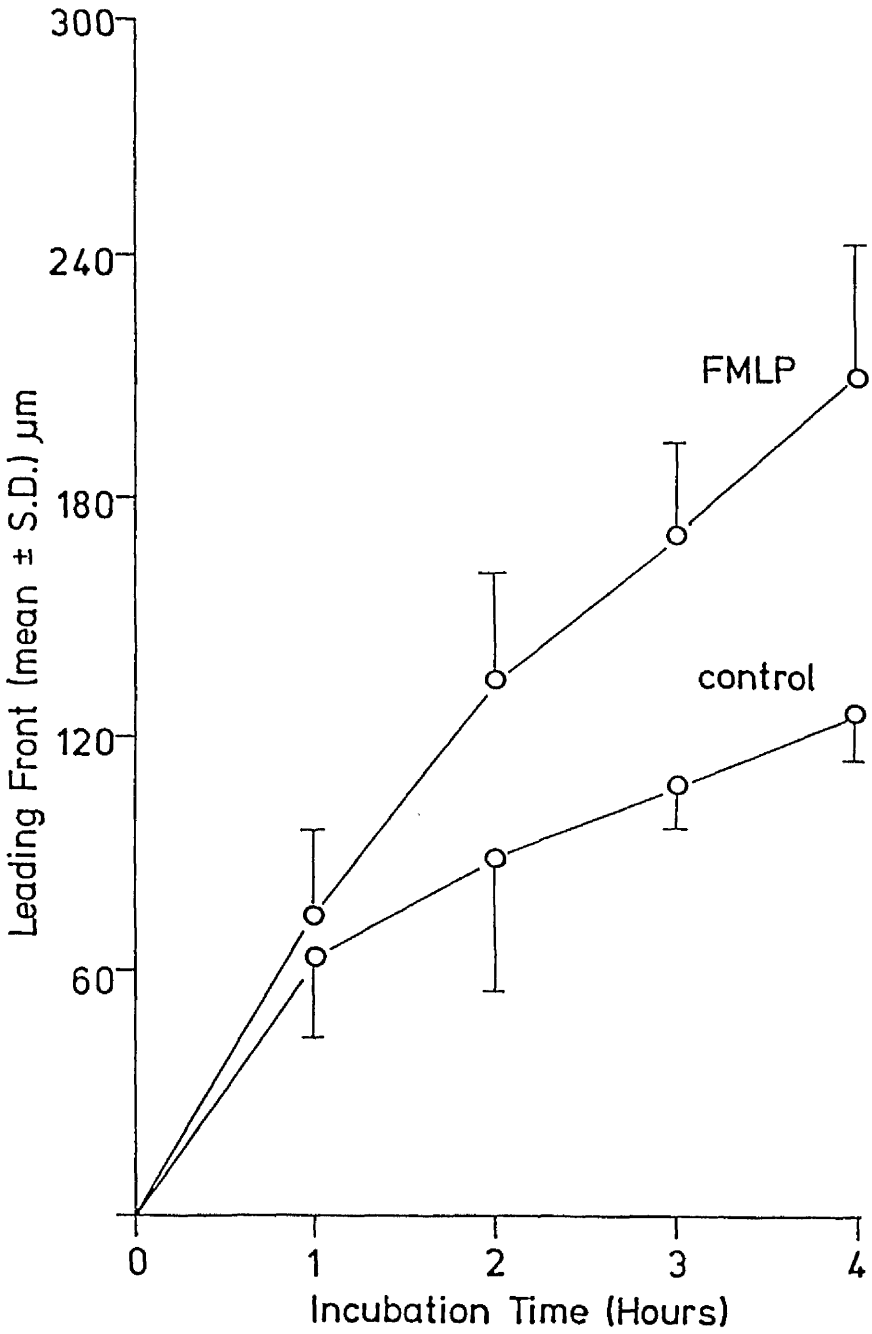



Fig. 48 Time course of invasion of monocytes into collagen gels (at 1.5 mg/ml) in the presence and absence of 5 X $10^{-9}$ M FMLP (throughout). The cells were suspended in HMSS-MOPS (±FMLP) for the invasion assay.

50-60% of the starting figures in both cases. Since monocytes were slow moving cells, an incubation time of 4 h was chosen for further experiments. This allowed reasonable cell penetration into the gel for accurate plotting of the cell number at different levels. A summary of the migration velocity of the cells at the leading front at different times in the presence and absence of 5 X $10^{-9}$ M FMLP is shown in Table 13.

**Proportion of monocytes invading gels**

As described earlier for the neutrophils, the number of monocytes recovered by washing the gel surface after cells had migrated in the presence and absence of FMLP was counted at the end of each incubation period. It was observed that more than 95% of the total monocytes initially placed on the gel surface had either migrated into or attached to the gel during the first hour of incubation. After 2 h, >99% cells were associated with gels in both cases.

**Morphology of monocytes in gels**

Monocytes migrating into the gel assumed rapid configurational changes like the neutrophils. About 5-8% of the total control cells anchored to the gel surface had locomotor morphology even after 3 h of incubation. Monocytes attached to a gel surface in the presence of 5 X $10^{-9}$ M FMLP (throughout) had variable morphology at different time of incubation. The results from 3 experiments are summarised in Table 13.

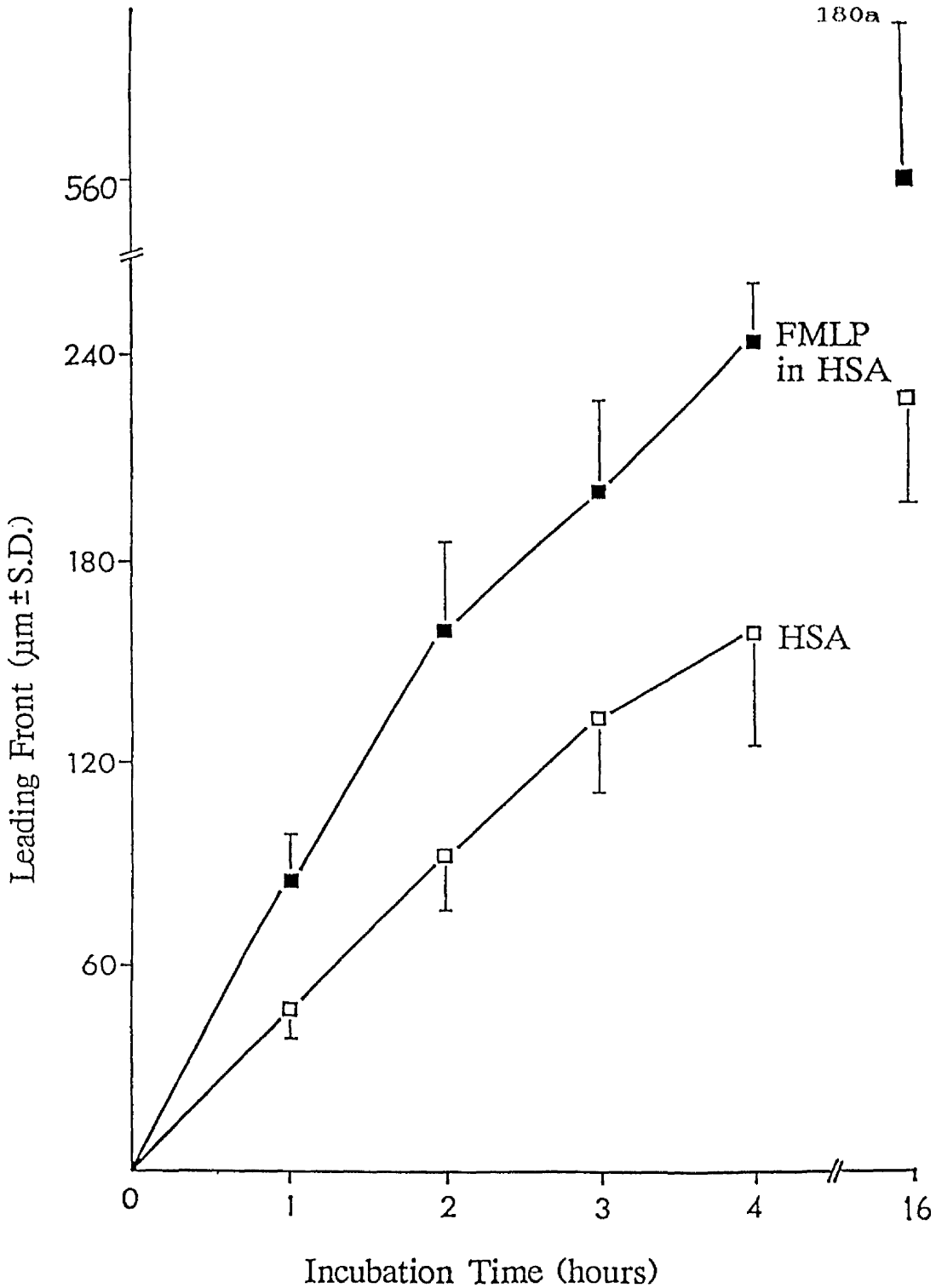



Fig. 49 Effect of suspending monocytes in HSA for invasion of collagen gels (at 1.5 mg/ml) in the presence and absence of FMLP (5 X $10^{-9}$ M, throughout). The cells were suspended in HSA (20 mg/ml) in HBSS-MOPS (±FMLP) for the invasion assay. The leading front values were slightly higher both in the presence and absence of FMLP when the cells were suspended in HSA in HBSS-MOPS than in HBSS-MOPS alone (cf. Fig. 48). HSA prevented formation of small aggregates by the cells during incubation and allowed further cell migration into the gels. The leading front value was higher both for FMLP and for the controls after overnight (16 h) incubation but many cells died during long-term invasion.

Table 13. Effects of time of incubation on invasion of collagen gels by monocytes in the presence and absence of uniform concentration of $5 \times 10^{-9}$ M FMLP

| Time of incubation (hours) | Velocity (μm/min) of cells at the leading front | | Morphology of cells at the gel surface in FMLP (% polarised cells) |
|---|---|---|---|
| | in control | in FMLP | |
| 1 | 0.67 - 1.00 | 1.13 - 1.67 | 80 - 85 |
| 2 | 0.63 - 0.93 | 1.08 - 1.58 | 70 - 75 |
| 3 | 0.67 - 0.94 | 0.94 - 1.28 | 45 - 50 |
| 4 | 0.52 - 0.81 | 0.92 - 1.12 | 30 - 40 |

Fig. 50 is a photomicrograph of monocytes attached to a 3-dimensional gel surface. It was interesting to see that over 80% of the total monocytes which attached to the top layer of gel in a uniform concentration of 5 X $10^{-9}$ M FMLP during the first hour of incubation had locomotor morphology (Table 13). It was mentioned earlier that 60% or so of monocytes were observed to have locomotor shapes when incubated in suspension in the presence of the same dose of FMLP. It is possible that attachment of the cells to a 3-D matrix caused more cells to change shape.

**Monocyte distribution within collagen gels**

The distribution of monocytes through collagen gels was plotted as mean cell number against distance from 40 μm below the top layer of the gel in the presence and absence of a uniform concentration of 5 X $10^{-9}$ M FMLP and is shown in Fig. 51. Similar results were obtained in 3 experiments.

The distribution of monocytes moving randomly into a gel would be identical to that expected for a simple diffusion (Zigmond and Hirsch, 1973); thus, if the logarithm of the cell number were plotted against the square of the distance migrated, a linear plot would be expected. The data of Fig. 51 were therefore transformed in this way and are shown in Fig. 52. This shows linear plots both for monocytes entering a gel in the presence and absence of attractant and monocytes responding to a uniform concentration of FMLP. These plots suggested that the cells did not respond chemotactically to collagen fibres or to any attractant and are suggestive of random

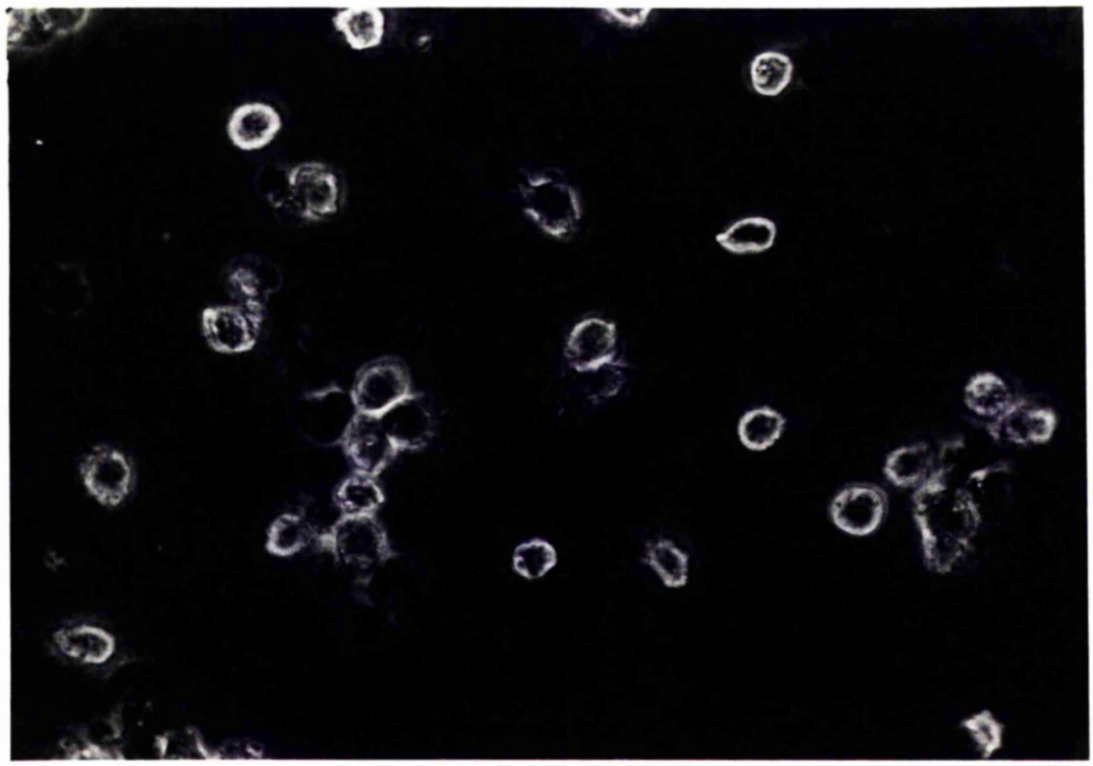


Fig. 50 Phase-contrast photomicrograph of monocytes attached to gel surface (1.5 mg/ml) containing a uniform concentration of 5 X $10^{-8}$ M FMLP. The photograph was taken after incubating the cells for 1 h at 37°C. Note about 80% monocytes showed locomotor morphology in FMLP after 1 h exposure while the cells were attached to a gel matrix. Loss of polarisation was observed earlier due to prolonged incubation of the monocytes in suspension with FMLP (cf. Fig. 8). Bar: 50 μm.

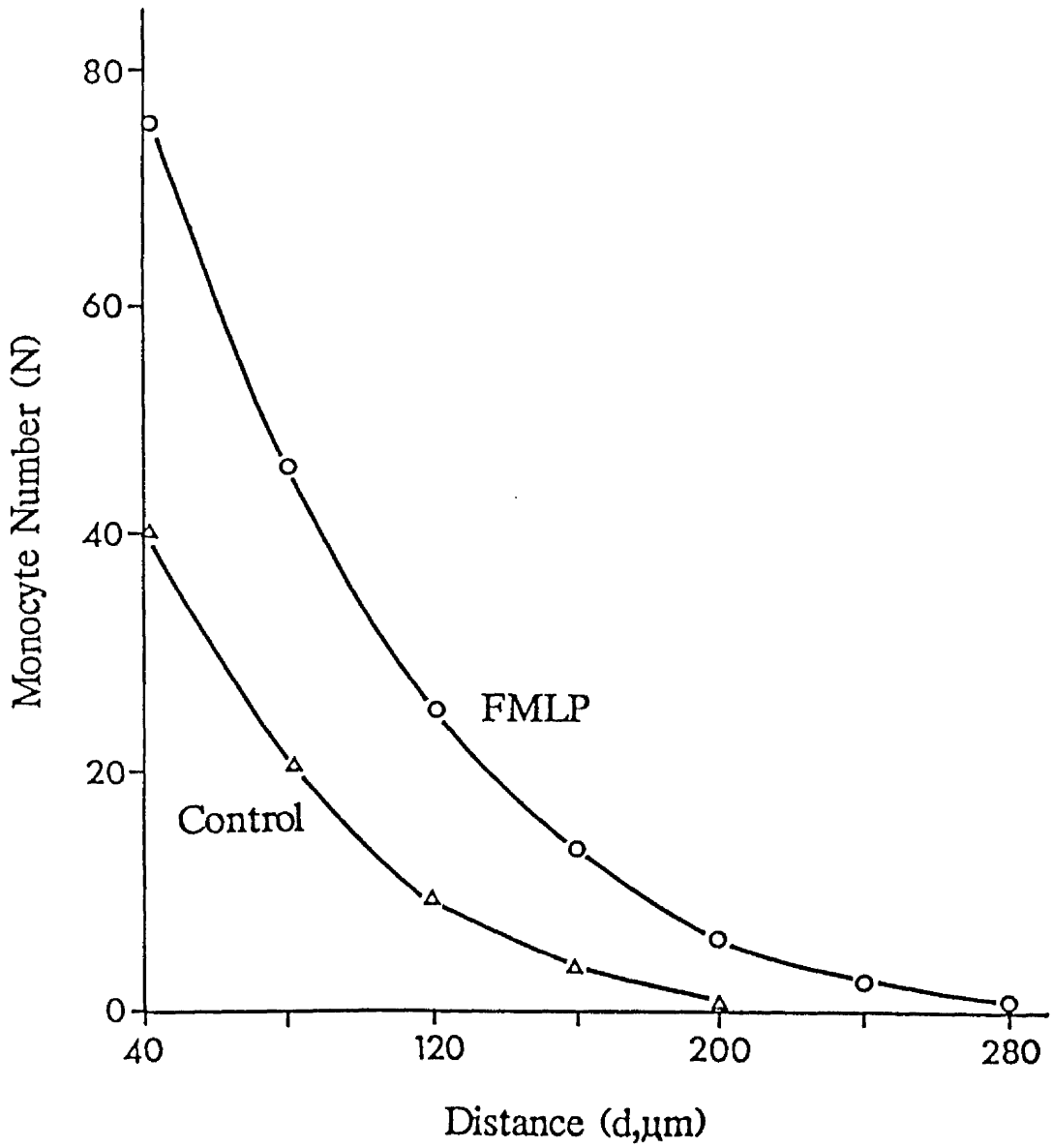



Fig. 51 Distribution of monocytes through collagen gels (1.5 mg/ml) after 4 h of incubation in the presence of an isotropic concentration of 5 X $10^{-9}$ M FMLP and in the control (without FMLP). The distribution patterns suggest that monocyte migration through collagen gels was random and FMLP increased the extent of random migration of the cells.

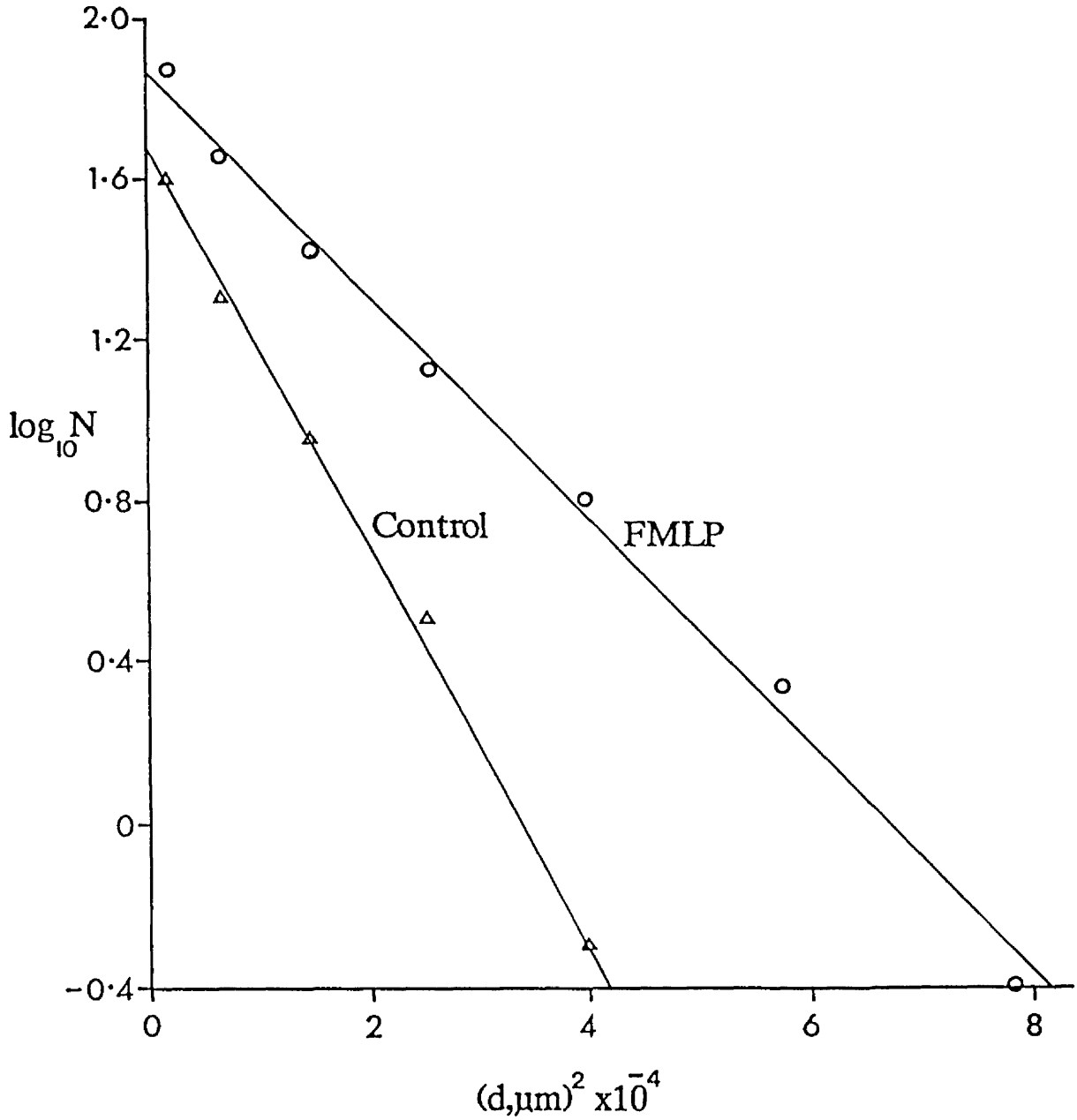



Fig. 52 Transformed plot of data from Fig. 51. Logarithm of cell numbers ($Log_{10}N$) against $d^2$. Monocytes followed the pattern of diffusion of particles from a boundary.

locomotion in the absence of an attractant and of chemokinesis (increased random locomotion either due to an increase in cell speed or to recruitment of more cells or to both) in the presence of uniform concentration of 5 X $10^{-9}$ M FMLP. In fact, the number of monocytes entering the gel was greater in the presence of FMLP than in its absence (Fig. 51)

The absolute number of monocytes inside the gel was calculated as described earlier for neutrophils. The results from 4 experiments suggested that 43% (range 29-50%) and 21% (range 17-24%) of the total cells layered on the gel surface were within the gel matrix in the presence and absence of uniform 5 X $10^{-9}$ M FMLP, respectively. This observation is also suggestive of chemokinetic locomotion of monocytes into collagen gels in the presence of FMLP.

Using collagenase to digest the upper 70-100 μm depth of gels from the top of the wells (after 4 h incubation with monocytes on) it was shown that 7-12% of the total cells placed on the control wells were within the remaining gel. Similar digestion of gels from wells where monocytes had migrated in the presence of uniform concentration of 5 X $10^{-9}$ M FMLP (for 4 h) showed that about 20-25% of the total cells added were within the remaining gels.

**Evidence that cells inside the gel were monocytes**

For identification of cells invading collagen gels, the purity of the monocyte preparations used in the invasion assays was routinely checked after incubating

the cells for α-napthyl acetate esterase (ANAE) stain. For all experiments described above, >96% of the total cells in the starting populations were monocytes. The rest of the cells in the initial populations were lymphocytes. After incubating the gels for 4 h with cells followed by partial digestion of the gels from the upper surface of the wells by using collagenase, i.e., after detachment of the cells associated with the top of the gel, the recovered cells were fixed and used for ANAE stain. At the same time a portion of the fixed gel (after partial digestion with collagenase) was transferred to a microscope slide and allowed to dry at room temperature. The dried gel was stained with Leishman stain, wet mounted followed by microscopic examination under phase objective.

It was observed that cells which were in the upper portion of the gel (stained with ANAE) consisted of >94% monocytes and that cells in the residual portion of the gel (after partial digestion with collagenase) were almost exclusively monocytes (>99%). It could be mentioned here that monocytes used for collagen gel invasion were purified on BHK microexudate-coated surfaces which utilised a reversible adhesion step. Hence lymphocytes which remained in the monocyte populations might be mostly B-lymphocytes as the majority of the lymphocytes present in the monocyte populations were negative to esterase. It was reported by previous workers (Shields et al., 1984) that B-enriched populations of mouse lymphocytes migrated more slowly than unseparated lymph node cells. Hence it is possible to conclude that

the very small proportion of lymphocytes present in the monocyte populations remained in the upper portion of the gel matrix. The cells which migrated further i.e., deep inside the gel were monocytes.

**Collagen gel invasion by monocytes in FMLP + AHS**

It was mentioned in the shape change assays that over 85% of circulating blood monocytes assumed polarised morphology when incubated in suspension in FMLP ($5 \times 10^{-9}$ M) combined with candida spore-activated human serum (AHS, 10%). It was hoped that adding the same dose of AHS to the monocytes (suspended in HSA containing $5 \times 10^{-9}$ M FMLP) might stimulate more cells to invade the gel, which contained the same dose of FMLP but no HSA or activated serum, during a 4 h incubation. The time course of invasion of the collagen matrices by monocytes, using the leading front method, is shown in Fig. 53. It was observed that the cells migrated almost the same distance within the gel as they did in the presence of FMLP alone.

**Distribution of monocytes in FMLP + AHS within collagen gels**

The distribution of monocytes suspended in FMLP + AHS through collagen gels was plotted as described before and is shown by Fig. 54. It is indicated from this figure that monocytes both in the presence and absence of chemoattractants were distributed inside collagen gels according to one half of a normal distribution curve. That is, the cells followed random migration.

