Neutrophil Leucocyte Locomotion in 3-D Gel Matrices

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DECLARATION

Except where otherwise stated, the work presented in this thesis is original, and has been carried out by me.

K.V. Crocket, November, 1987

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List of Abbreviations and Terms:

BSA	bovine serum albumin
cpm	counts per minute
d	day
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
f-MLP	N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine
f-NLP	N-Formyl-L-Norleucyl-L-Leucyl-L-Phenylalanine
g	gram
h	hour
Hepes	N-2-hydroxyethylpiperazine-N^1-2-ethane sulphonic acid
Ig	immunoglobulin
IU	international unit(s)
kD	kilodalton
litre(s)	litre(s)
M	molar
MBq	MegaBecquerels
m	metre(s)
min	minute(s)
mol	mole(s)
mol wt	molecular weight
ml	millilitre(s)
N	normal
NS	not significant
OD	optical density
P	probability
Pa	Pascal(s)
s	second(s)
PBS	phosphate-buffered saline
SD	standard deviation
SEM	standard error of mean
U	unit
SDS	sodium dodecyl sulphate
t test	Student's t-test
Tris	tris(hydroxymethyl)aminomethane
µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)
HS	Hepes Saline
H2	Hank's Hepes

Summary

The invasive and locomotory behaviour of neutrophil leucocytes was studied using 3-D collagen gel matrices made from reconstituted rat tail tendon. Other techniques used included time-lapse filming of neutrophils moving over protein-coated glass, and chemiluminescence.

The effect of pentoxifylline, a methyl xanthine, on the locomotion of neutrophils was looked at. This drug is used clinically for cases of peripheral vascular insufficiency, improving circulation without vasodilation, and there is evidence that it increases the deformability of erythrocytes. It was of interest to determine whether pentoxifylline had a similar effect on neutrophils. Pentoxifylline was found to enhance the penetration of collagen gels by rabbit neutrophils when used at 3.6×10^{-2} M (10 μg ml^{-1}), with higher concentrations of pentoxifylline inhibiting penetration. This enhancement was apparent over a range of gel concentrations. The same concentration of pentoxifylline enhanced the speed of rabbit neutrophils locomoting over protein-coated glass, while human neutrophils showed an enhancement of speed using pentoxifylline at 3.6×10^{-2} M and at 3.6×10^{-3} M (10 and 1.0 μg ml^{-1} respectively).

The locomotion of human neutrophils, pre-stimulated with f-MLP at 10^{-9} M was inhibited by pentoxifylline, with a decrease in speed of cells locomoting over protein-coated glass. An inhibitory effect of pentoxifylline was also seen using pre-stimulated rabbit neutrophils, and measuring

their chemiluminescence. The ID50 dose, the concentration of pentoxifylline required to reduce to 50% the light output, was calculated as being in the range 0.51 M - 0.65 M (0.14 - 0.18 mg ml^{-1}). Rabbit neutrophils pre-stimulated with f-MLP showed an enhancement of light output using another methyl xanthine, aminophylline.

There is debate about whether neutrophil leucocytes, invading a collagenous matrix, engage in any proteolytic activity. Collagen gels were therefore used as a convenient in vitro system for studying the locomotion of neutrophils into, and through, these gels.

Radio-labelled neutrophils were used to show that the passage of a primary wave of cells penetrating a collagen gel did not enhance the passage of a second wave. Using radio-labelled collagen gels, there was no detectable proteolytic activity by f-MLP-stimulated cells penetrating a collagen gel, in an assay capable of detecting degradation of the collagen from amounts as low as 10% of the predicted amount of collagenase released by the invading cells.

Neutrophils were allowed to penetrate collagen gels in the presence of two known metallo-protease inhibitors, EDTA and o-phenanthroline, which should decrease collagenase-mediated penetration of such a matrix. With EDTA at 10^{-5} M, there was a slight increase in penetration. With o-phenanthroline at 5 and 0.5 mM, there was also an increase in penetration, but an inhibition of penetration at a higher dose of 50 mM.

The effect of cell concentration on the speed and persistence of neutrophils locomoting over protein-coated glass was studied using time-lapse filming. At a high concentration of cells, 2×10^6 ml^{-1}, there was a reduction in speed. There was a graded relationship between persistence and cell concentration; the higher the cell concentration, the lower the persistence. Neutrophils were found to invade a cross-linked collagen gel (fixed with glutaraldehyde) to a lesser extent than they invaded a standard collagen gel.

Immune complexes were used to modify neutrophil locomotion in collagen gels and over protein-coated glass. The presence, in medium above a collagen gel, of either immune complex or complement, inhibited neutrophil invasion of the gel. Immune complexes at shallow depths in a gel also reduced the depth of invasion compared with controls, as measured by the leading front, while immune complexes at greater depth led to an enhancement of invasion. It is concluded that such complexes in a collagen gel have a trapping effect on an invading population of neutrophils. Different ratios of antigen to antibody had different modifying effects on cell locomotion. Immune complexes reduced neutrophil speed over protein-coated glass.

Neutrophils incorporated in collagen gels while gelation was taking place became immobilised. This immobilisation was not due to a cell-released factor, nor to a non-polymerised component of the collagen solution. Neutrophils attached to protein-coated glass under a collagen roof also became immobilised.

Chapter 1 Introduction

1.1 Natural history of neutrophil leucocytes

Mammalian white blood cells, including the neutrophil leucocyte, have evolved as part of the immune system. Their function is to seek out and inactivate foreign microorganisms such as bacteria. Various processes such as chemotaxis, phagocytosis, oxidant generation and lysosomal degranulation are central to neutrophil function, though all of these are worthless unless the neutrophil can invade and move efficiently through the various body tissues. This study seeks to examine some of the ways in which the polymorphonuclear neutrophil granulocyte, referred to here as the neutrophil for convenience, may perform such invasive behaviour.

The mammalian neutrophil derives from a committed stem cell or cells in the bone marrow. Other committed cells also give rise to the other polymorphonuclear cells, the basophil and the eosinophil. These three polymorphonuclear cells gain their names from their staining properties and their multilobed nucleus. The remaining white cells, the mononuclear cells, comprise the lymphocytes and monocytes. In the tissues the monocytes differentiate into macrophages, while the lymphocytes fall into two groups; the antibody producing B-lymphocytes, and the T-lymphocytes, involved in cellular

immunity. (Cline, 1975). Evidence from cell culture work, in which a single cell can give rise to colonies of both granulocytes and monocytes, suggests the existence of a pluripotent stem cell (See Appendix G).

The first recognisable precursor of the neutrophil is the myeloblast, which undergoes division while in the bone marrow. Further cell division leads to the promyelocyte, the cytoplasm of which contains a prominent Golgi apparatus. From this Golgi apparatus develop the primary granules, also known as the azurophilic granules due to their blue appearance when treated with a Romanovsky-type stain. The promyelocyte divides to produce the myelocyte, at which stage the secondary granules are formed. These secondary granules are also known as the specific granules, as they are to be found only in the neutrophil leucocytes. (Bainton & Farquhar, 1966).

The development period, from myeloblast appearance to myelocyte, takes about 4-6 days. The myelocyte now matures without further cell division into the mature neutrophil, taking a further 5-7 days. During this period, the diameter decreases by about 50-60%; the mature neutrophil is about 8 μm diameter in suspension (Schmid-Schönbein et al., (1980). White blood cells normally have to pass through capillaries whose diameters are smaller than 8 μm; they have a certain degree of deformability, though not as much as erythrocytes possess. The multilobed shape of the nucleus in the mature neutrophil may facilitate the cell's passage through narrow openings. Following maturation, leucocytes enter the circulation through the endothelial walls of the marrow sinuses. The theory, that in addition to circulating

neutrophils in the blood there was a pool of marginated neutrophils, adhering to the endothelium, was tested by Cartwright et al., (1964). They used a technique involving the incorporation of labelled diisopropylfluoro-32-phosphate (DFP^{32}), which labels neutrophil leucocytes selectively, and only neutrophils in the blood, the granulocytes and their precursors in the bone marrow remaining unlabelled. They found that the marginated pool accounted for about 56% of the total blood leucocyte population, the total pool in a healthy human subject being about 70×10^7 cells per kg. The half-life of a circulating neutrophil was 6.9 hours, while the reservoir in the marrow, consisting of immature and mature neutrophils, may number 8.8×10^9 cells per kg (Athens et al., 1961). Some doubt has been cast on the figure given above for the half-life in humans, other workers suggesting, using a ^3H-thymidine label, a figure of 17 hours (Steinbach et al., 1979).

One function of neutrophils is the clearance of bacteria and other infective organisms. In order to do this effectively it must be capable of recognising the presence of such organisms, moving quickly towards the source of infection, and disposing of the organism with the minimum of damage to healthy tissue. The neutrophil is usually the first host cell to arrive at a site of acute inflammation. The first observed reaction by neutrophils to an inflammatory event in vivo is an increase in neutrophil adherence to the endothelial wall, known as margination or pavmenting. It has been suggested that margination and

aggregation of neutrophils is initiated by chemotactic factors (MacGregor, 1980), but while likely this has not been rigorously proved *in vivo*. Lackie & Smith (1980), and Harlan (1985), have reviewed neutrophil-endothelial interactions.

Chemotaxis has been defined as: 'the directed movement of a cell (or organism) in response to a chemical substance in the environment, usually a diffusible substance' (Lackie, 1986). Keller *et al.*, (1977) have proposed definitions to cover cell locomotion. Several substances are known to be chemotactic for neutrophils, including components of the complement system; C3a, C5a, and its degradation product $C5a_{desarg}$.

Bacterial products were early shown to be chemotactic for neutrophils (Leber, 1888): subsequently, a group of synthesised biochemical compounds structurally related to bacterial products have been made, most notably the small peptide N-formyl-methionyl-leucyl-phenylalanine (f-MLP), (Schiffmann *et al.*, 1975).

Neutrophils respond to chemotactic factors by becoming morphologically polarised (Zigmond *et al.*, 1981; Davis *et al.*, 1982). This polarisation, it has been suggested, is controlled by the direction of waves of equatorial contraction, a process which corresponds to the well-known morphological feature known as the constriction ring (Haston & Shields, 1984). The polarity gives to the cell an asymmetry, with distinct anterior and posterior ends. There is evidence to suggest that a neutrophil can detect

gradients of a chemotactic substance as shallow as 1% from the front to the back of the cell. This directed response to gradients of chemoattractants could determine the directed locomotion of neutrophils (Zigmond, 1974).

Once the neutrophil reaches the site of inflammation it interacts with microorganisms present. The neutrophil has been shown to have Fc and C3b receptors, and will adhere to particles opsonised with IgG antibodies and complement components using these receptors. Attachment to the microorganisms is followed by phagocytosis, the bacteria being engulfed into an internal phagosome. The function of the granules was revealed by Hirsch & Cohn (1960). Following phagocytosis of bacteria by a neutrophil, the granules fuse with the internal phagosome, killing the bacteria. The fusion process was confirmed by electron microscopy (Zucker-Franklin & Hirsch, 1964), while Bainton (1973) demonstrated that the specific granules fused with phagosomes first, beginning about 30 seconds following phagocytosis. The primary, or azurophil granules begin to fuse after about 3 minutes, and then usually only into the larger phagosomes, suggesting some selective process at work.

Uptake of particles is also associated with a phenomenon known as the respiratory burst, the neutrophil exhibiting a marked increase in oxygen consumption and increased glucose metabolism via the hexose monophosphate shunt (Davis & Gallin, 1981). As part of the respiratory burst reactive oxygen metabolites are secreted; the superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen.

These oxygen metabolites are toxic for many organisms, including bacteria and parasites. They will also attack tumour cells (reviewed by Klebanoff & Clark, 1978). In certain circumstances normal tissues of the body may come under attack, as the oxygen metabolites are probably able to inactivate α1-antitrypsin and other protease inhibitors, thus allowing injury to the tissues under invasion (Janoff & Carp, 1982).

The respiratory burst in neutrophils is associated with a low level of cellular light release. This emission of light is due to the release of photons as an excited oxygen product returns to the ground state (DeChatelet et al., 1982; Briheim et al., 1984; Campbell, 1986). The level of chemiluminescence may involve the release of less than one photon per cell per second, and to be useful as a diagnostic tool it requires to be supplemented by the addition of synthetic compounds such as luminol or luciginen, which can increase the light emission by up to 10,000 times. In this way, the emission from as low a number as 5-10 cells may be measured, making the chemiluminescence assay a very sensitive indication of phagocytosis, and hence allowing some measurement of that cellular function.

This thesis is concerned with the motile behaviour of neutrophils, particularly their movement through tissue, and the next section will concentrate on these aspects.

1.2 Cell invasion

The phenomenon of intercellular invasion, 'the movement by a population of cells into an area already occupied by another population', (Abercrombie, 1979), is found in several situations in the vertebrate body. In all multicellular animals, early development in the embryo typically involves the movement of individual cells, groups of cells and entire tissues. The ultimate destination of these translocating cells is controlled by a variety of processes, influencing such factors as the onset of movement at a precise stage of development, the route taken by the moving cells, and the final destination relative to their surrounding tissues, which may be a considerable distance away from their origin (Trinkaus, 1976; 1984).

The process of cell invasion involves many complex factors, only some of which are beginning to be understood. What, for example, starts the active migration of a cell or group of cells? In some of the developmental systems, population pressures may initiate outward movement of cells. These cells now have to be guided to their destination, and there are numerous observations which would suggest that contact guidance plays a large part in this. (See Dunn, 1982 for a review.) Another control mechanism of importance is contact inhibition of movement, which for most cell types tends to prevent migration (Abercrombie, 1970). Chemotaxis as a control mechanism for neutrophil locomotion in particular has been widely studied (Wilkinson & Allan, 1978; Zigmond, 1978; Snyderman & Goetzl, 1981). The chemotaxis shown by neutrophils presumably directs their movement towards sites

of injury or infection, though it should be borne in mind that this is particularly difficult to prove *in vivo* (Lackie, 1986).

One 'long distance' movement of cells which has been intensively studied in the developing vertebrate embryo, and particularly in amphibians, is the migration of neural crest cells. (Armstrong, 1984). Another instance of cell migration over an appreciable distance in the developing embryo is that of the primordial germ cells. In vertebrates, these cells originate outside the gonad and migrate into it only secondarily, often by a vascular route (Ebert & Sussex, 1970; Heasman *et al.*, 1977; Heasman & Wylie, 1978).

Invasive behaviour of cells is to be found in systems outwith those of early development. Malignant tumours are often differentiated from benign tumours by the ability of the former to disseminate in the body by invasion and metastasis, the establishment of secondary tumours at sites distant to the parent tumour. The first stages of metastasis, the entry of tumour cells into the blood or lymph vessels, may involve invasion (Tickle *et al.*, 1978). Following attachment to the vascular endothelium, tumour cells undergo extravasation, invading the vascular endothelium and the underlying basement membrane (Kramer & Nicolson, 1979). The way in which tumour cells, neutrophils and other invasive cells pass through endothelia and basement membranes has been the subject of much investigation, with several possible mechanisms being suggested. These mechanisms include enzymic action by the

invading cells (Matzner et al., 1985; Levine et al., 1984), and movement through the endothelial wall by the extension of pseudopodia (Faustmann & Dermietzel, 1985; Beesley et al., 1979).

Schwann cells have been observed migrating in vivo, periods of visible activity involving the protrusion and growth of several pseudopodia, the attachment of these processes to an axonal surface, and finally the movement forward of the entire cell (Billings-Gagliardi, 1977). Fibroblasts and endothelial cells invade fibrin thrombi during the course of wound healing in vascularised tissue. (James & Taylor, 1969; Abercrombie, 1970; Harris et al., 1981).

The motile blood cells of vertebrates, including the leucocytes, lymphocytes, monocytes and macrophages can all, in certain circumstances, engage in invasive behaviour. Motile haemocytes have been described in insects such as the Orthoptera (Arnold, 1961). In the horseshoe crab Limulus polyphemus, an amoebocyte is the sole cell type found in the coelomic fluid. This amoebocyte can be stimulated to become motile, moving by the use of pseudopods and an extensive cytoplasmic flow. In this regard, the Limulus amoebocyte resembles the vertebrate neutrophil (Armstrong, 1979). In the vertebrates, invasive behaviour involves an active migration through the vascular endothelium, and a continuing migration through the underlying basement membrane and extracellular matrix (ECM).

Invasive behaviour is manifested during the inflammatory response, with leucocytes, monocytes and lymphocytes migrating principally from the post-capillary venules. Earlier in development, these blood cells have had to undergo a migration following haematopoiesis, leaving an extravascular compartment in the bone marrow and crossing the sinusoid endothelium to enter the vascular spaces of the marrow (De Bruyn et al., 1977). There also exists the phenomenon known as lymphocyte traffic, in which the small lymphocytes cross the wall of the high endothelial venule, migrate through the tissue of the lymph node, and finally re-cross the endothelium of the lymphatic sinuses (Ford & Gowans, 1969).

At the onset of an acute inflammatory response, neutrophils in the circulation adhere to the walls of the microcirculation in great numbers. It would seem likely that neutrophils circulate until small changes in the adhesive force between the cells and the endothelium are sufficient to overcome the wall shear stress in the blood vessels. Neutrophils will then begin to marginate (Lackie & Smith, 1980). The neutrophils then migrate across the endothelium (a process also known as diapedesis). The mode whereby they leave the circulation and cross the endothelium has for long been the subject of debate.

Examining leucocyte emigration during acute inflammation, Marchesi (1961) described the route taken by neutrophils moving through the endothelium. After flattening against the vessel wall, the neutrophils extended pseudopods through the intercellular junctions of the endothelial

cells. These pseudopods, as described by Marchesi, were sometimes of great length, and were followed by the main body of the leucocyte. Serial sections using electron micrography have confirmed that the route of emigration is intercellular and that the cell membranes of the neutrophils and endothelial cells are at all times separate and distinct (Marchesi & Florey, 1960).

This route of neutrophil migration through the endothelial wall was confirmed by Shaw (1980), using a lung inflammation model in rabbits. After adhering to the endothelium, leucocytes appeared to migrate to a subendothelial position by being enveloped by endothelial cytoplasmic extensions. The leucocytes extended a pseudopod through the endothelium at an intercellular junction, and the leucocyte then migrated between two separate endothelial cells. The endothelial cell junction appeared to reappose behind the migrating leucocyte.

As the observation of invasive behaviour is obviously difficult *in vivo*, several experimental *in vitro* models have been set up. Some of these *in vitro* methods involve cultured monolayers of endothelium (Beesley *et al.*, 1979), while other models have used a living amnion membrane, consisting of a single layer of epithelium bound to a continuous basement membrane interfacing an avascular collagenous stroma (Russo *et al.*, 1981). One of the most widely used models for infiltration of connective tissue uses gels made from reconstituted collagen (Elsdale & Bard, 1972). These gels, formed of hydrated collagen lattices, constitute a matrix similar to normal connective tissue. Their transparent

nature allows light microscopy of cells moving within the three-dimensional gel, and obviously several parameters can be varied so as to examine further the locomotory behaviour of cells in a three-dimensional matrix (Grinnell & Bennett, 1981; Brown, 1982; Islam et al., 1985).

The migration of leucocytes through endothelial cell layers has been conveniently studied by the use of cultured monolayers (Lackie & DeBono, 1977; Beesley et al., 1979; Russo et al., 1981; Evans et al., 1983; Cramer et al., 1986; Furie et al., 1987). Beesley et al., (1979) cultured porcine endothelial cells on glass coverslips then added neutrophils for 30 minutes before washing and fixing for microscopy. Essentially, neutrophils were found to adhere to endothelial cells, initially by microvillous processes, without spreading. A substantial proportion of adherent cells then migrated between the endothelial cells, the process taking between 15 and 30 seconds for complete passage of the monolayer.

Russo et al. (1981), examined the in vitro migration of human neutrophils through human amnion membrane. The membranes, consisting of a single layer of epithelium bound to a continuous basement membrane overlying an avascular collagenous stroma, were placed in plastic chambers over a 5 μm Millipore filter, stromal side against the filter. Neutrophils were introduced to the upper chamber and f-MLP in the lower chamber was used as a chemoattractant. The route of neutrophil migration was studied by transmission electron microscopy. This system models more closely than any previous one the in vivo state, where neutrophils, in

response to an inflammatory state, have to leave the vascular system and migrate both through the endothelium and through the underlying basement membrane. Russo et al., (1981) found that neutrophils placed on the epithelial layer of the amnion first aggregated and adhered over intercellular junctions. After extending pseudopods between adjacent epithelial cells the neutrophils appeared to migrate in groups around or through desmosomes.

A similar in vitro system, employing MDCK epithelial cells grown as a monolayer on a Millipore filter was used by Evans et al., (1983). They observed that rat peritoneal exudate neutrophils penetrated the epithelial monolayers by an intercellular route, with a consequent reversible dissolution of the junctional complex. Evans et al., (1983) modified this assay by incorporating collagen into the matrix of the Millipore filter, thus modelling more closely the in vivo situation. They were uncertain, however, as to whether the migrating neutrophils digested the collagen or pushed it aside.

The transepithelial migration of human neutrophils was investigated by Milks et al., (1983), using an in vitro model with MDCK cells cultured on Millipore filters. Neutrophils, migrating through the epithelium towards a source of f-MLP as a chemoattractant, crossed by a transcellular route, via the intercellular junctions. The junctions, following neutrophil passage, reapproximated, their resealing being shown to be complete by their being impervious to the ultrastructural tracers horseradish peroxidase and lanthanum nitrate. Additionally, the

permeability of the epithelium was correlated with neutrophil passage, measurements being made by conductance and by the use of the tracers mentioned above. Milks et al., (1983), found that the number of neutrophil invasion sites and the number of migrating cells were a function of the epithelial conductance.

Cramer et al., (1986) examined the effect of human serum and some of its components on the process of transepithelial migration of human neutrophils. Using an in vitro model with a monolayer of MDCK cells grown on micropore filters (Cramer et al., 1980), they were able to measure the transepithelial electrical resistance, a measure of epithelial permeability. With f-MLP as a chemoattractant in the lower chamber and 10% autologous serum in both chambers, there was a significant increase in adhesion to, and migration across, the monolayer. This contrasted with the situation in which serum was absent from both chambers and f-MLP was present in the lower chamber.