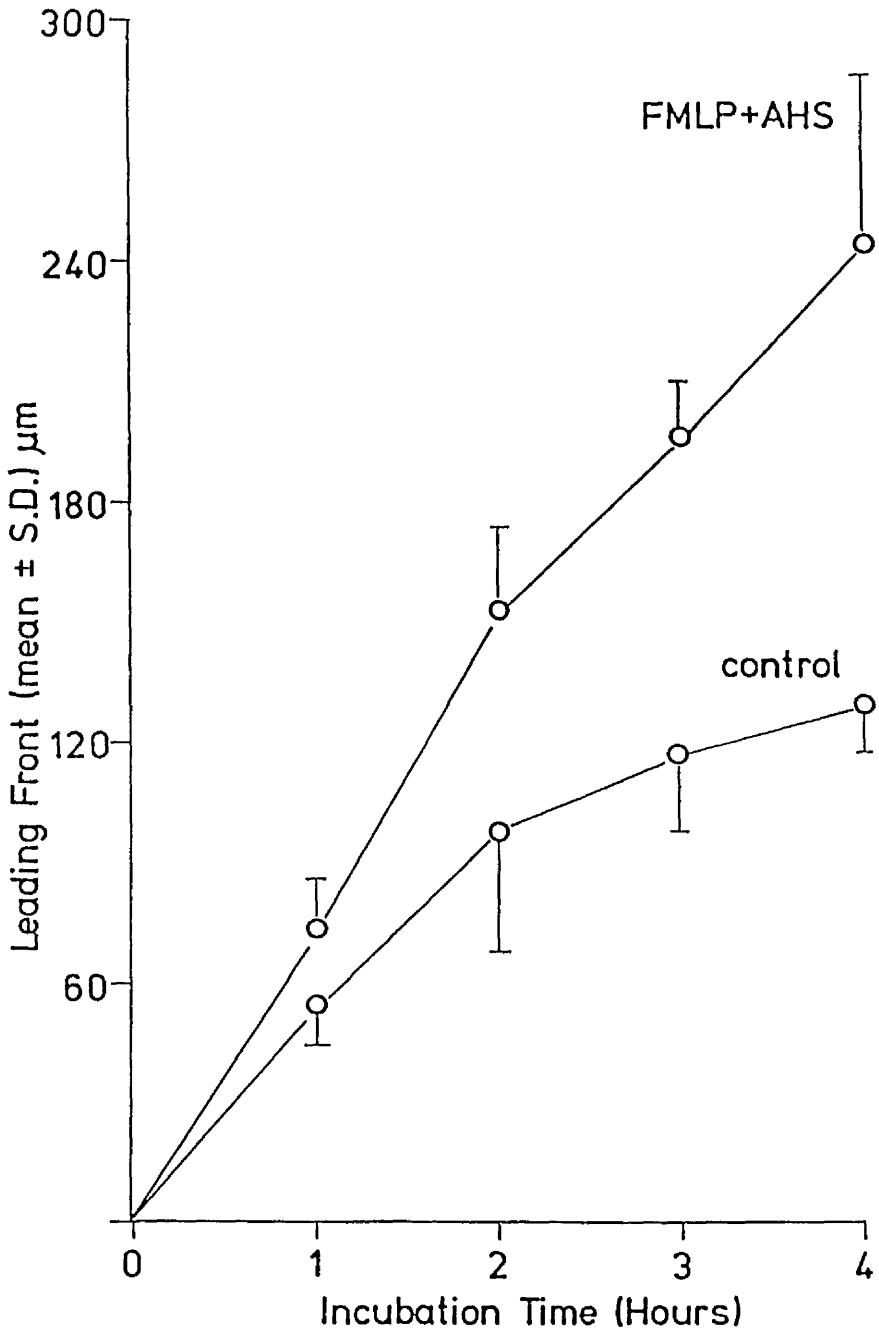



Fig. 53 Time course of invasion by monocytes of collagen gels (1.5 mg/ml) in the presence and absence of FMLP ($5 \times 10^{-9}$ M) + AHS (10%). FMLP was present both in the cell suspension and in the gels but AHS was present only in the cell suspension. Monocytes were suspended in HSA (20 mg/ml) in HBSS-MOPS for the invasion assay and the chemotactic factors, FMLP and AHS, were added after placing the cells on the gel surface.

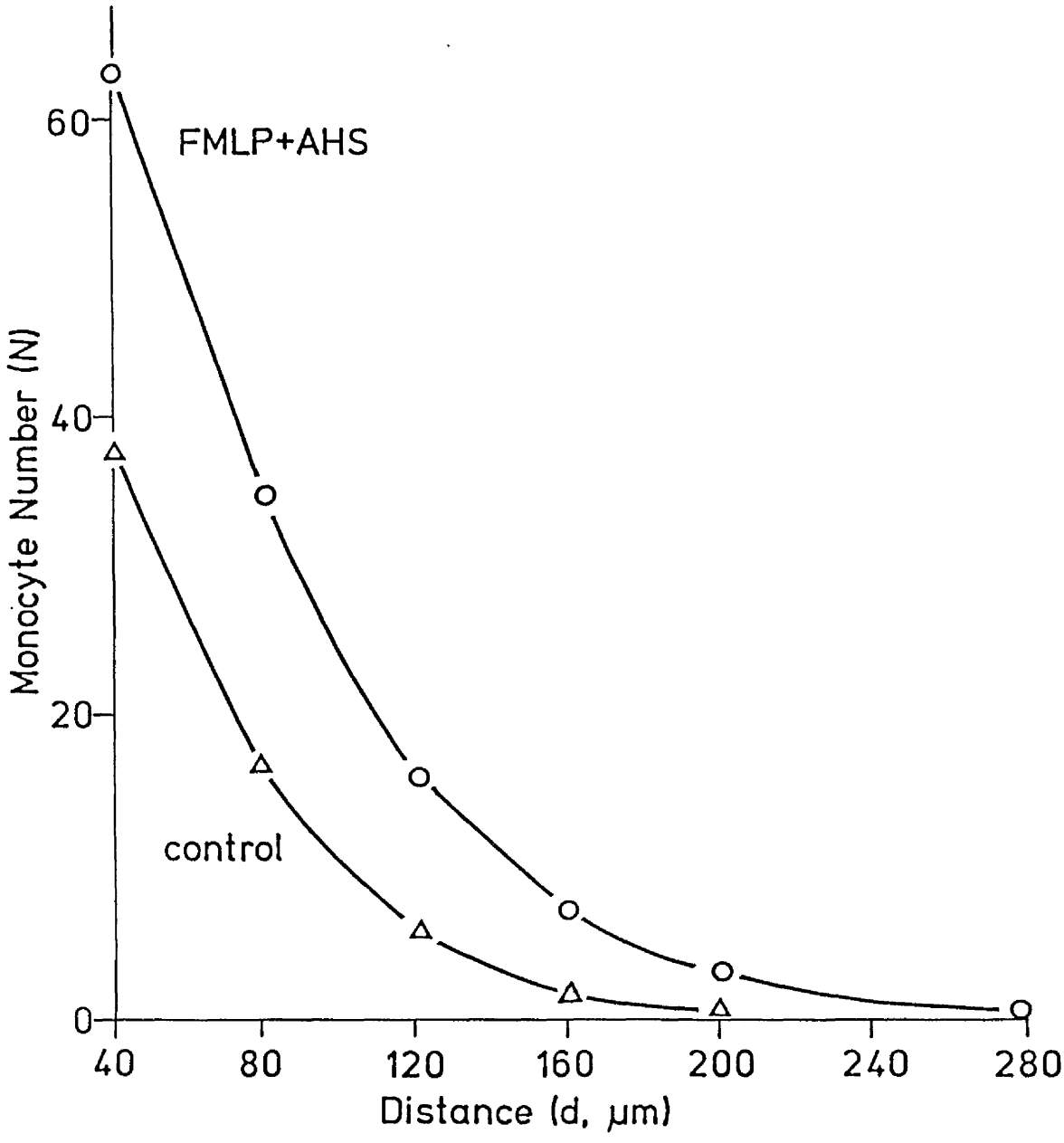



Fig. 54 Distribution of monocytes suspended in FMLP ($5 \times 10^{-9}$ M) + AHS (10%) through collagen gels with and without FMLP after 4 h. In both cases, the cells migrated through the gels according to simple diffusion of particles from a boundary. FMLP and AHS were absent both in the control gel and in the cell suspension.

The cells were counted at different levels of the gel starting from the top and at 40 μm intervals. It was observed that about 40% of the total cells suspended in FMLP + AHS were within the gel matrix i.e., at least 40 μm below the surface. This figure was almost the same as the proportion of monocytes present in the gel matrix in the presence of FMLP alone. For further experiments, activated serum was not incorporated into the collagen solution before it formed a firm gel.

**Behaviour of monocytes in three-dimensional collagen gels**

Invasion of collagen matrices by monocytes was an active process. Infiltration was completely prevented by low temp (4°C) and prior fixation of the cells. Once inside the gel matrix, monocytes moved randomly through the meshwork showing dynamic and pronounced morphological changes. The behaviour of the cells within gels was observed using phase-contrast and differential interference contrast (Nomarski) optics. Fig. 55 A and B shows photographs of monocytes invading collagen gels in the presence of uniform FMLP under Nomarski and phase-contrast optics. To quantify cell movement and the rapid configurational changes, time-lapse cinematography was employed.

*(a) Time-lapse cinematography analysis*

Frame by frame analysis of time-lapse films indicated that a wave of circular contraction was passing down the cells during migration through the gel. The position of the constriction remained fixed with

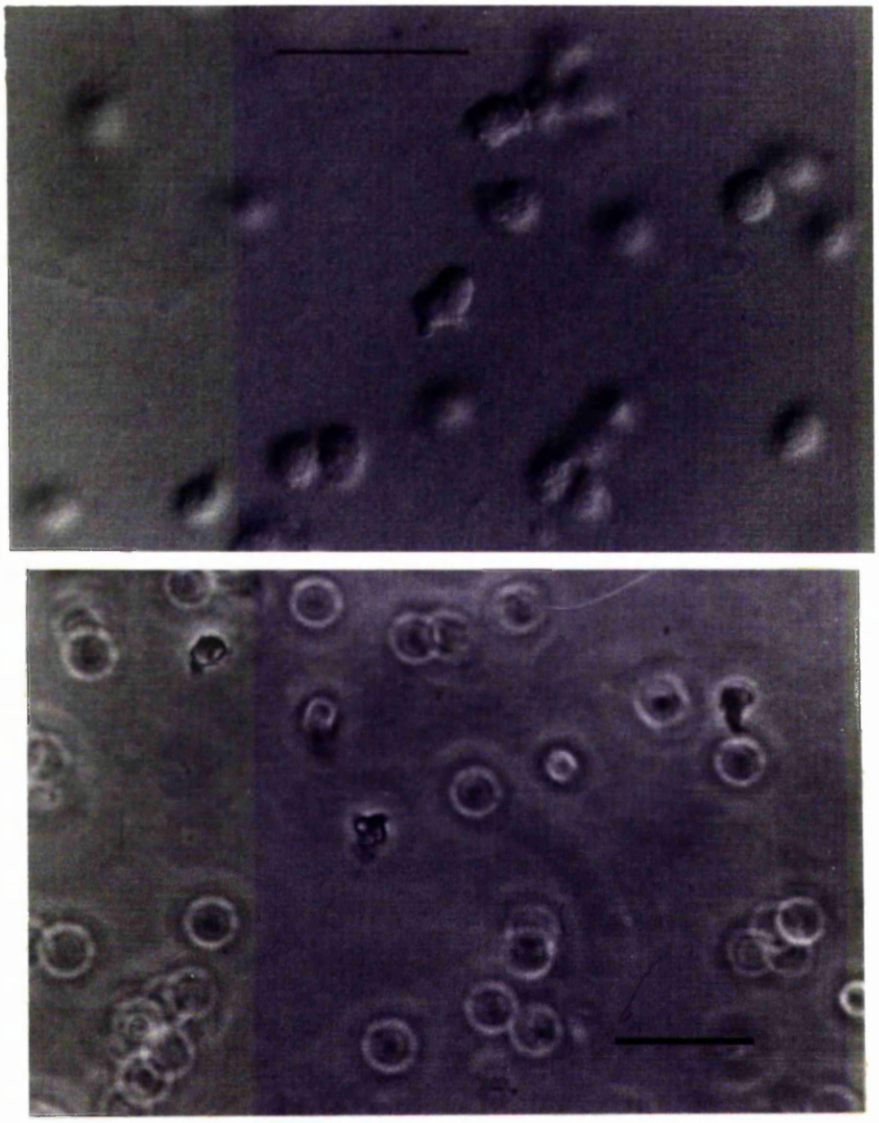



Fig. 55 Monocytes within the matrix of collagen gels (1.5 mg/ml) containing an isotropic concentration of 5 X $10^{-9}$ M FMLP. Collagen gels overlaid with cells were incubated at 37°C for 2 h before taking the photographs. A: Differential interference contrast photograph showing monocytes about 100 µm from the gel surface; B: phase-contrast photograph at 80 µm from the gel surface. Bar 50 µm.

respect to the external environment and the cell therefore appeared to move through it. Such locomotion of leucocytes was described by Lewis in 1931. More recently, the locomotion of lymphocytes (Haston and Shields, 1984) and neutrophils (Brown, 1982) through collagen gels has been reported to involve formation of constriction rings in the cell body through which they move forward.

Some monocytes moved through the constriction in 150-200 s whereas some cells took 400-500 s. The average time taken by a population of cells was 320 s i.e., the cells took 5.3 min to travel a distance equal to their lengths. Fig 56 A and B represent tracings from a time-lapse film of 2 monocytes where cell A took 464 s and cell B took 224 s to pass through their respective constrictions. It is possible to show that the constriction marking the contraction wave passes down the length of the cell by drawing sequential images of a moving cell, and arranging them so that the position of the cell is fixed and fell on a straight line (the Y-axis in Fig. 56). Fig. 56 also illustrates that cell A possessed complicated morphology with many side-projections while moving through the gel and in contrast to this, cell B was straightforward. Monocyte morphology inside collagen gels is described later which shows photographs of cells with side-projections like blebs. These may be necessary for anchoring the cell with collagen fibres. It is interesting to note here that the mean time for a lymphocyte to pass through the constriction when moving in a collagen gel is only 30 s (Haston and Shields, 1984).

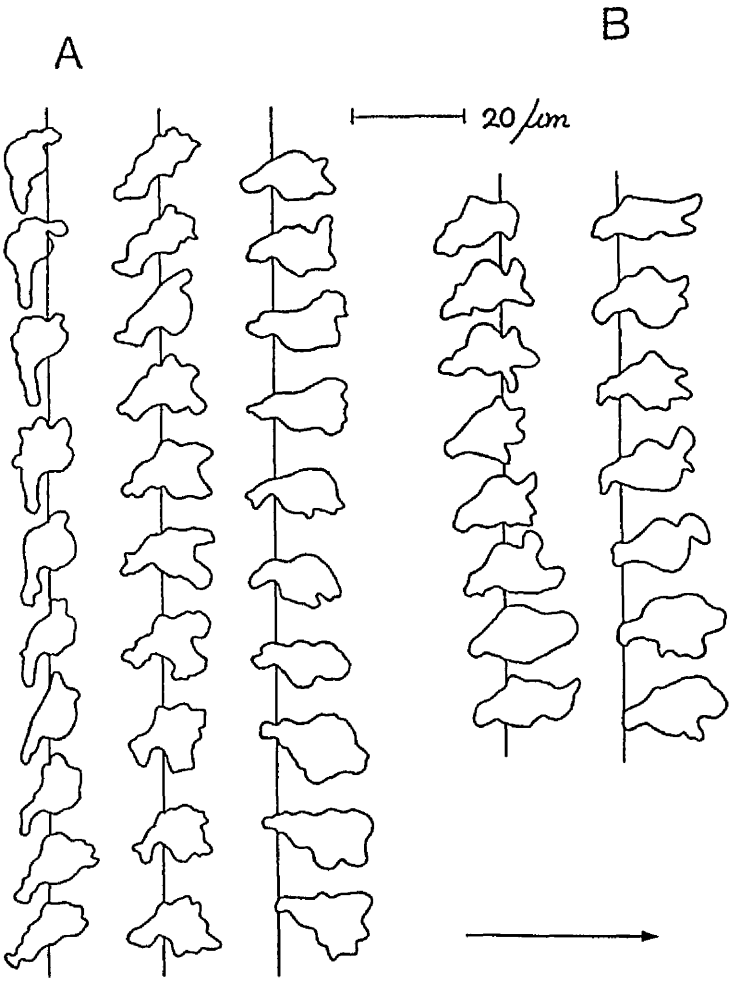
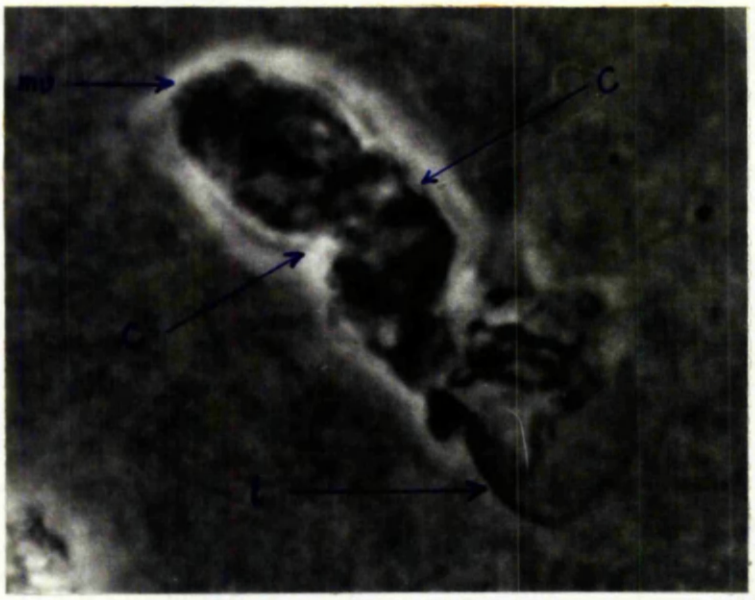



Fig. 56 Tracings from time-lapse films of monocytes moving through a collagen gel (1.5 mg/ml) containing an isotropic dose of 5 X $10^{-9}$ M FMLP at 37°C. The interval between drawings is 16 s. The drawings of cell A and B are spatially related by tracing the moving cells with reference to an external fixed point. The vertical line shows the constriction through which the cells passed. This line was fixed with respect to the environment. Cell A took 464 s and cell B took 224 s to pass through their respective constrictions. Bar represents 20 μm and arrow represents direction of migration.

Monocyte morphology inside a collagen gel in the presence of FMLP is illustrated by Fig. 57 (A to E). A and B are phase-contrast photographs (under high power) showing constrictions (c) that correspond to the contraction waves and huge lamellipodia (l) at the anterior of the monocytes (clear in Fig. 57 A). Unlike neutrophils , there were no tails at the posterior of these cells, rather, microvilli (mv) were seen at the back of moving monocytes which correspond to those observed in lymphocytes (Haston and Shields, 1984). Fig. 57 C represent the same features as A and B but under Nomarski optics. Fig. 57 D and E illustrate, using high power phase-contrast optics, the formation of 2 phase-dark, spherical blebs (b), in both sides of the contracted region in 2 monocytes. However, the functions of these blebs are not understood. Formation of blebs in lymphocytes, the pulling and pushing of collagen fibres by the blebs are known to be involved in lymphocyte locomotion through collagen gels (Haston et al., 1982).

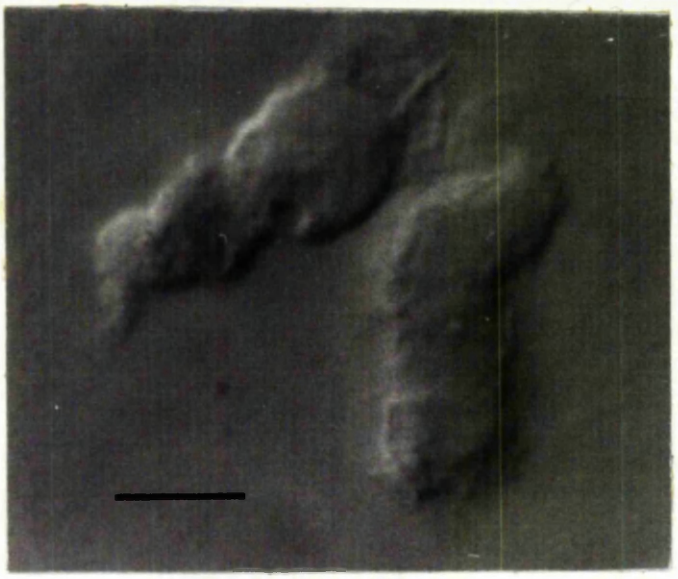
*(b) Analysis of cell tracks*

The paths taken by 43 monocytes were tracked from 5 separate film sequences. Fig. 58 illustrates the representative tracks of 12 monocytes moving within a 1.5 mg/ml collagen gel in the presence of an isotropic concentration of FMLP during 1½-2½ h incubation period. It is seen from the cell tracks that a wide variation of velocity occurred both between different cells in the same population and also within the path taken by individual cells. The monocyte at the top left




A

B



C

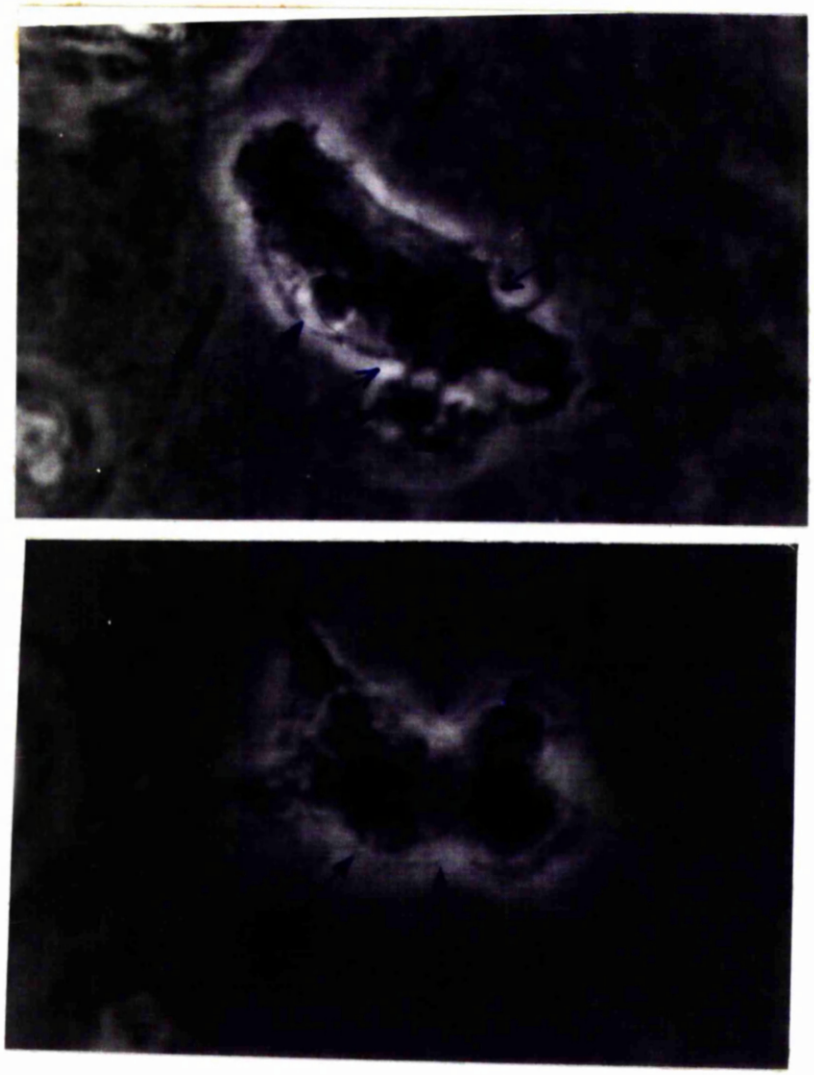



Fig. 57 A-E. High power photomicrographs of monocytes inside 3-D matrices of collagen gels (1.5 mg/ml) containing an isotropic concentration of 5 X $10^{-9}$ M FMLP. A and B: Phase-contrast photographs of monocytes showing constrictions (c) that correspond to the contraction waves; huge lamellipodia (l) at the anterior (clearer in A) and microvilli (mv) at the posterior end. Note monocytes inside collagen gels do not have tails as observed in neutrophils (cf. Fig. 42). C: Represents the same features as A and B except the two monocytes are under differential interference contrast optics. D and E: Phase-contrast photomicrographs of monocytes showing two spherical blebs (b) in both sides of the contracted region. The functions of these blebs were not understood. Bar, 10 μm.

corner was a fast moving cell with high persistence towards the bottom of the figure then it turned with a sharp angle and made a track which was more or less a straight path for the next 12 min. The cell at the bottom left was moving slowly and was not persistent at all, whereas the cell shown immediately above it, though migrating a long distance, had sections of track with high turning angles and little persistence. The monocyte at the bottom right started moving rapidly with high persistence which dramatically decreased near the end of the track.

The cell tracks were analysed for velocity and persistence of individual cells and for the population. The parameters S, P and R were Jackknife mean values determined as rms S and R, derived from line-fitting on the total data set, not from a simple mean ± S.E.M. for individual cells. These values were calculated by using a computer programme written by Dr. J. M. Lackie, Department of Cell Biology, University of Glasgow (details about the programme were described in chapter 2).

In pooled data involving 20 monocytes, the range of cell speeds was 1.91-4.40 μm/min with a Jackknife-mean speed of 2.68 ± 0.15 μm/min and Jackknife-mean persistence (P) = 1.76 ± 0.33 min and Jackknife-mean "diffusion coefficient" (R) = 25.28 ± 0.01 $\mu m^2$/min.

*(c) Turning angles*

For each track of monocyte invasion into the gel, the angle of turn between succeeding steps was measured.


Fig. 58 Representative tracks of 12 monocytes moving through a collagen gel (1.5 mg/ml) containing an isotropic dose of FMLP at 5 X $10^{-9}$ M. Each dot in the cell path indicates the position of the centre of a monocyte at an interval of 80 s. The arrows indicate the direction of locomotion. There were wide variations of velocities between different cells in the same population and also within the path taken by individual cells. The monocyte at the top left corner was a fast moving cell, the one at the bottom left was slow moving and the monocyte at the bottom right was initially moving rapidly and then slowed near the end of the track. Bar represents 20 μm.

Only angles of 10° or more were considered as discrete turns since it was difficult to measure narrower angles than this, particularly because of inaccuracies in the estimation of the mid-points of monocytes. The turning angles plotted as a histogram (Fig. 59) show that the distribution of angles of turn was not random, since the mean turn of the population was made at an angle within ±40° of the axis of the path of movement of the cell before turning. This suggested that monocytes moving chemokinetically through collagen gels tended to persist in a particular direction i.e., the cells moved in a relatively straight path. Similar results were obtained with neutrophils moving on plane substrata (Allan and Wilkinson, 1978; Shields and Haston, 1985) and with lymphocytes moving through collagen gels without any attractant (Haston and Shields, 1984)

In summary, like neutrophils and lymphocytes human monocytes straight from blood migrate through 3-dimensional gels of collagen. The work presented earlier (Brown, 1984) suggesting that monocyte cannot invade gels unless cultured for 48 h on protein-coated plane substrata cannot be true. It is demonstrated by the present study that human blood monocytes purified on BHK microexudate-coated surfaces (Ackerman and Douglas, 1978) migrate into collagen gels like other blood leucocytes. The cells migrated up to 200 μm inside gels during 4 h incubation. Using an isotropic dose of 5 X $10^{-9}$ M FMLP (the dose that induced maximum locomotor morphology in these cells as described in chapter 4) in gels and in the

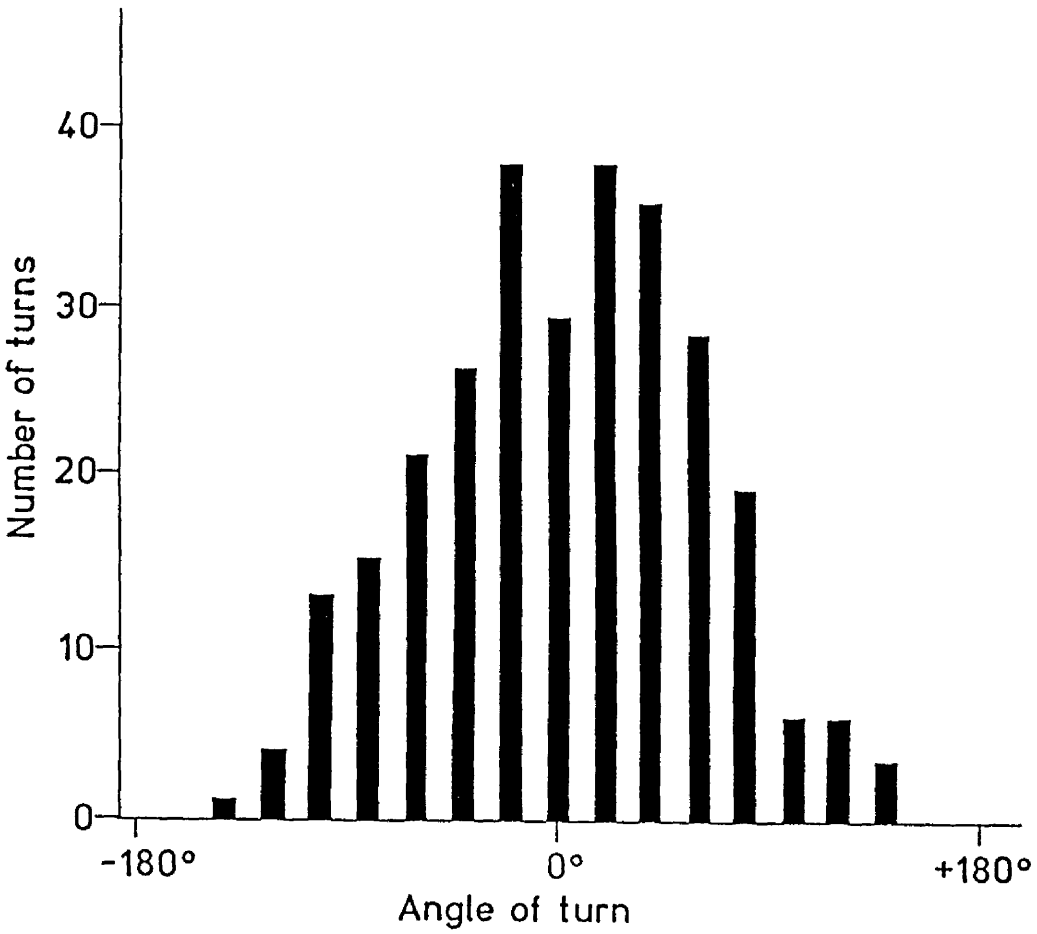



Fig. 59 Histogram of turning angles of monocytes migrating through collagen gels (1.5 mg/ml) containing an isotropic dose of 5 X $10^{-9}$ M FMLP. The turning angles were not random as the mean turn of the population was made at an angle within ±40° of the axis of the cell path before turning.

cell suspension, monocytes showed increased random migration into the gels i.e., chemokinesis. In the presence of uniform concentration of FMLP, the cells migrated further and also more cells were present inside the gel. A total of 20% monocytes direct from blood could invade the gels after 4 h of incubation and over 40% of the total cells would migrate into the gel during this interval in the presence of uniform concentration of FMLP. Addition of activated serum to monocytes in FMLP could not increase the proportion of cells invading the gel though the two chemoattractants combined together induced about 85% monocytes to change shape (described before) in suspension.

The cells inside gels showed rapid morphological changes. A wave of circular contraction or a constriction ring was observed in each monocyte moving inside the gel matrix. This constriction remained fixed with respect to the collagen fibres and the cell body was observed to pass through the constriction. Different cells in the same time-lapse film sequence migrated through the gel with different speeds and also there was marked variation in the speed of individual cells. It was interesting to see that the majority of the blood monocytes had a propensity to move in a fairly straight path through collagen gels containing FMLP throughout.

# CHAPTER 7

# VISUAL ASSAYS OF MONOCYTE LOCOMOTION ON TWO-DIMENSIONAL SUBSTRATA

This chapter describes monocyte locomotion in the presence and absence of uniform concentration of chemotactic factors and in the presence of gradients of chemotactic factors generated by *Candida albicans* in serum. Section I will consider experiments with and without uniform concentrations of chemoattractants and section II will consider experiments with *Candida albicans* in serum.

## SECTION I

### Monocyte locomotion in the presence and absence of uniform concentration of chemotactic factors

Adhesion of mononuclear phagocytes to glass or plastic surfaces is the major problem in the study of locomotion of these cells. If the surfaces are not coated with proteins, the cells irreversibly stick to them. Clean glass and collagen-coated glass coverslips were used to study monocyte adhesion. The result from 3 experiments suggested that about 95% of the total cells placed on clean glass coverslips were adherent to the surface during 30 min incubation. Considering this figure as 100% adhesion, about 84% of the total cells were adherent to collagen-coated glass coverslips. Studies on monocyte locomotion are described below.

*(a) Monocyte locomotion in the presence of HSA*

Monocytes were, generally, very well spread on clean glass with a phase-dark appearance. None of these cells could translocate either in the presence or absence of

uniform concentration of FMLP. However, if HSA was added to the filming chamber the cells were observed to adopt a more compact (less spread) shape and some of them move around actively with, very often, a distinct leading lamellipodium. Table 14 shows results from several filming sequences for monocyte locomotion on different concentration of HSA-coated glass coverslips. It is shown that the proportion of moving cells increased as the concentration of HSA increased (the results with 50 mg/ml HSA were not consistent for unknown reasons).

In Table 14 the term 'non-responding cells' was used to define monocytes which did not show polarisation and were unable to move i.e., these cells were static. However, these cells were viable as their veils were seen to move at the periphery. The term 'polarised cells, unable to translocate' defines those cells which were usually tethered to the coverslips by their posterior ends. Moving cells as the term implies, were actively translocating cells. Table 15 contains a summary of cell speed and persistence time at different concentration of HSA. It is shown that increasing the concentrations of HSA had no effect on mean cell speed.

Fig. 60 shows the representative tracks of 9 monocytes moving on glass coverslips in the presence of 100 mg/ml HSA. The cells were moving randomly with variable angles of turn. Some of the cells took small angles of turn within a short section of the track.

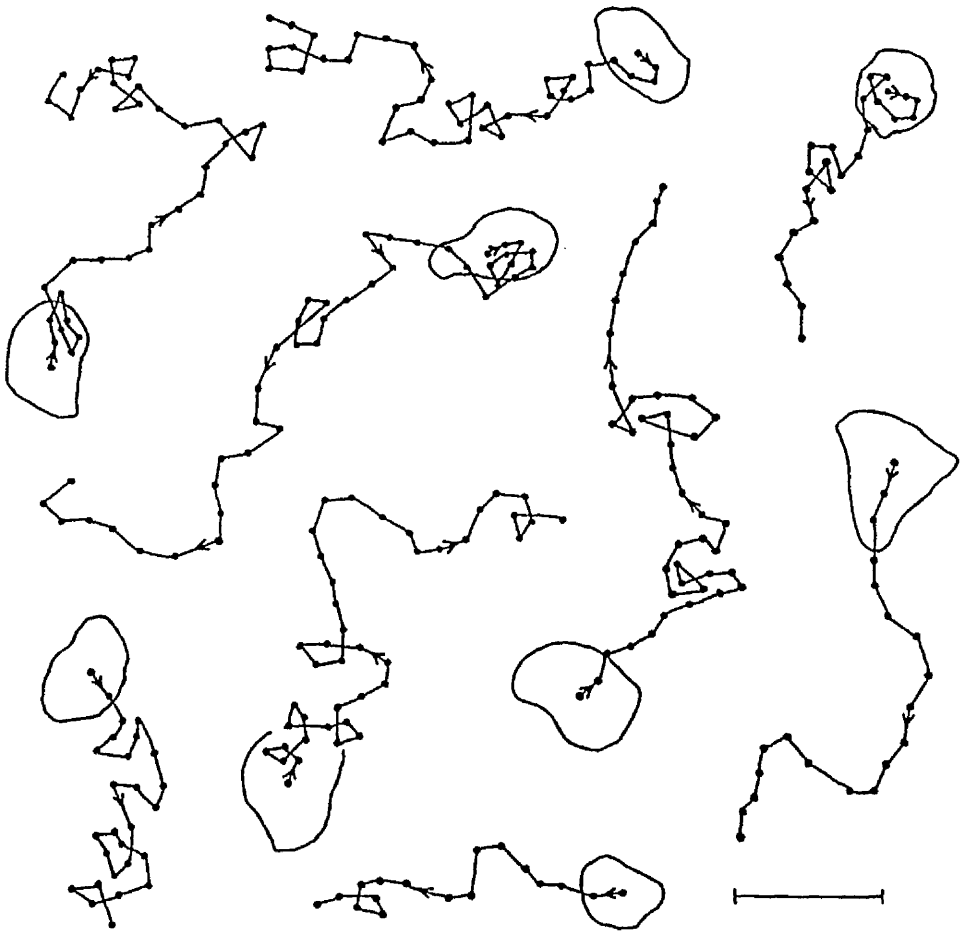


Fig. 60 Representative tracks of human blood monocytes moving randomly on glass coverslips in the presence of a uniform concentration of human serum albumin (HSA) at 100 mg/ml. The arrows indicate the direction of locomotion and the dots in the cell track represent the position of the cell centre at each 80 s interval. Note some cells took small angles of turn within a short section of the track. (Bar = 20 μm).

**Table 14. Monocyte locomotion on HSA-coated glass**

| Concentration of HSA in the medium (mg/ml) | Non-responding cells (%) | Polarised cells, unable to translocate (%) | Moving cells (%) | Total cells analysed |
|---|---|---|---|---|
| 0 | 87 | 13 | 0 | 68 |
| 5 | 68 | 24 | 8 | 78 |
| 20 | 57 | 30 | 13 | 103 |
| 50 | 58 | 33 | 9 | 60 |
| 100 | 55 | 31 | 14 | 149 |

**Table 15. Effect of different concentration of HSA on the speed (S) and the persistence time (P) of blood monocytes moving on glass coverslips**

| Concntration of HSA in the medium (mg/ml) | Number of moving cells analysed | Jackknife-(mean ± S.E.M.) values | | |
|---|---|---|---|---|
| | | speed, S (μm/min) | persistence time, P (min) | diffusion coefficent, R, ($\mu m^2$/min) |
| 5 | 6 | 4.58 ± 0.91 | 0.85 ± 0.20 | 35.66 ± 0.33 |
| 20 | 14 | 3.40 ± 0.10 | 1.11 ± 0.16 | 25.66 ± 0.01 |
| 50 | 5 | 3.38 ± 0.13 | 0.76 ± 0.11 | 17.36 ± 0.01 |
| 100 | 20 | 4.42 ± 0.28 | 0.58 ± 0.08 | 22.66 ± 0.01 |

The values given for speed (S), persistence time (P) and the rate of diffusion of the cells, R are the "Jackknife" mean values determined as rms S and R, derived from line-fitting on the total data set, not from a simple mean ± S.E.M. for individual cells. These values were calculated by using computer programmes written by Dr. J. M. Lackie, Department of Cell Biology, University of Glasgow. Details of this programme is published elsewhere (Lackie and Wilkinson, 1984). Here S is the rms speed of the cells, P is a measure of the directional persistence time and the third parameter R, is expresed as the rate of diffusion of the cells.

*(b) Chemokinetic locomotion of monocytes in the presence of FMLP*

After observing monocyte locomotion in the presence of HSA it was of considerable interest to study the locomotion of these cells in the presence of uniform concentration of FMLP. The polarising responses of monocytes, in suspension, towards different doses of FMLP were described in chapter 4. FMLP at $10^{-7}$ M induced about 35% of monocyte to change shape and at 5 X $10^{-9}$ M induced the maximum percentage of monocytes (about 60% or so) to change shape in those assays. The cells adopted different morphologies in the presence of these 2 different doses of FMLP. Hence it was interesting to study the locomotion of the cells which were polarised in the presence of supraoptimal and optimal doses of FMLP on coverslips coated with different concentrations of HSA. Table 16 represents results from several time-lapse filming sequences for monocyte locomotion in FMLP. All the parameters shown in Table 16 are identical to those described in Table 14. The total responding cells was the total of moving cells and of cells which were polarised but unable to translocate. The proportion of moving cells was greater in the presence of FMLP in HSA than in the presence of the corresponding dose of HSA alone (cf. Table 14). Still it is difficult to show convincing evidence for chemokinesis in the presence of FMLP as monocytes were sticky in the presence of low doses of HSA. The results with high concentrations of HSA (50 and 100 mg/ ml) clearly indicated the recruitment of extra

Table 16. Monocyte locomotion on different concentrations of HSA-coated glass in the presence of supraoptimal and optimal concentration of FMLP

| Composition of HSA in the medium (mg/ml) | | Non-responding cells (%) | Polarised cells unable to translocate (%) | Moving cells (%) | Total responding cells (%) | Total cells analysed |
|---|---|---|---|---|---|---|
| 5 | a | 72 | 28 | 0 | 28 | 69 |
| | b | 56 | 36 | 8 | 44 | 152 |
| 20 | a | 57 | 30 | 13 | 43 | 46 |
| | b | 47 | 36 | 16 | 52 | 224 |
| 50 | a | 58 | 36 | 6 | 42 | 72 |
| | b | 50 | 30 | 20 | 50 | 132 |
| 100 | a | 64 | 19 | 17 | 36 | 117 |
| | b | 34 | 32 | 34 | 66 | 362 |

(a) = $10^{-7}$ M FMLP i.e, supraoptimal concentration; (b) = 5 X $10^{-9}$ M FMLP i.e., optimal concentration.

locomotory cells on the plane substratum in the presence of FMLP.

Table 17 contains a summary of the speed and persistence time of monocytes moving in the presence of $10^{-7}$ M and 5 X $10^{-9}$ M FMLP. The values of S, P and R shown in Table 17 were obtained after analysing cell tracks as described for Table 15.

(i) Morphology of monocytes moving in uniform concentration of FMLP

Fig. 61 shows a photomicrograph of monocytes moving on plane substrata in the presence of (A) 20 mg/ml HSA, (B) 5 X $10^{-9}$ M FMLP in 20 mg/ml HSA, (C) 100 mg/ml HSA, (D) $10^{-7}$ M FMLP in 100 mg/ml HSA, (E) and (F) 5 X $10^{-9}$ M FMLP in 100 mg/ml HSA. The morphologies of monocytes moving in 5 X $10^{-9}$ M and in $10^{-7}$ M FMLP (both in 100 mg/ml HSA) on plane substrata were similar to those of the cells in suspension, respectively (cf. Fig. 6). Cells which were moving in 5 X $10^{-9}$ M FMLP/100 mg $ml^{-1}$ HSA quickly became elongated and asymmetric with an obvious leading edge and several contraction waves appeared in the cell body. Some cells at this dose of FMLP were continuously changing shapes and made several attempts to translocate in almost every direction. As these cells were adherent to the coverslips, mainly by their tails, they could not locomote (Table 16). Some of these cells were observed to be as elongated as 40 μm. Monocytes moving in $10^{-7}$ M FMLP/100 mg $ml^{-1}$ HSA (Fig, 61 D) could not achieve the same degree of polarity as those moving in 5 X $10^{-9}$ M FMLP/100 mg $ml^{-1}$ HSA (Fig. 61 E and F).

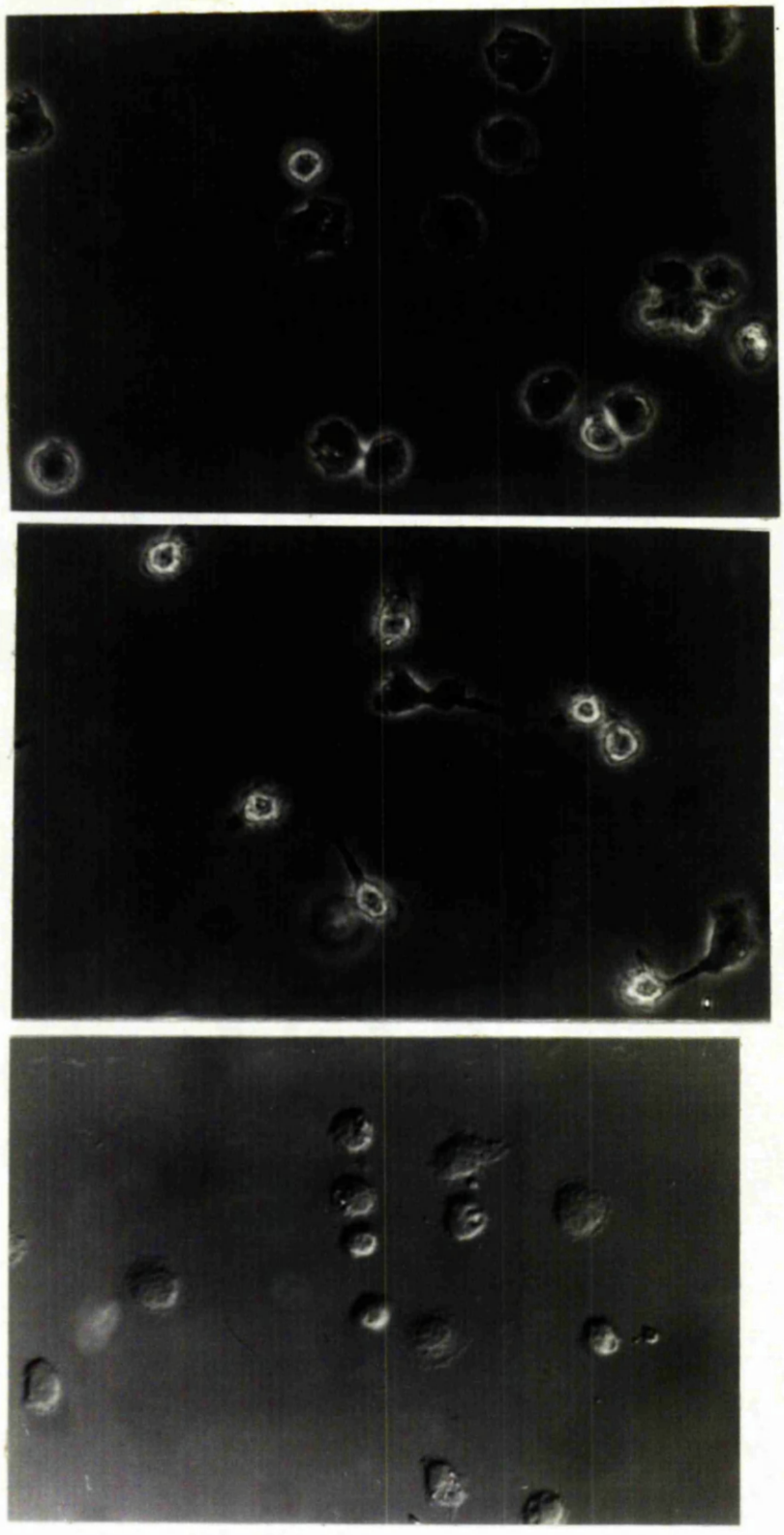
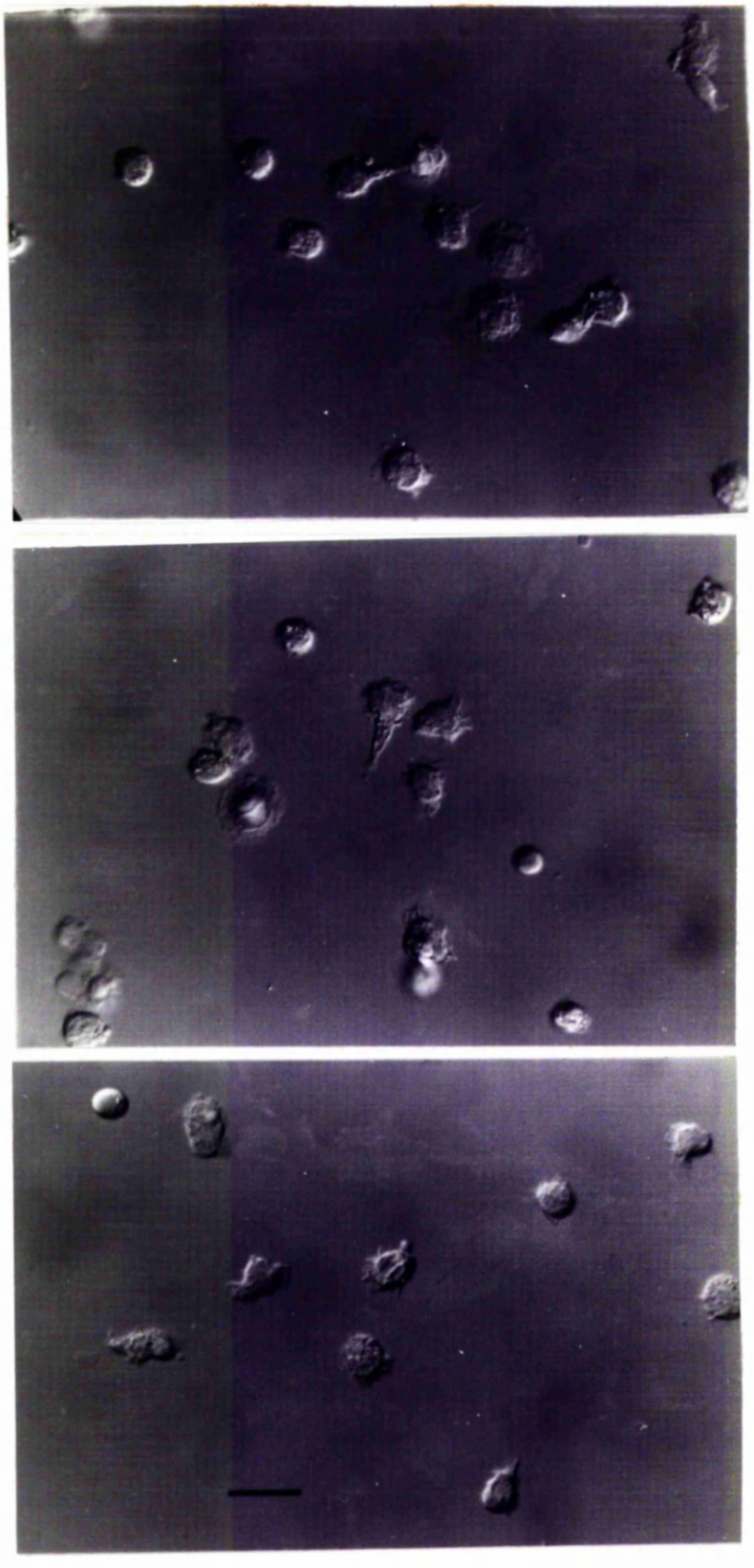



Fig. 61 A-F. Photomicrographs of monocytes moving on glass coverslips in the presence of HSA ± FMLP. A: Phase-contrast photograph of monocytes moving in HSA (20 mg/ml), Note phase-dark appearance of the spread cells. B: Phase-contrast photograph of monocytes moving in 5 X $10^{-9}$ M FMLP in HSA (20 mg/ml), monocytes tended to be adherent on the surface by their phase-dark tails. C: Differential interference contrast (DIC) photograph of monocytes moving in HSA (100 mg/ml). The cells are relatively less spread compared to those in 20 mg/ml HSA (Fig. 61 A).




D: DIC photograph of monocytes moving in $10^{-7}$ M FMLP in HSA (100 mg/ml). Note the irregular appearance of the responding cells with contraction waves emanating from all areas of the cell periphery. E and F: DIC photographs of monocytes moving in 5 X $10^{-9}$ M FMLP in HSA (100 mg/ml). Note the obvious anterior region and frequent contraction waves in the cell body of monocytes responding towards FMLP. Bar, 20 μm.