Cramer et al., (1986) concluded that a separate heat-stable factor(s) in serum was able to increase neutrophil migration across the epithelial monolayer. They hypothesised that this factor or factors, which increased the permeability of the epithelium (as measured by electrical resistance), could be responsible for the increase in neutrophil migration, through its permeability-increasing properties. This factor, they thought, worked by

opening epithelial-occluding junctions, thereby increasing the number of sites at which neutrophils could migrate (Cramer et al., 1986).

Two basic assay systems are employed to detect and measure chemotaxis, one measuring changes in the distribution of a population of cells, the other analysing the movement of individual cells (Zigmond, 1978). Population distribution can be measured by means of a Boyden chamber. In this technique, neutrophils are added to an upper cell chamber, separated from the lower chamber by a micropore filter. The test substance is contained in the lower chamber. After incubation, normally 2-3 hours, the filters are removed, fixed and stained, and the cell distribution in the filter determined (Boyden, 1962). Migration into the filter can be quantified either by counting the cells which have migrated predetermined distances, or by the leading front method, in which the cell 'front' in the filter is the maximum depth at which two cells are in focus (Zigmond & Hirsch, 1973). The position of the cell front, as measured by this latter method, was found to be a valid index of the invasive behaviour of the total cell population.

One note of caution must be raised about the Boyden chamber assay. In most circumstances it is difficult or impossible to distinguish between effects on locomotion (chemokinesis) and effects on chemotaxis. One way round this is to place the test substance on both sides of the chamber. Zigmond & Hirsch (1973) took this further, using different concentrations of the test substance, then defining chemotaxis in assays in which cells moved more rapidly into

the filter up a gradient of the substance than they would have done had the effect been on locomotion alone.

1.3 Collagen and collagen gels

Once invasive cells have left the microcirculation and crossed the endothelium, they are usually faced with the underlying extracellular matrix, including a basal lamina. Much interest has been aroused as to the mechanism by which the invading cells then cross this barrier. There is evidence to suggest that proteases are important for invasion and, perhaps more tentatively, for the ongoing process of locomotion.

1.3.1 Nature of Connective Tissues

Skeletal and other connective tissues consist of cells dispersed throughout an extracellular matrix secreted by resident cells, mainly collagen fibres, water, and small ions. The other main class of macromolecules found in the extracellular matrix besides the collagens are the polysaccharide glycosaminoglycans (GAGS, formerly known as mucopolysaccharides), usually covalently linked to form proteoglycans. One GAG which is not covalently linked to proteoglycans is hyaluronic acid, which is found as a single and very long carbohydrate chain of several thousand sugar residues.

Hyaluronic acid is produced in large amounts in tissues undergoing development or wound repair, with a concomitant cell migration, and there is some evidence that it may be associated with cell migration, its degradation by

hyaluronidase being associated with a cessation in cell migration (Trelstad et al., 1974; Toole, 1976: Rees, 1977; Lindhal & Hook, 1978; Chakrabarti & Park, 1980; Roden, 1980). In tendon, the tissue with the highest collagen content, collagen forms about 50% of the fresh weight, the remainder being mainly water. The function of collagen is to provide a strong but flexible framework for the cells and tissues of the organism. The derivation of the word collagen itself is instructive, coming from the Latin colla-gen, or gluemaker (Woodhead-Galloway, 1980).

There are ten distinct collagens described at present, closely related but differing genetically, chemically, and immunologically (Martin et al., 1985). Type I collagen, the first to be identified, is also the most abundant, being a major structural component in skin, bone, tendon and other fibrous tissues (Bornstein & Sage, 1980). Collagen gels used for in vitro work normally consist of Type I collagen, prepared from reconstituted rat tail tendon. Type IV collagen forms basement membranes (Chandrakasan et al., 1976).

Most cells in culture synthesise collagen, exceptions being lymphocytes, reticulocytes and other blood-borne cells (Green et al., 1966; Goldberg & Green, 1969; Langness & Udenfriend, 1974). The amount of collagen produced varies with the cell type. Most fibroblasts produce 5-10% of their total protein as collagen types I and III. Epithelial and endothelial cells produce type IV, consistent with the role these cells play in the production of the basement membrane (Kleinman et al., 1981). The collagen found in the

endoplasmic reticulum and Golgi vacuoles of fibroblasts is the precursor procollagen which can be secreted into the extracellular medium along with procollagen proteases. There the procollagen is cleaved, allowing polymerisation to occur (Olsen & Prockop, 1974; Prockop *et al.*, 1976; Chandrasekhar *et al.*, 1984). Viidik & Vuust (1980), and Hay (1981a; 1981b) have reviewed the structure and biosynthesis of collagen.

1.3.2 Leucocytes investigated *in vivo*

Leucocytes have been observed to cross the basement membrane (Shaw, 1980; Russo *et al.*, 1981; Faustmann & Dermietzel, 1985). The latter workers described neutrophil passage through the periendothelial basal lamina in the cat as resembling the endothelial migration. The leucocytes inserted pseudopodia into the basal lamina then penetrated that layer to enter the surrounding connective tissue (Faustmann & Dermietzel, 1985). Shaw (1980) observed leucocytes entering interstitial space by what he described as small channels in the vascular basement membrane. Dissolution of the basement membrane, such as described by Russo *et al.*, (1981), and Cochrane & Aikin (1966), was not seen by Shaw (1980).

The basement membrane can be damaged by polymorphonuclear leucocytes. Cochrane & Aikin (1966) showed the disrupting factors in the neutrophils to be cathepsins D and E. Neutrophils in the process of phagocytosing immune complexes release the proteolytic enzymes as well as certain cationic proteins. The cathepsins attack the

vascular basement membrane with a consequent loss of integrity of the vessel wall (Cochrane & Aikin, 1966).

Vissers et al., (1984) looked at the effect of human neutrophils on glomerular basement membranes, prepared from normal human kidney. When IgG aggregates were generated in situ by heating the membrane impregnated with IgG to 63°C, neutrophils adhered to the basement membrane surface. Electron micrographs also showed phagocytosis of smaller fragments. In the absence of IgG aggregates, Vissers et al., (1984) reported, neutrophils did not seem to associate strongly with the basement membrane.

Severe microvascular injury in rabbit dermis was induced in a study by Movat & Wasi (1985). The aim was to assess the damage caused by the injections of lysosomal products from stimulated neutrophils. Human neutrophils were stimulated to release their lysosomal contents by exposure to immune complex in the presence of cytochalasin B. Following incubation of the neutrophils with complex, the supernatant was injected intradermally. Movat & Wasi (1985) found the lysis and disappearance of vascular basement membrane to be a frequent occurrence, adding that the mild accumulation of neutrophils at the lesion sites did not contribute significantly to the microvascular injury. Ohlsson & Olsson (1977) have reported that human neutrophils, on incubation with immune complexes or yeast cells, in the presence of 10% fresh serum, release 25-30% of both their elastase (azurophil granules), and their collagenase (specific granules), as well as other lysosomal enzymes.

1.3.3 Role of hydrolytic/degradative enzymes in migration

Matzner et al., (1985) showed that neutrophils degrade heparan sulphate proteoglycans in the subendothelial extracellular matrix. Degradation was inhibited by heparin but not by protease inhibitors, and Matzner et al., (1985) concluded that neutrophils were releasing a heparanase. Mainardi et al., (1980) demonstrated that elastase purified from neutrophils degraded human type III collagen but not human type I collagen. These collagen reaction products were identical with those generated by human rheumatoid synovial collagenase, and they suggested that in pathologic conditions, neutrophil elastase can selectively deplete type III collagen from the extracellular matrix (Mainardi et al., 1980). (See Appendix F).

The effect of various proteases on the degradation of normal human skin was the subject of a study by Briggaman et al., (1984). Two neutrophil proteases, cathepsin G, and elastase, were examined, as well as a human skin chymotrypsin-like protease. All of these proteases produced damage and separation at the epidermal-dermal junction. Elastase in particular produced extensive degradation of the epidermal-dermal junction (Briggaman et al., 1984).

Weiss et al. (1986) examined the ability of human neutrophils to degrade the subendothelial matrix, employing neutrophils both from normal subjects and from patients with chronic granulomatous disease. In classic cases of this disease, phagocytic cells contain their normal complement of lysosomal enzymes, but are unable to generate

oxygen metabolites (Baehner et al., 1969; Tauber et al., 1983). Weiss et al., (1986) found that when normal neutrophils were triggered in vitro , in the presence of an excess of the plasma antiproteinase, α-1-protease inhibitor, they maintained their ability to degrade the matrix. The neutrophils in this case were shielding their released elastase from inactivation by using the chlorinated oxidants hypochlorous acid and endogenous N-chloroamines to suppress the activity of the antiprotease.

Neutrophils from patients with chronic granulomatous disease, also perhaps surprisingly, were able to degrade the subendothelial matrix to a comparable extent in the presence of excess α-1-protease inhibitor. Weiss et al., (1986) concluded that in the absence of chlorinated oxidants, both normal and chronic granulomatous disease neutrophils can continue to effect matrix degradation.

Cells other than leucocytes have the ability to degrade the extracellular matrix, including certain malignant cells (Roblin et al., 1975; Quigley, 1979; Kleinerman & Liotta, 1977; Kramer & Nicolson, 1979; Sherbet, 1980; Thorgeirsson et al., 1982, 1984; Mignatti et al., 1986). Spontaneously metastatic B16 melanoma sublines have been found to exhibit significant levels of type IV collagen-degrading activity (Liotta et al., 1980; Levine et al., 1984).

Mignatti et al., (1986) examined the role of proteases in tumour invasion, the cells used being a clone of B16 cells, BL6, grown on human amniotic membrane. Cells were labelled with ^{125}Iodine, the amount of invasion being measured by

the radioactivity associated with the basement membrane following the removal of cells on the surface. Mignatti et al., (1986) studied the effects of the various metallo-, serine-, and cysteine-protease inhibitors. Preadsorbing 1-10 phenanthroline, a tissue inhibitor of metallo-proteases, on to the basement membrane, completely prevented invasion, as did preadsorbing an anti-human skin fibroblast collagenase antiserum.

It has been suggested that proteolytic activity may assist the locomotion of neutrophils (Dierich et al., 1977; Wilkinson & Bradley, 1981). Dierich et al., 1977 used the chemotactic agent casein in a standard chemotaxis assay, finding that the binding of the casein to a solid substratum (cellulose nitrate filters) was sufficient for its attracting capacity. By analogy with fibroblasts, Dierich et al., (1977) argued, neutrophils would migrate to where there was a greater amount of bound attractive substance. Their results suggested that this was indeed the case, using filters to which there was a non-uniform concentration of bound casein. This movement, they argued, would only be possible if unoccupied receptors could be made available continuously either at the leading edge of the cell, or on the protrusions actively reaching out from the cell body. It was possible, they continued, that proteolytic activity might be involved in freeing these receptors from the bound substance (Dierich et al., 1977).

Wilkinson & Bradley (1981) looked at the effect of the purified amphipathic proteins, α-s-1-casein, β-casein, and

alkali-denatured serum albumin on the release of enzymes from neutrophils. All three proteins induced lysozyme release from the neutrophils in the absence of cytochalasin B. The proteins stimulated the release of neutrophil proteases able to digest casein and denatured albumin extracellularly. The cleavage of membrane-attached protein, especially on solid-phase protein gradients, could allow the neutrophil to detach itself from the substratum and locomote (Wilkinson & Bradley, 1981).

As an alternative mechanism to explain the passage of cells through the basal lamina, Simpson (1980) has proposed that this structure is biologically thixotropic; it has the capacity in certain circumstances to undergo isothermal, reversible gel-sol transformations. As a gel, its state is relatively stable, but reduces its viscosity in any localised region where a critical pressure is exceeded.

1.3.4 Use of collagen gels as an <u>in vitro</u> model

Gels consist of long-chain polymers which are cross-linked. The typical collagen gel, when examined with the scanning electron microscope, is seen as a tangled network of collagen fibres, with a random fibrillar orientation. Critical point drying and glutaraldehyde fixation give the same picture, making it probable that this is a true picture (Heath & Hedlund, 1984; Brown, 1984). The interstices are liquid, and depending on the composition of the gel, it may vary in consistency from a viscous fluid to a fairly rigid solid; typically they are jelly-like. The vitreous humour of the eye is a gel, as is the synovial

fluid of joints. The liquid component of gels allows for the free diffusion of oxygen and nutrients, while the polymer mesh holds the liquid in place (Tanaka, 1978.) The most common source of collagen for such reconstituted gels is from rat-tail tendon.

Commercially available collagen is from bovine skin; Heath & Hedlund (1984) found no appreciable differences in the behaviour of cells using both sources of collagen. The transparency of these reconstituted collagen gels allows observations to be made on the locomotion and invasive behaviour of cells within the gels. Collagen gels containing invasive cells can be fixed for light or electron microscopy, allowing observations on the locomotory parameters of invasive cells in a three-dimensional matrix resembling that found in vivo.

Numerous workers have used collagen gels to study the locomotory behaviour of various cell types: normal fibroblasts and simian virus-transformed rat cells (Elsdale & Bard, 1972); corneal fibroblasts from the chick (Bard & Hay, 1975); Chinese hamster ovary cells (a fibroblastoid cell line), and RPMI-3460 melanoma cells (a tumourigenic cell line from Syrian hamster), (Schor et al., 1980); Schor et al., (1980) were able to compare the gel surface with the gel interior by scanning electron microscopy. They found the fibre organisation within the gel to be slightly looser than on the surface. Though they made no comment on this point, it probably reflects a partial dehydration of the surface layers of the gel, with a concomitant partial shrinking and increase in density of fibres. This is also

suggested by Haston et al., (1982), working with lymphocytes (see below). As far as Schor et al., (1980) could judge using fixed preparations, the invasion of cells into the collagen gel matrix was done with no disruption of the collagen fibres, suggesting that invasion was not accompanied by lytic action.

The use of collagen gels as an experimental tool was continued by Schor et al., (1982). Two cell lines were again used, RPMI-3460 Syrian hamster melanoma, and human fibroblasts. Type I collagen from rat-tail tendon was used to prepare collagen gels and various parameters were changed such as gel concentration. Invasion by the melanoma cells seemed to be directly proportional to initial cell number, while fibroblast migration showed an inverse relationship to initial cell density.

Discussing these results, Schor et al., (1982) thought it reasonable to assume that the inhibition of migration of cells at the higher concentrations of collagen would be due to the higher density of collagen fibre packing, making penetration and/or movement of the invading cells more difficult. This is supported by work on neutrophils invading collagen gels, where invasion is reduced by increasing the concentration of collagen in the gels (Brown, 1982), although Docherty et al., (in preparation) have shown that requirements of fibroblasts are different from those of neutrophils.

Looking at the effect of initial cell numbers on the invasion of gels, Schor et al., (1982) suggested that

melanoma cells released an auto-stimulatory substance. The stimulation of invasion was not due to overcrowding of the cells, as at the densities used a confluent cell layer was not produced. With the fibroblast cells, on the other hand, they suggested that the inhibition of invasion seen with increasing cell numbers might be correlated with fibronectin produced by the cells.

Schor et al., (1983) used collagen gels to study the invasive behaviour of human lymphocytes. In essence, the invasion of lymphocytes appeared similar to that by neutrophils, the lymphocytes moving through the gel in a 'random walk' manner, as determined by examining the distribution of cells in the gel. The results of Schor et al., (1983) confirmed those of Haston et al., (1982), conclusions being that movement of lymphocytes into and through the collagen gel probably involved no collagenolytic activity by the cells. This latter point was arrived at using both scanning electron microscopy and radio-labelled collagen. Regarding the actual penetration of the collagen matrix by the lymphocytes, Schor et al., (1983) agreed with Haston et al., (1982) in concluding that the cells moved into the gel by inserting processes between the randomly disposed collagen fibres.

1.3.5 Neutrophils in collagen gels

Grinnell (1982) made time-lapse films of human neutrophils invading and migrating through hydrated collagen gels. Cells that were tracked for a minimum of five minutes moving through the gel were found to have a migration rate

of from 5 to 8 $\mu m\ min^{-1}$, though as this did not take into account movement in a third dimension, Grinnell considers it to be an underestimate. Collagen fibres observed in the collagen gels seemed to be more phase-dense in the region of the moving neutrophils, an indication, perhaps, that the fibres were under stress. Scanning electron microscopy however, showed no changes in the gel matrices adjacent to cells. Grinnell confirmed the finding by Hoffstein et al., (1981) of the glycocalyx on neutrophil surfaces, and postulated that this glycocalyx might be important in neutrophil adhesion to collagen, though others have indicated that neutrophils have very little adhesion to collagen-coated substrata.

Brown (1982) investigated the locomotion of neutrophils both on collagen-coated glass, and in three-dimensional collagen gels. While cells were unable to locomote over the collagen-coated substratum, they readily invaded the gels. Gels of collagen concentration less than 1 mg ml^{-1} were unstable and very liable to rupture, while at collagen concentrations of over about 2.5 mg ml^{-1} the optical properties of the gel became unsuitable. Scanning electron microscopy showed neutrophils in collagen gels with pseudopodia extended between collagen fibres, though the direction of motion cannot be known in such preparations.

The incorporation of two broad-spectrum plasma anti-proteases, α-1-anti-trypsin, and α-2-macroglobulin into collagen gels, had no effect on neutrophil invasion, suggesting that invasion of these gels by the neutrophils was not dependent on the proteolytic digestion of the

collagen fibres, in addition to which, Brown (1982) noted, scanning electron micrographs showed no apparent changes in the structure of the collagen gels, following neutrophil invasion. Discussing the possible mechanisms whereby neutrophils might invade the collagen gels, Brown (1982) proposed that neutrophils attached to collagen gel surfaces, then locomoted within the gel, by extending pseudopodia into gaps in the matrix which pseudopodia, when dilated, firmly anchored the cell. The cell would then be able to follow the jammed pseudopod with the rest of its body.

One very interesting morphological feature encountered in the movement of neutrophils and lymphocytes is the constriction ring (De Bruyn, 1946; Lewis, 1931; Norberg, 1971; Senda et al., 1975; Brown, 1982, 1984; Grinnell, 1982; Haston et al., 1982; Haston & Shields, 1984). This constriction ring was first reported by Lewis (1931), who observed the movement of lymphocytes in plasma clots. The constriction ring appears to be a narrow band, or waist, of cytoplasm, through which the cell appears to move. De Bruyn (1946) observed the presence of the constriction ring in lymphocytes and neutrophils moving over substrata. Norberg (1971) and Senda et al., (1975) found that moving neutrophils possessed a posteriorly moving wave of contraction, the concave area of which corresponded to the contraction ring. These contraction rings have also been shown to be present in neutrophils in suspension (Keller & Cottier, 1981), and in lymphocytes in suspension (Haston & Shields, 1984).

Senda et al., (1975), in discussing the movement of neutrophils, have remarked on the similarity between the cell's constriction ring and the muscular movements seen in moving Planaria and Lumbricus. This suggested a similar mode of movement in the cells, with a hydrostatic mechanism as a basis. Haston & Shields (1984) supported this theory in discussing the movement of lymphocytes through collagen gels.

Tucker & Erickson (1984) cultured quail neural crest cells in collagen gels containing various macromolecules, known to be encountered in vivo by the cells used in their series of experiments. They found that the crest cells, which readily migrated in the gels, showed a decrease in speed as the concentration of collagen was increased. This is in agreement with several workers (Davis & Trinkaus, 1981; Brown, 1982).

Islam et al., (1985) investigated the chemotactic responses of human neutrophils using collagen and fibrin gels, incorporating f-MLP as an attractant. Using the leading front method for scoring invasion of collagen gels with a uniform distribution of the attractant, a dose-response curve for f-MLP was obtained. The optimum concentration was found to be 10^{-8} M. The distribution of cells through the gel was found to be strongly suggestive of random locomotion by the invading cells, though the number of cells entering the gels was greater when f-MLP was present as a chemokinetic agent, i.e. at a uniform concentration throughout the gel.

Islam et al., (1985) also prepared gels with gradients of f-MLP, showing that the plot of log number of cells against the square of the distance migrated was non-linear. This demonstrated a chemotactic response. (If cells start from a plane surface of a gel and move into the gel 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:

$$N = \frac{a}{s} \left(\exp \left(\frac{-(d - vt)^2}{2s^2} \right) + \exp \left(\frac{-(d + vt)^2}{2s^2} \right) \right)$$

where **N** is the number of cells in focus at a depth **d** in the gel, **a** is a constant proportional to the total number of cells in the gel, **s** is the root-mean-square displacement attributable to random migration alone, **t** is the incubation time and **v** is the velocity of directional migration (Islam et al., 1985).

1.4 Mechanism of cell locomotion in a 3-D matrix

The morphology of migrating neutrophil leucocytes has been described by several workers: (Marchesi & Florey, 1960; Marchesi, 1961; Ford & Gowans, 1969; Shaw, 1980; Brown, 1982; Schmalstieg et al., 1986). Brown (1982) has discussed the locomotion of neutrophils in a three-dimensional matrix of reconstituted collagen gel, arguing that adhesion is of minor importance in the locomotion of these cells through such a matrix, cells moving by a process of pseudopod extension, jamming, and pulling.

Senda et al., (1975) made a particular study of the mechanism of movement of human leucocytes over glass, describing what is obviously the constriction ring of other workers but referring to it as a contraction wave. They noticed that this contraction wave did not move with respect to a fixed point on the substratum, despite the movement of the cell, a point also made by Haston & Shields (1984) who examined the locomotion of lymphocytes in collagen gels, describing the constriction ring as being stationary with respect to the matrix, the locomoting cell appearing to move through the ring in its passage through the matrix.

Further investigating the mechanism of movement of neutrophil leucocytes, Senda et al., (1975) argued that as the motile forms and functions of leucocytes are coordinated and maintained by the intracellular levels of ATP, these cells may have a mechanochemical transfer system similar to muscle contractile protein, and functioning with ATP as an energy source. Haston & Shields (1984) attempted to find a correlation between the cytoskeleton and the constriction ring, staining locomoting cells with a marker for F-actin. There was no obvious correlation, the F-actin of the cells being uniformly distributed throughout the cytoplasm.