Table 17. Effect of supraoptimal and optimal concentration of FMLP in different doses of HSA on the speed (S) and persistence time (P) of blood monocytes moving on glass coverslips

| Concentration of HSA in the medium (mg/ml) | | Number of moving cells analysed | Jackknife-(mean ± S.E.M.) values | | |
|---|---|---|---|---|---|
| | | | speed, S (μm/min) | persistence time, P (min) | diffusion coefficent, R, ($\mu m^2$/min) |
| 5 | a | 0 | * | * | * |
| | b | 11 | 3.89 ± 0.25 | 1.64 ± 0.33 | 49.63 ± 0.04 |
| 20 | a | 6 | 3.01 ± 0.10 | 0.96 ± 0.23 | 17.39 ± 0.01 |
| | b | 20 | 4.79 ± 0.53 | 0.91 ± 0.20 | 41.76 ± 0.11 |
| 50 | a | 4 | 4.42 ± 0.27 | 0.38 ± 0.06 | 14.85 ± 0.01 |
| | b | 20 | 4.22 ± 0.35 | 0.62 ± 0.10 | 22.08 ± 0.02 |
| 100 | a | 19 | 3.98 ± 0.16 | 0.54 ± 0.06 | 17.10 ± 0.01 |
| | b | 20 | 4.51 ± 0.46 | 1.11 ± 0.24 | 45.15 ± 0.10 |

(a) = $10^{-7}$ M FMLP i.e, supraoptimal concentration; (b) = 5 X $10^{-9}$ M FMLP i.e., optimal concentration.
* monocytes were not moving in the presence of $10^{-7}$ M FMLP on coverslips coated with 5 mg/ml HSA. Hence the values of S, P and R could not be calculated. The values of S, P and R were obtained after analysing cell tracks as described for Table 15.

Fig. 62 shows representative tracks of monocytes moving in the presence of (A) 5 X $10^{-9}$ M FMLP and (B) $10^{-7}$ M FMLP, both in HSA at 100 mg/ml. Analysis of the tracks of moving cells in 5 X $10^{-9}$ M FMLP/100 mg $ml^{-1}$ HSA demonstrated that the cells tended to move in relatively straight paths (mean angle of turn = 43°), making narrow angles of turn. The waves of circular contractions were apparently moving down the length of the cell, although in relation to the substratum the wave was stationary but the cell was moving forward. Monocytes moving in $10^{-7}$ M FMLP/100 mg $ml^{-1}$ HSA were not well polarised (Fig. 61 D). Cells made wide angles of turn (mean angle of turn = 73°, Fig. 62 B) and occasionally changed direction by changing their polarity. This lack of persistence in polarity resulted in a lack of persistence in direction, reflected in the "zig-zag" tracks made by the cells. The histograms of the turning angles for monocytes moving in 5 X $10^{-9}$ M FMLP/100 mg $ml^{-1}$ HSA and $10^{-7}$ M FMLP/100 mg $ml^{-1}$ HSA are shown in Fig. 63. The mean turning angles of individual cells moving in these 2 different concentrations of FMLP were determined. These were significantly different from each other without formal statistical test (Fig. 64). However, using Mann-Whitney U test for comparison, the difference in turning behaviour of monocytes moving in 5 X $10^{-9}$ M and $10^{-7}$ M FMLP was demonstrated to be highly significant ($P<0.0001$).

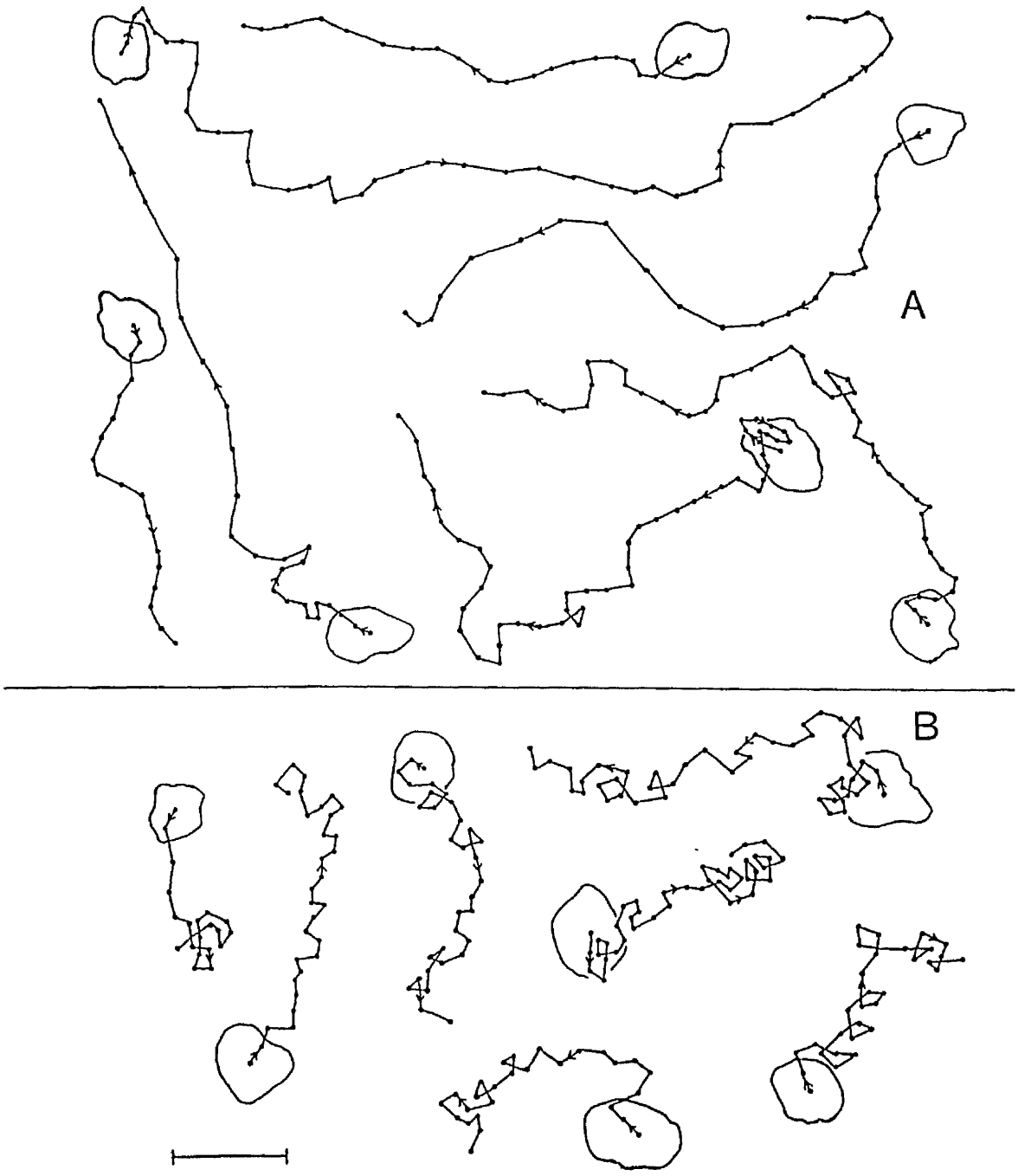



Fig 62 Tracks of human blood monocytes moving in uniform concentrations of FMLP in 100 mg/ml HSA. A: 5 X $10^{-9}$ M FMLP in HSA; B: $10^{-7}$ M FMLP in HSA. The arrows and dots in the cell tracks are as described earlier for Fig. 60. Bar = 20 μm. Monocytes in an optimal dose of FMLP (5 X $10^{-8}$ M) tended to locomote in a relatively straight path.

*(c) Monocyte locomotion in the presence of different chemoattractants*

Monocyte locomotion was studied on plane substrata in the presence of neat human serum (autologous), 5 X $10^{-9}$ M FMLP in serum and C5a at $10^{-8}$ M in 20 mg/ml HSA. The results are summarised in Table 18. A total of 70 monocytes were filmed in the presence of neat serum. None of these cells translocated. The reason for this was not understood. In earlier experiments, about 30-35% of the monocytes changed shape in suspension after incubating with 10-100% of serum.

Of the 32 cells (49% of total cells) translocating in the presence of 5 X $10^{-9}$ M FMLP in serum, 20 were analysed. They had a Jackknife-mean value of speed = 3.40 ± 0.19 μm/ min, persistence time = 0.84 ± 0.06 min and diffusion coefficient =19.42 ± 0.01 $\mu m^2$/min. Similarly, the 5 monocytes (about 6% of the total cells) moving in the presence of $10^{-8}$ M C5a/20 mg $ml^{-1}$ HSA had Jackknife-mean speed = 3.38 ± 0.25 μm, persistence time = 1.39 ± 0.29 min and diffusion coefficient =31.76 ± 0.04 $\mu m^2$/min. These values were determined as described before.

*(d) Locomotion of Fc rosetted cells in FMLP*

It was of considerable interest to study the locomotion of monocytes bound to antibody coated sheep red cells. Monocytes were rosetted on coverslips (as described before) and the coverslips were later used in the filming chamber. The chamber was filled with 5 X 10-9 M FMLP/100 mg $ml^{-1}$ HSA and the cells were filmed. About

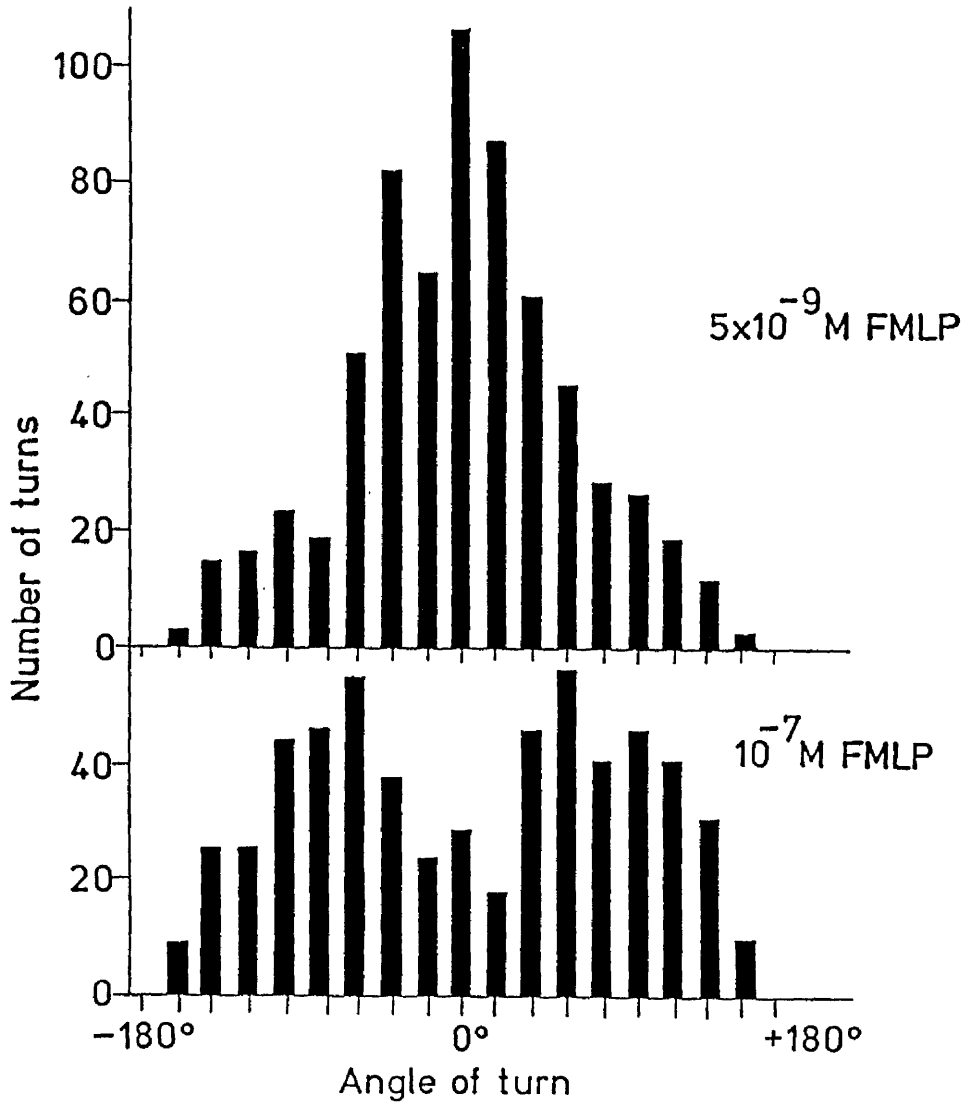



Fig. 63 Histogram of turning angles of monocytes moving in uniform concentrations of FMLP in HSA at 100 mg/ml during a filming period of 1 h. In $10^{-7}$ M FMLP the angles were spread fairly uniformly throughout the range of ±140° with a mean turning angle of the population at ±73°, whereas at 5 X $10^{-9}$ M FMLP the angles were peaking prominently at zero degrees with a mean angle of turn at ±43°.

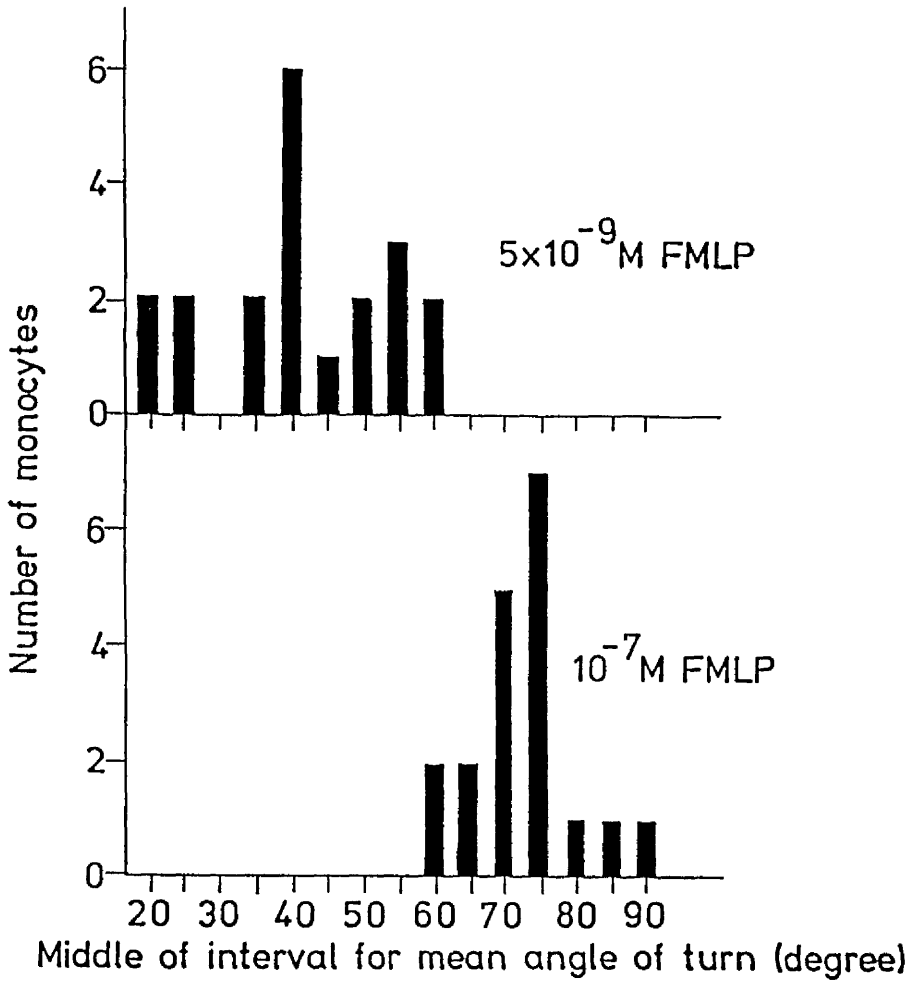



Fig. 64 Histogram of the middle of the interval for the mean angle of turn for monocytes moving in uniform concentrations of FMLP in HSA at 100 mg/ml. The mean angles of turn of 20 cells analysed while moving in 5 X $10^{-9}$ M FMLP were in a range between ±20-60°, whereas in $10^{-7}$ M FMLP a total of 19 cells analysed had a mean angle of turn in a range between ±60-90°. Using a Mann-Whitney U test for comparison, the difference in turning behaviour of monocytes moving in 5 X $10^{-9}$M and $10^{-7}$ M FMLP was highly significant ($P<0.0001$).

Table 18. **Monocyte locomotion on glass surface coated with serum, 5 X $10^{-9}$ M FMLP in serum and $10^{-8}$ M C5a in HSA**

| Composition of medium | Non-responding cells (%) | polarised cells, unable to translocate (%) | Moving cells (%) | Total cells analysed |
|---|---|---|---|---|
| Serum (autologous, neat) | 93 | 7 | 0 | 70 |
| 5 X $10^{-9}$ M FMLP in 90% autologous serum | 36 | 15 | 49 | 65 |
| $10^{-8}$ M C5a in HSA at 20 mg/ml | 86 | 8 | 6 | 90 |

50 Fc-rosetted monocytes were filmed at 20°C for 30 min in the presence of FMLP. None of these cells could locomote. Some of these cells had locomotor morphology, few of them phagocytosed the red cells and only 3 cells attempted to translocate. A total of 30 cells were filmed at 30°C in the presence of 5 X $10^{-9}$ M FMLP in 100 mg/ml HSA. About 17% of these cells moved slowly with the bound red cells accumulated at their posterior end, 33% monocytes phagocytosed the attached red cells and the remaining 50% cells were stuck to the coverslips and were inactive. Fc-rosetted neutrophils are known to translocate in the presence of $10^{-8}$ M FMLP with the red cells at their tail (Wilkinson et al., 1980).

In summary, monocyte adhesion to glass surface is the major problem in the study of locomotion of these cells *in vitro*. If the surface were coated with HSA, a proportion of cells were able to translocate. Some of the monocytes (about 16%) had locomotor morphology immediately after purification. This could be the reason for a proportion of monocytes moving around when placed on an HSA coated surface.

In the presence of FMLP in HSA, the locomotion of monocytes on a 2-D substratum increased. Monocytes moving in the presence of uniform $10^{-7}$ M and 5 X $10^{-9}$ M FMLP both in HSA at 100 mg/ml had similar morphological features to those of the cells in suspension at the same dose of the peptide in HSA. Cells moving in the presence of 5 X $10^{-9}$ M FMLP made narrow angles of turn and moved in a relatively straight path. Monocytes moving in the

presence of $10^{-7}$ M FMLP made wide angles of turn and were not persistent. The difference between the angle of turns made by the cells at these 2 doses of FMLP were highly significant.

## SECTION II

### Monocyte chemotaxis towards *Candida albicans*

Human monocytes in normal serum move directionally towards, and phagocytose, blastospores of the yeast *Candida albicans*. This is illustrated by a series of sequential photographs, presented by Fig. 65 (A to E), where one monocyte is seen responding to the presence of blastospores. This reaction was taken as the basis for an assay of chemotaxis.

*(a) Initial response of the cells towards the spores*

Most monocytes were initially at a fairly short distance from the nearest blastospore (less than 40 μm in all cases, ranging from 12-35 μm) under the conditions of the assay. The cells responded within seconds to the presence of a blastospore by the formation of a membrane ruffle and then of a distinct leading edge, facing the source of the gradient . The cells then migrated towards the spore maintaining a compact oriented morphology throughout their path (Fig. 65 A and B). Some cells put forward their anterior veil extending 20-30 μm ahead towards the spore without translocation. Also some cells could stretch about 40 μm and phagocytose the spores around them. Such events were not considered as

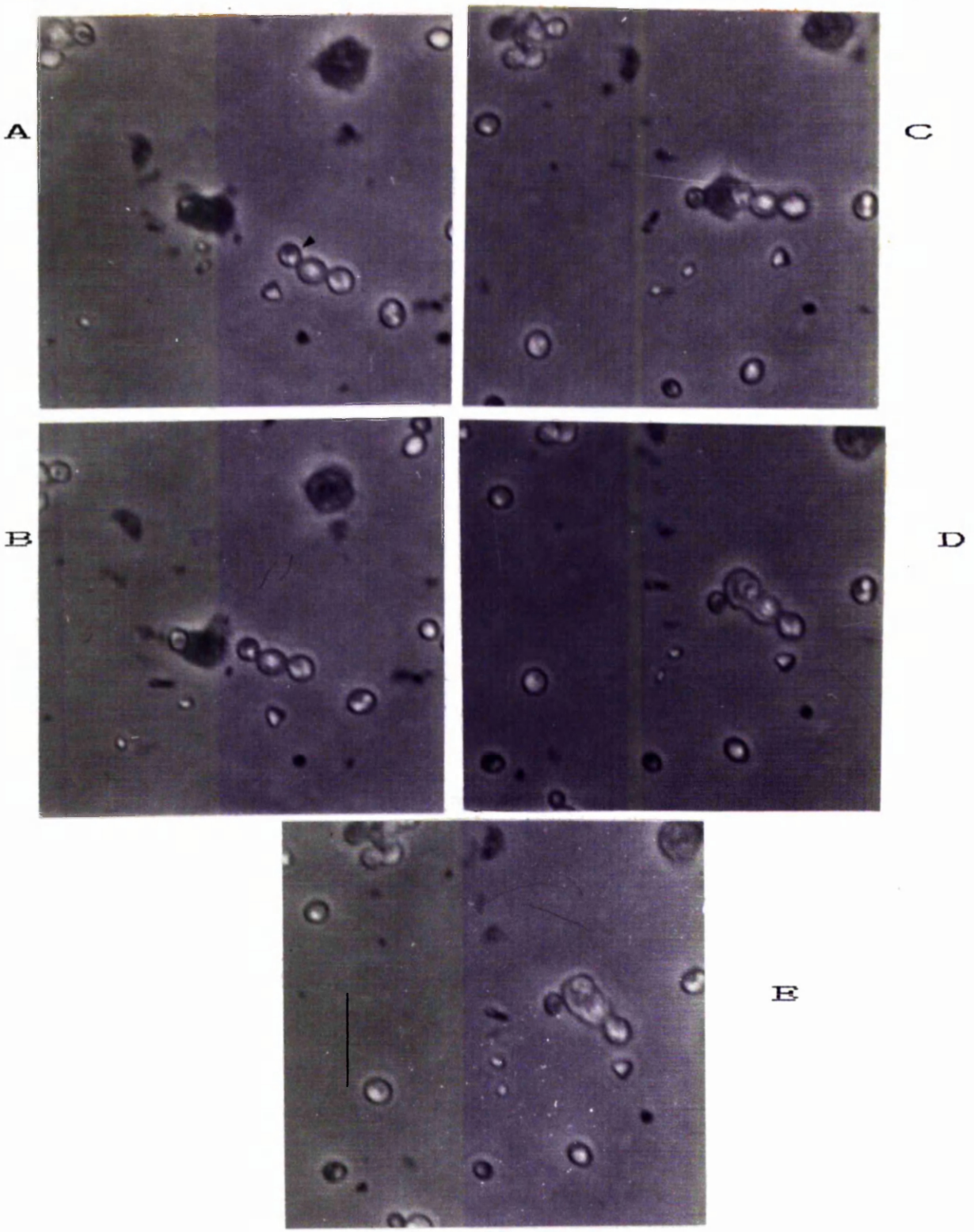



Fig. 65 A-E. Sequential photographs of chemotaxis of human monocyte towards and phagocytosis of *Candida albicans* blastospores. A group of blastospores is present in the centre of the field (arrowed in A). Figures A-C represent the positions of monocytes at intervals of about 30 s. The interval between C and D and that between D and E is about 1 min. Note the locomotion of the monocyte directly towards the spores followed by engulfment of the whole group of spores. The cell took a little pause after phagocytosing one spore before starting a new one (D and E). Bar = 20 μm.

chemotaxis as the cells did not locomote to their positions. In every case observed, having reached a spore, whether the cell had translocated or not, it ingested the spore. The whole response usually took not more than 5 min. After ingesting a spore, the cells often rounded up for a short-time, then, if there was another spore around the cells, they re-oriented to ingest the new spore. If there was a spore close by, cells which had completed phagocytosis usually oriented and moved on towards the second spore without delay without rounding up, and then engulfed it (65 C to E). In this way, monocytes could during a period of 45 or so minutes filming, ingest up to 15 spores.

*(b) Requirement of serum for phagocytosis*

The locomotion of monocytes towards *Candida albicans* was serum dependent. Very few monocytes could move in the absence of serum and only 2-3% of them could phagocytose the spores which came in contact with them. If serum was present, changes in its concentration (5-40% v/v) did not affect the velocity of moving cells towards the spores or their ability to phagocytose them. The presence of serum in the assay chamber greatly increased the proportion of monocytes phagocytosing the spores. For unknown reasons monocytes were observed to be strongly adherent to neat serum-coated substrata (described before). Hence the concentration of serum used in the candida assay was not allowed to exceed more than 20% (the generally used concentration was 10%). Also candida spore-activated human serum (AHS, at 10% v/v) was used instead of fresh

human serum in the filming chamber. It was interesting to note that AHS had no effect upon the ability of the monocytes to ingest the spores (Table 19). It was reported earlier that about 60% monocytes could change shape in suspension in the presence of 10% AHS. However, the nature of chemotactic factor present in AHS is not known.

Monocytes were suspended in HSA at 5-20 mg/ml for the candida assay. There was no detectable difference between the ability of the cells in 5 mg/ml and 20 mg/ml HSA to ingest the spores in the presence of serum. For further experiments, monocytes were suspended in 10 mg/ml HSA in HBSS-MOPS for the candida assay.

*(c) Pre-opsonisation of spores not necessary for phagocytosis*

*Candida albicans* blastospores were usually opsonised by incubating with normal human serum (containing anti-candida IgG) before using for chemotactic assays with monocytes. In some experiments, non-preopsonised spores were used to see if the cells could detect the presence of these spores in the surrounding medium. There was no difference between the proportion of monocytes phagocytosing opsonised spores and spores that had not been preopsonised. The results presented in Table 19 suggested that opsonisation of the spores was an instant process occurring when the spores came in contact with the serum.

*(d) Phagocytosis by cultured monocytes*

To see if culturing the cells had any effect upon their responsiveness towards candida spores, monocytes were cultured in RPMI + 20% FCS containing 2mM L-glutamine, 100 IU/ml of penicillin and 100 μg/ml of streptomycin in siliconized glass tubes for 18 and 72 h. Then the culture supernatant was removed and the cells were suspended in HSA at 10 mg/ml for candida phagocytosis. The results from several experiments suggested that there was no difference between the proportion of monocytes directionally responding towards and phagocytosing opsonised spores and spores that had not been preopsonised, both in the presence of fresh human serum when the cells were precultured for 18 h (Table 19). However, these values were significantly lower than the proportion of monocytes direct from blood capable of phagocytosing the spores. When monocytes were cultured for 72 h, none of them could phagocytose a single spore. The cells were totally non-responsive when few spores settled at less than 10 μm from the nearest monocyte. These cells appeared similar to resident peritoneal mouse macrophages which were mostly non-responsive towards candida spores (Wilkinson, 1982b).

*(e) Paths taken by monocytes towards **Candida albicans***

In order to test the usefulness of the candida assays as a measure of chemotaxis, the chemotactic ratios were calculated in 60 events observed in 40 randomly selected monocytes from 3 separate filming sequences. Fig. 66 illustrates the representative tracks made by 11

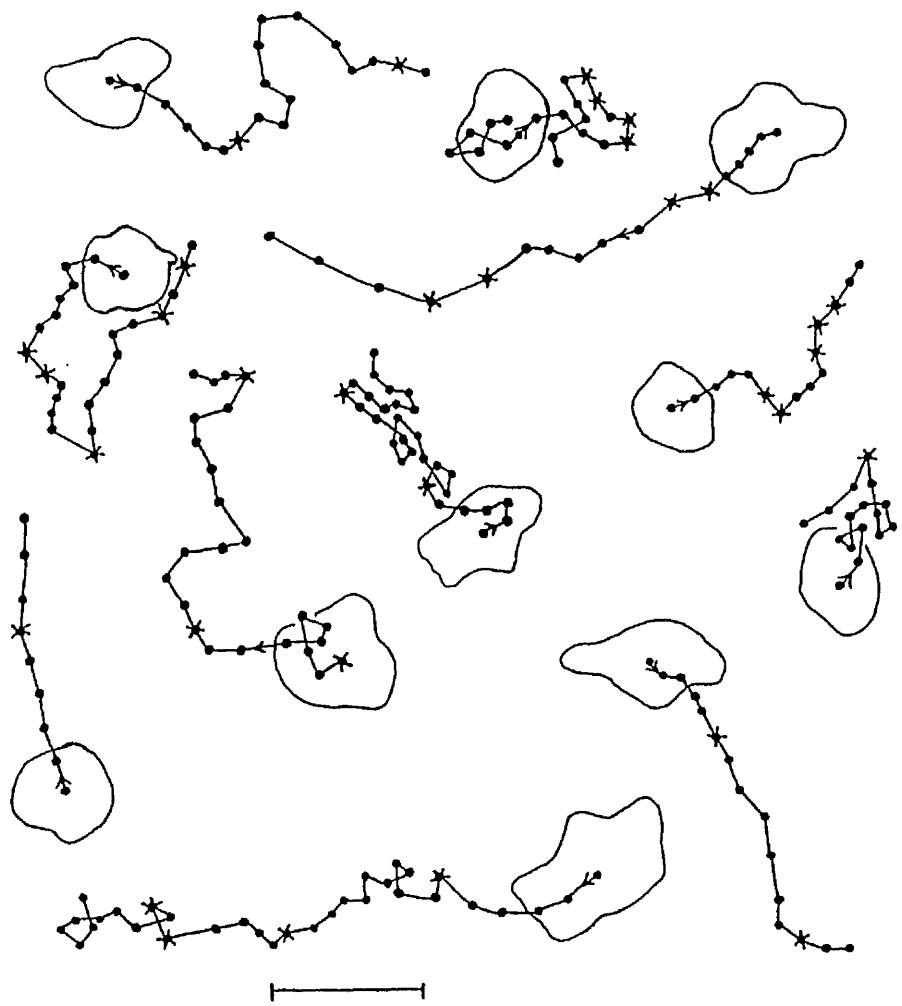


Fig. 66 Tracks of human blood monocytes moving towards spores of *Candida albicans* in human serum. The arrows in the cell track indicate the direction of locomotion, the dots indicate the position of the cell centre at intervals of 40 s and ( **x** ) indicates contact with, and ingestion of, a spore. Bar = 20 $\mu$m.

Table 19. Phagocytosis of *Candida albicans* by monocytes at different conditions

| Monocytes | Candida spores opsonised or non-preopsonised | Medium consisted of 10% v/v FHS/AHS | % Monocytes phagocytosing candida spores | Total cells studied |
|---|---|---|---|---|
| Direct from blood | op | FHS | 86-90 | 96 |
| | npo | FHS | 82-87 | 23 |
| | npo | AHS | 85-90 | 61 |
| Cultured for 18 h | op | FHS | 65-70 | 35 |
| | npo | FHS | 60-65 | 32 |
| Cultured for 72 h | op | FHS | 0 | 28 |

op = opsonised, npo = non-preopsonised, FHS = fresh human serum, AHS = activated human serum.

Table 20. Distribution of chemotactic ratios of monocytes phagocytosing candida spores in serum

| Chemotactic ratio (range) | No. of events observed |
|---|---|
| 1.0 | 15 |
| 0.90 - 0.99 | 13 |
| 0.80 - 0.89 | 9 |
| 0.70 - 0.79 | 9 |
| 0.60 - 0.69 | 7 |
| 0.50 - 0.59 | 7 |

monocytes towards candida spores and Table 20 contains a summary of the distribution of chemotactic ratios made by 40 cells. Monocytes direct from blood migrated towards *Candida albicans* blastospores produced a mean chemotactic ratio = 0.82 (S.D. = 0.16, n = 40, considering the mean chemotactic ratio of cells which showed more than one event of chemotaxis). Of the total events analysed, 25% (15 out of 60, observed in 13 cells, Table 20) gave the maximum possible ratio of +1.0. Cells with this ratio did not, within the limits of the analytical technique, show any turning behaviour at all, but moved directly towards the gradient source.

In summary, monocyte chemotaxis towards candida spores is a clearly defined process. Within seconds of appearance, the cells could detect the presence of the spores in their environment and migrated towards them. In some cases if the spores were close by, the cells shot their processes towards them while the posterior part of the cells were attached to the substratum. Monocytes showed chemotaxis towards opsonised/non-preopsonised spores provided fresh serum or candida spore-activated serum was present in the medium. The cells produced a positive chemotactic ratio 0.82 ± 0.16 while many of them showed the maximum ratio of 1.0. Culturing monocytes for 18 h reduced the proportion of cells capable of phagocytosing the spores to 65-70% from the value of 86-90% for uncultured cells. When cultured for prolonged times (3 days) the phagocytic capacity of the monocytes was totaly lost.

# CHAPTER 8

# GENERAL DISCUSSION

It has been understood for a long time that the circulating leucocytes leave the blood stream and migrate through tissues at sites of inflammation which is of the greatest importance for the protection of the body against infection. Under normal conditions the polymorphonuclear leucocytes, mononuclear phagocytes and lymphocytes provide a broad defence mechanism which can cope with most potential pathogens.

Blood monocytes enter the tissues to become macrophages and hence to carry out their principal phagocytic functions. The fate, function and activity of macrophages varies depending on the inflammatory or immune stimulus which has evoked their differentiation. It is, therefore, not surprising that the locomotion and chemotaxis of different populations of mononuclear phagocytes may vary considerably from one another. Nevertheless, there are still difficulties in obtaining purified and functionally intact blood monocytes or tissue macrophages in a state suitable for studying their locomotion.

**Purification of monocytes**

Most of the reported methods for purifying peripheral blood monocytes are clinically unattractive due to poor reproducibility, low yield and long separation time. The cells may be isolated by different methods based on (i) their ability to adhere to glass or plastic surfaces (Bloom and Bennett, 1966), plastic surfaces coated with serum protein (Kumagai et al., 1979), plasma proteins (Freundlich and Avdalovic, 1983)

or microexudate (Ackerman and Douglas, 1978), (ii) on their density (Bennett and Cohn, 1966; Loos et al., 1976; Nathanson et al., 1977; Johnson et al., 1977; Hardin and Downs, 1981; Fluks, 1981; Böyum, 1983) and (iii) on their cell size (Bont et al., 1979; Norris et al., 1979; Figdor et al., 1981). Although a considerable purification and yield of monocyte is obtained by the adherence method, this method has several disadvantages including enhanced adherence due to activation of cells by factors such as migration inhibition factor (David, 1975), and damage to the cell surface when the cells are recovered by mechanical scraping or chemical removal from a glass or plastic surface (Koller et al., 1973; Rinehart et al., 1978). Velocity sedimentation has been successfully used by Bont et al. (1979) for collecting large number of purified monocytes. The general disadvantage of this method is that it is not suitable forwork with a small volume of blood and also relatively long separation times are required (up to 10 h). Various gradient media used for monocyte purification are based on isokinetic, linear or discontinuous density centrifugation principles. For these methods a choice has to be made either for high cell recovery or for high purity of the cell population. Higher monocyte recoveries were obtained by using bovine serum albumin (BSA) density gradients (Bennett and Cohn, 1966; Johnson et al., 1977) with variable monocyte purity and poor reproducibility.

Fluks (1981) described the use of discontinuous density gradients of percoll (DDGP) for isolation of blood monocytes in which he centrifuged the mononuclear

cells in hyper-osmotic and iso-osmotic DDGP for 90 min in a swing-out rotor at 2250 g and 2700 g, respectively. In practice, it was observed that cells tended to assume non-spherical morphology if centrifuged over 600 g. Such a starting population of monocytes would be unsuitable for the shape change assay used in this thesis. For this reason, monocytes were not centrifuged over 500 g for the percoll separation procedure tried in the present study. This could be one reason for the poor separation of the cells. However, Fluks (1981) reported the presence of monocytes in 2 fractions even after centrifugation of the mononuclear cells at a very high speed. Also the average yield was 56% of the monocytes present in whole blood.

In another study, the commercially available gradient medium, Nycodenz-monocyte was used to separate the cells. It was not understood why monocyte purity in this study was only about 50% using this procedure, described by Böyum (1983), in which he separated 95-98% pure monocytes. Moreover, if the cells had been separated well, they would not be suitable for use in the present study because of platelet adherence to the monocytes during separation.

Leb et al. (1983) made a comparative study of percoll density gradient and other separation procedures for monocyte purification. They described the use of gelatin-coated plastic tissue culture dishes for cell adherence followed by dislodgement of the adherent cells at low temperature (4°C) and with lidocaine at 15 mM and 30 mM. These workers also incubated mononuclear cells on plastic tissue culture dishes and later detached the

adherent cells with 3 mM EDTA at 4°C. Finally, Leb et al. (1983) demonstrated that monocyte purification based on techniques which use dislodgement of adherent cells at low temperature only, or 3 mM EDTA at low temperature, yielded monocytes with better plasma membrane function in comparison with methods which employ lidocaine for cell detachment or separation by percoll density gradient.

Freundlich and Avdalovic (1983) used gelatin-treated flasks coated with plasma for active adhesion of monocytes and suggested that the ability of monocytes to adhere to such a surface is most likely dependent upon their membrane receptors for fibronectin (FN). Bevilacqua et al. (1981) found that blood monocytes would not adhere to gelatin surfaces coated with FN-free serum, but if serum is reconstituted with FN, binding, which is a magnesium dependent process, occurs. Thus the use of gelatin-plasma-coated flasks is an example of the FN-receptor mediated adherence of the monocytes on the surface. When these flasks were used for cell purification (described in chapter 3 of this thesis), it was observed that the adherent cells were not highly enriched in monocytes (maximum purity was about 60%) and also the yield was not attractive. The reason for this could be the use of diluted autologous plasma (50%) as there were limitations in obtaining 10-12 ml of platelet free plasma for routine use. Perhaps the concentration of FN present in 50% autologous plasma was not enough to cover the gelatin-coated surfaces. Freundlich and Avdalovic (1983) recommanded the use of neat plasma since any bare area, not coated with FN, could result in

passive adhesion by the cells. Hence neat FCS was used to coat the gelatin-treated flasks which were used for monocyte purification. This modified procedure also suffered similar problems as described for gelatin-plasma-coated flasks.

Another separation procedure, described by Kumagai et al. (1979), was followed to purify monocytes. This procedure is based upon the ability of human peripheral blood monocytes and mouse peritoneal exudate macrophages to adhere to FCS-coated plastic dishes from which they were detached by EDTA treatment. Adhesion and spreading of monocytes and macrophages to serum- or plasma-coated surface are temperature- and divalent cation-dependent (Rabinovitch and DeStefano, 1973a and b; Grinnell, 1978). In monocyte isolation procedures involving an adherence step, therefore, several workers regularly used serum in the culture medium to avoid irreversible adhesion of the cell. Kumagai et al. (1979) found that direct coating of the plastic surface with serum (FCS) rather than addition of serum alone in the culture medium would prevent the passive adhesion of monocytes more effectively. However, it is not known which factor(s) of serum bound to the plastic surface favours the change of adhesiveness and later detachment of the monocytes from the surface. Kumagai et al. (1979) described that an acid glycoprotein of bovine serum (fetuin) if used to coat the flasks produced similar results though to a lesser extent than with neat FCS.

It was observed that a proportion of lymphoid cells were capable of attaching to the FCS-coated surfaces and

these could not be readily removed by washing. These cells were detached during incubation of the adherent cells with 4mM EDTA and these lymphocytes reduced the purity of the monocyte fraction. Monocyte population separated by this procedure contained 25-30% lymphocytes. These are probably mostly B-lymphocytes as described by van Voorhis et al. (1982). The total recovery of monocytes from FCS-coated surfaces was only about 50%, the rest of the cells remained adherent to the flask. Moreover, like other separation procedures involving an adhesion step, a proportion of monocytes, 6-20%, were not able to bind to the FCS-coated surface. Kumagai et al. (1979) reported similar results and also Leb et al. (1983) found the same proportion of monocytes present in the non-adherent cell population when gelatin-coated surfaces were used for separation of the mononuclear cells. Leb et al. (1983) suggested that the non-adherent monocytes are due to lower adherence to gelatin-coated flasks by a subset of the cells.

It was observed that a high proportion of lymphocytes, 25-40% were present in the monocyte fraction separated on a gelatin-plasma, gelatin-FCS or FCS-coated surface. Previous workers, who introduced these surfaces to purify monocytes did not report this. In the case of FCS-coated flasks (described by Kumagai et al., 1979), two different batches of FCS were used which gave similar proportions of lymphoid cell contamination in the monocyte preparation. It is now known that lymphocytes straight from blood can change shape in suspension in the presence of FCS (Wilkinson, 1986). It might be possible that some

of the polarised lymphocytes could stick to the plastic surface and would not then be readily removed with the non-adherent cells during washing.

Monocytes isolated on BHK microexudate-coated flasks were obtained in a highly pure form with better yield, 55-80% (see chapter 3). An average of 30-35% of the total monocytes present in the peripheral blood were lost by this procedure. A proportion of these cells, 1-15%, remained attached to the microexudate-coated flask after EDTA treatment. It was observed that strong adhesion of these cells were not related to the storage of the microexudate-coated surface prior to use. Monocytes were inconsistently found to remain attached to a freshly prepared microexudate-coated surface and, maximum detachment of adherent cells was sometimes possible from microexudate-coated flasks stored at 4°C for 2 weeks. The rest of the monocytes, 10-20%, were not possible to purify were non-adherent to the surface used for culture. This was similar to findings observed in other adhesion procedures used to purify monocytes. Further, it is possible that the adhesion technique removes a proportion of the total mononuclear phagocytes from the cell suspension applied to a protein-coated or uncoated-surface.

Ackerman and Douglas (1978) described that monocyte detachment from a microexudate-coated surface is reversible and, by alternate additions of media and EDTA to a single flask, the cells could be induced to undergo at least 3 cycles of attachment and detachment. The mechanism is a chelater-mediated detachment of the monocytes from their substratum, and Ackerman and Douglas

(1978) found that EGTA at 3 mM is nearly as effective as EDTA in detaching the cells from the surface. They also found that $Ca^{2+}$ at 4 mM does not completely prevent monocyte detachment either in the presence of EDTA or EGTA, but $Mg^{2+}$ at 4mM does so.

Since certain negatively charged serum proteins as well as acid glycoprotein (fetuin) of FCS can bind to plastic surfaces (Kumagai et al., 1979) not coated with microexudate, the role of microexudate in connection with the active adhesion of monocytes is unclear. Ackerman and Douglas (1978) suggested that monocytes might bind to certain component(s) of the microexudate in an EDTA/EGTA- dependent fashion or the presence of microexudate could alter some physical property of the substratum which would then non-specifically reduce its interaction with the cells.

About 84% of the monocytes isolated by the above procedure showed spherical morphology after separation. It was not known why the rest of the cells in the unstimulated population had locomotor morphology. However, after EDTA detachment of the monocytes from the microexudate-coated surface, the cells were washed with different media including RPMI alone, HSA in RPMI and RPMI +10% FCS. Each time a similar proportion of monocytes with locomotor morphology was observed among populations of cells separated on the same surface. Since monocytes were purified by a procedure involving an adhesion step, it might be possible that some of the cells were already activated by the process of adherence to the plastic surface, as reported by David (1975).

**The polarisation or shape change assay**

It is now generally accepted that the majority of the circulating blood monocytes are capable of adherence to plastic or glass surfaces or on the top of micropore filters used to study the locomotor response of the cells. From this point of view, the **polarisation or shape change assay** is an ideal assay to study monocytes in suspension. Since there is no interaction between the cells and the substratum, any alteration in the morphology of the cells is directly related to the presence of the chemotactic factor in the environment. The polarisation assay was first used by Cianciolo and Snyderman (1981) in which they tested a series of N-formylated oligopeptides, zymosan-activated human serum and a chemotactic lymphokine against monocytes. The cells changed shape from a spherical to a characteristic polar configuration in response to those chemoattractants. The effect was dependent upon the dose of the test substance and the time of exposure. The polarisation of monocytes towards different chemoattractants is an early event that precedes their chemotactic response. Cianciolo and Snyderman (1981) found a correlation between the proportion of monocytes polarised in response to different chemotactic factors and the proportion of cells migrated into the polycarbonate filters in response to those substances.

Human blood neutrophils in suspension also assume a polarised morphology when exposed to chemotactic stimuli (Smith et al., 1979; Smith and Hollers, 1980). The polarised morphology of neutrophils in suspension in

presence of optimal and supraoptimal doses of the chemotactic peptide, FMLP, is identical to the morphologies of the cells locomoting on protein-coated glass surfaces in the presence of the respective doses of the peptide (Shields and Haston, 1985). Human blood lymphocytes also assume a polarised morphology when incubated with colchicine (at $10^{-5}$ M) for 30 min (Wilkinson 1986). The proportion of polarised blood monocytes and neutrophils was observed to be maximal after 20 and 30 min exposure of the cells, respectively, to FMLP when tested by the polarisation assay. Thus, the polarisation or shape change assay is a useful method for studying responses of the cells towards known or supposed chemotactic factors.

**Monocyte polarisation and receptor redistribution**

Monocytes in suspension in siliconized glass tubes were incubated with a wide range of concentrations of different chemotactic factors. These factors included FMLP, leukotriene $B_4$ ($LTB_4$), complement peptides, candida spore-activated human serum (AHS), alkali denatured HSA and many others. A proportion of the cells responded towards these chemotactic factors by assuming a polarised or non-round morphology from an initial spherical outline. The maximum percentage of monocytes responding towards different chemotactic factors was dependent upon the efficacy of the factor and the cells responded in a dose- and time-dependent fashion towards these factors (see chapter 4 of this thesis). The maximum percentage of monocytes, usually, 60-66%, changed shape in FMLP at

5 X $10^{-9}$ M after 20 min incubation. Further incubation of the cells in FMLP resulted in loss of polarisation of the cells. A similar trend of the time course of monocyte polarisation was observed not only with FMLP but with other chemotactic factors like C5a, AHS and $LTB_4$, all of which had an optimum period of incubation of 10 min at 37°C. This has not been reported by previous workers. It is likely that monocytes perhaps lose their surface receptors or internalise them due to prolonged incubation in the presence of chemotactic factors. This is supported by the studies of the proportion of Fc-receptor bearing monocytes after different times in FMLP, reported in this thesis. These observations suggested that due to prolonged exposure to chemotactic factors, monocytes internalise their surface receptors and thus lose their responsiveness towards the factors.

Incubating monocytes for a prolonged time in 5 X $10^{-9}$ M FMLP in presence of the metabolic inhibitor 2-deoxy glucose (2DG at 5 X $10^{-2}$ M) showed that the proportion of polarised cells did not decline with time of exposure as observed without 2DG. It is known that 2DG is an inhibitor of glycolysis and hence would prevent additional energy mediated internalisation of the surface receptors of monocytes. The cells were still able to polarise towards FMLP even after incubating for 1 h with 2DG present in the environment. This supports the idea that loss of polarisation on prolonged exposure to FMLP is due to an energy-dependent internalization and loss of receptors which can be prevented by blocking glycolysis.

Neutrophils and monocytes have fewer surface receptors for FMLP than for Fc and C3b . Also FMLP receptors are technically relatively difficult to localize on the surface (personal communication of Dr. W. S. Haston and Mr. J. M. Shields, Department of Bacteriology and Immunology, University of Glasgow) compared to Fc and C3b receptors. Recently, Sullivan et al. (1984) coupled FMLP to haemocyanin and demonstrated by scanning electron microscopy that the receptors for FMLP are asymmetrically distributed on neutrophils. Also Shields and Haston (1985) demonstrated that neutrophils polarised in FMLP bind opsonised yeast (C3b receptor) or antibody-coated sheep red cells (Fc receptors) at the head region. This was also shown by Walter et al. (1980) and Wilkinson et al. (1980). Haston and Shields (1984) also found that the surface marker Thy 1-2 is located at the front of all locomoting T-lymphocytes. These observations suggest that there is asymmetry in receptor distribution in locomoting human neutrophils and lymphocytes. However, no report is available so far describing distribution of receptors on human monocytes.

The localisation of Fc receptors on monocytes prestimulated for various time intervals in FMLP was studied using antibody-coated sheep red cells (see chapter 4). Monocytes polarised in FMLP for an optimum period capped the ligand bound Fc receptors at the head region (cf. Fig. 33). This observation suggests that there is asymmetry in receptor distribution (in this case Fc receptors) on human monocytes with locomotor morphology, as observed in other blood leucocytes. Most

interestingly, after prolonged incubation in FMLP, many monocytes did not form rosettes with antibody-coated sheep red cells (see Fig. 32 D). This also supports the finding discussed above that monocytes incubated for a prolonged period in FMLP in the presence of 2-deoxy glucose (2DG), did not lose their polarization, probably due to inhibition of receptor internalisation by 2DG. Monocytes lose their chemotactic receptors due to prolonged exposure to chemotactic factors. A similar loss of response of mouse peritoneal macrophages has been reported by Wilkinson (1985b) who collected macrophages 4 days after injection of mice with *Corynebacterium parvum*, a strong immunopotentiating agent. The author reported that very few of these macrophages showed locomotor or chemotactic responses towards *Candida albicans* blastospores in mouse serum. He suggested that the macrophage population lost the typical locomotor characteristics of inflammatory macrophages, possibly in response to lymphocyte secretion products like MIF. For the present study, it is not known what metabolic process could activate the monocytes to phagocytose or internalise their surface receptors in the *in vitro* system.

**Monocyte responses to chemotactic factors**

Although monocytes responded over a wide range of FMLP concentrations (see Fig. 7), there were differences in both concentration and time in their responsiveness. The cells were most polarised at 5 X $10^{-9}$ M FMLP at which concentration the maximum proportion of monocytes changed shape (Fig 6 C). In contrast, cells in high

concentrations of FMLP ($10^{-6}$ and $10^{-7}$ M) mostly had an irregular outline and also very few of them were polarised (Fig. 6 B). The difference in cell morphology at different concentrations of FMLP was quantified by measuring the length of each monocyte. Since there is heterogeneity in monocyte size as reported by earlier workers (Norris et al., 1979; Arenson et al., 1980; Normann and Weiner, 1981), also observed in the present study, quantifying polarisation of monocytes of different sizes by measuring their lengths was not accurate. Hence the term 'polarisation index' (P.I.) was used to compare the cell morphology where P.I. is the ratio of the length and breadth (perpendicular drawn at the middle of the length) of the monocyte. Thus the P.I. ratios of monocytes at different doses of the chemotactic factor was the better criterion for comparing their degree of polarisation. A cell with P.I. value less than 1.35 was not considered as polarised.

It was interesting to see that polarisation of neutrophils, stimulated by an optimal dose of FMLP, could also be scored by measuring their P.I. values ($\geq$1.35 as considered for a polarised monocyte). Neutrophils responding towards their respective dose of FMLP were more polarised than the monocytes at their respective optimum dose of the peptide. It was observed that the average length of a polarised neutrophil was twice the breadth of the cell and that of polarised monocyte was 1.77 times of its breadth (see Table 3).

It was described in detail in chapter 4 of this thesis that a maximum of 66% of circulating blood

monocytes were capable of responding towards the optimal dose of FMLP. The maximum percentage of monocytes polarised towards other chemotactic factors was less than that observed for FMLP. Several chemotactic factors were combined together to see if there was any additive effect on monocyte polarisation. It was observed that when pure chemotactic factors like FMLP, C5a or $LTB_4$ were combined together, there was no additive effect. When candida spore-activated human serum (AHS) was combined with FMLP, C5a or $LTB_4$, a higher proportion of monocytes responded. With FMLP + AHS more than 85% of blood monocytes assumed polarised morphology. This is in contrast to the findings described by Cianciolo and Snyderman (1981) who found that a maximum of 60% of blood monocytes were capable of responding to a combination of a wide range of chemotactic factors. These authors also proposed that the rest of the circulating blood monocytes (about 40%) lack chemotactic receptors. A similar study was also reported by Falk and Leonard (1980) who reported that only about 40% of blood monocytes were capable of responding to chemoattractants. In contrast, the present study demonstrates that about 90% monocytes (see Table 5) are stimulable by a mixture of FMLP and AHS. However, the nature of the chemotactic factor present in AHS is not known. Cianciolo and Snyderman (1981) combined zymosan-activated human serum (where the chemotactic factor was C5a) with FMLP and tested against monocytes. There was no additive effect of the mixture towards the maximum proportion of monocytes polarised. Similar results were obtained for the mixture of pure C5a and FMLP when

combined together in the present study (Table 5). The additive effect of monocyte polarisation in the presence of FMLP + AHS suggests that the chemotactic properties of candida spore-activated serum cannot be ascribed to C5a alone. Since most human sera contain at least some anti-candida antibody and the polysaccharides present in the cell wall of yeast could activate other complement components than C5a alone. However, a combination of C5a and C3a with FMLP could not induce monocyte polarisation above the value obtainable with FMLP alone (see Table 5).