Senda et al., (1975) were struck by the similarity between the contraction wave in locomoting leucocytes, and the whole body movements of Aplysia, Planaria, and Lumbricus. In the earthworms, by way of example, locomotion involves neurally controlled waves of muscle contraction which pass

anteriorly along the worm, adding posteriorly to each bulge where the longitudinal muscles are contracted and the setae protruded. The bulges therefore move slowly back, while the body of the earthworm moves forward (Russell-Hunter, 1969). Although the putative arrangement of contractile elements in leucocytes may not be as systematic as in muscular fibres, Senda et al., (1975) felt that they might be arranged so as to exert circular and longitudinal contraction forces, as in these soft-bodied invertebrates When the leucocyte begins to move circular contractions would elongate the forming pseudopod; this would then contract due to longitudinal contraction, firmly jamming the extended pseudopod if in a three-dimensional matrix, as postulated by Brown (1982). It is possible, Senda et al., (1975) concluded, that the motive force in leucocytes could be due to association-dissociation of myosin A and F-actin sited in the superficial layer of the granuloplasm.

In a study of the active deformation of leucocytes, Chien et al., (1984) found that during active deformation the mechanical properties of leucocytes changed markedly in the region of the pseudopod, which became stiffer than the remainder of the cell body. Gelation of actinlike and myosinlike proteins present in the leucocytes, according to Chien et al., (1984), appeared to generate the formation of these pseudopods. In the presence of 2 mM Ca^{2+} the neutrophils had higher viscoelastic coefficients than in Ca^{2+}-free medium, in addition to which the cells formed pseudopods which had a greater resistance than the main cell body to deformation by micropipette aspiration. It has

been shown that neutrophil pseudopods contain actin networks, and that processes of gelation and solation are involved (Stossel, 1982). Oster (1984) has reviewed this model of cell locomotion in greater detail.

Schmid-Schönbein & Skalak (1984) have postulated that polymerisation of the actin matrix is triggered by the influx of Ca^{2+} across specialised regions of the cell membrane, and that polymerisation occurs on an interface at the base of the pseudopod where it connects to the cell. Considering the active motion of leucocytes, Skalak (1984) pointed out one important feature of pseudopods, namely, that they include no granules, nuclei or other structures. This feature, he remarked, could help support the gelation theory of pseudopod formation. The details of this myosin-actin interaction, however, remain to be fully elucidated on a molecular level (Schmid-Schönbein & Skalak, 1984).

Locomotory mechanisms have been studied in other cells: for example amoeboid haemocytes in the giant cockroach Blaberus giganteus (Arnold, 1961); mesenchymal cells in the avian cornea (Bard et al., 1975); Schwann cells in Xenopus tadpole tail (Billings-Gagliardi, 1977); primordial germ cells in Xenopus (Heasman et al., 1977; Heasman & Wylie, 1978); amoebocytes in Limulus (Armstrong, 1979); mesenchymal cells in the killifish Aphyosemion scheeli (Wood & Thorogood, 1984). The latter study concentrated on the role of contact guidance in cell behaviour, in which migrating cells use cues from their immediate surroundings in order to be guided towards their ultimate destinations. Wilkinson & Lackie (1983) looked at the role of contact

guidance on chemotaxis of human neutrophil leucocytes, using aligned 3-D collagen or fibrin gels and concluded that tissue architecture might have a significant effect on migration during an inflammatory reaction. Contact guidance in the control of cell behaviour has also been studied by Curtis & Varde (1964), and reviewed by Dunn (1982).

1.5 Pentoxifylline and cell locomotion

The use of pentoxifylline, a methyl xanthine, for the treatment of peripheral vascular disorders such as intermittent claudication (Reich et al., 1984; Roekaerts & Deleers, 1984), cerebral insufficiency (Koppenhagen et al., 1977; Harwart, 1979), and leg ulcers (Weitgasser, 1983), has led to interest in the mechanism of action of the substance. Müller (1983) has reviewed its potential and actual therapeutic properties with regards to modifying the disturbed flow properties of blood. It increases peripheral circulation, reduces blood viscosity, and elevates rat erythrocyte ATP levels (Stefanovich, 1978).

Pentoxifylline increases the penetration of neutrophils through 5μm Nuclepore filters, Schmalzer & Chien (1984) suggesting that the most likely cause of this effect was a change in the cell cytoplasm. They also found that pentoxifylline treated neutrophils lacked protopods (pseudopods), and as protopods have been found to be stiffer than the rest of the cell (Schmid-Schönbein et al., 1981), they thought that the removal or reduction in protopod formation by pentoxifylline accounted for the increased penetration. This study confirmed the work on

erythrocyte passage through Nuclepore filters, done by Ehrly (1978), Isogai et al., (1981), and Dormandy et al., (1981).

Sheetz et al., (1984) demonstrated that pentoxifylline increases rabbit peritoneal neutrophil motility through a rigid matrix, in this case a standard Boyden chamber system. Movement of the cells was enhanced at low concentrations of pentoxifylline (0.014 mg ml^{-1}). This enhancement occurred both in the presence and absence of f-MLP, a known chemoattractant, and knowing from related studies that pentoxifylline was not chemotactic for neutrophils, they suggested that the effect of the drug was on cell motility. (See Appendix E).

1.6 Immune Complexes and cell behaviour

Neutrophils emigrate from blood vessels, most often in response to a chemotactic signal originating from an area of inflammation. It is likely that complement activation provides the strongest signal for neutrophils, with C5a being generated either at bacterial surfaces, or via products released from tissue cells by either classical or alternative pathway activation (Wilkinson, 1981). Complement activation however, which may lead to release of enzymes by the neutrophils, may be unnecessary in cases where there is minimal injury to tissue. In such a case, recognition by neutrophils without a potentially damaging release of enzyme may be a more appropriate reaction, and there is evidence that neutrophils show an antigen-specific stimulation of locomotion and chemotaxis (Jensen &

Esquenazi, 1975; Wilkinson et al., 1977).

Neutrophils have receptors for the Fc portion of IgG (Alexander et al., 1979): receptor-ligand interactions might therefore trap cells where IgG is immobilised (Wilkinson et al., 1984). Under conditions of flow, immune complexes (IC) proved to be negatively chemokinetic in comparison with albumin coated surfaces, neutrophils moving more slowly over the IC coated surface. Neutrophils moving on an IC lost their ability to form rosettes with IgG-coated erythrocytes, probably because of redistribution of their Fc receptors to the under surface, the resulting tethering of neutrophils by substratum-bound IgG-Fc showing in a reduction in speed (Wilkinson et al., 1984). Wilkinson (1980) had previously shown that although native IgG was not chemotactic for neutrophils, heat-denatured IgG was, suggesting that heating the IgG molecule caused a conformational change, allowing neutrophils to recognise the altered molecule and undergo a chemotactic response.

Soluble IC have been shown to be chemotactic for neutrophils (Hawkins & Peeters, 1971; Leung-Tack et al., 1977; Weiss & Ward, 1982). Using radiolabelled antigen (BSA) and rabbit antiserum to the BSA, Weiss & Ward (1982) studied the effect of various ratios of antigen:antibody mixes on human neutrophils. They found that complexes prepared close to equivalence were the most extensively internalised by the neutrophils. With increasing antigen, this internalisation fell off, though at an antigen excess of 2 to 4 times equivalence, enzyme release by the neutrophils was almost as high as that found at

equivalence.

Weiss & Ward (1982) also found that neutrophil production of oxygen metabolites O^{2-} and H_2O_2 were maximal when antigen was in excess, confirming the chemiluminescence study on neutrophils by Starkebaum et al., (1981). Their conclusions were that the larger IC fix complement, which is important in attracting and activating neutrophils. The small IC, containing antigen excess, may lead to a more intense production of O^{2-} and H_2O_2.

Leung-Tack et al., (1977) showed that although small, soluble IC, formed in conditions of antigen excess, induced little or no complement fixation, they were chemotactic for neutrophils. This had been previously suggested by Boyden (1962). Hawkins & Peeters (1971) had shown that IC, formed both at equivalence and in antigen excess of some 20 times equivalence, were effective at inducing release of enzymes from neutrophils incubated in vitro in the presence of the IC.

Chemotactic substances such as f-MLP have been incorporated into collagen gels (Islam et al., 1985), though detailed descriptions of the use of collagen gels for assaying neutrophil chemotaxis had not been previously described. The use of immune complexes in collagen gels has not been described in the literature to date; its obvious relevance to the in vivo situation would make it an interesting and attractive in vitro model.

This thesis sets out further studies on the locomotion of neutrophil leucocytes, using three-dimensional collagen gel

matrices as an *in vitro* model for neutrophil invasion and migration. The effects of invading cells on the gel matrix are investigated, while the use of immune complexes in conjunction with collagen gels to modify cell invasion is described.

Chapter 2 Materials & Methods

2.1 Cells

2.1.1 Isolation of Neutrophil Leucocytes:

Rabbit peritoneal neutrophil leucocytes were obtained from female New Zealand White animals. A sterile suspension of 0.1% (w/v) oyster glycogen (Sigma) in 500 ml of 0.9% (w/v) NaCl was injected intraperitoneally. Four hours later the peritoneal exudate was collected by the same route (Lackie, 1977). The exudate was filtered through gauze to remove fibrin clots, if necessary, and stored in plastic containers at 4°C for use within 3 d.

Before use, the cells were washed once in HS (Calcium- and Magnesium-salts free medium; see Appendix A for recipe) then resuspended in H2 at the desired concentration. Any contaminating erythrocytes were removed following the first wash by hypotonic lysis: 0.5 ml distilled water for 30 s. Cell suspensions so prepared contained >95% neutrophils, while cell viability was >95%, as determined by Trypan Blue dye exclusion, dead cells being stained. Normally, some 2-300 ml of exudate fluid, containing 2-6 x 10^6 ml^{-1} were recovered from each animal. Rabbits were allowed four weeks to recover between procedures.

Human neutrophil leucocytes were isolated from peripheral venous blood samples taken from normal, healthy volunteers. Some 20 ml of whole blood was allowed to settle in a plastic universal bottle with heparin (10 U ml^{-1} blood, Evans Medical Ltd., Middlesex), and 2 ml Dextran 110 (Fisons plc, Loughborough) at room temperature or at 37°C

for 40-60 min. The supernatant was gently layered by pipette over an equal volume of Ficoll-Hypaque (Pharmacia, Uppsala), then centrifuged (Boyum, 1968). The cell pellet was washed in HS, and contaminating erythrocytes removed by hypotonic lysis, and resuspended in H2 at the desired concentration.

2.1.2 ^{51}Chromate labelling of neutrophils:

For labelling with ^{51}Chromate, neutrophils were prepared as above and the cell pellet resuspended in 1 ml of 0.5% w/v Bovine Serum Albumin (BSA) in H2 and 4 MBq of $Na_2{}^{51}CrO_4$ activity added. Cells were incubated for 30-45 m at 37°C, washed three times in H2 to remove BSA and free ^{51}Chromate. then resuspended in H2 at a concentration of 1 x 10^6 ml^{-1}. Typically, cells were labelled with 30-40 x 10^3 cpm 10^6 $cells^{-1}$.

2.2 Collagen

2.2.1 Isolation of Collagen:

Type I collagen was purified from rat tail tendons (Schor, 1980). Isolated tendons were solubilised in 3% v/v acetic acid for 2 d at 4°C. Insoluble material was removed by centrifugation at 3000 g for 30 m and the dissolved tropocollagen precipitated by mixing with an equal volume of 20% (w/v) NaCl. The collagen was pelleted by centrifugation at 3000 g for 45 m, washed twice in distilled water, then resuspended in 3% (v/v) acetic acid at a final concentration of 3 mg ml^{-1}. The concentration was best calculated by freeze-drying a known volume of the

solute and weighing the residue.

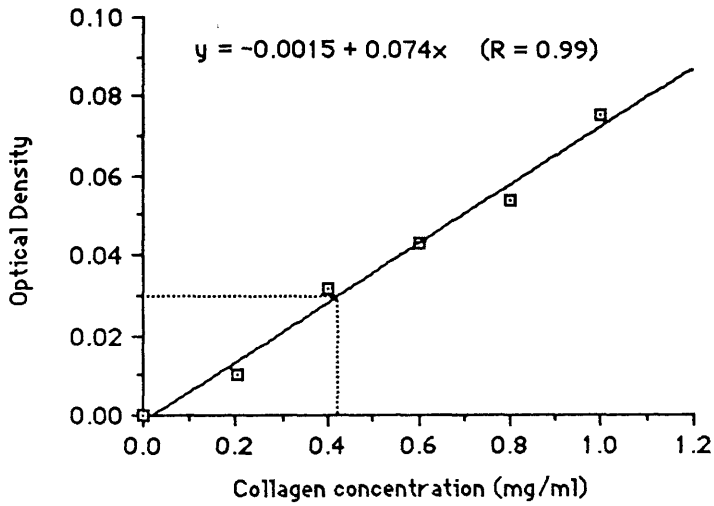
Optical densitometry with a collagen solution was found to be unreliable, frequently giving a density figure too high, presumably due to either fibrous collagen or some contaminating matter. See Figure 2.1 for one typical calibration plot, which indicates the collagen stock sample as being about 8 mg ml^{-1}, whereas in fact it is closer to 4 mg ml^{-1}, a normal density following the above preparation method.

The collagen was dialysed exhaustively against distilled water adjusted to pH 4.0 with HCl, divided into aliquots of about 4 ml. and stored at -20°C before use. Collagen, even when stored at this temperature, begins to show changes in properties after 3-4 months, and is then unsuitable for the production of stable gels. (Islam et al., 1985, reported a similar effect). New batches of collagen were therefore prepared at appropriate intervals.

Commercially prepared Type I collagen, derived from pepsin-digested bovine dermis, was used in some experiments (Vitrogen 100, Collagen Corporation, Palo Alto, California).

2.2.2 Preparation of tritium-labelled collagen:

Labelled collagen was prepared by the method of Rice & Means (1971). Some 3.5 ml of Vitrogen, 3 mg ml^{-1} stock, was made up to 5.0 ml with 0.2 M borate buffer, pH 9.0. Addition of 80 MBq ^{3}H-formaldehyde (80 μl) was followed by four sequential additions of 0.5 mg Sodium borohydride.



Calibration lines for collagen stock at 1:20 dilution

Fig. 2.1 Calibration of Collagen stock by Optical Densitometry at 230 nm

stirred until dissolved. After an interval of 60 s, 2.5 mg of Sodium borohydride was added. The collagen was then dialysed exhaustively at 5°C against 0.012 N HCl with continuous stirring. Aliquots from the wash were counted until the counts were negligible. Typically, 30–40 x 10^3 cpm 10 μl^{-1} of labelled collagen were obtained. The Vitrogen was mixed with rat tail collagen before use. Figure 2.2 shows the release of labelled fragments of collagen into the medium above a newly made gel. Samples were taken at intervals, with minimum agitation. For assays using tritiated collagen, gels were washed overnight before incubating with cells, to minimise background release.

2.2.3 Rheological Tests on Collagen

Samples of rat tail collagen stock, approximate density 4.0 mg ml^{-1} were forwarded to the Agricultural & Food Research Council Institute at Norwich. Viscosity and other measurements were carried out by Dr G. Brownsey on both undiluted stock, and prepared gels at a concentration of 1 mg ml^{-1}, under conditions of constant rotation; additionally measurements were made using a pulse shearometer (Rank Bros., Bottisham, Cambridgeshire). Gels were set <u>in situ</u> to facilitate measurements. Density measurements were also carried out. The dependence of gel strength on the age of the gel was studied over a period of 8.5 h after plating the collagen solution. Figure 2.3 shows the dependence of collagen gel strength on age. This provides a post-hoc justification for local practice with collagen gels, which are usually allowed one-hour to set

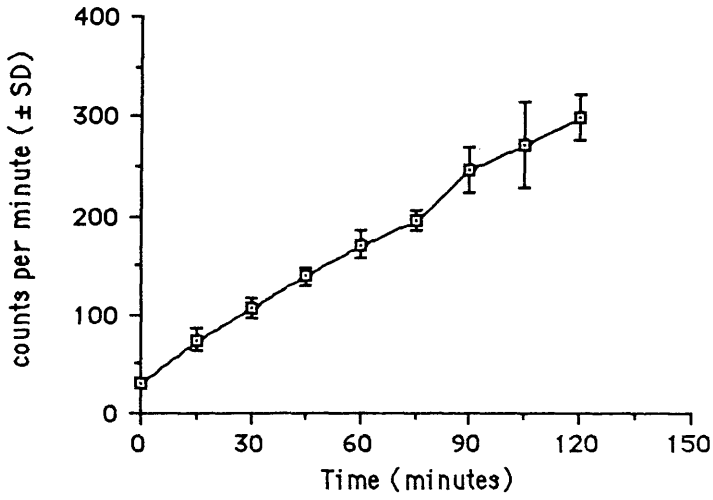


Fig. 2.2 Background release of tritium-labelled collagen fragments from a collagen gel. Counts made from a gel newly plated and in triplicate samples.

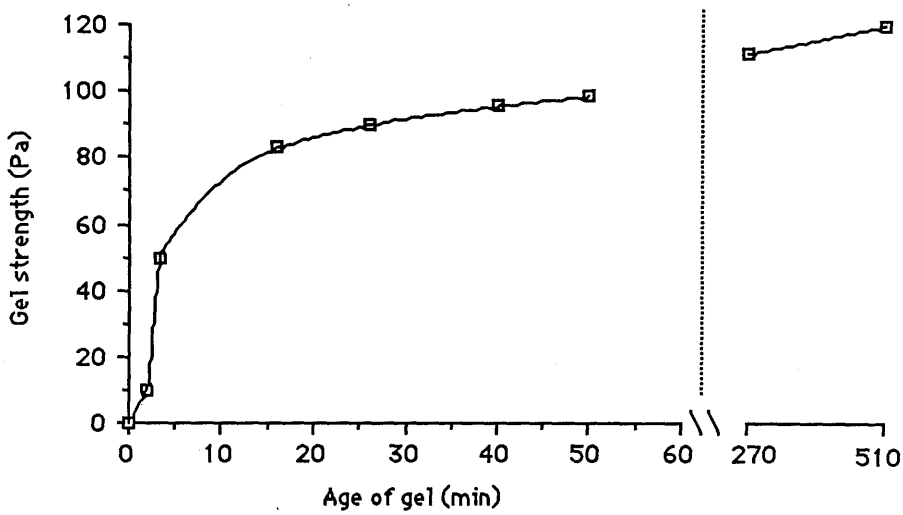


Fig. 2.3 Dependence of collagen gel strength on age; 1 mg/ml gel used and readings taken using pulse shearometer. (See Appendix, p. 182.)

before addition of medium. Appendix C gives more details on the rheological testing of collagen.

2.3 Preparation of Collagen Gels:

Type I rat tail collagen was extracted as described above. Three-dimensional collagen matrices were prepared as described by Brown (1982). The stock collagen, stored sterile at -20°C, was brought to 4°C 24 h before use, in order to thaw. The osmolarity and pH of the collagen solution was adjusted to physiological levels by mixing with 1:9 parts by volume of 10 x BHK21 Glasgow modified medium (Gibco, Paisley), and adjusting the pH by careful addition of NaOH. Final dilution was made by addition of H2. All mixing was done at 4°C. The stirred solution was poured into the appropriate tissue culture dishes and allowed to set at 37°C for 1 h. H2 was then added and the system left to equilibriate at 37°C for 2 h before removing the medium and adding the cell suspension. Collagen gels using Vitrogen (Collagen Corp., Palo Alto, California), were prepared in a similar fashion.

2.4 Statistical Analysis

Student's t (2-tailed) test as described in Campbell (1974) was used to evaluate the significance of differences between the population means in various experiments. This test was used only if the data had been obtained by random sampling from a normal, or approximately normal, distribution, and the variances of the populations did not differ significantly when tested using the F-test. If the variances of the two groups differed significantly, the Mann-Whitney U test was

then used to evaluate any differences between the population means. For testing the distribution of cells across an area in the slab assays described in Chapter 5, a one-way analysis of variance was used. The degree of difference between the means of the control and experimental groups was either not significant ($P > 0.05$), marked NS, or significant at the levels $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, denoted *, **, and *** respectively.

2.5 Assays

2.5.1 Visual Migration Assays (collagen gel invasion):

Type I rat tail collagen was prepared as described. For standard visual migration assays, 24-well tissue culture dishes were used (Nunclon, Kamstrup, Denmark). To each 16 mm diameter well 0.5 ml of collagen solution was added. The collagen formed a gel within about 15 min at 37°C, but full strength, as determined by rheological measurements, was not attained until 60 min after pouring. One hour after pouring, H2 was added and left for 2 h to allow equilibrium to occur. The medium was then carefully removed and the cell suspension, with or without test reagents, was added. The assay was incubated for 2 h at 37°C then fixed in formol saline for scoring.

Measurements were made from two or more wells of each type, five replicates per well, using a x 20 long-working distance objective on a Leitz Diavert microscope. Distances migrated into a gel by a cell population were measured using the leading front method of Zigmond & Hirsch (1973). In this method, the leading front is taken to be the

distance that the leading two cells in the same focal plane in a microscope field had migrated from the gel surface. Student's t-test (unpaired) was used to test for any significant differences between experimental and control wells. Significances are marked thus: NS (Not significant, $P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). No correction was applied for refractive indices of the gel and medium. Fuller descriptions of the different visual migration assays carried out are given in Results.

Gels using tritiated collagen were made in the same fashion as above, but instead of 24-well tissue culture dishes, 35 mm diameter dishes were used and collagen was plated in thin layers (1 ml of 1 mg ml^{-1}, giving a layer of collagen ca. 1 mm thick). After allowing the collagen 1 h to set fully at 37°C, 5 ml of H2 was added to each well. Wells were kept at 4°C overnight and carefully washed several times with buffer to reduce the background count. The medium was then removed and 2 ml of 1 x 10^7 cells ml^{-1} were added to the experimental wells. Wells containing cells also contained f-MLP from 10^{-6} to 10^{-10} M. Control wells contained medium but no cells. After incubation for 2 h at 37°C, triplicate or quadruplicate 100 μl aliquots were taken from each well and counted in a Packard scintillation counter.

For the collagenase-treated thin layer assays, gels were prepared as above, but instead of adding cells to the experimental wells, collagenase (Type II, Sigma) at concentrations of 0.1, 1.0 and 10.0 μg ml^{-1} was used. Wells containing collagenase were incubated for 2 h at 37°C.

Triplicate or quadruplicate 100 μl aliquots were taken from each well and counted.

To examine whether the passage of a first wave of neutrophils into a collagen gel would help or hinder the passage of a second, ^{51}Chromate-labelled cells were used. The method is outlined in Figure 4.1 and explained in more detail in the Results section.

Assays 16 onwards employed a new assay system (the slab assay) for chemotactic and distribution scoring (see Figure 5.19). A stainless steel slide with a cut-out was used to provide a means of plating collagen in a rectangular slab of approximate dimensions 18 x 20 x 1.6 mm. This was enclosed by glass coverslips and was completely sealed, allowing observation of cells throughout the gel by normal inverted microscopy. Additionally, the intimate juxtaposition of two collagen areas was readily permitted by this system, allowing, for example, one collagen to contain cells and the adjoining collagen to be without cells, or one collagen to contain a chemoattractant and the other to be without, both collagens containing cells, or not, as desired. The boundary between the two collagen slabs was normally visible through the microscope as a faint line.

Typically, about 200 μl of collagen was used for each area. delivered between the coverslips by careful pipetting. For plating the slide was held in a vertical position, the first collagen plated and allowed to set in a horizontal position (about 10 minutes at 37°C) then the second

collagen plated on top of the first, maintaining the slide in a horizontal position for 10 minutes during gelation before returning the slide to a vertical position. After sealing each slide with paraffin wax and agarose, slides were kept in a humid box at 37°C for the incubation period. The boundary between two collagens in this assay system was just visible (Plate 5.1). Collagen was plated at a concentration of 1.0 mg ml^{-1}, and in some assays at 1.5 and 0.5 mg ml^{-1}.

For scoring this assay system for cell distribution, an eyepiece graticule of 10 x 10 squares was used in conjunction with a x 10 or x 20 objective on an inverted microscope. Using a x 10 objective each small square covered an area 120 x 120 μm, i.e. 14.4 x 10^3 square microns. Starting at the boundary between the collagen areas, cells in focus that fell within the graticule squares were counted in rows of 10 squares parallel to the boundary, and in columns of 8 or 16 squares stretching away from the boundary in both directions. Depending on the assay, counts were made starting at one or more sections of the boundary, and also at one or more depths of focus within the collagen matrix.

Data obtained from cell distribution assays were tested for significance using a one-way analysis of variance. Depending on the magnification used to score the assays, cell counts were pooled in pairs of rows stretching away from the collagen boundary and four or six of these pooled rows were then tested for any deviation from the population mean.

2.5.2 Time-lapse filming (Cine):

This followed the procedure of Wilkinson et al. (1982). Film sequences of neutrophils were taken using a Bolex H16 Reflex 16 mm cine camera (Bolex, Switzerland), mounted on a Leitz Ortholux microscope. Shutter release and film wind on were controlled through a Variometer (Wilkinson et al., 1982), sequences involving one frame advancing every six seconds, with an exposure time of 0.2 s. Kodak (Rochester, N.Y.) Plus-X reversal film 7276 (ASA 40, tungsten light) was used for filming and the exposure level monitored using a Wild exposure meter via a compensating telescopic eyepiece. Sequences were routinely of 30 minutes total duration (300 frames per sequence).

Neutrophils were contained in a filming chamber; a stainless steel slide 70 x 40 x 1 mm with a 15 mm-diameter circular aperture drilled through the slide and positioned centrally. A square, 32 x 32 mm coverslip was affixed over the aperture with vacuum grease (Dow Corning, Seneffe, Belgium), the slide turned over and the cell suspension pipetted into the shallow well thus formed, with a second coverslip sealed over the well using hot paraffin wax/vaseline brushed on over the coverslip margins.

The film chamber was placed on the microscope stage and allowed 10-15 minutes to warm up. A constant temperature of 37°C ± 0.1°C was provided by a thermostatically controlled fan heater, blowing a warm air curtain over a thermistor positioned adjacent to the stage. Cells, normally prepared to a final concentration of 0.5×10^6 ml^{-1}, were filmed

moving on the upper surface of the lower coverslip; while not as good optically as using the lower surface of the upper coverslip, this obviated the problem of cell detachment. The volume of cell suspension in each chamber was 200-300 μl with cells suspended in a 50:50 peritoneal exudate: H2 medium. Film was processed commercially.

For assays performed to study the effect of cell concentration on locomotion over a 2-D substratum, five concentrations were used; 2.0, 1.0, 0.5, 0.25 and 0.01 x 10^6 ml^{-1}. Film chambers were as described above and cells were filmed in 30 minute sequences at 37°C using an automated tracking system described below. Cells were suspended in a 50:50 peritoneal exudate: H2 medium.

2.5.3 Film Analysis (Computer-assisted)

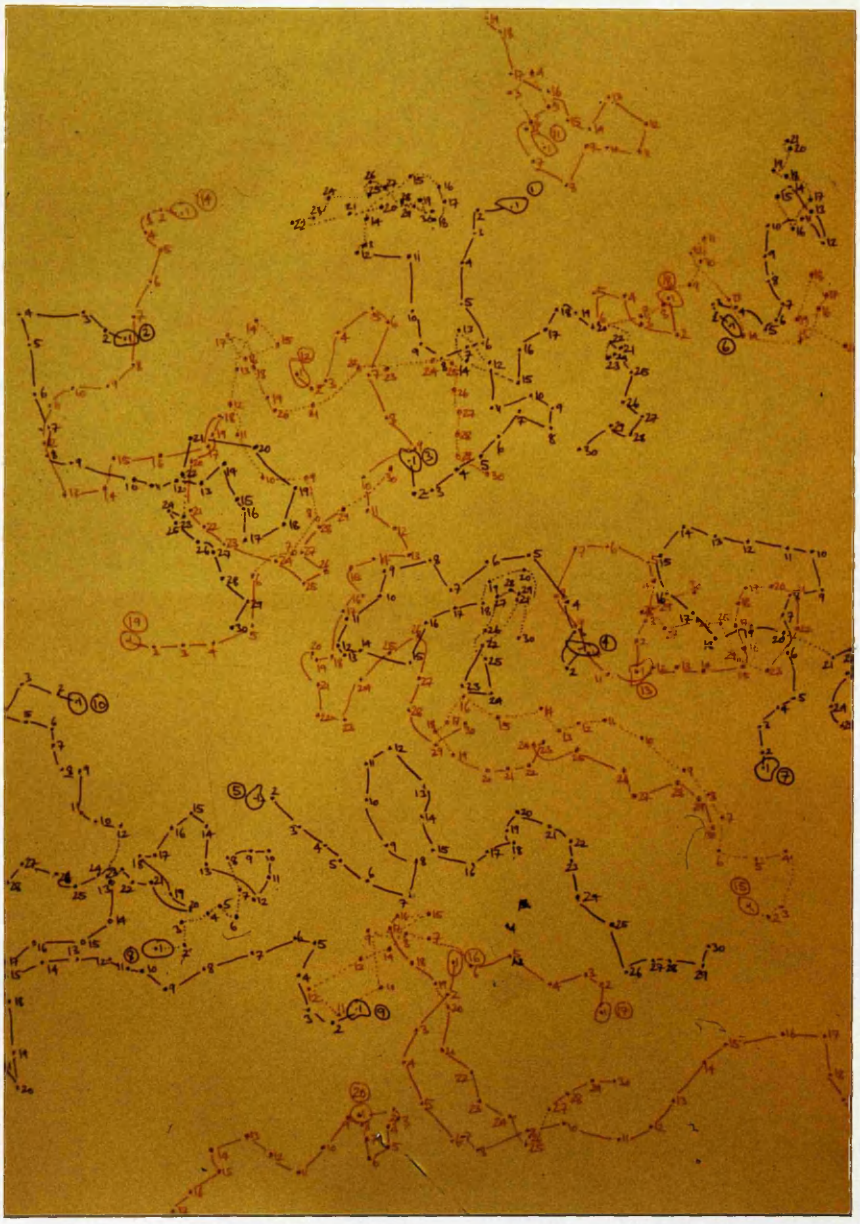
Analytical methods for cell tracks have been described before (Lackie & Burns, 1983; Wilkinson et al., 1984). A stop-action projector (L & W Photo-Optical Data Analyser, California) was set up so as to project the film horizontally on to a 45° front-silvered mirror; the image was then conveniently thrown on to the bench. Cells were selected at random on the first frame of each sequence and the positions of their centres marked at every tenth frame, i.e. at 60 s intervals. A hand control allowed the film to be stopped if necessary. For statistical reasons a minimum of 20 cell tracks, each with a minimum of 10 marked positions, was preferred. Cells which did not move more than two cell diameters from their starting point were regarded as anchored and were not used. Fine-point pens of different colours simplified the marking of cell tracks on

sheets of drawing paper. Plate 2.1 shows one such sequence, in this case human neutrophils filmed with pentoxifylline at 0.02 mg ml^{-1} for 30 minutes. Colours have no significance; pens were switched after marking the first ten tracks for convenient distinction later.

The marked sheets were then transferred to a graphic digitising tablet (Summagraphics Ltd.; Bit Pad One) linked to a Superbrain microcomputer (Intertec Data Systems, Columbia), or a BBC microcomputer (Acorn Computers, Cambridge). A scale bar was marked on the sheet, utilising the projected image of a scale micrometer filmed for a few frames between cell sequences. Sequential positions of each cell were then marked using the bit pad pen, which incorporates a contact switch, so automatically transferring the position coordinates to the microcomputer where data could be stored for computation.

2.5.4 Automated cell tracking

At a later stage, cell tracking was further automated using a Panasonic Newvicon-tube video camera on a beam-splitting eyepiece, and a 12-inch monochrome monitor (Panasonic). The video camera was attached to a video digitiser unit (Watford Electronics) connected to a BBC Master microcomputer. Successive frames were stored at 30 s intervals for 30 minutes; each frame was acquired as a 640 x 256 pixel array and was stored in < 2 s. The system was capable of randomly selecting and tracking up to 50 cells over the 30 minute sequence, with a print-out of the population and individual cell locomotory parameters being



Scale bar: 100 µm

Plate 2.1 Manual marking of neutrophil tracks; human neutrophils moving over protein-coated glass with 0.001 mg/ml pentoxifylline in 10% human serum. Colours are not significant, and are for convenience of marking only

possible shortly after the end of a sequence (Dow, Lackie & Crocket, 1987).

Plate 2.2 is a photograph taken from the monitor 11 minutes into a 30-minute sequence; rabbit cells at a concentration of 0.5×10^6 ml^{-1} locomoting over protein-coated glass. Cells in this sequence are locomoting vigorously, and 23 cells have been lost, partly due to collisions and partly to moving out of the field of view. As a point of interest, tracks in this sequence are suggestive of non-random movement. If so (and subjective impressions are not relied upon) this would be shown on subsequent analysis of the tracks.

For statistical analysis of film sequences, population speeds and persistences were compared using the non-parametric Mann-Whitney U-test (Campbell, 1974).

2.5.5 Luminometry

Metabolic activation of neutrophils and any possible effect on this by pentoxifylline was assessed by luminol-enhanced chemiluminescence using an LKB 1251 Luminescence Photometer (Luminometer; LKB Wallac Oy, Turku, Finland). The luminometer was connected to a 1291 Dispenser, a peristaltic pump capable of delivering small volumes of liquid. The luminometer was controlled by a BBC microcomputer. Washed neutrophils were used at a final concentration of 1×10^6 ml^{-1}. Reagents used included:

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma)

f-MLP (Sigma)

H2

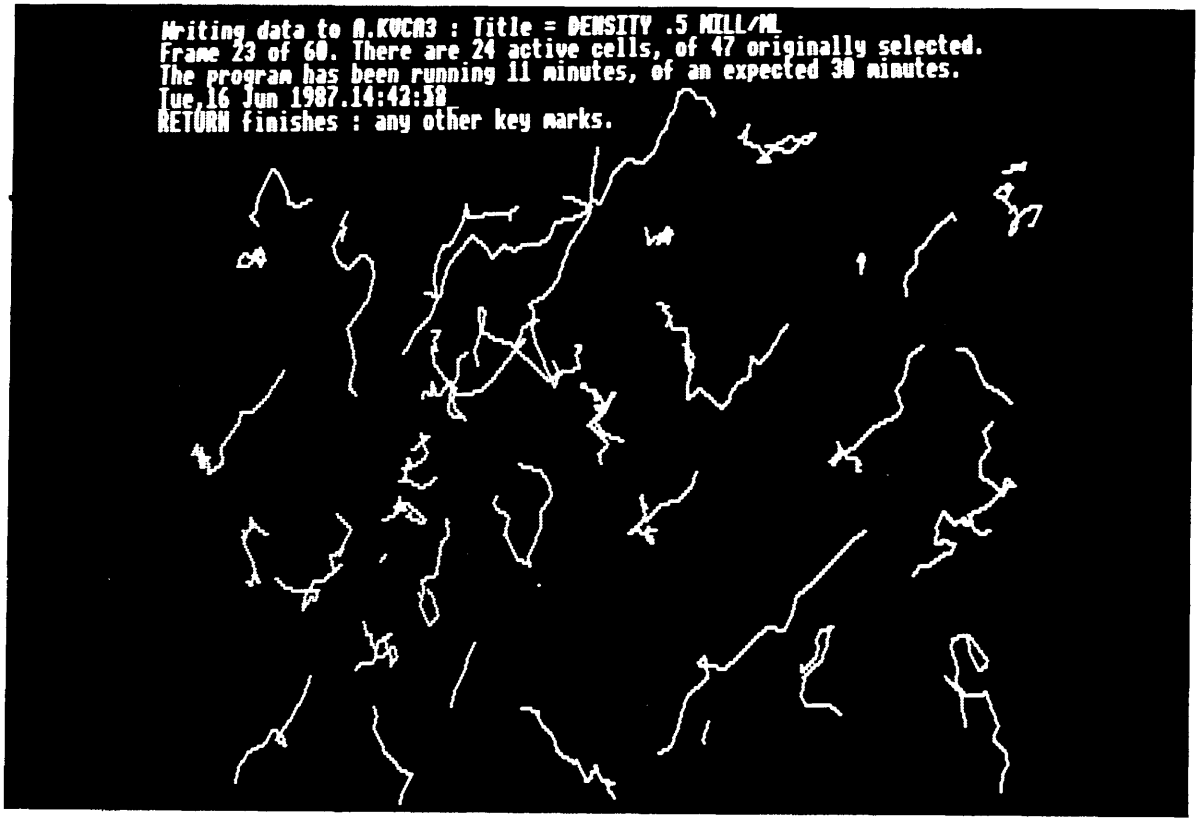


Plate 2.2 Automated cell tracking. Photograph taken of monitor 11 minutes into a 30-min sequence. Cell tracks, with a x 10 objective as here, cover an area 540 x 360 µm. Stepped appearance of individual tracks is an artifact caused by the monitor screen.

Cytochalasin B, $C_{29}H_{37}NO_5$, from Helminthosporium dematioideum (Sigma).

Pentoxifylline; Oxpentoxifylline (Trental®), 3,7-Dimethyl-1(5-oxohexyl) xanthine, $C_{13}H_{18}N_4O_3$. Mol Wt 278.3. The pentoxifylline was a gift from Hoechst Pharmaceuticals.

The luminol and f-MLP were dissolved in Dimethyl sulphoxide (C_2H_6OS; DMSO) at stock concentrations of 10^{-2} M and diluted immediately before use. Luminol was used at a final concentration of 10^{-5} M; f-MLP at 10^{-7} to 10^{-9} M, with appropriate amounts of test reagent being added as required, the volume in each test tube being made up to 1.0 ml with phenol red-free H2. (Inclusion of the phenol red dye indicator affects light output and hence would bias readings). Chemiluminescence response of the cells was measured in millivolts at 37°C and plotted against time. Peak readings for control and experimental tubes were plotted against the log dose of pentoxifylline and the straight line curves so obtained used to calculate the concentration of pentoxifylline which inhibits 50% of the response to f-MLP.

2.6 Immune Complexes

2.6.1 Preparation of Immune complexes:

Sheep anti-human albumin was supplied by the Scottish Antibody Production Unit (SAPU), Carluke, in a lyophilised form. The anti-albumin serum was from a pool of selected bleedings from sheep immunised with highly purified human albumin obtained from a pool of normal sera. As the

antiserum was of unknown titre, standard precipitation and immunodiffusion tests were performed, using human albumin (Sigma) as the antigen.

Rabbit anti-human albumin was purchased from Sigma Chemical Company (St. Louis, Miss.). This antiserum is developed in rabbits using purified human protein as immunogen, and is the IgG fraction of serum in a lyophilised form. The titre given was 1:16, 5 μl of serially diluted antiserum reacting against 5 μl of 1 mg ml^{-1} human albumin (Sigma).

Finally, high titre rabbit anti-ovotransferrin (conalbumin) was a kind gift of Dr Alvarez-Hernandez, Department of Bacteriology & Immunology, Glasgow University. Ovotransferrin saturated with iron (1 mg of protein binding with 1.5 μg ferric iron) was emulsified with the same volume of Freund's complete adjuvant (Difco, Detroit, Mich.), and injected in 2 ml volumes intramuscularly. Injections were done at 15 d intervals, the titre being tested after the fourth injection. The equivalence point, with complement, is 1 μl antiserum to 4 μg ovotransferrin. When inactivated, the equivalence point is 1 μl antiserum to 8 μg ovotransferrin. Visual migration assays carried out using immune complexes are described in Results.

2.7 Immunodiffusion Test:

Pure HEEO agarose powder (Miles Laboratories Ltd., Stoke Poges), was dissolved in near-boiling borate-saline (10.0 g $litre^{-1}$). The agar was stored in aliquots at 4°C until use.

When needed, the agar was melted in a microwave oven (10 s) then poured into a 9 cm petri dish. When set, a pattern of 2 mm diameter wells was cut with a punch; one central well and six circumferential wells. The centre-to-centre distance of the wells was 5 mm. The wells were charged with 5 - 10 μl of antigen or antiserum and a filter paper placed under the lid to effect a tight seal. Dishes were incubated overnight at room temperature then stained briefly (< 5 min) with Kenacid Blue. Plate 2.3 shows an Ouchterlony done using SAPU antiserum (purified fractions 1 + 2, see below) in the central well, and different amounts of human albumin (Sigma) in the circumferential wells.

2.8 Ion-exchange Column Chromatography:

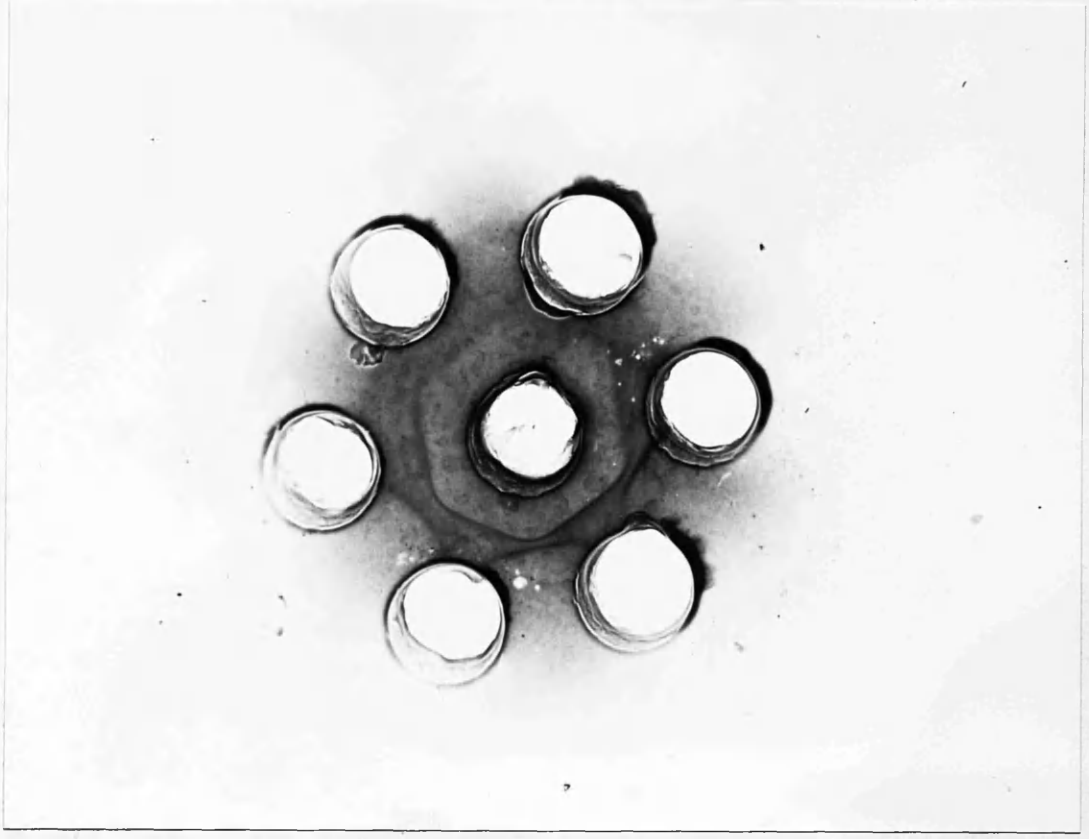
This method was used to isolate sheep anti-human IgG from the antiserum supplied by SAPU, using QAE-Sephadex (Sigma), bead size 40-120 μm, bed volume 30-40 ml g^{-1} in Tris HCl buffer, pH 6.5. One gram of the QAE-Sephadex A50 was swollen for 3 d in the buffer and degassed just before use. The antiserum was dialysed overnight against the buffer. A K9/15 Pharmacia column was used, and Ouchterlony assays were run to check the titres of the eluted samples. Fractions 1 and 2 were pooled, as were fractions 3 and 4.