The results presented in this thesis clearly show that human blood monocytes bear surface receptors for different chemotactic factors. The cells can respond towards combinations of these factors so that the accumulated response is higher than that obtainable towards any single chemotactic factor tested alone. These results suggest, in contrast to earlier reports, that almost all the monocytes of blood have locomotor capacity, but that some of them lack receptors for FMLP or C5a. It would be interesting to explore whether the monocytes with different chemotaxin receptors are at different stages of differentiation as the study of lymphokine-induced expression of FMLP receptors in cultured monocytes by Pike et al. (1980) would suggest.

**Monocytes in culture**

Several workers cultured monocytes to study their *in vitro* differentiation (Balner, 1963; Goodman, 1964; Metcalf and Moore, 1971; Goud et al., 1975). These studies lead to the finding that macrophages found in

various tissues and body spaces were derived from circulating blood monocytes. Human peripheral blood monocytes have been repeatedly reported by several workers to have appreciable levels of cytotoxic activity against murine and human tumor cell lines (Shaw et al., 1978; Mantovani et al., 1979a and b; Mukherji, 1980; Weiss and Slivka, 1982). Different workers tried to induce an enhancement of the cytotoxic activity in monocytes by *in vitro* culturing of the cells and also using various stimuli (Hammerström, 1979a and b; Jett et al., 1980; Mantovani et al., 1980). In a majority of these studies, the monocytes tested were isolated and cultured by adherence to glass or plastic surfaces. Van der Meer et al. (1978) described the use of a teflon surface which would prevent monocyte adherence during culture. Kaplan and Gaudernack (1982) demonstrated that the *in vitro* differentiation of human peripheral blood monocytes to macrophages is dependent on the conditions of monocyte culture. Monocytes cultured on glass are cytotoxic to tumor cells but monocytes cultured on collagen are not (Kaplan, 1983). Both glass- and collagen differentiated monocytes became much larger with time of culture which reflected the protein content of the culture. Kaplan (1983) found that both the cultures contained about 4 times the original amount of protein after *in vitro* culturing for 20-22 days. Nakagawara et al. (1981) found similar results with glass differentiated monocytes. Kaplan (1983) described that the incorporation of glucosamine into monocytes cultured on glass continued to rise to about twice that

incorporated by collagen -differentiated monocytes. This suggested that monocytes cultured on glass were more activated than monocytes cultured on collagen. The morphological differences between the two types of cultures showed that monocytes cultured on glass differentiated into epithelioid-like cells while monocytes on collagen differentiated into cells similar to resting tissue macrophages (Kaplan and Gaudernack, 1982). Kaplan (1983) suggested that human monocytes, although potentially capable of differentiating into cytotoxic cells, might require a stimulus such as contact with a foreign surface (glass) to do so.

In the present study, monocytes were cultured in siliconized glass tubes in which cell adherence during culture was not observed. About 90% monocytes assumed polarised morphology after culturing for 3 days in RPMI-FCS. In general, cultured monocytes showed an increase in cell size with cytoplasmic spreading and an eccentrically located nucleus. Cultured cells with locomotor morphology was much more polarised than chemoattractant induced polarised cells though the former cells were bigger in size. An average length of the cultured monocytes was more than 18 μm and their mean P.I. value (ratio of length and breadth) was 1.79 (see Fig. 37).

The present study also demonstrates that monocytes in culture synthesise proteins to assume polarised morphology. When cycloheximide, an inhibitor of protein synthesis, was added to the monocytes in culture, the cells could not assume locomotor morphology. Wilkinson (1986) found similar results with lymphocytes in culture.

Pike et al. (1980) described that a human monocyte cell line, U937, would poorly respond to FMLP unless cultured for 4 days with a lymphokine. This showed that the cell line synthesised chemotactic receptors for FMLP during culture.

Monocytes kept in culture for 18 h and 72 h were allowed to phagocytose *Candida albicans* blastospores in normal human serum. It was interesting to see that the phagocytic capacity of cultured monocytes was lower than that observed in monocytes straight from blood (cf. Table 19). About 90% of freshly isolated monocytes were capable of phagocytosing the blastospores of candida whereas 70% of the cells kept in culture for 18 h would do so and monocytes cultured for 72 h were totally inactive even for spores sitting next to them. This suggested that since the cultured monocytes were not attached to a foreign surface (in this case siliconized glass tubes where suspensions of monocytes were introduced for culturing and subsequent adherence of cells was not observed) during culture, they differentiated into a form perhaps identical to tissue macrophages, as Kaplan (1983) described for monocytes cultured on a collagen gel.

**Neutrophil chemotaxis through collagen gels**

The most widely used assays for measuring leucocyte chemotaxis are filter assays, agarose assays and visual assays using plane glass or plastic surfaces. These assays do not take account of the architecture of the tissues through which leucocytes move *in vivo*. To obtain new insights into mechanisms for the accumulation of

leucocytes at inflammatory sites *in vivo* new assays are needed. Recently, a few workers have used modified chemotaxis assays which approach this problem. Among them, Cramer et al. (1980) grew renal epithelial cells on micropore filters to form a monolayer and then placed leucocytes on the top of the epithelium so that the leucocytes had to invade the monolayer of cells to respond to the chemotactic factor placed below the filter. Russo et al. (1981) overlaid filters with amnion, an epithelial layer overlaying a collagenous stroma, also requiring penetration of a tissue for the chemotactic response by the leucocytes.

Neutrophil leucocytes, after leaving the blood vessels, move through a matrix of which fibronectin and collagen constitute the major components. Brown and Lackie (1981) reported that the adhesion of neutrophils to native collagen-coated surfaces is weaker than their adhesion on fibronectin-coated surfaces. As a result neutrophils are unable to locomote over collagen-coated glass, possibly due to lack of traction between the cells and collagen, but can migrate through 3-dimensional matrices of collagen gels. Haston et al. (1982) found similar results with lymphocytes. On the other hand, fibroblast locomotion seems to be similar on 2-dimensional substrata and in 3-dimensional collagen matrices though the cell morphology is different in the two situations. Fibroblasts form strong adhesions with protein-coated glass (Abercrombie and Dunn, 1975) or collagen fibers within a gel (Grinnell and Bennett, 1981) which allows the locomotory force of the cell to be

translated into forward movement, producing some deformation of the collagen fibres in gels as fibroblasts contract (Bard and Hay, 1975). Harris (1982) found that while normal fibroblasts can deform silicone rubber substrata, forming wrinkles, neutrophils do not. Brown (1982) described that the invasion of collagen matrices by neutrophils appeared to be independent of neutrophil-collagen adhesion, except under conditions of very high adhesion (in high $Mn^{2+}$ concentration), which are likely to be non-physiological.

Three-dimensional fibrous matrices such as collagen and fibrin provide a milieu for studying cell locomotion which is closer in character to those which cells encounter *in vivo* than the artificial substrata in more common use. Assays of chemotaxis of leucocytes in such gels has the advantage that this, unlike other assays, can be used both to study the distribution of a cell population following exposure to a stimulus and as a visual assay in which the detailed behaviour of individual cells during the response can be followed. Population assays have the advantage that a very large sample is taken. However, owing to the 3 dimensions of the collagen gel assay and probably to chance factors that affect both diffusion of the chemotactic factor and penetration of the gel by the cells, the distinction between population distribution in a gradient, in which a flux of cells responding chemotactically should be seen, and population distribution in isotropic attractant concentrations, which should be normal, is in practice a fine one, and is clearer in some experiments than in others (Table 12).

This is equally true of the filter assay and the agarose assay but the collagen gel assay has advantages over these assays in certain respects. It has been shown for example that lymphocytes (Haston et al., 1982) and neutrophils (Brown, 1982) move through 3-dimensional matrices of collagen by a mechanism which is largely independent of adhesive interactions with the substratum. Therefore, assays of chemotaxis using collagen gels are not confused by the cell substratum interactions which are unavoidable using the glass or cellulose nitrate substrata of other assay systems.

There are certain technical problems with gel assays. For example, protein gels are fragile, cannot be manipulated readily, and collapse if cut. They may become detached from the sides of tissue culture wells, and the sides of the wells can be scratched to improve gel attachment. Counting cells in gels is not easy when there is any vibration, since the gels are prone to wobble. Because of this it was found that counting was best done when the laboratory was quiet. Another difficulty that may be encountered after a gel has been poured and has set is that cells placed on the surface of the gel sometimes do not move in, even though they are in locomotor morphology. This is due to some undefined change at the gel surface, possibly contraction or horizontal alignment of the gel. It was found that covering the gel with fluid as soon as possible after it had set, rather than leaving it exposed to air reduced the incidence of this difficulty.

The difficulty with which chemotaxis towards FMLP through collagen gels was demonstrated in the population distribution assay highlights the problem of showing chemotaxis convincingly with this type of assay. The micropore filter assay is the classical population distribution assay and had been used in the great majority of studies of neutrophil chemotaxis. It is a good assay for demonstrating that locomotion has been stimulated by a given factor, but is not an ideal assay for showing that the stimulated locomotion is chemotactic. The status of many of the putative chemotactic factors reported in the literature needs further evaluation using assays which give more direct evidence of chemotaxis.

**Monocyte chemokinesis through collagen gel**

Human blood neutrophils (Brown, 1982) and lymphocytes (Haston et al., 1982) are known to invade 3-dimensional matrices of collagen gels *in vitro*. There is no reason why human blood monocytes should not do so, since these cells have to migrate through tissues which are made of collagenous stroma, in response to inflammation, *in vivo*. Though there is a delay before monocytes are found to accumulate at an inflammatory site, it is not known whether this is due to specific delay in the recruitment of these cells , or to the fact that there are fewer monocytes than neutrophils in the blood and that their migration rate is much slower. The ability of blood monocytes to migrate through 3-D matrices of collagen was not studied previously, except

by Brown (1984), who concluded that monocytes straight from blood could not migrate through gels unless cultured on a protein-coated surface for 48 h. The present study demonstrates that freshly isolated monocytes not only invade collagen gels like neutrophils and lymphocytes but show chemokinesis, i.e., increased random migration with recruitment of extra locomotory cells, in the presence of FMLP, as observed earlier with neutrophils.

Unlike lymphocytes (Haston et al., 1982) and neutrophils (Brown and Lackie, 1981) which do not normally adhere to collagen-coated surfaces, about 85% of freshly isolated monocytes could do so. Also cells on the surface of collagen gels had well spread morphology (cf. Fig. 50). These characteristics of monocytes resemble those of fibroblasts in locomotion on a plane surface (Abercrombie and Dunn, 1975) and through a 3-D matrix (Grinnell and Bennett, 1981). Both are adhesive as well as slow moving cells.

Analysis of time-lapse films for the mechanism of monocyte invasion through gels showed that some of the cells took several more complicated stages of morphological change than the others (cf. Fig. 56 A and Fig. 57 B). Like neutrophils (Brown, 1982) and lymphocytes (Haston et al., 1982; Haston and Shields, 1984) a wave of circular contraction or a constriction ring was observed in the cell body of migrating monocytes. The constriction remained fixed with respect to the environment and the monocyte was observed to pass through the constriction. Formation of blebs in both sides of the contracted region was seen in some monocytes

(cf. Fig 57 D and E), but their role in forward movement of the cell was not understood. However, the extension and expansion of blebs through gaps in the gel frameworks are instrumental in providing anchorage points during lymphocyte invasion (Haston et al., 1982).

On the basis of the data shown in the present sudy, it is demonstrated that monocytes straight from blood can migrate through collagen gels by extending their cellular processes (pseudopods) into the meshwork of the matrix to act as anchors; the cells could then gain sufficient traction (by adhering to collagen fibres) to pull the rest of its body through the gaps. A similar mechanism was proposed for neutrophil migration through collagen gels (Brown, 1982).

The ease with which invasion of collagen gels by freshly isolated human monocytes is demonstrated in the present study and the difficulty with which Brown (1984) reported that 'monocytes would not migrate through gels unless cultured' perhaps highlights the problem of monocyte purification, though it is not understood why it should be so. Brown (1984) purified the monocytes on FCS-coated surfaces (method described by Kumagai et al., 1979) and the cells in the present study were purified on BHK microexudate-coated surfaces (method described by Ackerman and Douglas, 1978), both of which utilised an active adhesion step. However, it remains to be shown that cells which migrated into collagen gel after culturing for 48 h are typical monocytes, since a proportion of the cells which detach from a plastic surface after 16 h are dendritic cells (van Voorhis et

al., 1982), which are also capable of invading collagen gels (personal communication of Dr. W.S. Haston, Department of Bacteriology and Immunology, University of Glasgow).

**Visual assays of monocytes locomotion**

It was apparent from the experiments with visual assays that monocytes could not locomote over clean glass due to strong adhesion of the cells to the substratum. However, a small proportion of the cells were able to move about on HSA-coated glass surfaces. Leucocyte locomotion on clean glass is non-physiological. Human serum albumin (HSA) reduces adhesion of the cells to the substratum. HSA is not chemokinetic to the cells but is necessary to coat the surface for optimal adhesion required for locomotion, since the cells cannot locomote on a less adhesive substratum like a collagen-coated surface, on which neutrophils usually do not adhere (Brown and Lackie, 1981) and hence cannot locomote.

Monocytes in suspension showed distinct morphological responsiveness over a wide range of FMLP concentrations. About 66% of the cells showed polarised morphology in 5 X $10^{-9}$ M FMLP and about 35% or so of them were responding in $10^{-7}$ M FMLP. Monocytes were more polarised in 5 X $10^{-9}$ M FMLP then they were in $10^{-7}$ M FMLP. When these cells were allowed to settle on an HSA-coated glass surface they showed locomotion which was recorded by time-lapse cinematography. Analyses of the films showed that cells in optimal dose of FMLP (5 X $10^{-9}$ M) moved in a relatively straight path with a small angle

of turn whereas, cells in supraoptimal dose of FMLP ($10^{-7}$ M) moved in a zig-zag path with a wide angle of turn (described in chapter 7 of this study). Shields and Haston (1985) found similar results with neutrophils where the cells were moving in a nearly-straight path, making narrow angles of turn in the optimal dose of FMLP ($10^{-8}$ M), in supraoptimal dose of FMLP ($10^{-6}$ M) the cells were moving with frequent changes in direction. These observations, together with the observations on Fc receptors distribution discussed previously (P. 222), suggest that FMLP receptors are located at the front of the cell while moving in the presence of an optimal dose of FMLP. These receptors would bind new molecules of FMLP at the front of the cell, which would result in a narrow angle of turn made by the cells during locomotion. In the presence of the supraoptimal dose of FMLP, it has been suggested (Shields and Haston, 1985) that overstimulation would prevent receptors from redistributing to the front of the cell and thus result in a weakly polarised morphology. This is reflected by a wide angle of turn made by the cells during locomotion.

Monocyte chemokinesis through 3-D matrices of collagen gels (in the presence of uniform concentration of FMLP at optimal dose) showed similar features as observed for cells locomoting on a protein-coated plane surface. The histogram for the distribution of turning angles of a population of monocytes showed that the mean angle of turn of the population was within $\pm 40^{o}$ of the axis of the path of the cell before turning. This showed that the cells migrating through a fibrous matrix in the

presence of uniform concentration of FMLP tended to be persistent in random locomotion. That is, monocytes migrating in chemokinetic concentrations of FMLP do not show pure random locomotion, but persist in straight paths and show a higher than expected incidence of narrow angled turns .

Fc rosetted monocytes allowed to locomote in the presence of 5 X $10^{-9}$ M FMLP on HSA-coated glass surface showed capping of rosettes to the tail of the moving monocytes. In fact, the cells initially formed general rosettes with antibody-coated sheep red cells and as the monocytes changed shape in the presence of FMLP, the rosettes remained stationary relative to the substratum and the cap formed because the head of the locomoting monocytes moved away from the rosettes. Wilkinson et al. (1980) showed similar results with neutrophils and suggested that the cross-linked receptors formed relatively immobile patches of membrane which did not flow forwards with the rest of the cell during locomotion.

The visual assay of monocyte locomotion towards *Candida albicans* blastospores showed a purely chemotactic reaction. The cells responded within seconds towards the nearby spores by assuming an oriented morphology. Then they started locomotion in a relatively straight path towards the spores and phagocytosed them. This suggested that the gradient produced around the spores was locally very stable and intense. It is expected that candida spores will continuously generate chemotactic factors from the surrounding serum by conversion of precursor

molecules into active chemotactic molecules at the gradient source, thus effectively preventing decay of the gradient. The exact mechanism by which *Candida albicans* generates chemotactic gradients is unknown. A large amount of evidence suggest that the major chemotactic factor generated from serum is the complement peptide C5a. Normal human serum contains anti-candida antibody, which on binding to the spores may activate the complement cascade leading to release of C5a and the phagocytic attachment component C3b. Also the polysaccharides present in the cell wall of the yeast are capable of complement activation through the alternative pathway, a mechanism which would activate the same complement components. This activation would take place even in the absence of anti-candida antibody in serum. Monocyte chemotaxis towards *Candida albicans* blastospores suggests that the capacity of a chemotactic factor to be generated at a source is an important criterion of activity and serum derived factors released by contact with candida spores are very potent.

Allan and Wilkinson (1978) studied the chemotactic locomotion of human neutrophils towards candida spores and found that the cells were migrating in a nearly straight line path towards the spores with chemotactic ratio +0.96. Wilkinson and Allan (1980) found the chemotactic ratio of monocytes to be +0.89 when the cells were allowed to phagocytose candida spores. In the present study, the ratio was found to be slightly lower, +0.82.

**Concluding Comments**

Much of the work reported in this thesis supports the view that cellular responses to external stimuli form the basis of cell behaviour. Understanding cell behaviour is of paramount importance in many aspects of biology, for example, in the immune response, in embryogenesis, in wound healing and in neoplastic disease. Fibroblasts have been used as the paradigm for the study of cell locomotion but in fact leucocytes provide a model system for the study of motile responses which is superior in many ways. This is in spite of the difficulties, outlined in this thesis, in obtaining cell populations which are homogenous in both cell type and responsiveness. The major advantage of leucocytes as a model system is that the surface receptors or ligands which stimulate the locomotor response have, to a large extent, been well characterised.

# REFERENCES

ABERCROMBIE, M. (1965) The locomotory behaviour of cells. In *Cells and Tissues in Culture*, ed. WILLMER, E. N. Vol. 1, pp. 177-202. Academic Press, London.

ABERCROMBIE, M. (1967) Contact inhibition: The phenomenon and its biological implications. *National Cancer Institute Monographs*, **26**, 249-277.

ABERCROMBIE, M and DUNN, G. A. (1975) Adhesion of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. *Experimental Cell Research*, **92**, 57-62.

ABERCROMBIE, M., HEAYSMAN, J. E. M. and PEGRUM, S. M. (1971) The locomotion of fibroblasts in culture. IV Electron microscopy of the leading lamella. *Experimental Cell Research*, **67**, 359-367.

ABRAMSON, N., LOBUGLIO, A. F., JANDL, J. H. and COTRAN, R. S. (1970) The interaction between human monocytes and red cells: binding characteristics. *Journal of Experimental medicine*, **132**, 1191-1206.

ACKERMAN, S. K. and DOUGLAS, S. D. (1978) Purification of human monocytes on microexudate-coated surface. *Journal of Immunology*, **120**, 1372-1374.

ADDISON, W. (1849) *On Healthy and Diseased Structure*, Churchill, London.

ALITALO, K, HOVI, T. and VAHERI, A. (1980) Fibronectin is produced by human macrophages. *Journal of Experimental medicine*, **151**, 602-613.

ALLAN, R. B. and WILKINSON, P. C. (1978) A visual analysis of chemotactic and chemokinetic locomotion of human neutrophil leucocytes : Use of a new chemotaxis assay with *Candida albicans* as gradient source. *Experimntal Cell Research*, **111**, 191-203.

ALTMAN, L. C., SNYDERMAN, R., OPPENHEIM, J. J. and MERGENHAGEN, S. E. (1973) A human mononuclear leukocyte chemotactic factor. Characterization, specificity and kinetics of production of homologous leukocytes. *Journal of Immunology*, **110**, 801-810.

ARENSON Jr., E. B., EPSTEIN, M. B. and SEEGER, R. C. (1980) Volumetric and functional heterogeneity of human monocytes. *Journal of Clinical Investigation*, **65**, 613-618.

ARMSTRONG, P. B. and LACKIE, J. M. (1975) Studies on intercellular invasion *in vitro* using rabbit peritoneal neutrophil granulocytes (PMNs) I. Role of contact inhibition in locomotion. *Journal of Cell Biology*, **65**, 439-462.

BAEHNER, R. L. and JOHNSTON Jr., R. B. (1972) Monocyte function in children with neutropenia and chronic infections. *Blood*, **40**, 31-41.

BAINTON, D. F., ULLYOT, J. L. and FARQUHAR, M. G. (1971) The development of neutrophilic polymorphonuclear leucocytes in human bone marrow. *Journal of Experimental Medicine*, **134**, 907-934.

BALDRIDGE, C. W. and GERRARD, R. W. (1933) The extra respiration of phagocytosis. *American Journal of Physiology*, **103**, 235-236.

BALNER, H. (1963) Identification of peritoneal macrophages in mouse radiation chimeras. *Transplantation*, **1**, 217-223.

BAR, R. S., GORDEN, P., ROTH, J., KHAN, C. R. and DeMEYTS, P. (1976) Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients. *Journal of Clinical Investigation*, **58**, 1123-1135.

BARD, J. B. L. and HAY, E. D. ( 1975 ) The behaviour of fibroblasts from the developing avian cornea: Morphology and movement *in situ* and *in vitro*. *Journal of Cell Biology*, **67**, 400-418.

BAYNES, J. W. and WOLD, F. (1976) Effect of glycosylation on the *in vitro* circulating half-life of ribonuclease. *Journal of Biological Chemistry*, **251**, 6016-6024.

BECKER, A. M., McCULLOCH, E. A. and TILL, J. E. ( 1963 ) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature (London)*, **197**, 452-454.

BECKER, E. L., DAVIS, A. T., ESTENSEN, R. D. and QUIE, P. G. (1972) Cytochalasin B. IV. Inhibition and stimulation of chemotaxis of rabbit and human polymorphonuclear leucocytes. *Journal of Immunology*, **108**, 396-402.

BENNETT, W. E. and COHN, Z. A. (1966) The isolation and selected properties of blood monocytes. *Journal of Experimental Medicine*, **123**, 145-160.

BERKEN, A. and BENACERRAF, B. (1966) Properties of antibodies cytophilic for macrophages. *Journal of Experimental medicine*, **123**, 119-144.

BESSIS, M. (1974) Necrotaxis : Chemotaxis towards an injured cell. *Antibiotics and Chemotherapy*, **19**, 369-381.

BESSIS, M. and BURTÉ, B. (1965) Positive and negative chemotaxis as observed after the destruction of a cell by U.V. or laser microbeams. *Texas Reports in Biology and Medicine*, **23**, 204-212.

BEVILACQUA, M. P., AMRANI, D., MOSESSON, M. W. and BIANCO, C. ( 1981 ) Receptors for cold-insoluble

globulin (plasma Fibronectin) on human monocytes. *Journal of Experimental medicine*, **153**, 42-60.

BIANCO, C., GRIFFIN Jr., F. M. and SILVERSTEIN, S. C. (1975) Studies of the macrophage complement receptor. Alteration of receptor function upon macrophage activation. *Journal of Experimental medicine*, **141**, 1278-1290.

BIBERFELD, P. (1971) Uropod formation in phytohaemagglutinin (PHA) stimulated lymphocytes. *Experimental Cell Research*, **66**, 433-445.

BIGGAR, W. D., HOLMES, B., PAGE, A. R., DEINARD, A. S., L'ESPERANCE, P. and GOOD, R. A. (1974) Metabolic and functional studies of monocytes in congenital neutropenia. *British Journal of Haematology*, **28**, 233-243.

BLECHER, M. and GOLDSTEIN, S. (1977) Hormone receptors: VI. On the nature of the binding of glucagon and insulin to human circulating mononuclear leukocytes. *Molecular and Cellular Endocrinology*, **8**, 301-315

BLOOM, B. R. and BENNETT, B. (1966) Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. *Science*, **153**, 80-82.

BOETCHER, D. A. and LEONARD, E. J. (1974) Abnormal monocyte chemotactic response in cancer patients. *Journal of National Cancer Institute*, **52**, 1091-1099.

BONT, W. S., DeVRIES, J. E., GEEL, M., van DONGEN, A. and LOOS, H. A. (1979) Separation of human lymphocytes and monocytes by velocity sedimentation at unit gravity. *Journal of Immunological Methods*, **29**, 1-16.

BOREL, J. F. (1970) Studies on chemotaxis : Effect of subcellular leukocyte fractions on neutrophils and

macrophages. *International Archives of Allergy and Applied Immunology*, **39**, 247-271.

BOTAZZI, B., POLENTARUTTI, N., BALSARI, A., BORASCHI, D., GHEZZI, P., SALMONA, M. and MANTOVANI, A. (1983) Chemotactic activity for mononuclear phagocytes of culture supernatants from murine and human tumor cells: Evidence for a role in the regulation of the macrophage content of neoplastic tissues. *International Journal of Cancer*, **31**, 55-63.

BOXER, L. A., RICHARDSON, S. and FLOYD, A. ( 1976 ) Identification of actin-binding protein in membrane of polymorphonuclear leukocytes. *Nature*, **263**, 249-251.

BOXER, L. A. and STOSSEL, T. P. (1976) Interactions of actin, myosin and an actin-binding protein of chronic myelogenous leukemia leukocytes. *Journal of Clinical Investigation*, **57**, 964-976.

BOYDEN, S. V. (1962) The chemotactic effect of mixure of antibody and antigen on polymorphonuclear leucocytes. *Journal of Experimental Medicine*, ***115***, 453-466.

BOYLES, J. and BAINTON, D. F. (1979) Changing patterns of plasma membrane-associated filaments during the initial phases of polymorphonuclear leucocyte adherence. *Journal of Cell Biology*, **82**, 347-368.

BöYUM, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scandinavian Journal of Clinical and Laboratory Investigation*, **97** (supplement), 77-89.

BöYUM, A. (1983) Isolation of human blood monocytes with nycodenz, a new non-ionic iodinated gradient medium. *Scandinavian Journal of Immunology*, **17**, 429-436.

BRAUN, J., FUJIWARA, K., POLLARD, T. D. and UNANUE, E. (1978) Two distinct mechanisms for redistribution of lymphocyte surface macromolecules. I Relationship to cytoplasmic myosin. *Journal of Cell Biology*, 79, 409-418.