2.9 Polyacrylamide Gel Electrophoresis (PAGE):

Reagents used were electrophoretically pure. They included:

acrylamide (Koch-Light)

bis-acrylamide (Koch-Light)



Key: antigen amounts given in µg/ml;
10 µl antiserum placed in centre

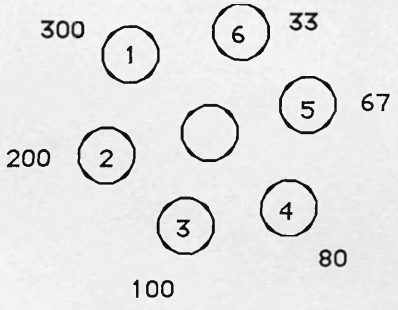
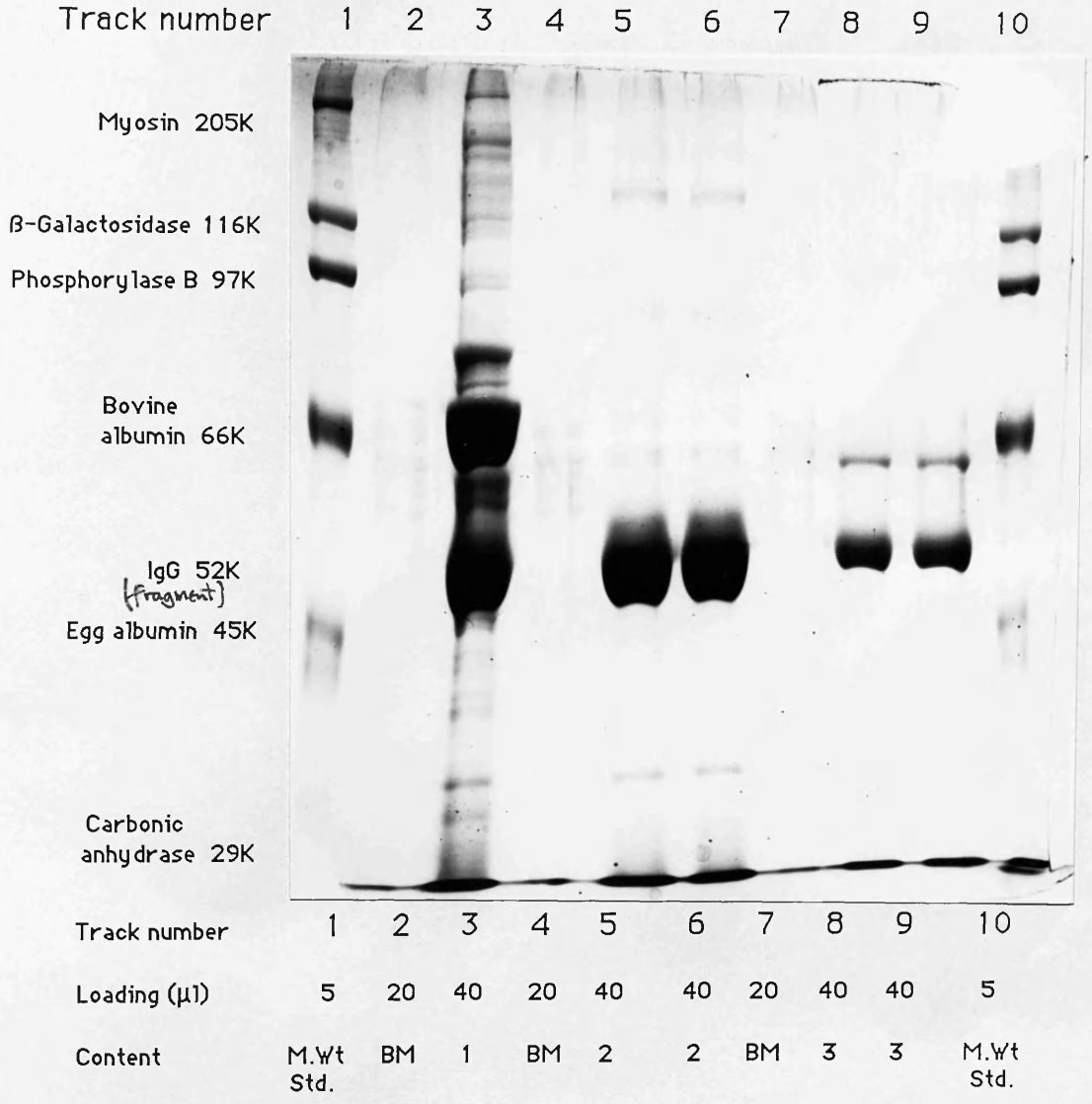


Plate 2.3 Ouchterlony Immunodiffusion Test. Deposits of immune complex are very evident in wells 2 - 5, much less so in wells 1 & 6. Well 1 was taken to indicate the equivalence point, with antigen excess and precipitation just visible . Wells are about 6 mm apart



Key: M.Wt Std. - Molecular Weight Standards
BM - Boiling Mix
1 - Whole antiserum
2 - Pooled fractions 1 + 2
3 - Pooled fractions 3 + 4

Plate 2.4 SDS gel with SAPU sheep antiserum (anti-human albumin)

sodium dodecyl sulphate (SDS) (BDH)

Kenacid Blue R (BDH)

ammonium persulphate (BDH)

bromophenol blue (BDH)

N,N,N¹,N¹,-tetramethylethylenediamine (TEMED) (Sigma)

Tris (hydroxymethyl) aminomethane (Tris) (Boehringer, Mannheim)

glycine (Sigma).

Plate 2.4 shows the SDS gel made using SAPU antiserum purified by column chromatography. Track 3 was loaded with whole antiserum, tracks 5 & 6 (duplicates) with pooled fractions 1 + 2, and tracks 8 & 9 (duplicates) with pooled fractions 3 + 4. Tracks from both pooled fractions show strong banding at about 52 Kdaltons, presumably due to IgG fragments. Fractions 1 + 2 were used for further work.

RESULTS

Chapter 3

Effect of pentoxifylline on neutrophil locomotion

Introduction

Pentoxifylline, a drug used clinically to improve peripheral blood flow ('Trental' 400, trade mark Hoechst), may decrease neutrophil deformability, and there is preliminary evidence showing that low concentrations of pentoxifylline improve penetration of a rigid matrix, possibly by a general increase in cellular motility. Pentoxifylline is not chemotactic for neutrophils (Sheetz et al, 1984). The outcome of an increase in deformability on penetration into a matrix composed of reconstituted collagen is uncertain. A cell must be deformable, to pass through small passages, and yet have a certain amount of rigidity, in order to displace small obstacles such as collagen fibrils. Invasion assays using collagen gels incorporating pentoxifylline were therefore carried out, to study the effect of this drug on cell invasion. To test whether pentoxifylline stimulates locomotion over a 2-D substratum, time-lapse filming was employed. Finally, the effects of pentoxifylline and aminophylline, another methyl xanthine, on the metabolism of neutrophils were studied by measuring the chemiluminescence of stimulated cells in a luminometer.

3.1.1 Effect of pentoxifylline on invasion of collagen gels

To examine the possible effect of pentoxifylline on cell invasion of a collagen gel, 1 x 10^6 cells in 1 ml of H2 were placed above the collagen gels. Pentoxifylline was present in the medium in various concentrations, while control wells contained no pentoxifylline. No chemoattractant was used in any well.

Initial assays used pentoxifylline at concentrations of 0.01 - 5.0 mg ml^{-1} (0.036 - 18 mM). Five readings were made from two duplicate wells of each type and the readings compared with controls using Student's t test (unpaired). An enhancement of invasion was seen in wells containing pentoxifylline at 0.01 mg ml^{-1} ($t = 4.1$; d.f. 18, $P < 0.001$). At the higher concentrations of pentoxifylline an inhibitory effect on cell invasion was seen, with reduced values for the leading fronts compared with control values. (Table 3.1, Fig. 3.1). This was assumed to be due to toxicity effects, and further assays were done using pentoxifylline at concentrations of 0.1 - 20 μg ml^{-1} (3.6 - 72 μM)

Three assays were carried out using pentoxifylline at concentrations of 0.1 - 20 μg ml^{-1}. Five readings were made from each of 16 control wells and 12 experimental wells. In wells containing pentoxifylline at a concentration of 10 μg ml^{-1}, the mean depth of the leading front was significantly increased compared with control wells ($t = 8.15$; d.f. 138, $P < 0.001$). In the wells with 20 μg ml^{-1} pentoxifylline, the mean depth of the leading front

Table 3.1

Effect of pentoxifylline on collagen gel invasion

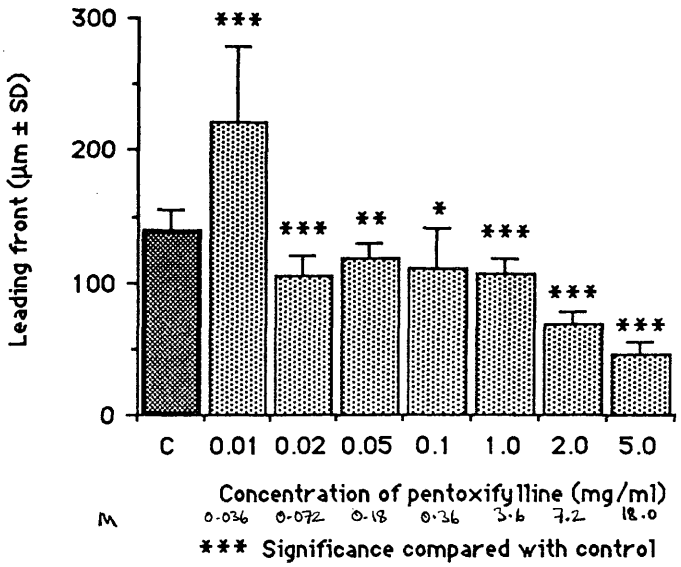
Control	Pentoxifylline (mg ml^{-1})						
	0.01 (0.036M)	0.02 (0.072M)	0.05 (0.18M)	0.1 (0.36M)	1.0 (3.6M)	2.0 (7.2M)	5.0 (18.0M)
Leading front (μm) 139	221	105.4	117.8	111.2	106.6	69	45.8
SD 16.1	57.1	15.9	12.3	29.9	12.8	8.8	9.7
t	4.1	4.46	3.14	2.45	4.73	11.45	14.91
d.f.	18	18	18	18	18	18	18
P <	0.001	0.001	0.01	0.05	0.001	0.001	0.001

Table 3.2

Effect of collagen concentration & pentoxifylline on cell invasion

		Collagen concentration (mg. ml^{-1})		
		1.0	1.5	2.0
Leading front (μm ± SD)	Control	65.9 ± 16.9	79.4 ± 18.7	159.8 ± 30.8
	pentox. 0.036M (10 mg ml^{-1})	74.0 ± 13.4	106.9 ± 25.8	193.9 ± 38.3
% increase		12.3	34.6	21.3
t		1.64	3.76	3.03
d.f.		38	38	38
P <		0.05	0.001	0.01

Note: Assays used in Table 3.2 were run on different days and using different batches of cells; they are therefore not directly comparable.



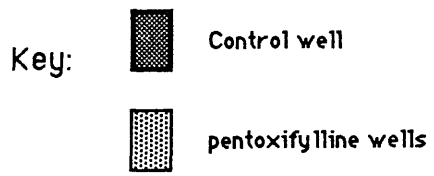


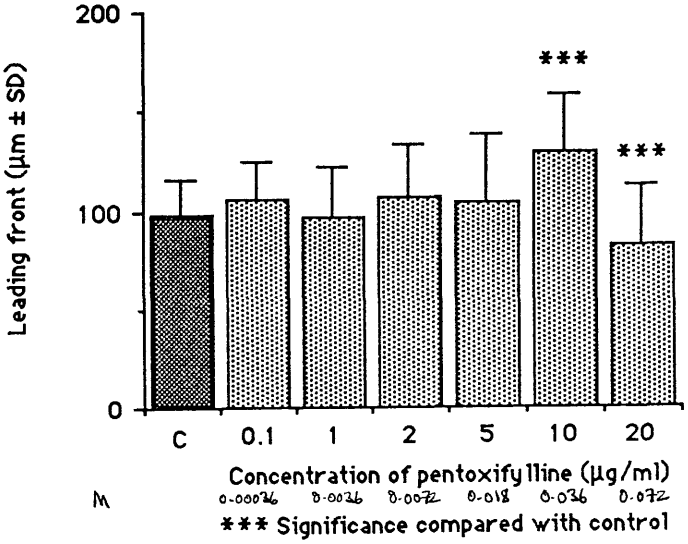
Fig. 3.1 Effect of pentoxifylline on neutrophil invasion of collagen gels

was significantly reduced compared with control wells ($t = 3.74$; d.f. 138, $P < 0.001$). In the remaining experimental wells containing pentoxifylline, the mean depth of the leading front was not significantly different from the control mean ($P > 0.05$, Fig. 3.2).

These assays indicate that pentoxifylline is enhancing the invasion of collagen gels, though in a very narrow dose range. Obviously, any mechanism which permits neutrophils to move more quickly through the ECM in an inflammatory response will be of benefit to the host animal, though as yet it is not clear whether this enhancement of invasion is due to a simple increase in speed of the neutrophils, or some other change, such as in cell deformability.

3.1.2 Effect of collagen concentration and pentoxifylline on invasion

If pentoxifylline, by increasing the deformability of neutrophils, is aiding their invasion of collagen gels, then it would be interesting to examine the effect of the drug over a range of gel concentrations, predicting that any enhancement of invasion might be more marked at higher concentrations of the collagen, where presumably passages in the gel matrix are narrower. Collagen gels are normally made at a concentration of 1.0 mg ml^{-1}, with gels at concentrations of 2.5 mg ml^{-1} showing a marked reduction in neutrophil invasion, presumably due to the higher density of collagen fibres physically obstructing the passage of the cells (Brown, 1982).



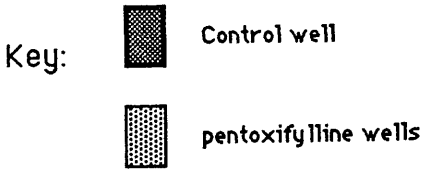


Fig.3.2 Effect of pentoxifylline on neutrophil invasion of collagen gels

Invasion assays were carried out in gels prepared at 1.0, 1.5 and 2.0 mg ml^{-1}. Experimental wells contained pentoxifylline at a concentration of 10 μg ml^{-1}, as this concentration had been found to be optimum for enhancing the invasion of gels. Measurements were made from four wells of each category, five replicates per well. In all cases invasion of the collagen gels was significantly enhanced in the wells containing pentoxifylline (t values of 1.64; d.f. 38, 3.76; d.f. 38, and 3.03; d.f. 38, $P < 0.05$, 0.001 and 0.01 for gels of 1.0, 1.5 and 2.0 mg ml^{-1} respectively. The percentage increases in the depth of the leading front in the experimental wells over control wells were 12.3%, 34.6%, and 21.3% for gels of 1.0, 1.5 and 2.0 mg ml^{-1} respectively (Table 3.2).

Though these results are from different batches of cells, and should therefore be treated with some caution, they nonetheless suggest that pentoxifylline enhances neutrophil invasion of collagen gels, though slightly less so at higher gel concentrations. This finding does not clearly indicate, however, whether the enhancement is due to a simple increase in speed, or a change in cell deformability. To investigate this further, it was decided to use time-lapse filming.

3.1.3 Effect of pentoxifylline on neutrophil locomotion over a 2-D substratum

The enhanced invasion of collagen gels by pentoxifylline-stimulated neutrophils might be as a result of the direct stimulation of cellular motility, rather than an increase

in deformability. Time-lapse filming was carried out, using rabbit and human neutrophils, in order to investigate the possible effect of pentoxifylline on neutrophil speed and/or persistence over a 2-D substratum. Speed is given in microns min^{-1}, while persistence, in seconds, may be defined as 'the tendency to continue moving in the same direction' (Lackie, 1986). Rabbit cells were prepared from peritoneal exudate, human cells from peripheral blood. In some assays cells were pre-stimulated with f-MLP at 10^{-9}M. (See Appendix D).

Nine assays were carried out. (Table 3.3). As the speed and persistence data from neutrophil populations showed a non-Gaussian distribution (see Figure 3.3), the Mann-Whitney U-test was used for comparing the speed and persistence of the experimental population with control. The three assays utilising rabbit cells showed no inter-assay variation, and these were then pooled. There was a significant increase in mean speed between the control sequence and the experimental sequence using pentoxifylline at a concentration of 10 $\mu g\ ml^{-1}$ ($U = 2.1$; $P < 0.05$). At the highest concentration of pentoxifylline, 500 $\mu g\ ml^{-1}$, speed was significantly reduced ($U = 2.42$; $P < 0.05$). At high levels of the drug speed decreases, which is likely to be attributable to toxicity. There were no significant differences in persistence times except at the highest concentration of pentoxifylline, when the persistence was significantly reduced, again presumably due to toxicity ($U = 3.21$; $P < 0.001$). (Fig. 3.4).

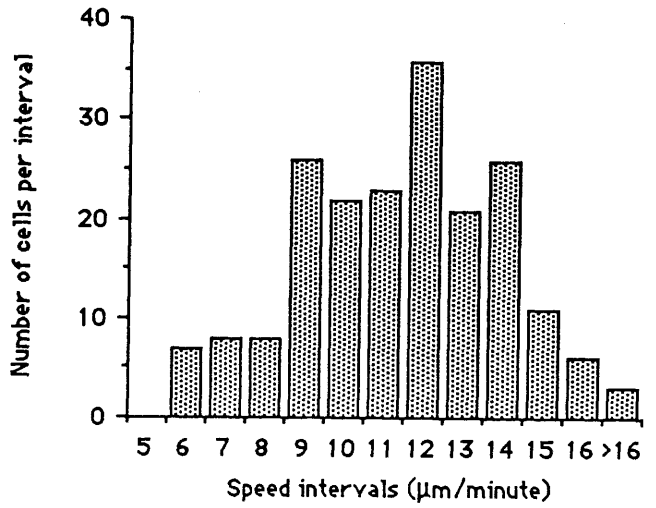
In three separate assays using unstimulated human neutrophils, there was no sign of inter-assay variation,

Table 3.3

Effect of pentoxifylline on neutrophil speed and persistence over a 2-D substratum

Cell Origin	No.	Treatment f-MLP (10^{-9} M)	Treatment pentoxifylline ($\mu g\ ml^{-1}$)		Speed ($\mu m\ min^{-1}$)	Persistence (s)	
					Means ± SEM		
Rabbit	87	–	–		9.23 ± 0.3	69.55 ± 5.17	
	62	–	1		10.06 ± 0.45	68.97 ± 7.9	
	50	–	5		10.64 ± 0.7	70.03 ± 5.63	
	50	–	10	*	11.81 ± 0.9	61~2 ± 6.42	
	49	–	20		10.38 ± 0.61	77.22 ± 6.13	
	29	–	500	*	8.06 ± 0.54	41.73 ± 4.73	***
Human	120	–	–		11.09 ± 0.2	76.77 ± 3.5	
	59	–	1	***	12.7 ± 0.31	72.1 ± 4.75	
	60	–	5		10.61 ± 0.3	90.13 ± 4.58	
	60	–	10	**	12.08 ± 0.26	65.82 ± 4.95	*
	60	–	20		10.68 ± 0.33	71.34 ± 7.27	*
	60	–	500	***	7.76 ± 0.24	87.8 ± 5.7	
Human	120	–	–		11.19 ± 0.2	49.9 ± 3.0	
	60	+	–	***	13.52 ± 0.36	57.3 ± 3.5	
	60	+	1		13.54 ± 0.34	61.46 ± 3.8	
	60	+	5		12.98 ± 0.44	57.4 ± 3.5	
	60	+	10	**	11.77 ± 0.46	56.5 ± 3.9	
	60	+	20	***	10.56 ± 0.57	56.1 ± 3.5	
	60	+	500	**	8.53 ± 0.35	51.9 ± 4.7	

Significant differences between experimental means and control means are denoted by asterisks; levels of significance being $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, given by *, **, and *** respectively. Control figures are the top row of each group.



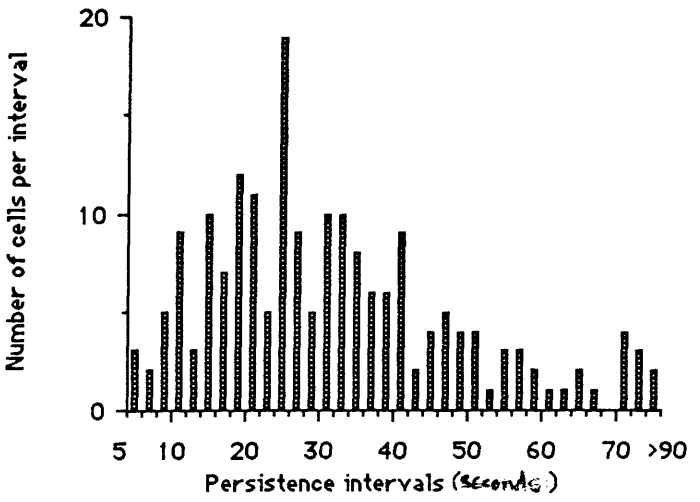
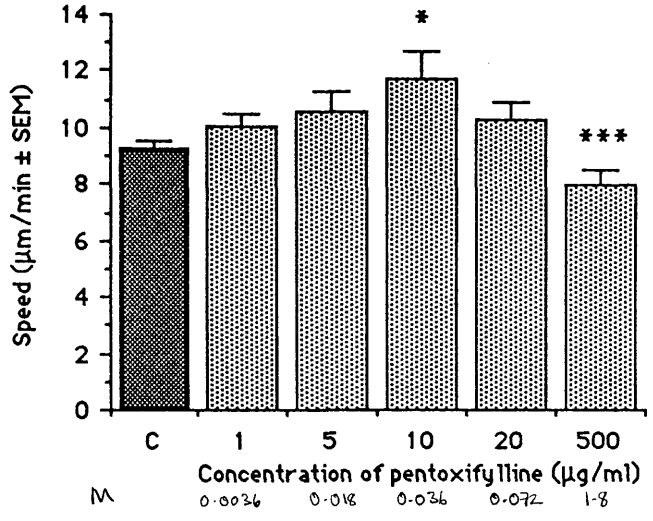
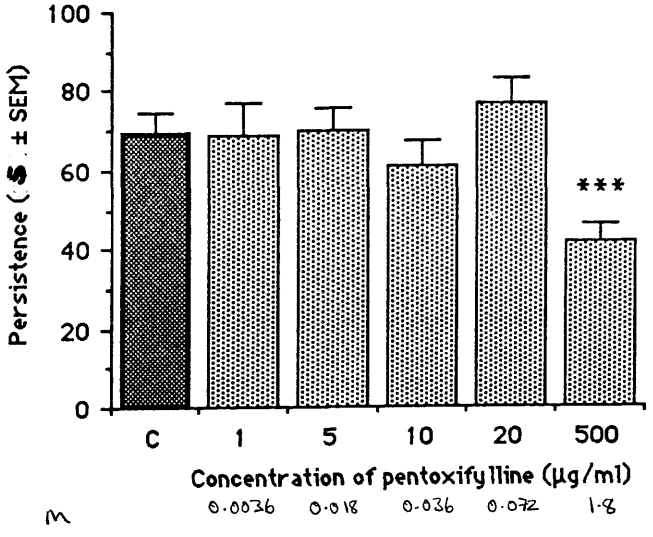


Fig. 3.3 Frequency distributions for neutrophil speed and persistence over a 2-D substratum. Data taken from pooled control populations in Section 5.3. Cells were filmed in 50% peritoneal exudate, 50% H2, at a concentration of 0.5 million/ml.





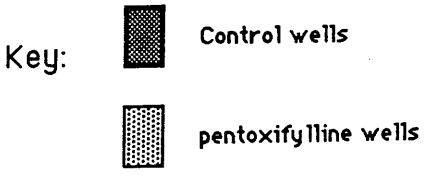
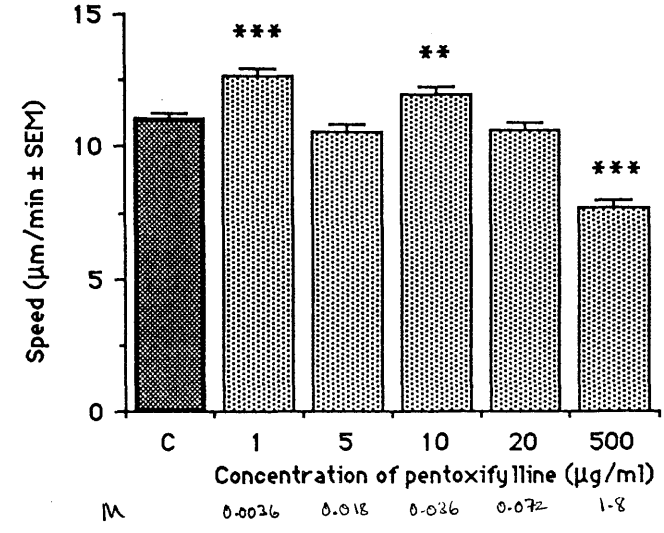


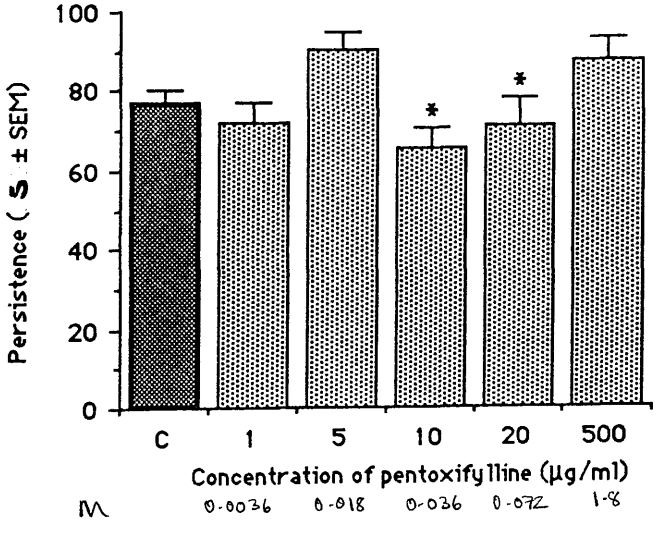
Fig. 3.4 Effect of pentoxifylline on rabbit neutrophil speed and persistence over a 2-D substratum

*** significance compared with controls

and results were then pooled. Comparing experimental sequences to control, there was a significant increase in speed with pentoxifylline at a concentration of 1.0 $\mu g\ ml^{-1}$ ($U = 3.83$; $P < 0.001$) and also at 10.0 $\mu g\ ml^{-1}$ ($U = 2.59$; $P < 0.01$), and a significant decrease in speed using pentoxifylline at a concentration of 500 $\mu g\ ml^{-1}$ ($U = 8.08$; $P < 0.001$). There were small though significant decreases in persistence using pentoxifylline at concentrations of 10 and 20 $\mu g\ ml^{-1}$ ($U = 2.4$; $P < 0.05$, $U = 2.17$; $P < 0.05$. Table 3.3, Fig. 3.5.

In three assays with human neutrophils stimulated by 10^{-9} M f-MLP, identical end controls were used, containing 10% human serum. A second control contained f-MLP at 10^{-9} M, as did experimental sequences. There was no significant inter-assay variation, and assays were accordingly pooled. As expected, the control set of cells treated with f-MLP but no pentoxifylline showed a significant increase in speed compared with the end control sets of cells with no f-MLP ($U = 4.61$; $P < 0.001$). Cells treated with f-MLP and pentoxifylline showed a progressive decrease in speed with increasing dosage of pentoxifylline, suggesting an inhibitory effect on pre-stimulated cells. The cells treated with pentoxifylline at 10, 20 and 500 $\mu g\ ml^{-1}$ showed a significant decrease in speed compared to the control set of cells treated with f-MLP but not pentoxifylline ($U = 2.97$, 4.17 and 7.32 respectively, significant at $P < 0.01$, 0.001 and 0.001). There were no detectable effects on persistence. (Table 3.3, Fig. 3.6).





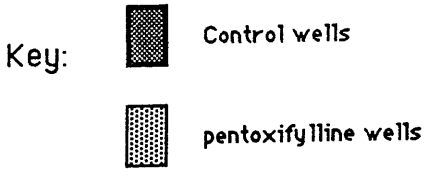


Fig. 3.5 Effect of pentoxifylline on unstimulated human neutrophil speed and persistence over a 2-D substratum

*** significance compared with controls

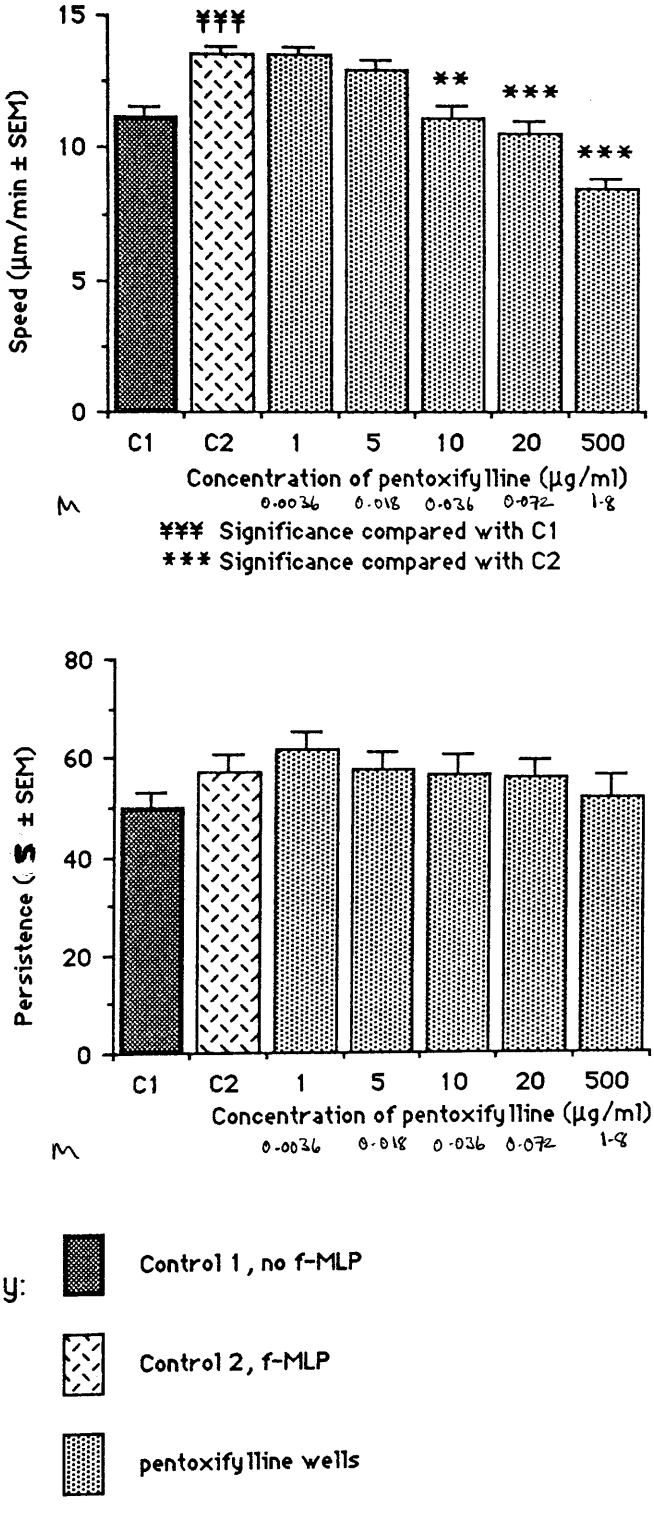


Fig. 3.6 Effect of pentoxifylline on stimulated human neutrophil speed and persistence

Unstimulated cells treated with pentoxifylline, whether rabbit peritoneal or human peripheral in origin, showed an increase in speed over a narrow dose range. This simple increase in speed may explain the enhancement of invasion found in the collagen gel assays. The human neutrophils showed a slight increase in sensitivity when compared to the rabbit neutrophils; this could be a species specific difference, or because the rabbit neutrophils were pre-stimulated to some degree, being elicited to invade the peritoneal cavity by injection of sterile saline containing oyster glycogen.

Pentoxifylline has no effect on the adhesive properties of neutrophils, rabbit or human (Crocket et al., 1987), though adhesion is probably of minor importance when considering neutrophil movement through a collagen gel matrix. Chemiluminescence studies were next carried out, to examine the effect of pentoxifylline on the respiratory burst of neutrophils, when during phagocytosis the cells show an increase in oxygen consumption and glucose metabolism via the hexose monophosphate shunt (Davis & Gallin, 1981).

3.1.4 Effect of pentoxifylline and aminophylline on the neutrophil metabolic burst; measured by chemiluminescence

During an acute inflammatory episode, neutrophils respond to a variety of signals in a number of ways, depending on the strength and nature of the signal. The chemotactic peptide f-MLP is commonly used as an activator of neutrophil metabolism (Wilkinson, 1982), with optimum stimulation of locomotion at a concentration of 10^{-8} M, and

optimal stimulation of secretion at 10^{-6} M (Lackie & Lawrence, 1987). It was decided to use f-MLP at a concentration of 10^{-7} M.

Initial assay results (not shown), using pentoxifylline at concentrations of 1.0 mg ml^{-1} and over, indicated a strong inhibitory effect on the metabolic burst of the neutrophils, and subsequent assays used pentoxifylline at lower concentrations. The first assay for which results are presented used six tubes; a control with cells and f-MLP at 10^{-7} M, a control with cells, f-MLP at 10^{-7} M, and cytochalasin B at a concentration of 10 μg ml^{-1}, and four experimental tubes containing, in addition to f-MLP and cytochalasin B at the same concentrations as above, different concentrations of pentoxifylline (0.2, 0.25, 0.3 and 0.35 mg ml^{-1}). The tubes were made up to 1 ml with cells and reagents, the f-MLP being added by automatic dispenser in the luminometer immediately before counting commenced. Readings were taken every 30 s for 7 minutes and the response of the cells, expressed in millivolts, was plotted against time. A graded inhibitory effect was seen (Figure 3.7).

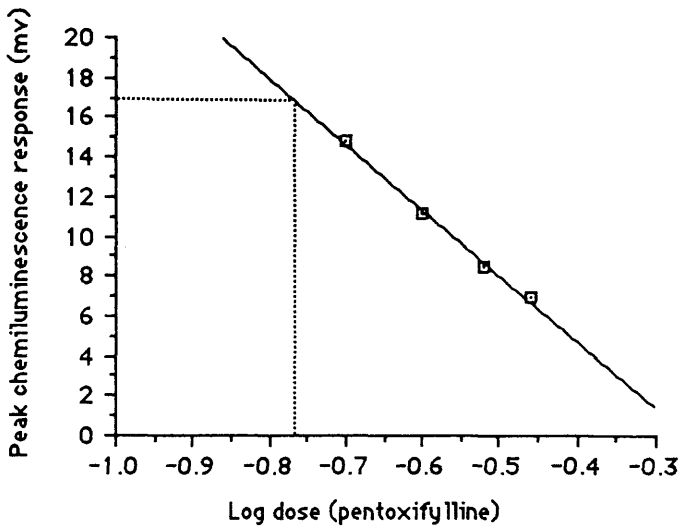
An effect on the time course of the response of cells to pentoxifylline was seen in this assay, with pentoxifylline-treated neutrophils showing a faster response compared with controls (Fig. 3.7). In this assay, experimental cells gave a peak chemiluminescence response at about 100 s, compared with control cells which gave a peak response at about 140s. This may be due to a membrane disruption effect, allowing luminol to cross the cell membrane more quickly.

The same effect was seen in repeat assays (Fig. 3.9). There is evidence that pentoxifylline treatment of leucocytes is correlated with increased polyphosphoinositides levels, causing an increase in membrane destabilisation (Sheetz et al., 1984).

When the log of each dose of pentoxifylline was plotted against the peak chemiluminescence response, the straight line curve so obtained allowed a calculation of the ID50; the concentration of pentoxifylline which would inhibit 50% of the response obtained with the fixed concentration of the f-MLP. In this assay, the ID50 was calculated as being 0.17 mg ml^{-1}. (Figure 3.8). This is an estimate by extrapolation

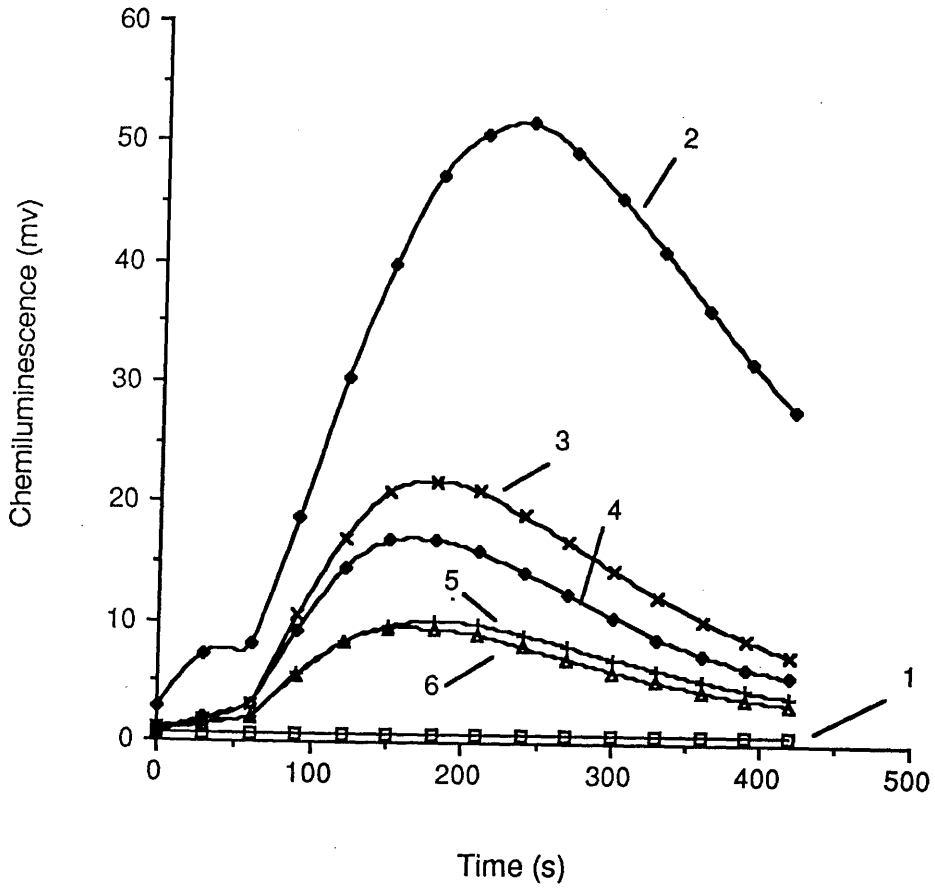
Similar results were obtained from repeat assays, one of which is given here. Depending on the batch of cells and its age, the ID50 varied from 0.14 - 0.18 mg ml^{-1}. Results from this assay for example, in which pentoxifylline was used at concentrations from 0.16 - 0.19 mg ml^{-1}, indicated an ID50 of 0.15 mg ml^{-1}. (Figures 3.9, 3.10). If pentoxifylline was used in concentrations below the ID50, the peak response gradually approached that found for the control curve, using f-MLP and cytochalasin B. An assay employing pentoxifylline at 0.01 mg ml^{-1} gave results indistinguishable from the control curve. (Figure 3.11).

In the collagen gel invasion assays with pentoxifylline, a dose of 10 μg ml^{-1} of the drug significantly enhanced invasion of the gels. Unusually, no other dose of pentoxifylline seemed to have any effect on cell invasion, except at high doses, where there was an inhibitory



ID50 dose from graph = 0.17 mg/ml (0.6 M)

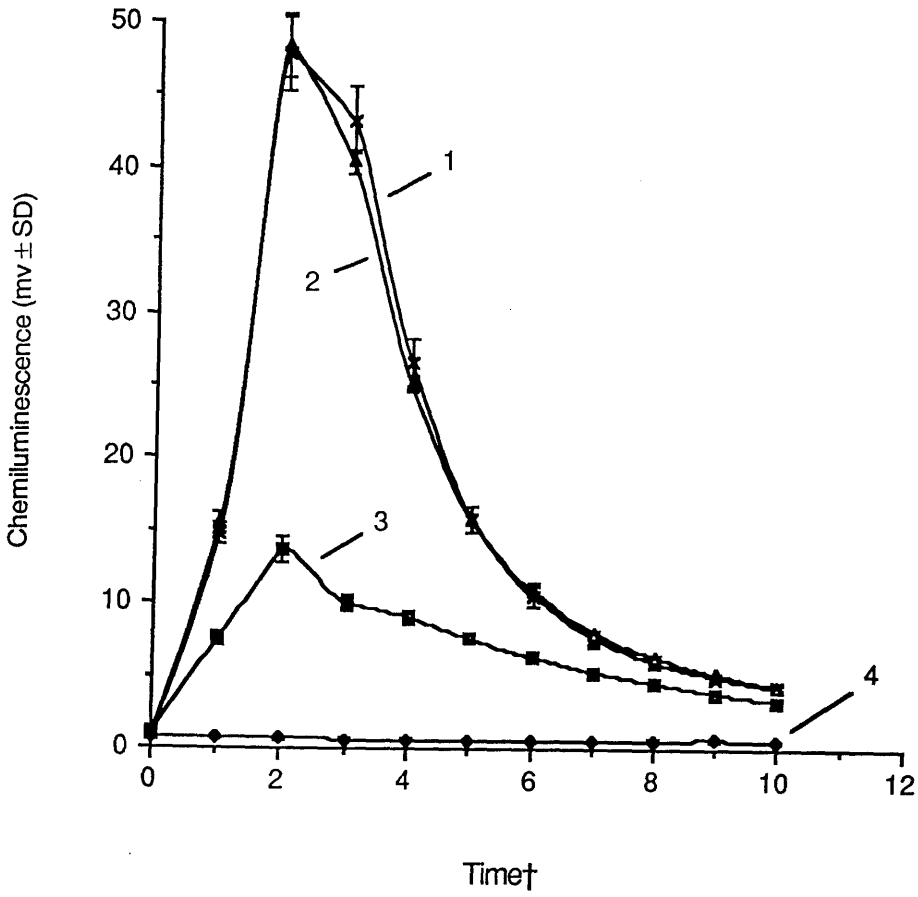
Fig. 3.8 Log dose (pentoxifylline) versus chemiluminescent response of cells



Key:
1 background, no cells
2 f-MLP + Cytochalasin B
3 f-MLP + Cytochalasin B + pentoxifylline (0.58 mM)
4 f-MLP + Cytochalasin B + pentoxifylline (0.61 mM)
5 f-MLP + Cytochalasin B + pentoxifylline (0.65 mM)
6 f-MLP + Cytochalasin B + pentoxifylline (0.68 mM)

f-MLP used at 10^{-7}M , Cytochalasin B at 2.08×10^{-5}M (10 µg/ml)

Fig. 3.9 Chemiluminescent response of cells to different concentrations of pentoxifylline



Key: 1 f-MLP + Cytochalasin B

2 f-MLP + Cytochalasin B + pentoxifylline (0.036 mM)

3 f-MLP + Cytochalasin B + pentoxifylline (0.36 mM)

4 f-MLP + Cytochalasin B + pentoxifylline (3.6 mM)

f-MLP used at 10^{-7}M , Cytochalasin B at 2.08×10^{-5}M (10 µg/ml)

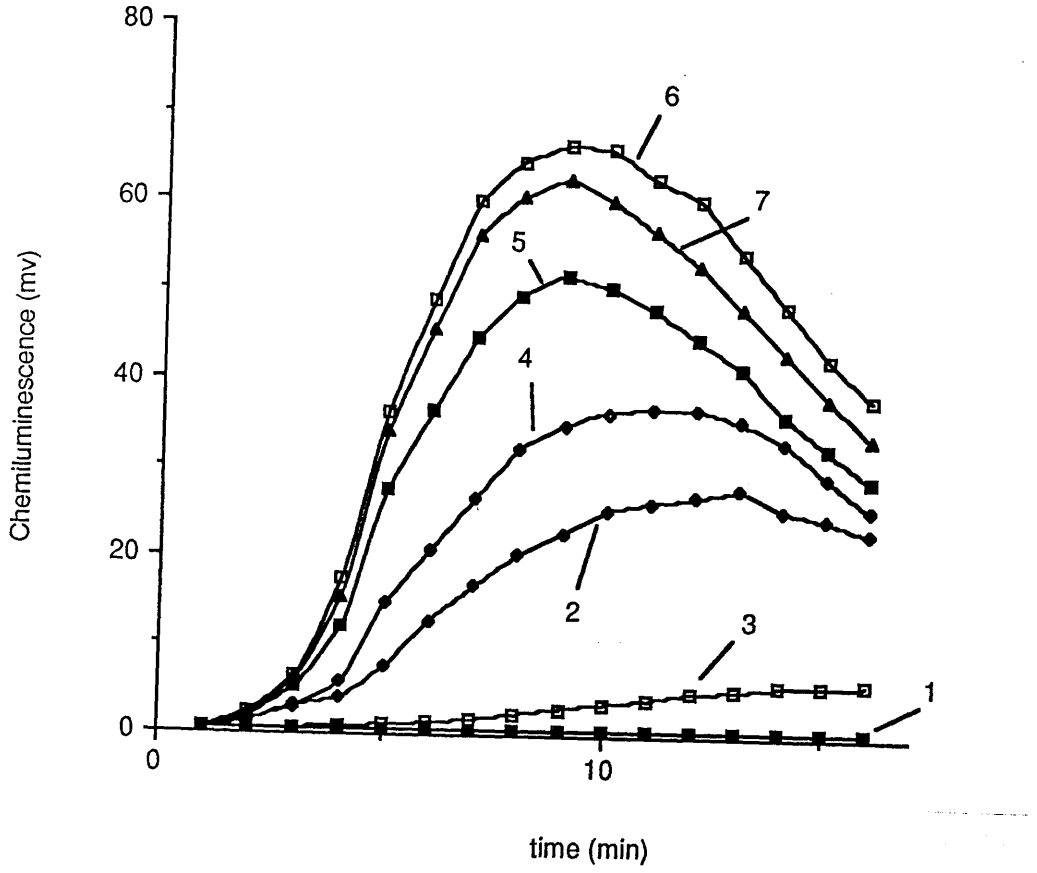
† Numbers on x-abscissa refer to sampling points; total duration of assay was 26 minutes

Fig. 3.11 Chemiluminescence: dose response of cells to pentoxifylline

effect, attributed to toxicity. This inhibitory effect was also seen in a reduced light output during luminometry, where a dose of approximately 0.15 mg ml^{-1} resulted in a halving of the light output compared with cells in control tubes.