BRAY, M. A., FORD-HUTCHINSON, A. W. and SMITH, M. J. H. (1980) Generation of chemotactic activity from rat

polymorphonuclear leucocytes treated with calcium ionophore A23187. *British Journal of Pharmacology*, **70**, 84P.

BROWN, A. F. (1982) Neutrophil granulocytes: Adhesion and locomotion on collagen substrata and in collagen matrices. *Journal of Cell Science*, **58**, 455-467.

BROWN, A. F. (1984) Neutrophil and monocyte behaviour in three-dimensional collagen matrices. *Scanning Electron Microscopy*, **11**, 747-754.

BROWN, A. F. and LACKIE, J. M. (1981) Fibronectin and collagen inhibit cell-substratum adhesion of neutrophil granulocytes. *Experimental Cell Research*, **136**, 225-231.

BUCKLEY, I. K. (1963) Delayed secondary damage and leucocyte chemotaxis following focal aseptic heat injury *in vivo*. *Experimental and Molecular Pathology*. **2**, 402-417.

BUMOL, T. F. and DOUGLAS, S. D. (1977) Human monocyte cytoplasmic spreading *in vitro* : Early kinetics and scanning electron microscopy. *Cellular Immunology*, **34**, 70-78.

CARTWRIGHT, G. E., ATHENS, J. W. and WINTROBE, M. M. (1964) The kinetics of granulopoesis in normal man. *Blood*, **24**, 780-803.

CHABAUD, O. and LISSITZKY, S. (1977) Thyrotropin-specific binding to human peripheral blood monocytes and polymorphonuclear leucocytes. *Molecular and Cellular Endocrinology*, **7**, 79-87.

CHENOWETH, D. E., GOODMAN, M. G. and WEIGLE, W. O. (1982) Demonstration of a specific receptor for human C5a anaphylatoxin on murine macrophages. *Journal of Experimental Medicine*, **156**, 68-78.

CIANCIOLO, G. J. and SNYDERMAN, R. (1981) Monocyte responsiveness to chemotactic stimuli is a property of a subpopulation of cells that can respond to multiple chemoattractants. *Journal of Clinical Investigation*, **67**, 60-68.

CLARK, E. R. and CLARK, E. L. (1920) Reactions of cells in the tail of amphibian larvae to injected corton oil (asceptic inflammation). *American Journal of Anatomy*, **27**, 221-254.

CLARK, E. R., CLARK, E. L. and REX, R. O. (1936) Observations on polymorphoneuclear leucocytes in the living animal. *American Journal of Anatomy*, **59**, 123-173.

CLINE, M. J. (1975) *The White Cell*. Harvard University Press, Cambridge, Mass.

COMANDON, J. (1917) Phagocytose *in vitro* des hématozoaires des Séances et mémoires de la Société de Biologie, **80**, 314-316.

COMANDON, J. (1919) Tactisme produit par l'amidon sur les leucocytes. Enrobement du charbon. Comptes rendus hebdomadaires des Séances et mémoires de la Société de Biologie, **82**, 1171-1174.

COUCHMAN, J. R. and REES, D. A. (1979) The behaviour of fibroblasts migrating from chick heart explants: Changes in adhesion, locomotion, and growth and in the distribution of actomyosin and fibronectin. *Journal of Cell Science*, **39**, 149-165.

CRAMER, E. B., MILKS, L. C. and OJAKIAN, G. K. ( 1980 ) Transepithelial migration of human neutrophils : An *in vitro* model system. *Proceedings of the National Academy of Sciences of the U.S.A.*, **77**, 4069-4073.

CSABA, G., KAPA, E. and CSERHALMI, M. (1975) Hormone receptor studies on frog macrophage cells by means of histamine, serotonin and indoletacetic acid. *Endokrinologie*, **65**, 219-223.

CUTLER, J. (1974) A simple *in vitro* method for studies on chemotaxis. *Proceedings of the Society for Experimental Biology and Medicine*, **147**, 471-474.

DAMERAU, B., ZIMMERMANN, B., GRUNEFELD, E., CZORMAK, K. and VOGT, W. (1980) Biological activities of C5a and C5a des Arg from hog serum. *International Archives of Allergy and Applied Immunology*, **63**, 408-414.

DAVID, J. R. (1975) Macrophage activation of lymphocyte mediators. *Federation Proceedings*, **34**, 1730-1736.

DAVIES, P., BONNEY, R.J., HUMES, J. L. and KUEHL Jr., F. F. (1980) The synthesis of arachidonic acid oxygenation products by various mononuclear phagocytic populations. In *The Mononuclear Phagocytes: Functional Aspects*, ed. FURTH, R. van, pp. 1317-1345. Martinus Nijhoff, The Hague.

DAVIES, W. A. and STOSSEL, T. P. (1977) Peripheral hayline blebs (podosomes) of macrophages, *Journal of Cell Biology*, **75**, 941-955.

DAVIES, W. C,. HUBER, H., DOUGLAS, D. and FUDENBERG, H. H. (1968) A defect in circulating mononuclear phagocytes in chronic granulomations disease of childhood. *Journal of Immunology*, **101**, 1093-1095.

DAY, J. F., THORNBERG, R. W., THORPE, S. R. and BAYNES, J. (1980) Carbohydrate-mediated clearance of antibody antigen complexes from the circulation. The role of high mannose oligosaccharides in the hepatic uptake of IgM antigen. *Journal of Biological Chemistry*, **255**, 2360-2365.

DE BRUYN, P. P. H. (1946) The amoeboid movement of the mammalian leucocyte in tissue culture. *Anatomical Record*, **95**, 177-191.

DIXON, H. M. and McCUTCHEON, M. (1935) Absence of chemotropism in leucocytes. *Archives of Pathology*, **19**, 679-683.

DIXON, H. M. McCUTCHEON, M. and CZARNETZKY, E. J. (1937) The chemotropic attraction of leucocytes by fractions of *Streptococcus haemolyticus*. *American Journal of Pathology*. **13**, 645-646.

DOHLMAN, J. G. and GOETZL, E. J. ( 1978 ) Unique determinants of alveolar macrophage spontaneous and chemokinetically stimulated migration. *Cellular Immunology*, **39**, 36-46.

DOUGLAS, S. D. (1976) Human monocyte spreading *in vitro*- Inducers and effects on Fc and C3 receptors. *Cellular Immunology*, **21**, 344-349.

DUNN, G. A. (1981) Chemotaxis as a form of directed cell behaviour : Some theoretical considerations. In *Biology of the Chemotactic Response*, ed. LACKIE, J. M. and WILKINSON, P. C. pp. 1-26. Cambridge University Press, Cambridge.

ELSDALE, T. and BARD, J. B. L. (1972) Collagen substrata for studies on cell behaviour. *Jurnal of Cell Biology*, **54**, 627-637.

FALK, W. and LEONARD, E. J. (1980) Human monocyte chemotaxis: Migrating cells are a subpopulation with multiple chemotaxin specificities on each cell. *Infection and Immunity*, **29**, 953-959.

FALK, W. and LEONARD, E. J. (1981) A functional subpopulation among normal peripheral blood monocytes. In

*Heterogeneity of Mononuclear Phagocytes*, ed. FöRSTER, O. and LANDY, M. pp. 156-158. Academic Press, London.

FIGDOR, C. G., BONT, W. S., De VRIES, J. E. and van ES, W. L. (1981) Isolation of large numbers of highly purified lymphocytes and monocytes with a modified centrifugal elutriation technique. *Journal of Immunological methods*, **40**, 275-288.

FLUKS, A. J. (1981) Three-step isolation of human blood monocytes using discontinuous density gradients of percoll. *Journal of Immunological Methods*, **41**, 225-233.

FORD-HUTCHINSON, A. W., BRAY, M. A., DOIG, M. V., SHIPLEY, M. E. and SMITH, M. J. H. (1980) Leukotriene B, a potent chemotactic and aggregating substance released from polymorphonuclear leukocytes. *Nature (London)*, **286**, 264-265.

FORRESTER, J. V., WILKINSON, P. C. and LACKIE, J. M. (1983) Effect of modified $\alpha_2$-macroglobulin on leucocyte locomotion and chemotaxis. *Immunology*, **50**, 251-259.

FRAENKEL, G. S. and GUNN, D. L. (1940) *The Orientation of Animals : Kineses, Taxes and Compass Reactions.* Claredon Press, Oxford.

FREUNDLICH, B. and AVDALOVIC, N. (1983) Use of gelatin/plasma coated flasks for isolating human peripheral blood monocytes. *Journal of Immunological Methods*, **62**, 31-37.

van FURTH, R. (1978) Mononuclear phagocytes in inflammation. In *Handbook of Experimental Pharmacology*, ed. VANE, J. R. and FERREIRA, S. H. Vol. 50/I, pp. 68-99. Springer Verlag, Berlin - Heidelberg - New York.

van FURTH, R. and COHN, Z. A. (1968) The origin and kinetics of mononuclear phagocytes. *Journal of Experimental Medicine*, **128**, 415-435.

van FURTH, R., HIRSCH, J. G. and FEDORKO, M. E. ( 1970 ) Morphology and peroxidase cytochemistry of mouse promonocytes and macrophages, *Journal of Experimental Medicine*, **132**, 794-812.

van FURTH, R., COHN, Z. A., HIRSCH, J. G., HUMPHREY, J. H., SPECTOR, W. G. and LANGEVOORT, H. L. (1972) The mononuclear phagocyte system : a new classification of macrophages, monocytes and their precursor cells. *Bulletin of the World Health Organization*, **46**, 845-852.

van FURTH, R., COHN, Z. A., HIRSCH, J. G., HUMPHREY, J. H., SPECTOR, W. G. and LANGEVOORT, H. L. (1972) The mononuclear phagocyte system : a new classification of macrophages, monocytes and their precursor cells. *Bulletin of the World Health Organization*, **46**, 845-852.

GABBIANI, G., CHAPPONIER, C., ZUMBE, A. and VASSALLI, P. (1977) Actin and tubulin co-cap with surface immunoglobulins in mouse B lymphocytes. *Nature*, **269**, 697-698.

GAIL, M. H. AND BOONE, C. W. (1970) The locomotion of mouse fibroblasts in tissue culture. *Biophysical Journal*, **10**, 980-993.

GALLIN, J. I. and QUIE, P. G. (1978) Leukocyte chemotaxis: Methods. physiology and clinical implications. Raven Press, New York.

GERISCH, G. and KELLER, H. U. ( 1981 ) Chemotactic reorientation of granulocytes stimulated with micropipettes containing fMet-Leu-Phe. *Journal of Cell Science*, **52**, 1-10.

GINKEL, R. J. W. van and AKEN, W. G. van, (1980) Generation of tissue thromboplastin by human monocytes. In *The Mononuclear Phagocytes: Functional aspects*, ed. FURTH, R. van, pp. 1351-1365. Martinus Nijhoff, The Hague.

GOETZL, E. J., DERAIN, C. K., TAUBER, A. I. and VALONE, F. H. (1980) Novel effects of 1-O-hexadecyl-2-acyl-SN-glycero-3-phosphorylcholine mediators on human leukocyte function. *Biochemical and Biophysical Research Communications*, **94**, 881-888.

GOETZL, E. J. and SUN, F. F. (1979) Generation of unique mono-hydroxy-eicosatetraenoic acids from arachidonic acid by human neutrophils. *Journal of Experimental Medicine*, **150**, 406-411.

GOODMAN, J. W. (1964) On the origin of peritoneal fluid cells. *Blood*, **23**, 18-26.

GORDON, S. (1980) Lysozyme and plasminogen activator: constitutive and induced secretory products of mononuclear phagocytes. In *The Mononuclear Phagocytes: Functional aspects*, ed. FURTH, R. van, pp. 1273-1294. Martinus Nijhoff, The Hague.

GORDON, S., TODD, J. and COHN, Z. A. (1974) *In vitro* synthesis and secretion of lysozyme by mononuclear phagocytes. *Journal of Experimental medicine*, **139**, 1228-1248.

GOUD, Th. J. L. M. and van FURTH, R. (1975) Proliferative characterestics of monoblasts grown *in vitro*. *Journal of Experimental Medicine*, **142**, 1200-1217.

GOUD, Th. J. L. M., SCHOTTE, C. and van FURTH, R. (1975) Identification and characterization of the monoblast in mononuclear phagocyte colonies grown *in vitro*. *Journal of Experimental Medicine*, **142**, 1180-1199.

GRIFFIN Jr., F. M., BIANCO, C. and SILVERSTEIN, S. C. (1975) Characterization of the macrophage receptor for complement and demonstration of its functional independence from the receptor for the Fc portion of immunoglobulin G. *Journal of Experimental medicine*, **141**, 1269-1277.

GRINNELL, F. (1978) Cellular adhesiveness and extracellular substrata. *International Review of Cytology*, **53**, 65-144.

GRINNELL, F. and BENNETT, M. H. (1981) Fibroblast adhesion on collagen substrata in the presence and absence of plasma fibronectin. *Journal of Cell Science*, **48**, 19-34.

HAMMERSTRöM, J. (1979a) Human monocyte-mediated cytotoxicity to K-562 cells: activation by lymphokines. *Scandinavian Journal of Immunology*, **10**, 575-585.

HAMMERSTRöM, J. (1979b) *In vitro* influence of endotoxin on human mononuclear phagocyte structure and function. 2. Enhancement of the expression of cytostatic and cytolytic activity of normal and lymphokine-activated monocytes. *Acta Pathologica ET Microbiologica Scandinavica Section C Immunology*, **87**, 391-399.

HARDIN, J. A. and DOWNS, J. T. (1981) Isolation of human monocytes on re-orienting gradients of percoll. *Journal of Immunological Methods*, **40**, 1-6.

HARRIS, A. K. (1973) Cell surface movements related to cell locomotion. *Ciba Foundation Symposium*, **14**, 3-26.

HARRIS, A. K. (1982) Traction and its relations to contraction in tissue cell locomotion. In *Cell Behaviour*, ed., BELLAIRS, R; CURTIS, A. and DUNN, G. pp. 109-134. Cambridge University Press, Cambridge.

HARRIS, H. (1954) Role of chemotaxis in inflammation. *Physiological Reviews*, **34**, 529-562.

HARRIS, H. ( 1961 ) Chemotaxis and phagocytosis. In *Functions of the Blood*, ed. McFARLANE, R. G. and ROBB-SMITH, A. T. H., pp. 463-494, Blackweel, Oxford.

HARTWIG, J. H. and STOSSEL, T. P. (1975) Isolation and properties of actin, myosin and a new actin-binding protein in rabbit alveolar macrophages. *Journal of Biological Chemistry*, **250**, 5696-5705.

HARTWIG, J. H., TYLER, J. and STOSSEL, T. P. (1980a) Actin-binding protein promotes the bipolar and perpendicular branching of actin filaments. *Journal of Cell Biology*, **87**, 841-848.

HARTWIG, J. H., YIN, H. L. and STOSSEL, T. P. ( 1980b ) Contractile proteins and the mechanism of phagocytosis in macrophages. In *Mononuclear Phagocytes : Functional Aspects*, ed. van FURTH, R., pp. 971-996. Marfinus Nijhoff, The Hague.

HASTON, W. S. (1979) A study of lymphocyte behaviour in cultures of fibroblast-like reticular cells. *Cellular Immunology*, **45**, 74-84.

HASTON, W. S. and SHIELDS, J. M. (1984) Contraction waves in lymphocyte locomotion. *Journal of Cell Science*, **68**, 227-241.

HASTON, W. S. and SHIELDS, J. M. (1985) Neutrophil leucocyte chemotaxis: a simplified assay for measuring polarising responses to chemotactic factors. *Journal of Immunological Methods*, **81**, 229-237.

HASTON, W. S., SHIELDS, J. M. and WILKINSON, P. C. (1982) Lymphocyte locomotion and attachment on two-dimensional surfaces and in three-dimensional matrices. *Journal of Cell Biology*, **92**, 747-752.

HEATH, J. P. and DUNN, G. A. (1978) Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high-voltage electron microscope study. *Journal of Cell Science*, **29**, 197-212.

HESS, C. (1887) Untersuchungen zur Phagocytenlehre. *Virchows Archiv für Pathologische Medizin*, **109**, 365.

HIBBS, J. B., CHAPMAN, H. A. and WEINBERG, J. B. (1980) Regulation of macrophage non-specific tumoricidal capability. In *Mononuclear Phagocytes: Functional Aspects*, ed. van FURTH, R., pp. 1681-1720. Martinus Nijhoff, The Hague.

HUBER, H. AND DOUGLAS, S. D. (1970) Receptor sites on human monocytes for complement: Binding of red cells sensitized by cold autoantibodies. *British Journal of Haematology*, **19**, 19-26.

HUBER, H., DOUGLAS, S. D. and FUDENBERG, H. H. (1969) The IgG receptor: and immunological marker for the characterization of mononuclear cells. *Immunology*, **17**, 7-21.

HUBER, H. and FUDENBERG, H. H. (1968) Receptor sites of human monocytes for IgG. *International Archives of Allergy and Applied Immunology*, **34**, 18-31.

ISLAM, L. N., McKAY, I. C. and WILKINSON, P. C. (1985) The use of collagen or fibrin gels for the assay of human neutrophil chemotaxis. *Journal of Immunological Methods*, **85**, 137-151.

JETT, J. R., MANTOVANI, A. and HERBERMAN, R. B. (1980) Augmentation of human monocyte-mediated cytolysis by interferon. *Cellular Immunology*, **54**, 425-434.

JOHNSON, W. D., MEI, B. and COHN, Z. A. (1977) The separation, long-term cultivation, and maturation of the human monocytes. *Journal of Experimental Medicine*, **146**, 1613-1626.

JOHANSSON, S., RUBIN, K., HÖÖK, M., AHLGREN, T. and SELJELID, R. (1979) *In vitro* biosynthesis of cold insoluble globulin (fibronectin) by mouse peritoneal macrophages. FEBS LETTERS, **105**, 313-316.

JOHNSTON Jr., R. B., LEHMEYER, J. E. and GUTHRIE, L. A. (1976) Generation of superoxide anion and chemilumine-scence of human monocytes during phagocytosis and on contact with surface-bound immmunoglobulin G. *Journal of Experimental Medicine*, **143**, 1551-1556.

KAPLAN, G. (1983) *In vitro* differentiation of human monocytes. Monocytes cultured on glass are cytotoxic to tumor cells but monocytes cultured on collagen are not. *Journal of Experimental Medicine*, **157**, 2061-2072.

KAPLAN, G. and GAUDERNACK, G. ( 1982 ) *In vitro* differentiation of human monocytes. Differences in monocyte phenotypes induced by cultivation on glass or on collagen. *Journal of Experimental Medicine*, **156**, 1101-1114.

KELLER, H. U. (1983) Motility, cell shape, and locomotion of neutrophil granulocytes. *Cell motility*, **3**, 47-60.

KELLER, H. U., BARANDUN, S., KISTLER, P. and PLOEEM, J. S. (1979) Locomotion and adhesion of neutrophil granulocytes : effects of albumin, fibrinogen, and gamma-globulin studied by reflection contrast microscopy. *Experimental Cell research*, **122**, 351-362.

KELLER, H. U. and BESSIS, M. (1975) Migration and chemotaxis of anucleate cytoplasmic leucocyte fragments. *Nature*, **258**, 723-724.

KELLER, H. U., BOREL, J. F., WILKINSON, P. C., HESS, M. and COTTIER, H. (1972) Reassessment of Boyden's technique for measuring chemotaxis. *Journal of Immunological Methods*, **1**, 165-168.

KELLER, H. U., NEAF, A. and ZIMMERMANN, A. (1984) Effect of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leucocytes. *Experimental Cell Research*, **153**, 173-185.

KELLER, H. U. and SORKIN, E. ( 1967 ) Studies on chemotaxis. V On the chemotactic effect of bacteria. *International Archives of Allergy and Applied Immunology*, **31**, 505-517.

KELLER, H. U., WILKINSON, P. C., ABERCROMBIE, M., BECKER, E. L., HIRSCH, J. G., MILLER, M. E., RAMSEY, W. S. and ZIGMOND, S. H. (1977) A proposal for the definition of terms related to locomotion of leucocytes and other cells. *Clinical and Experimental Immunology*, **27**, 377-380.

KELLER, H. U., WILKINSON, P. C., ABERCROMBIE, M., BECKER, E. L., HIRSCH, J. G., MILLER, M. E., RAMSEY, W. S. and ZIGMOND, S. H., AUSTEN, K. F., BAUM, J., BOREL, J. F., CURTIS, A. S. G., DUNN, G. A., GALLIN, J. I., GOETZL, E. J., HARRIS, A. K., HUMBERT, J. R., SORKIN, E., TRINKAUS, J. P., VASILIEV, J. M., WEISS, L. and WISSLER, J. H. (1980) A proposal for the definition of terms related to locomotion of leucocytes and other cells. *Bulletin of the World Health Organization*, **58**, 505-509.

KELLER, R. (1980) Distinctive characteristics of host tumor resistance in a rat fibrosarcoma model system. In *Mononuclear Phagocytes: Functional Aspects*, ed. van Furth, R., pp. 1725-1740. Martinus Nijhoff, The Hague.

KLEBANOFF, S. J. (1975) Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Seminars in Haematology*, **12**, 117-142.

KLEBANOFF, S. J. (1980) Oxygen intermediates and the microbicidal event. In *Mononuclear Phagocytes: Functional Aspects*, ed. van FURTH, R., pp. 1105-1137. Martinus Nijhoff, The hague.

KLEBANOFF, S. J. and HAMON, C. B. (1975) Antimicrobial systems of mononuclear phagocytes. In *Mononuclear Phagocytes in Immunity, Infection and Pathology*, ed. van FURTH, R., pp. 507-529, Blackwell Scientific Publications, Ltd. Oxford.

KOLLER, C. A., KING, G. W., HURTUBISE, P. E., SAGONE, A. L. and LoBUGLIO, A. F. (1973) Characterization of glass adherent human mononuclear cells. *Journal of Immunology*, **111**, 1610-1612.

KUMAGAI, K., ITOH, K., HINUMA, S. and TADA, M. (1979) Pretreatment of plastic petri dishes with fetal calf serum. A simple method for macrophage isolation. *Journal of Immunological Methods*. **29**, 17-25.

LACKIE, J. M. and BURNS, M. D. ( 1983 ) Leucocyte locomotion: Comparison of random and directed paths using a modified time-lapse film analysis. *Journal of Immunological Methods*,**62**, 109-122.

LACKIE, J. M. and WILKINSON, P. C. (1984) Adhesion and locomotion of neutrophil leucocytes on 2-D substrata and in in 3-D matrices. In *White Cell Mechanics: Basic Science and Clinical Aspects*, pp. 237-254. Alan R. Liss, Inc., New York.

LEB, L., CRUSBERG, T., FORTIER, N. and SNYDER, L. M. (1983) Evaluation of methods using adherence to substrate and density gradient for the isolation of

human monocytes. *Journal of Immunological Methods*, **58**, 309-321.

LEBER, T. (1888) über die Entstehung der Entzündung und die Wirkung der entzündungerregenden Schädlichkeiten. *Fortschritte der Medizin*, **4**, 460-464.

LEHRER, R. I. (1970) The functional activity of human monocytes: a myeloperoxidase-linked mechanism. *Clinical Research*, **18**, 408A

LEHRER, R. I. (1975) The fungicidal mechanisms of human monocytes. I. Evidence for myeloperoxidase-linked and myeloperoxidase-independent candidacidal mechanisms. *Journal of Clinical Investigation*, **55**, 338-346.

LEONARD, E. J. and SKEEL, A. H. (1978) Isolation of macrophage stimulating protein (MSP) from human serum. *Experimental Cell Research*, **114**, 117-126.

LEONARD, E. J. and SKEEL, A. H. (1980) Functional differences between resident and exudate peritoneal mouse macrophages: Specific serum protein requirements for responsiveness to chemotaxins. *Journal of the Reticuloendothelial Society*, **28**, 437-447.

LEWIS, W. H. (1931) Locomotion of lymphocytes. *Bulletin of the Johns Hopkins Hospital*, **49**, 29-36.

LEWIS, W. H. (1934) On the locomotion of the polymorphonuclear neutrophils of the rat in autoplasma cultures. *Bulletin of the Johns Hopkins Hospital*, **55**, 273-279.

LEWIS, W. H. and WEBSTER, L. T. (1921) Migration of lymphocytes in plasma cultures of human lymph nodes. *Journal of Experimental Medicine*, **33**, 261-269.

LOOS, J. A., BLOCK-SCHUT, B., van DOORN, R., HOKSBERGEN, R., BRUTEL, de la RIVIERE, A. and MEERHOF, L. (1976) A method for the recognition and separation of human

blood monocytes on density gradients. *blood*, **48**, 731-742.

MANTOVANI, A., DEAN, J. H., JERRELLS, T. R. and HERBERMAN, R. B. (1980) Augmentation of tumoricidal activity of human monocytes and macrophages by lymphokines. *International Journal of Cancer*, **25**, 691-699.

MANTOVANI, A., JERRELLS, T. R., DEAN, J. H. and HERBERMAN, R. B. (1979a) Cytolytic and cytostatic activity on tumor cells of circulating human monocytes. *International Journal of Cancer*, **23**, 18-27.

MANTOVANI, A., JERRELLS, T. R., DEAN, J. H. and HERBERMAN, R. B. (1979b) Cytolytic activity of circulating human monocytes on transformed and untransformed fibroblasts. *International Journal of Cancer*, **23**, 28-31.

MARONI, E. S. and WILKINSON, P. C. (1971) Selective chemotaxis of macrophages towards human and guinea pig spermatozoa. *Journal of Reproduction and Fertility*, **27**, 149-152.

MARONI, E. S., SYMON, D. N. K. and WILKINSON, P. C. (1972) Chemotaxis of neutrophil leucocytes towards spermatozoa and seminal fluid. *Journal of Reproduction and Fertility*, **28**, 359-368.

McCUTCHEON, M. (1923) Studies on the locomotion of leucocytes. I The normal rate of locomotion of human neutrophilic leucocytes *in vitro*. *American Journal of Physiology*, **66**, 180-190.

McCUTCHEON, M. (1924) Studies on the locomotion of leucocytes. III The normal rate of locomotion of human lymphocytes *in vitro*. *American Journal of Physiology*, **69**, 279-282.

McCUTCHEON, M. ( 1946 ) Chemotaxis in leucocytes. *Physiological Reviews*, **26**, 319-336.

McCUTCHEON, M. and DIXON, H. M. (1936) Chemotropic reactions of polymorphonuclear leucocytes to various microorganisms. *Archives of Pathology*, **21**, 749-755.

McCUTCHEON, M., WARTMAN, W. B. and DIXON, H. M. (1934) Chemotropism of leucocytes *in vitro*. *Archives of Pathology*, **17**, 607-614.

MEER, J. W. M. van der, BULTERMAN, D., van ZWET, Th. L., ELZENGA-CLAASEN, I. and van FURTH, R. (1978) Culture of mononuclear phagocytes on a teflon surface to prevent adherence. *Journal of Experimental Medicine*, **147**, 271-276.

MEER, J. W. M. van der, van de GEVEL, J. S., ELZENGA-CLAASEN, I. and van FURTH, R. (1979) Suspension culture of mononuclear phagocytes in the teflon culture bag. *Cellular Immunology*, **42**, 208-212.

MELEWICZ, F. M., BOLTZ-NITULESCU, G. and SPIEGELBERG, H. L. (1981) Fc receptors for IgE on human monocytes and rat macrophages. In *Heterogeneity of Mononuclear Phagocytes*, ed. FÖRSTER, O. and LANDY, M., pp. 102-108. Academic Press, London.

MELTZER, M. S. and STEVENSON, M. M. (1977) Macrophage function in tumor-bearing mice. Tumoricidal and chemotactic responses of macrophages activated by infection with *Mycobacterium bovis*, strain BCG. *Journal of Immunology*, **118**, 2176-2181.

MELTZER, M. S., STEVENSON, M. M. and LEONARD, E. J. (1977) Characterization of macrophage chemotaxins in tumor cell cultures and comparison with lymphocyte-derived chemotactic factors. *Cancer Research*, **37**, 721-725.

METCALF, D. and MOORE, M. A. S. (1971) *Haemopoietic Cells.* North-Holland Publishing Company, Amsterdam-London.

METCHNIKOFF, E. (1893) *Lectures on the Comparative Pathology of Inflammation.* Kegan Paul, London.

MICHL, J., UNKELESS, J. C. and SILVERSTEIN, S. C. (1980) Modulation of macrophage plasma membrane receptors for IgG and complement. In *Mononuclear Phagocytes: Functional Aspects*, ed. van FURTH, R., pp. 921-937. Martinus Nijhoff, The Hague.

MINKIN, C., BLACKMAN, L., NEWBREY, J., POKRESS, S., POSEK, R. and WALLING, M. (1977) Effects of parathyroid hormone and calcitonin on adenylate cyclease in murine mononuclear phagocytes. *Biochemical and Biophysical Research Communications*, **76**, 875-881.

MIZEL, S. B. and FARRAR, J. F. (1979) Revised nomenclature for antigen-nonspecific T-cell proliferation and helper factors. *Cellular Immunology*, **48**, 433-436.

MUELLER, J., BRUN DEL RE, G., BUERKI, H., KELLER, H. U., HESS, M. W. and COTTIER, H. (1975) Nonspecific acid esterase activity: a criterion for differentiation of T and B lymphocytes in mouse lymph nodes. *European Journal of Immunology*, **5**, 270-274.

MUKHERJI, B. ( 1980 ) *In vitro* assay of spontaneous cytotoxicity by human monocytes and macrophages against tumor cells. *Journal of Immunological Methods*, **37**, 233-247.

MUNDY, G. R., VARANI, J., ORR, W., GONDEK, M. D. and WARD, P. A. (1978) Resorbing bone is chemotactic for monocytes. *Nature*, **275**, 132-135.

MURPHY, P. (1976) The Neutrophil. Plenum Medical Book Company, New York.

NAKAGAWARA, A., NATLAN, C. F. and COHN, Z. A. (1981) Hydrogen peroxide metabolism in human monocytes during differentiation *in vitro*. *Journal of Cloinical Investigation*, **68**, 1243-1252.

NATHAN, C. F. and ROOT, R. K. (1977) Hydrogen peroxidase release from mouse peritoneal macrophages: Dependence of sequential activation and triggering. *Journal of Experimental Medicine*, **146**, 1648-1662.

NATHANSON, S. D., ZAMFIRESCU, P. L., DREW, S. I. and WILBUR, S. M. (1977) Two-step separation of human peripheral blood monocytes on discontinuous density gradients of colloidal silica-polyvinylpyrrolidone. *Journal of Immunological Methods*, **18**, 225-234.

NELSON, R. D., QUIE, P. G. and SIMMONS, R. L. (1975) Chemotaxis under agarose : a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leucocytes and monocytes. *Journal of Immonology*, **115**, 1650-1656.

NORMANN, S. J. and SORKIN, E. (1976) Cell-specific defect in monocyte function during tumor growth. *Journal of the National Cancer Institute*, **57**, 135-140.

NORMANN, S. J. and SORKIN, E. (1977) Inhibition of macrophage chemotaxis by neoplastic and other rapidly proliferating cells *in vitro*. *Cancer Research*, **37**, 505-711.

NORMANN, S. J. and WEINER, R. (1981) Isolation of two subsets of human peripheral blood monocytes differing in tumor cytotoxicity. In *Heterogeneity of Mononuclear Phagocytes*, ed. FORSTER, O. and LANDY, M. pp. 496-500. Academic Press, London.

NORRIS, D. A., MORRIS, R. M., SANDERSON, R. J. and KOHLER, P. F. (1979) Isolation of functional subsets

of human peripheral blood monocytes. *Journal of Immunology*, **123**, 166-172.

OLIVER, J. M., KRAWIEC. J. A. and BECKER, E. L. (1978) The distribution of actin during chemotaxis in rabbit neutrophils. *Journal of the Reticuloendothelial Society*, **24**, 697-704.

O'NEILL, G. J. and PARROTT, D. M. V. (1977) Locomotion of human lymphoid cells. I Effect of culture and con A on T and non-T lymphocytes. *Cellular Immunology*, **33**, 257-267.

OTU, A. A., RUSSELL, R. J., WILKINSON, P. C. and WHITE, R. G. (1977) Alterations of mononuclear phagocyte function induced by Lewis lung carcinoma in C57BL mice. *British Journal of Cancer*, **36**, 330-340.

PIKE, M. C., FISCHER, D. G., KOREN, H. S. and SNYDERMAN, R. (1980) Development of specific receptors for N-formylated chemotactic peptides in a human monocyte cell line stimulated with lymphokines. *Journal of Experimental medicine*, **152**, 31-40.

POSTE, G., GREENHAM, L. W., MALLUCCI, L., REEVE, P. and ALEXANDER, J. D. (1973) The study of cellular "micro-exudates" by ellipsometry and their relationship to the cell coat. *Experimental Cell Research*, **78**, 303-313.

RABINOVITCH, M. and DESTEFANO, M. J. (1973a) Macrophage Spreading *in vitro*. I Inducers of spreading. *Experimental Cell Research*, **77**, 323-334.

RABINOVITCH, M. and DESTEFANO, M. J. (1973b) Macrophage Spreading *in vitro*. II. Manganese and other metals as inducers or as co-factors for induced spreading. *Experimental Cell Research*, **79**, 423-430.

RABINOVITCH, M., MANEJIAS, R. E. and NUSSENZWEIG, (1975) Selective phagocytic paralysis induced by immobilized immune complexes. *Journal of Experimental Medicine*, **142**, 827-838.

RAMSEY, W. S. (1972a) Analysis of individual leucocyte behaviour during chemotaxis. *Experimental Cell Research*, **70**, 129-139

RAMSEY, W. S. (1972b) Locomotion of human polymorphonuclear leucocytes. *Experimental Cell Research*, **72**, 489-501.

RAMSEY, W. S. and HARRIS, H. (1973) Leucocyte locomotion and its inhibition by antimitotic drugs. *Experimental Cell Research*, **82**, 262-270.

REAVEN, E. P. and AXLINE, S. G. (1973) Subplasmalemmal microfilaments and microtubules in resting and phagocytizing cultivated macrophages. *Journal of Cell Biology*, **59**, 12-27.

REBUCK, J. W. and CROWLEY, J. H. (1955) A method for studying leucocyte functions *in vivo*, *Annals in the New York Academy of Science*, 59, 757-794.

RINEHART, J. J., GORMUS, B. J., LANGE, P. and KAPLAN, M. E. (1978) A new method for isolation of human monocytes. *Journal of Immunological Methods*, **23**, 207-212.

ROBINEAUX, R. (1964) Movements of cells involved in inflammation and immunity. In *Primitive Motile Systems in Cell Bilogy*. ed. ALLEA, R. D. and KAMIYA, K. p. 351. Academic Press, New York.

RODEY, G. E., PARK, B. H., WINDHORST, D. B. and GOOD, R. A. (1969) Defective bactericidal activity of monocytes in fatal granulomatous disease. *Blood*, **33**, 813-820.

RUSSELL, R. J., WILKINSON, P. C., SLESS, F. and PARROTT, D.M. V. (1975) Chemotaxis of lymphoblasts. *Nature*, **256**, 646-648.

RUSSO, R. G., LIOTTA, L. A., THORGEIRSSON, U., BRUNDAGE, R. and SCHIFFMAN, E. ( 1981 ) Polymorphonuclear leucocytes migration through human amnion membrane. *Journal of Cell Biology*, **91**, 459-467.

RYAN, G. B., BORYSENKO, J. Z. and KARNOVSKY, M. J. (1974) Factors affecting the redistribution of surface bound concanavalin A on human polymorphonuclear leucocytes. *Journal of Cell Biology*, **62**, 351-365.

RYAN, G. B. and MAJNO, G. (1977) Acute inflammation. *American Journal of Pathology*, **86**, 185-276.

SANDISON, J. C. (1928) The transparent chamber of the rabbit's ear giving a complete description of improved technic of constraction and introduction and general account of growth and behaviour of living cells and tissues as seen with the microscope. *American Journal of Anatomy*, **41**, 447-472.

SBARRA, A. J. and KARNOVSKY, M. L. (1959) The biochemical basis of phagocytosis. I Metabolic changes during the ingestion of particles by polymorphonuclear leukocutes. *Journal of Biological Chemistry*, **234** 1355-1362.

SCHLESINGER, P.H., DOEBBER, T., MANDELL, B., RICHARD, W., DESCHRYVER, C., RODMAN, J. S., MILLER, M. J. and STAHL, P. (1978) Plasma clearance of glycoproteins with terminal mannose and N-acetylglucosamine by liver non-parenchymal cells. Studies with β-glucuronidase, N-acetyl-β-glucosaminidase, ribonuclease B and agalacto-orosomucoid. *Biochemical journal*, **176**, 103-109.

SCHREINER, G. F., FUJIWARA, K., POLLARD, T. D., and UNANUE, E. R. (1977) Redistribution of myosin accompanying capping of surface immunoglobulin. *Journal of Experimental Medicine*, **145**, 1393-1398.

SCHOR, S. L. (1980) Cell proliferation and migration on collagen substrata *in vitro*. *Journal of Cell Science*, **41**, 159-175.

SCHOR, S. L., ALLEN, T. D. and WINN, B. (1983) Lymphocyte migration into three-dimensional collagen matrices : A quantitative study. *Journal of Cell Biology*, **96**, 1089-1096

SENDA, N. (1977) The mechanism of ingestion by leucocytes based on their motility. In *Movement, Metabolism and Bactericidal Mechanisms of phagocytes*, ed. ROSSI, F., PATRIARCA, P. L. and REMO, D. pp. 5-14. Piccin, Padua.

SENDA, N., SHIBATA, N., TATSUMI, N., KONDO, K. and HAMADA K. (1969) A contractile protein from leukocytes. Its extraction and some of its properties. *Biochimica et Biophysica Acta*, **181**, 191-200.

SENDA, N., TAMURA, H., SHIBATA, N., YOSHITAKE, J., KONDO, K. and TANAKA, K. (1975) The mechanism of the movement of leucocytes. *Experimental Cell Research*, **91**, 393-407.

SHAW, G. M., LEVY, P. C. and LOBUGLIO, A. F. (1978) Human monocyte cytotoxicity to tumor cells. I. Antibody-dependent cytotoxicity. *Journal of Immunology*, **121**, 573-578.

SHIBATA, N., TATSUMI, N., TANAKA, K., OKAMURA, Y. and SENDA, N. (1972) A contractile protein possessing $Ca^{+2}$ sensitivity (natural actomyosin) from leukocytes. Its extraction and some of its properties. *Biochimica et Biophysica Acta*, **256**, 565-576.

SHIELDS, J. M. and HASTON, W. S. (1985) Behaviour of neutrophil leucocytes in uniform concentrations of chemotactic factors: contraction waves, cell polarity and persistence. *Journal of Cell Science*, **74**, 75-93.

SHIELDS. J. M., HASTON, W. S. and WILKINSON, P. C. (1984) Invasion of collagen gels by mouse lymphoid cells. *Immunology*, **51**, 259-268.

SILVERSTEIN, S. C. (1982) Membrane receptors and regulation of mononeuclear phagocyte effector functions. In *Macrophages and Natural Killer Cells: Regulation and Function*, ed. NORMANN, S. J. and SORKIN, E. pp. 21-31. Plenum Press. New York.

SMITH, C. W. and HOLLERS, J. C. (1980) Motility and adhesiveness in human neutrophils. Redistribution of chemotactic factor-induced adhesion sites. *Journal of Clinical Investigation*, **65**, 804-812.

SMITH, C. W., HOLLERS, J. C., PATRICK, R. A. and HASSETT, C. (1979) Motility and adhesiveness in human neutrophils. Effects of chemotactic factors. *Journal of Clinical Investigation*, **63**, 221-229.

SMITH, T. J. and WAGNER, R. M. (1967) Rabbit macrophage interferons. I Conditions for biosynthesis by virus-infected and uninfected cells. *Journal of Experimental Medicine*, **125**, 559-577.

SNODGRASS, M. J., HARRIS, T. M. and KAPLAN, A. M. (1978) Chemotactic responses of activated macrophages to soluble products of neoplastic cells. *Cancer Research*, **38**, 2925-2929.

SNYDERMAN, R., ALTMAN, L. C., HAUSMAN, M. S. and MERGENHAGEN, S. E. (1972) Human mononuclear leucocyte chemotaxis: a quantitative assay for humoral and cellular chemotactic factors. *Journal of Immunology*, **108**, 857-860.

SNYDERMAN, R. and FUDMAN, E. J. (1980) Demonstration of a chemotactic factor receptor on macrophages. *Journal of Immunology*, **124**, 2754-2757.

SNYDERMAN, R., SEIGLER, H. F. and MEADOWS, L. (1977) Abnormalities of monocyte chemotaxis in patients with melanoma. Effects of immunotherapy and tumor removal. *Journal of the National Cancer Institute*, **58**, 37-41.

SNYDERMAN, R., SHIN, H. S., PHILLIPS, J. K., GEWURZ, H. and MERGENHAGEN, S. E. (1969) A neutrophil chemotactic factor derived from C'5 upon interaction of guinea pig serum with endotoxin. *Journal of Immunology*, **103**, 413-422.

SNYDERMAN, R. and PIKE, M. C. (1976a) An inhibitor of macrophage function produced by neoplasms. *Science*, **192**, 370-372.

SNYDERMAN, R. and PIKE, M. C. ( 1976b ) Defective macrophage migration produced by neoplasms: identification of an inhibitor of macrophage chemotaxis. In *The Macrophage in Neoplasia*, ed. FINK, M. A. pp. 49-65. Academic Press, New York.

SNYDERMAN, R., PIKE, M. C. McCARLEY, D. and LANG, L. (1975) Quantification of mouse macrophage chemotaxis *in vitro*. Role of C5 for the production of chemotactic activity. *Infection and Immunity*, **11**, 488-492.

SPILBERG, I., MEHTA, J., DAUGHADAY, C. and SIMCHOWITZ, L. (1981) Determination of specific receptor for formyl-methionyl-leucyl-phenylalanine on the pulmonary alveolar macrophage and its relationship to chemotaxis and superoxide production. *Journal of Laboratory and Clinical Medicine*, **97**, 602-609.

STADECKER, M. J., CALDERON, J., KARNOVSKY, M. L. and UNANUE, E. R. ( 1977 ) Synthesis and release of thymidine by macrophages. *Journal of Immunology*, **119**, 1738-1743.

STAHL, P., RODMAN, J., MILLER, J. and SCHLESINGER, P. (1978) Evidence for receptor-mediated binding of

glycoproteins, glucoconjugates, and lysosomal glycosidase by alveolar macrophages. *Proceedings of the National Academy of Sciences of the U.S.A.*, **75**, 1399-1403.

STAHL, P., SCHLESINGER, P., RODMAN, J. and DOEBBER, T. (1976) Recognition of lysosomal glycosidase *in vivo* inhibited by modified glycoproteins. *Nature*, **264**, 86-88.

STAHL, P., TIETZE, C., TOWNSEND, R., SHEPHERD, V. and SCHLESINGER, P. (1981) Plasma membrane receptors on macrophages which bind and internalize mannose or L-fucose terminated glycoproteins. In *Heterogeneity of Mononuclear Phagocytes*, ed. FORSTER, O. and LANDY, M. pp. 134-140. αcademic Press, London.

STENDAHL, O. I., HARTWIG, J. H., BROTSCHI, E. A. and STOSSEL, T. P. (1980) Distribution of actin-binding protein and myosin in macrophages during spreading and phagocytosis. *Journal of Cell Biology*, **84**, 215-224.

STOCKERT, R., MORELL, A. and SCHIENBERG, I. (1976) The existence of a second route for the transfer of certain glycoproteins from the circulation into the liver. *Biochemical and Biophysical Research Communications*, **68**, 988-993.

STOSSEL, T. P. (1978) The mechanism of leukocyte locomotion. In Leukocyte Chemotaxis, ed. GALLIN, J. I. and QUIE, P. G. pp. 143-160. Raven Press, New York.

STOSSEL, T. P. and HARTWIG, J. H. (1975) Intractions between actin, myosin and an actin-binding protein from rabbit alveolar macrophages. Alveolar macrophage myosin $Mg^{2+}$ adenosine triphosphatase requires a cofactor for activation by actin. *Journal of Biological Chemistry*, **250**, 5706-5712.

STOSSEL, T. P. and HARTWIG, J. H. (1976) Interactions of actin, myosin and a new actin-binding protein of rabbit pulmonary macrophages. II Role in cytoplasmic movement and phagocytosis. *Journal of Cell Biology*, **68**, 602-619.

STOSSEL, T. P. and POLLARD, T. D. (1973) Myosin in polymorphonuclear leucocytes. *Journal of Biological Chemistry*, **248**, 8288-8294.

SULLIVAN, S. J., DAUKAS, G. and ZIGMOND, S. H. (1984) Asymmetric distribution of the chemotactic peptide receptor on polymorphonuclear leukocytes. *Journal of Cell Biology*, **99**, 1461-1467.

SYMON, D. N. K., McKAY, I. C. and WILKINSON, P. C. (1972) Plasma-dependent chemotaxis of macrophages towards *Mycobacterium tuberculosis* and other organisms. *Immunology*, **22**, 267-276.

TATSUMI, N., SHIBATA, N., OKAMURA, Y., TAKEUCHI, K. and SENDA, N. (1973) Actin and myosin from leucocytes. *Biochimica et Biophysica Acta*, **305**, 433-444.

THORSSON, A. V. and HINTZ, R. L. (1977) Specific $^{125}$I-somatomedin receptor on circulating human mononuclear cells. *Biochemical and Biophysical Research Communications*, **74**, 1566-1573.

ULMER,, A. J. and FLAD, H. D. (1979) Discontinuous density gradient separation of human mononuclear leucocytes using percoll as gradient medium. *Journal of Immunological Methods*, **30**, 1-10.

VASSALLI, J-D., HAMILTON, J. and REICH, E. (1976) Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, metabolic inhibitors, and cyclic nucleotides. *Cell*, **8**, 271-281.

VERNON-ROBERTS, B. (1969) The efect of steroid hormones on macrophage activity. *International Review of Cytology*, **25**, 131-159.

VIROLAINEN, M. (1968) Hematopoietic origin of macrophages as studied by chromosome markers in mice. *Journal of Experimental Medicine*, **127**, 943-952.

van VOORHIS, W. C., HAIR, L. S., STEINMAN, R. M. and KAPLAN, G. (1982) Human dendritic cells. Enrichment and characterization from peripheral blood. *Journal of Experimental Medicine*, **155**, 1172-1187.

WALTER, R. J., BERLIN, R. D. and OLIVER, J. M. (1980) Asymmetric Fc receptor distribution on human PMN oriented in a chemotactic gradient. *Nature*, 286, 724-725.

WALLER, A. ( 1846 ) Microscopic observations on the perforation of the capillaries by the corpuscles of the blood and on the origin of mucus and pus globules. *London and Edinburgh Philosophical Magazine and Journal of Science*, **29**, 271-287 and 379-405.

WARD, P. A., REMOLD, H. G. and DAVID, J. R. (1969) Leukotactic factor produced by sensitized lymphocytes. *Science*, 163, 1079-1081.

WARD, P. A., REMOLD, H. G. and DAVID, J. R. (1970) The production from antigen-stimulated lymphocytes of a leukotactic factor distinct from migration inhibition factors. *Cellular Immunology*, **1**, 162-174.

WEENING, R. S., ROSS, D. and LOOS, J. A. (1974) Oxygen consumption of phagocytizing cells in human leukocyte and granulocyte preparations: a comparative study. *Journal of Laboratory and Clinical Medicine*, **83**, 570-576.

WEINBERG, J. B., MUSCATO, J. J. and NIEDEL, J. E. (1981) Monocyte chemotactic peptide receptor. Functional characteristics and ligand-induced regulation. *Journal of Clinical Investigation*, **68**, 621-630.

WEISS, S. J. and SLIVKA, A. ( 1982 ) Monocyte and granulocyte-mediated tumor cell destruction. *Journal of Clinical Investigation*, **69**, 255-262.

WERB, Z., FOLEY, R. and MUNCK, A. (1978a) Interaction of glucocorticoids with macrophages. Identification of glucocorticoid receptors in monocytes and macrophages. *Journal of Experimental Medicine*, **147**, 1684-1693.

WERB, Z., FOLEY, R. and MUNCK, A. (1978b) Glucocorticoid receptors and glucocorticoid-sensitive secretion of neutral proteinases in a macrophage line. *Journal of Immunology*, **121**, 115-121.

WERB, Z. and GORDON, S. (1975) Secretion of specific collagenase by stimulated macrophages. *Journal of Experimental Medicine*, **142**, 346-360.

WHALEY, K. ( 1980 ) Biosynthesis of the complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. *Journal of Experimental Medicine*, **151**, 501-516.

WILKINSON, P. C. (1973) Recognition of protein structure in leukocyte chemotaxis. *Nature*, **244**, 512-513.

WILKINSON, P. C. (1982a) *Chemotaxis and Inflammation*, 2nd ed. Churchill-Livingstone, Edinburgh.

WILKINSON, P. C. (1982b) Visual observation of chemotaxis in mouse macrophages. *Immunobiology*, **161**, 376-384.

WILKINSON, P. C. (1985a) A visual study of chemotaxis of human lymphocytes using a collagen-gel assay. *Journal of Immunological Methods*, **76**, 105-120.

WILKINSON, P. C. (1985b) Locomotion and chemotaxis of mononuclear phagocytes. *Comparative Immunology, Microbiology, and Infectious Diseases*, **8**, 213-224.

WILKINSON, P. C. (1986) The locomotor capacity of human lymphocytes and its enhancement by cell growth. *Immunology*, **57**, 281-289.

WILKINSON, P. C. and ALLAN, R. B. (1978) Assay systems for measuring leucocytes locomotion : an overview. In *Leucocyte Chemotaxis*, ed. GALLIN, J. I. and QUIE, P. G. pp. 1-24. Raven Press, New York.

WILKINSON, P. C. and ALLAN, R. B. (1980) The locomotor behaviour of human blood monocytes in chemokinetic and chemotactic environments and the role of the substratum in monocyte locomotion. In *Mononuclear Phagocytes, Fuctional Aspects*, ed. van FURTH, R., pp. 475-500. Martinus Nijhoff, The Hague.

WILKINSON, P. C. and BRADLEY, G. (1981) Chemotactic and enzyme-releasing activity of amphipathic proteins for neutrophils. *Immunology*, **42**, 637-648.

WILKINSON, P. C., LACKIE, J. M. and ALLAN, R. B. (1982) Methods for measuring leucocyte locomotion. In *Cell Analysis*, ed. CATSIMPOOLAS, N. Vol. 1, pp. 145-193. Plenum, New York.

WILKINSON, P. C., LACKIE, J. M., FORRESTER, J. V. and DUNN, G. A. (1984) Chemotactic accumulation of human neutrophils on immune complex-coated substrata: Analysis at a boundary. *Journal of Cell Biology*, **99**, 1761-1768.

WILKINSON, P. C., MICHL, J. and SILVERSTEIN, S. C. (1980) Receptor distribution in locomoting neutrophils. *Cell Biology International Reports*, **4**, 736.

YIN, H. L. and STOSSEL, T. P. (1979) Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature*, *281*, 583-586.

ZIGMOND, S. H. (1974) Mechanisms of sensing chemical gradients by polymorphonuclear leucocytes. *Nature*, *249*, 450-452.

ZIGMOND, S. H. (1977) Ability of polymorphonuclear leucocytes to orient in gradients of chemotactic factors. *Journal of Cell Biology*, **75**, 606-616.

ZIGMOND, S. H., and HIRSCH, J. G. (1972) Effects of cytochalasin B on polymorphoneuclear leucocyte locomotion, phagocytosis, and glycolysis. *Experimental Cell Research*, **73**, 383-393.

ZIGMOND, S. H. and HIRSCH, J. G. ( 1973 ) Leucocyte locomotion and chemotaxis : new methods for evalution, and demonstration of a cell derived chemotactic factor. *Journal of Experimental Medicine*, **137**, 387-410.

ZIGMOND, S. H., LEVITSKY, H. I. and KREEL, B. J. (1981) Cell polarity : an examination of its behavioral expression and its consequences for polymorphoneuclear leucocyte chemotaxis. *Journal of Cell Biology*, **89**, 585-592.

ZIGMOND, S. H. and SULLIVAN, S. J. (1979) Sensory adaptation of leucocytes to chemotactic peptides. *Journal of Cell Biology*, **82**, 517-527.