In addition to examining the effect of pentoxifylline on neutrophil chemiluminescence, another methyl xanthine, aminophylline, was studied using luminometry. Assay conditions were similar to those described for pentoxifylline, with f-MLP at 10^{-7} M and cytochalasin B at 10 μg ml^{-1}. Aminophylline was used at concentrations of from 10^{-3} M to 10^{-13} M, and the metabolic burst of the neutrophils was plotted as mv against time in minutes (Fig. 3.12). There was complete inhibition of light output using aminophylline at 10^{-3} M, with enhancement using aminophylline at concentrations of from 10^{-4} M to 10^{-10} M.

Maximum enhancement of chemiluminescence was obtained using aminophylline at 10^{-10} M, with more dilute solutions giving a reduced response. The inhibition using aminophylline at 10^{-3} M may be attributed to toxicity. The assay was repeated and gave the same results. This finding, that aminophylline causes an enhancement of the neutrophil metabolic burst, is in direct contrast to the situation using pentoxifylline, which inhibits at all doses. Interestingly, while it has been found that pentoxifylline causes an increased motility, this increase was not seen with either caffeine or aminophylline, both of which are methyl xanthines (Sheetz et al., 1984). This difference cannot be explained without further work.



Key:
1 f-MLP
2 f-MLP + cytochalasin B
3 f-MLP + cytochalasin B + aminophylline at 10^{-3} M
4 f-MLP + cytochalasin B + aminophylline at 10^{-4} M
5 f-MLP + cytochalasin B + aminophylline at 10^{-5} M
6 f-MLP + cytochalasin B + aminophylline at 10^{-10} M
7 f-MLP + cytochalasin B + aminophylline at 10^{-13} M

f-MLP used at 10^{-7}M , Cytochalasin B at 2.08×10^{-5}M (10 µg/ml)

Fig. 3.12 Effect of aminophylline on chemiluminescent response of neutrophils

Summary

1. The effect of the methyl xanthine pentoxifylline on neutrophil locomotion was studied.

2. Pentoxifylline at a concentration of 10 $\mu g\ ml^{-1}$ enhanced the invasion of collagen gels by rabbit neutrophils.

3. Pentoxifylline at the same concentration enhanced neutrophil invasion of collagen gels over a range of gel concentrations, though less so at the highest density of gel.

4. Pentoxifylline at a concentration of 10 $\mu g\ ml^{-1}$ enhanced the speed of rabbit neutrophils over glass. Similarly, using human neutrophils, there was an enhancement of speed over glass with pentoxifylline at 1.0 and 10 $\mu g\ ml^{-1}$. Human neutrophils pre-stimulated with f-MLP showed an inhibitory effect with pentoxifylline.

5. Chemiluminescence studies on neutrophils indicated an inhibitory effect on cells pre-stimulated with f-MLP. The ID50 dose, the concentration of pentoxifylline required to reduce to 50% the metabolic burst as measured by chemiluminescence, was in the range 0.14 - 0.18 mg ml^{-1}, by estimation. (0.51-0.65M).

6. Chemiluminescence studies using aminophylline indicated an enhancement of light output on rabbit cells stimulated with f-MLP.

Chapter 4

Effects of neutrophil invasion on a gel matrix

Introduction

Invasiveness has been linked to the possibility of extracellular matrix degradation by protease activity (Matzner et al. 1985), and also to other cooperative processes such as some cell released migration-stimulating factor; such processes could result in the formation of tunnels or marked paths through a matrix. If either or both mechanisms were operating during neutrophil invasion of native collagen gels, a second set of cells might then benefit from the passage of a first set. Accordingly, labelled cells were used to determine the percentage of first and second set cell populations entering a gel, and secondly, labelled collagen was used to determine whether invading cells degraded the matrix.

4.1 Second set invasion: effect of a primary invasion wave on a second

In order to investigate whether the passage of a first wave of neutrophils into a collagen gel would facilitate the passage of a second, later wave, ^{51}Chromate-labelled neutrophils were employed. Collagen gels (0.5 ml vol., one mg ml^{-1} collagen) were prepared in the wells of Nunclon multiwell dishes (diameter 16mm). Four sets of wells were used and were incubated at 37°C. The wells are described in Figure 4.1.

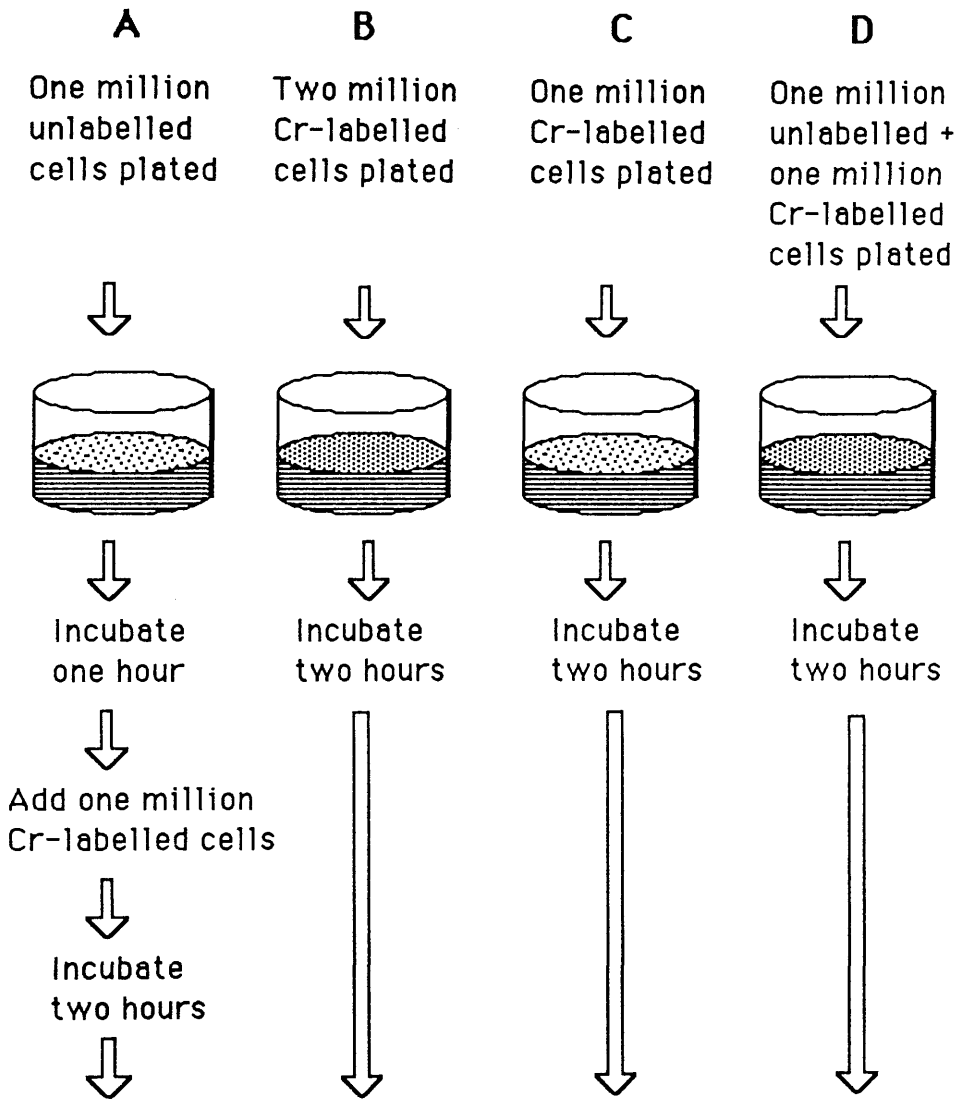


Fig. 4.1 Second set assay

At the end of the incubation period the medium was removed and one ml of 0.02% collagenase added to each well. The gels were monitored under the microscope until the cells on the gel surface floated freely after gentle agitation, usually after 10 minutes at 37°C. The collagenase was then removed and the surface of the gel washed three times with H2 to remove surface cells. The bulk of the gel was then dissolved in one ml of 10% acetic acid and 0.4 ml of this shaken suspension, including any labelled cells which had entered the gel, was counted on a Wilj 2001 gamma counter. Knowing the total counts added to each well and the residual counts in the gel, the percentage of cells invading the gels can be calculated.

Six assays were carried out; each assay employed 16 wells, four of each set. Counts were done in duplicate from each well and percentages of invasion calculated from these were compared for significant differences using the Mann-Whitney U-test. There were no significant differences between wells A, B or D. In well C, the only set with a final total of 1 x 10^6 cells per well, as opposed to 2 x 10^6 cells per well, there was a slight but significant decrease in the amount of invasion (6.89% ± 0.28%, c.f. 8.17% ± 1.17%, 9.74% ± 1.46%, and 9.84% ± 1.25%, means and SD for C, A, B, and D respectively. ($t = 1.83$; d.f. 22, $P < 0.05$, comparing data from C against pooled data from A, B and D. Figure 4.2). (See section 7.1 for further data on concentration effects).

The invasion assays described above allowed one hour between waves of invasion and used acellular gels. If

invading neutrophils migrating through such a gel employed enzymic activity to dissolve a pathway, then in all probability this pathway should remain after the cells had moved on. It might be expected that some neutrophils, invading a collagen gel, would in preference move through preformed passages suited for their size and mode of locomotion, rather than force their own passage. Studies using SEM have seen no such changes (Brown, 1982), and from the invasion assays described here no benefit seems to have been conferred on a second set population by a first.

The increased migration of PMN in the present study, seen in wells with an initial density of 2×10^6 cells ml^{-1} compared with 1×10^6 cells ml^{-1}, does not result from a lack of available area at the gel surface. It may be that the more densely plated cells produce a factor which stimulates cell invasion. Cell migratory factors have previously been shown to be produced by a variety of transformed cells (Burk, 1973), and there is evidence that PMN release factors which stimulate the movement of other PMN (Zigmond & Hirsch, 1973; Venge, P., 1979). It was decided to study this finding further by time-lapse filming, results of which are provided in Section 4.4.

4.2 Neutrophil invasion of labelled collagen gels

It has been demonstrated that human neutrophils can release 25-30% of their neutral proteases (collagenase and elastase) when stimulated by damaged tissue or immune complex (Henson, 1971). Cellular levels of collagenase in human neutrophils are about 40 μg per 10^7 cells, so

assuming rabbit neutrophils to be similar, and 25% of cellular collagenase to be released on stimulation, this would provide some 20 μg of released collagenase in each experimental well of 2×10^7 cells. Seven assays were carried out; four from assays using cells (Table 4.1), and three from assays using collagenase (Table 4.2). Cells were stimulated with f-MLP at concentrations of 10^{-6} to 10^{-10} M. The assays done with collagenase should demonstrate whether rabbit neutrophils, if releasing the same quantity of available and active collagenase on stimulation as do human cells, would degrade the labelled collagen in measurable amounts. Assays were stopped after two hours' incubation with cells, as in the standard invasion assay.

There were no significant differences in counts between any of the wells in which cells were stimulated with f-MLP, and these results were then pooled. In none of the assays employing cells was any significant increase in counts found in the experimental wells, when compared with counts from control wells ($t = 0.19$; d.f. 94, $P > 0.05$, Table 4.1). The collagenase on the other hand, at concentrations of 1.0 and 10.0 μg ml^{-1}, significantly degraded the collagen gels, as indicated by the increased counts found in the medium above the gels ($t = 8.87$; d.f. 18, $P < 0.001$, $t = 33.9$; d.f. 22, $P < 0.001$ respectively). The counts in the medium above gels with collagenase at 0.1 μg ml^{-1}, though increased, were just outside significance levels ($t = 1.59$; d.f. 19, $P > 0.05$, Table 4.2). It was concluded that radiolabelled collagen gels, despite possible cross-linking, are degraded by collagenase.

As the assays using collagenase with levels as low as 1 μg ml^{-1} showed a significant increase in counts, it was concluded that no detectable proteolytic activity by the neutrophils was occurring. In a parallel set of standard invasion assays, neutrophils readily invaded gels made from the same batch of radiolabelled collagen, and to appropriate depths; additionally, neutrophils were seen to have invaded the thin layers of collagen used in the degradation assays. As a further test to examine the possible role of proteolytic activity by invading neutrophils, standard invasion assays were carried out, in the presence of known protease inhibitors.

4.3 Effect of protease inhibitors on gel invasion

There is controversy as to whether invading neutrophils are aided in their passage through extracellular matrix by proteolytic activity. To test whether neutrophils are aided in such a manner in their invasion of gels formed with reconstituted rat-tail collagen, visual invasion assays were carried out in the presence of the known metallo-protease inhibitors EDTA, and o-phenanthroline.

4.3.1 Effect of EDTA on gel invasion

Three standard visual invasion assays were carried out, using collagen at 1 mg ml^{-1}, and cells at 1 x 10^6 ml^{-1}. Control wells contained no EDTA, while experimental wells contained collagen with EDTA incorporated at 10^{-4}, 10^{-5}, and 10^{-6} M. After allowing the gels one hour to set, HS with the appropriate concentration of EDTA was pipetted

onto the gels and the culture dishes incubated for 2 h at 37°C. Medium was then removed and replaced with fresh medium containing cells and EDTA. After 2 h the assays were stopped and scored for the leading front. Five readings were made from three duplicate wells of each type. Three assays were pooled. Student's t test was used to test for significance between wells. The only experimental sequence to show any significant difference from control wells were the wells containing EDTA at 10^{-5}, where there was a slight increase in invasion ($t = 2.7$; d.f. 88, $P < 0.05$. Corresponding figures for EDTA at 10^{-4} and 10^{-6} were $t = 1.54$; d.f. 88, $P > 0.05$, $t = 0.8$; d.f. 88, $P > 0.05$. Figure 4.3).

The slight enhancement of invasion using EDTA at 10^{-5} has been seen in a study by Patten et al. (1973), who looked at the effects of cell concentration and various anticoagulents, including EDTA, on neutrophil migration. In some assays, they found a slight enhancement in the chemotactic response of neutrophils, with EDTA at 1×10^{-2} M. This effect is unexplained. Conclusions from this series of assays are that neutrophil invasion in the presence of EDTA, a scavenger of Zinc at pH 7.6 and hence a protease inhibitor, is not affected, suggesting that proteolytic activity is not necessary for neutrophil invasion in this assay system. EDTA might also reduce adhesion, though this has been suggested as being of little importance in neutrophil locomotion through a collagen gel (Brown, 1982).

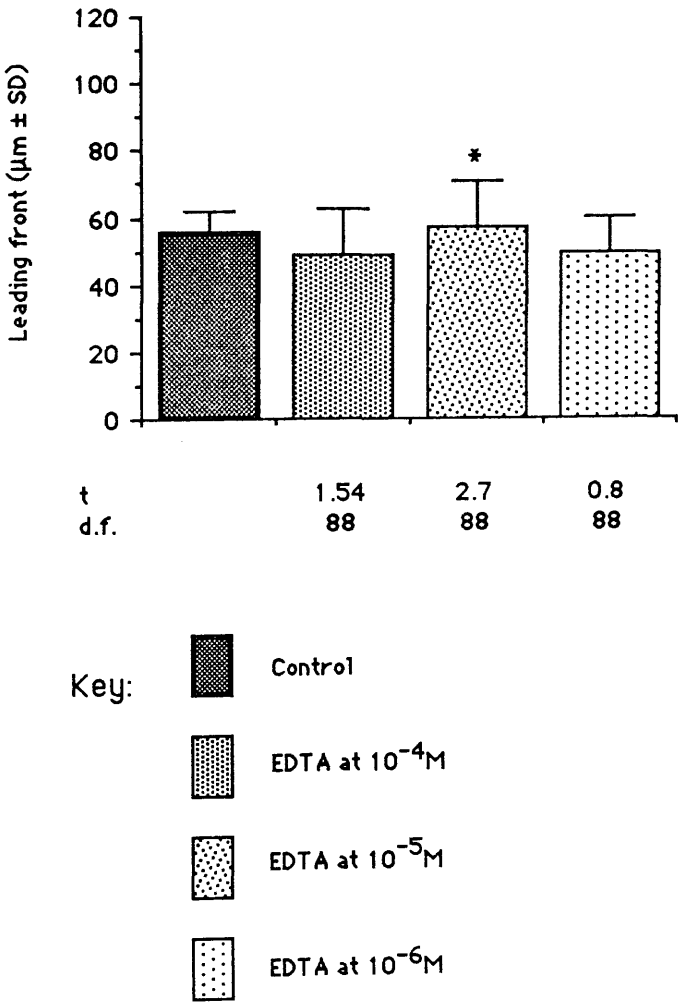


Fig. 4.3 Effect of EDTA on neutrophil invasion of collagen gels

4.3.2 Effect of o-phenanthroline on gel invasion

Two assays were carried out, using o-phenanthroline, another known metallo-protease inhibitor, at concentrations of 10, 1 and 0.1 $\mu g\ ml^{-1}$ (50, 5 and 0.5 mM). Assay conditions were similar to the EDTA assays, and assays were scored for the leading front. There was no significant difference between similar well types in the assays so the data were pooled and Student's t test used to look for significance between control and experimental wells. There was a significant inhibition of invasion in wells with the highest concentration of o-phenanthroline, which is attributed to toxicity effects ($t = 4.08$; d.f. 58, $P < 0.001$). In the wells containing o-phenanthroline at 5 and 0.5 mM strength, there was actually a paradoxical increase in invasion when compared with control wells ($t = 7.39$; d.f. 58, $P < 0.001$, and $t = 2.21$; d.f. 58, $P < 0.05$ respectively). (Figure 4.4). It can be concluded from the experiments with o-phenanthroline that proteolytic activity is not necessary for neutrophil invasion in this assay system.

4.4 Effect of cell concentration on locomotion over a substratum

It was observed that in the second set experiment, involving invasion assays of collagen gels by ^{51}Cr-labelled cells, an enhancement of invasion was possibly linked to cell concentration, as wells containing cells at 2×10^6 ml^{-1} showed a greater percentage of the cell population had invaded the gels when compared with wells containing cells

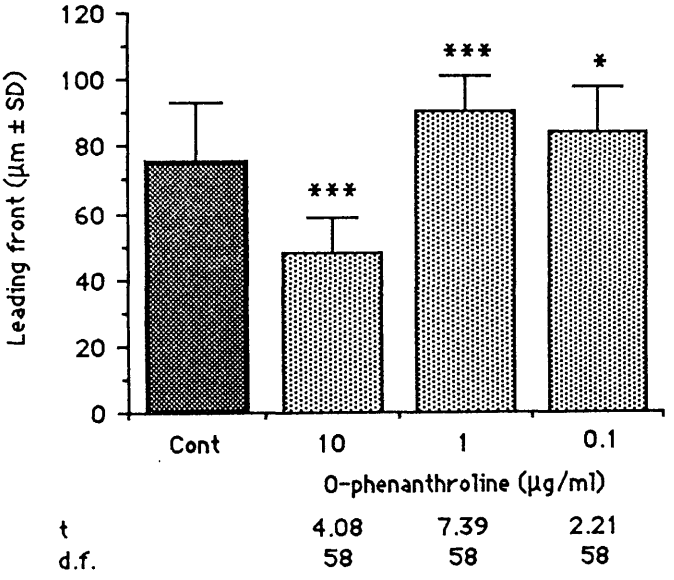


Fig. 4.4 Effect of o-phenanthroline on neutrophil invasion of collagen gels.

*** significance compared with control

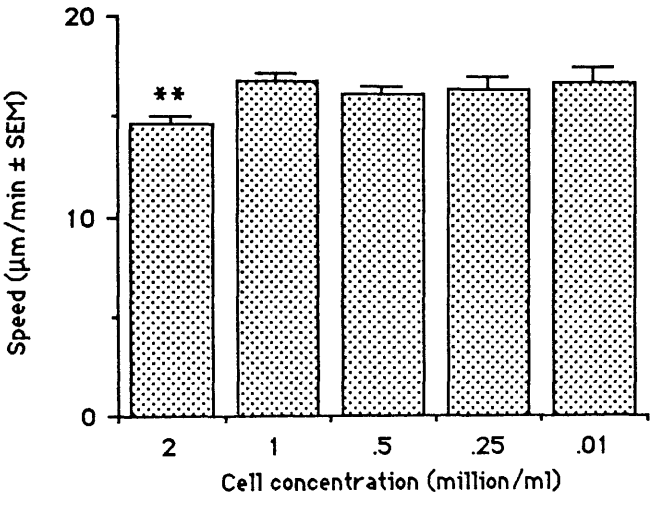
at 1 x 10^6 ml^{-1}. The automated cell tracking system allowed a study of the effect of cell concentration on population speed and persistence, as an increase in the mean speed of a population is one obvious way in which invasion could be increased.

Three assays were carried out, each consisting of 5, 30-minute sequences. Cells were filmed at concentrations of 2.0, 1.0, 0.5, 0.25 and 0.01 x 10^6 ml^{-1} in filming chambers in 50% H2, 50% peritoneal exudate.

On finding no inter-assay variation, data from sequences of the same concentrations were pooled for statistical analysis using the Mann-Whitney U-test, while the speed and persistence data obtained from the cell concentration of 1.0 x 10^6 ml^{-1} were compared to the speeds and persistences obtained at the other cell concentrations. The effect of cell concentration on speed became apparent only at the highest concentration used, 2.0 x 10^6 ml^{-1}. At this concentration there was a significant reduction in the population speed ($U = 2.96$, $P < 0.01$, Figure 4.5).

The effect of cell concentration on population persistence showed a graded response; persistence being lowest at the highest cell concentration (U-values 5.08; $P < 0.001$, 2.34; $P < 0.05$, 4.42; $P < 0.001$ and 3.97; $P < 0.001$ for cell concentrations 2.0, 0.5, 0.25 and 0.01 x 10^6 ml^{-1} respectively, Figure 4.6).

These results, showing that cells at a concentration of 1 x 10^6 ml^{-1} were moving more quickly over protein-coated glass than were cells at 2 x 10^6 ml^{-1}, do not explain the results

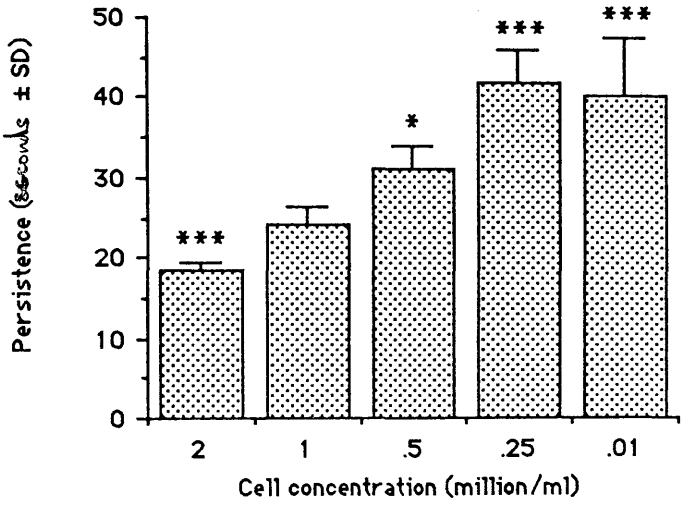


number of cells	129	118	115	80	51
U	2.96				

** significance compared with value for 1.0 million/ml

(Note: Standard Error of Median is given by the approximation $1.25 \times \Sigma/\sqrt{n}$, where Σ = SD of population)

Fig. 4.5 Effect of cell concentration on neutrophil speed over a 2-D substratum (values given are medians)



number of cells	129	118	115	80	51
U	5.08		2.34	4.42	3.97

*** significance compared with value for 1.0 million/ml

Fig. 4.6 Effect of cell concentration on neutrophil persistence over a 2-D substratum

from section 4.1, concerning the second set experiment, where a greater percentage of cells were found to invade collagen gels at a cell concentration of 2×10^6 ml^{-1} than at 1×10^6 ml^{-1}. Clearly, the invasion of a collagen gel by a population of cells, while proportional to its initial density on the gel surface, is not due simply to an increase in population speed. Syrian hamster melanoma cells have also been shown to exhibit a cell density effect when invading collagen gels (Schor et al., 1982), and the suggestion has been made that some cell-released factor from more densely-packed cells stimulates invasion.

It is difficult to see how invasion could be stimulated without an increase in speed. Neutrophils, however, do not readily move over a collagen surface, and it may be that it is wrong to extrapolate the situation on protein-coated glass to the situation on a collagen gel surface. It may be possible that neutrophils, moving at high densities over protein-coated glass, are activated, but because of collisions with other cells this increased activity is not being reflected in an increase in speed. The fact that an increase in cell concentration correlates well with a decrease in persistence would support this collision explanation. On invasion into the collagen gel however, the considerable reduction in interaction with neighbouring cells could allow the increase in speed to show, by an increase in the leading front.

4.5 Neutrophil invasion of a rigid collagen matrix

If neutrophils invading a collagen gel require to be able to push aside small obstacles in their path, then movement through a rigid collagen matrix should be restricted to some degree. Glutaraldehyde fixation of a collagen gel renders the matrix rigid (and hence suitable for SEM), and one such assay was done, also incorporating immune complex in the medium above some of the wells, as a further inhibitor of invasion. Collagen was prepared as for a standard invasion assay, at a concentration of 1.0 mg ml^{-1}. After incubation of the gel and removal of medium, one ml of 4% glutaraldehyde was added to each well, left for one hour then removed. The wells were washed overnight. Free aldehyde groups were blocked by addition of 0.1M glycine for 2 h, the glycine solution was changed and left overnight, before re-introducing H2 and using the gel as for a standard invasion assay.

For immune complex wells, SAPU antibody was incubated with an appropriate amount of conalbumin for 2 h at 37°C before adding to the wells with cells. Neutrophils were added at a concentration of 1 x 10^6 ml^{-1}, incubated for 2 h, fixed in formol saline then scored for the leading front assay. Five readings were taken from each of three control wells, which contained no immune complex, and five readings from each of the 3 wells containing IC.

A standard collagen gel invasion assay was run in parallel with the glutaraldehyde-fixed gel, using the same batch of collagen and the same batch of neutrophils. Readings from

this assay were taken from 3 control wells, five from each well, and from 3 wells containing IC prepared in the same way as in the glutaraldehyde-fixed assay. There was a significant reduction in invasion in all glutaraldehyde-fixed wells, both in the IC wells and in the IC-free wells, when compared with invasion in the wells from the standard assay run in parallel (67.7 ± 12.73 μm and 60.4 ± 16.96 μm, versus 114.5 ± 10.34 μm; $t = 11.59$, d.f. 28, and $t = 10.2$, d.f. 28, $P < 0.001$ in both cases).

There was no significant difference between the glutaraldehyde-fixed wells with and without IC ($t = 0.59$, d.f. 28, $P > 0.05$). In the standard assay run in parallel, there was a significant reduction in invasion in the wells containing IC, when compared with control wells in the same assay (89.1 ± 16.6 μm versus 114.5 ± 10.34 μm; $t = 4.88$, d.f. 28, $P < 0.1\%$). Data are shown in Figure 4.7.

It was expected that neutrophil invasion of a rigid collagen gel would be reduced, when compared with control wells from a normal collagen gel. The progress of a cell, moving by a process of extension, expansion, and contraction, is probably aided to some extent by a degree of give in the matrix, small fibrils being pushed aside. If this is prohibited, by stiffening the matrix, we would expect the cell to take longer to pass constricting obstacles. There was no further reduction in invasion in the stiff gels with IC in the medium. Any reduction so induced would probably have been small, and so did not show over the primary reduction induced by the IC in the medium above the gel.

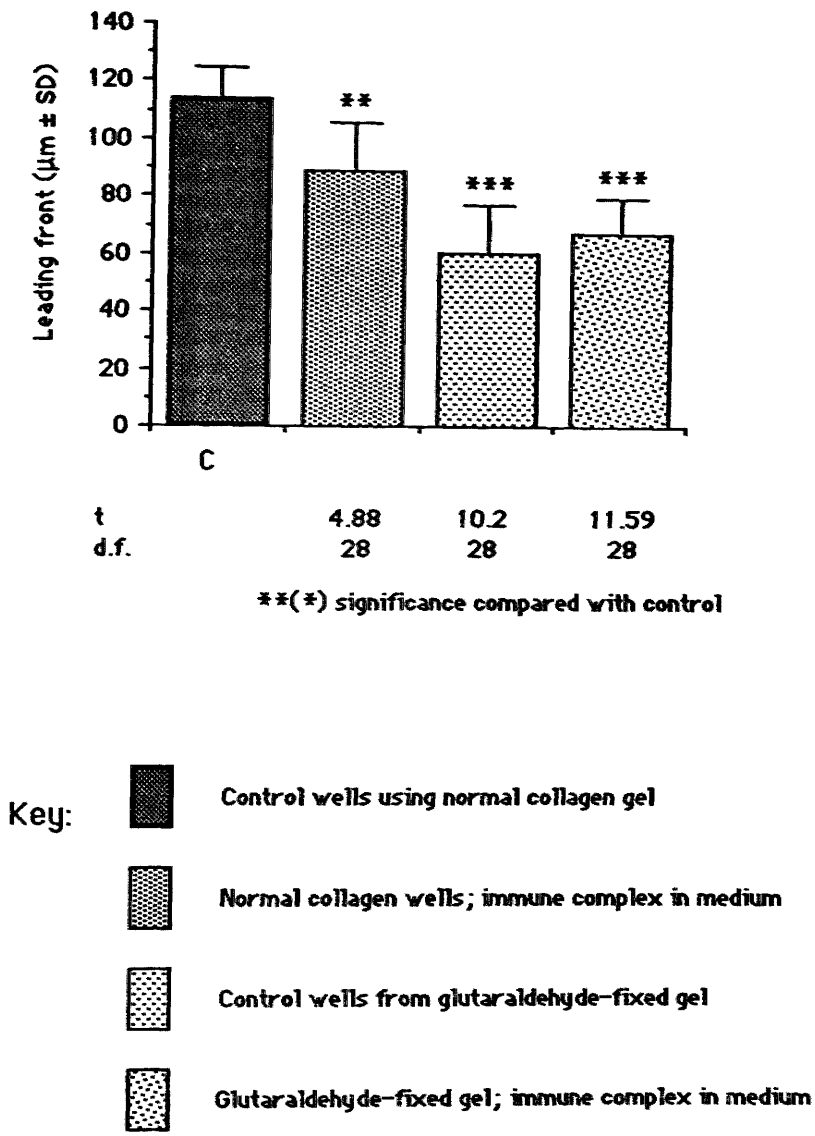


Fig. 4.7 Neutrophil invasion of normal and rigid collagen gels

Summary

1. The passage of a primary invasive wave of neutrophils does not confer any detectable benefit on the passage of a secondary wave.

2. Using labelled collagen gels, there was no detectable proteolytic activity by stimulated, invading neutrophils, in an assay capable of detecting degradation of the collagen from amounts as low as 5% of the predicted amount of collagenase released by the invading neutrophils.

3. In standard invasion assays of collagen gels, the presence of two known metallo-protease inhibitors, EDTA and o-phenanthroline, made contrasting differences to the degree of invasion, as measured by the leading front.

4. The effect of cell concentration on the speed and persistence over protein-coated glass was looked at. At a high concentration of cells, 2×10^6 ml^{-1}, there was a significant drop in speed. There was a graded response in persistence to cell concentration; the higher the cell concentration, the lower the persistence.

5. Neutrophils invade a cross-linked collagen gel to a lesser degree than they do a standard collagen gel.

Chapter 5

Visual Migration Assays using Immune Complexes and Chemotactic Peptides

Introduction

Neutrophil leucocytes are the first host white blood cells to appear at a site of acute inflammation, having perceived some chemotactic signal during their passage through the circulation. The complement system has been shown to provide several such chemotactic components attractive to neutrophils, C3a and the more active C5a. Also proposed as activators of neutrophil chemotaxis are IgG, IgG + C3, and C5b67 (Roitt et al., 1985). Formation of immune complexes (IC), with antigen and IgG or IgM, can lead to complement activation and a consequent acute inflammation attracting neutrophils. It has been shown that both soluble and insoluble IC are capable of attracting neutrophils (Wilkinson, 1982; Leung-Tack et al., 1977). These cells phagocytose the larger IC, releasing lysosomal enzymes with resultant damage to local tissue - the Arthus reaction. Small soluble complexes, on the other hand, formed in situations of excess antigen, are not readily phagocytosed (Anderson, 1985). Persisting in the circulation, they are slowly removed by the mononuclear phagocyte population. IC trapped in tissues may not readily be phagocytosed by attracted neutrophils, these cells then causing tissue damage by release of lysosomal enzymes. Complement can also solubilise precipitated IC. (Roitt et al., 1985).

The mechanism of formation and degradation of immune complexes is obviously not a simple one, and it would be of interest to attempt to produce an in vitro model. One such model involves collagen gels, in which invasion of these gels by neutrophils might be modified by the presence of an antigen, an antiserum to that antigen, or the product of the interaction between an antigen and an antiserum - an immune complex (IC), with or without complement. An IC present in the medium above a gel, for example, might inhibit invasion of that gel by neutrophils, particularly if the IC has activated the complement system. Conversely, an IC incorporated at some depth in the gel might stimulate invasion, if a gradient of chemoattractant were detectable by invading cells. As the system involving IC's, neutrophils and their enzymes, and complement is so complex, it was felt that experiments such as those outlined above, in which conditions are well-defined, might produce useful results.

Visual migration assays were carried out using collagen gels prepared as described earlier, with initial trials employing sheep anti-human albumin serum, and later assays using a high titre rabbit anti-ovotransferrin as the antiserum, and ovotransferrin (conalbumin) as the antigen. The sheep serum was purified using Ion-Exchange Column Chromatography and collected fractions were run on a Polyacrylamide Gel Electrophoresis assay to verify the presence of IgG. (See Plate 2.4, following page 68.)

For work with the anti-albumin serum, a quantitative precipitation test was carried out using purified fractions

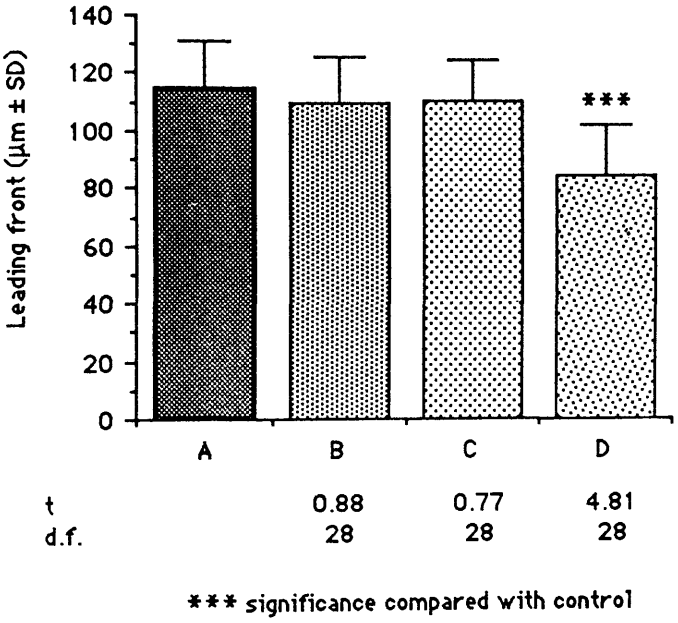
to determine the approximate equivalence point (Table 5.1). Additionally, an Immunodiffusion test, using the Ouchterlony technique described in Materials and Methods, was performed, suggesting that equivalence was obtained with 300 μg ml^{-1} of human albumin (Sigma) and 10 μl sheep serum. For running invasion assays, the amount of antigen was increased to 400 μg ml^{-1}, to facilitate the formation of a soluble IC (i.e. antigen excess). In an *in vivo* situation, neutrophils are attracted to the site of damaged tissue by recognition of, and response to, a chemotactic gradient. In this *in vitro* model, using collagen gels, a soluble IC was preferred, as offering more likelihood of being available at the surface of a collagen gel. Whether or not IC are chemotactic for neutrophils should be easily verified, by looking for an effect on invasion by complement-free IC. The situation is not a simple one however, and as the model is, for the first hours at least, a dynamic one, with several substances diffusing in different directions, it is difficult to predict conditions in any one assay.

Results

In the first two assays performed, the presence of a substance chemotactic for neutrophils in the medium above the gels should result in an inhibition of invasion, as measured by the leading front. Assay 1 contained four types of well (Figure 5.1); control wells containing only collagen and cells in H2, wells with 50 μl of pre-diluted serum, wells with 20 μl of 400 μg ml^{-1} albumin, and wells

with both. Five readings were made from three duplicate wells of each type. On scoring for the leading front after incubating the wells with cells for 2 h at 37°C, wells containing serum only and wells with albumin only showed no difference compared with controls (leading fronts 120.1, 119.3 and 110.9 μm respectively, $t = 0.11$; d.f. 28, $P > 0.05$, $t = 1.41$; d.f. 28, $P > 0.05$). Wells containing albumin and serum showed a small but significant inhibition of invasion, with the leading front mean of 92.7 μm ($t = 3.96$; d.f. 28, $P < 0.001$). (Figure 5.1). This result is consistent with there being a chemoattractant present in the medium above the collagen gels; giving a slight inhibition of invasion. The serum was not heat-treated, and the inhibition could have been due to either IC or complement.

Assay 2 was a repeat of assay 1, with similar results. Wells containing serum only and wells with albumin only showed no significant difference compared with controls (leading fronts 115.2, 109.9, 110.8 μm respectively, $t = 0.88$; d.f. 28, $P > 0.05$, $t = 0.77$; d.f. 28, $P > 0.05$). Wells containing albumin and serum showed a small but significant inhibition of invasion, with the leading front mean of 84.7 μm ($t = 4.81$; d.f. 28, $P < 0.001$). (Figure 5.2). Following repeat assays in which there was no consistent effect on cell invasion, with in some cases no inhibition of invasion in wells containing both serum and albumin, the use of the low titre anti-albumin serum was switched to a high titre rabbit anti-conalbumin serum.



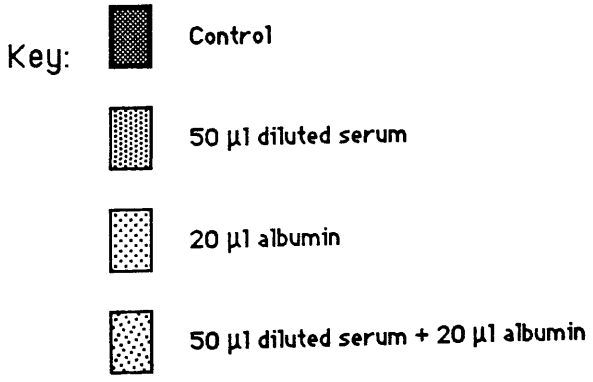


Fig. 5.2 Leading front scores from assay 2

It should be stated , however, that these two assays, while not dramatic in their effects, are consistent with the prediction that formation of an immune complex and/or complement activation will modify cell invasion of a collagen gel; in this case with the presence of an IC in the medium above a gel inhibiting invasion of that gel. The inhibition is perhaps weak and inconsistent due to a very low titre of antibody, this despite purification with Ion-exchange column chromatography. It has been demonstrated that soluble antigen-antibody complexes made in antigen excess of 20 times equivalence proved virtually equally effective as insoluble IC at inducing the release of intracellular enzymes by neutrophils (Hawkins & Peeters, 1971).

For the following invasion assays, using the high titre prepared anti-conalbumin serum, several modifications were attempted. Invasion assays were run, in which experimental wells contained collagen with incorporated conalbumin, at either 10, 100, or 500 $\mu g\ ml^{-1}$. Four separate collagen mixes were prepared; three with different concentrations of conalbumin, and one with no conalbumin. The final concentration of collagen was identical in each mix. Each collagen solution was kept in a glass universal bottle on ice until pouring into tissue culture wells.

After incubating the collagen for 60 m at 37°C, one ml of medium was added to each well, medium containing the appropriate concentration of conalbumin for each type of well. This ensured full equilibrium of conalbumin in experimental wells, control wells containing no conalbumin in the

medium. Gels were then incubated for 2 h at 37°C before changing the medium and adding cells at 1 x 10^6 ml^{-1}.

The principal intention with this series of invasion assays was to attempt the formation of immune complexes either at or near the surface of the gels, or at some depth in the gels. Neutrophil invasion of the gels should then be modified accordingly. It is known, for example, that neutrophils adhere rapidly and strongly to immune complexes (Lackie et al., 1987), and that their movement is reduced on complex-coated surfaces (Wilkinson et al., 1984). Secondly, it should be possible to test for any chemotactic properties possessed by antigen or antibody, or IC without complement, formed by using heat-inactivated serum.

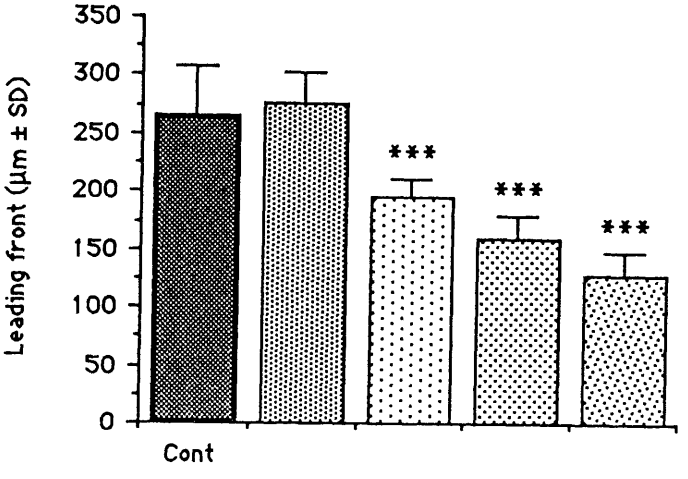
Experimental wells each had 10 µl or more of serum added to the medium along with the cells. Some wells had heat-inactivated serum added, serum having been previously heated for 30 m at 56°C to inactivate serum complement, while some conalbumin-containing wells had no serum added to the cell suspension. Finally, some wells had no conalbumin incorporated in the collagen but had serum added with the cells. Assays were scored for the leading front, and in some cases the cell distribution was examined, counting the number of cells in focus per field in planes at 20 µm intervals down through the gel. The equivalent point, using the high-titre antiserum with complement, was 1 µl antiserum to 4 µg conalbumin. When inactivated, the equivalence point was 1 µl antiserum to 8 µg conalbumin.

Assays were of two types; a standard invasion assay adapted for immune complexes (13 assays described), and the slab assay, employing thin slabs of collagen (11 assays described). In addition, the standard assay was designed for use with either a single, or a double layer of collagen (9 and 4 assays respectively).

5.1 Standard invasion assay (Assays 3 - 15)

Assays 3 - 6 Conalbumin in experimental gels only

This series of assays was scored for leading fronts. Assay 3 was scored both for leading fronts and for cell distribution. Five measurements were made from duplicate wells in each of the five types of well. Conalbumin was incorporated into the collagen gels before plating, while 10 μl of serum was added to the medium overlying the gel in some wells. Control wells contained neither conalbumin nor serum. There was no difference in the leading front between the control wells and wells containing 10 μg ml^{-1} conalbumin plus normal serum ($t = 0.66$; d.f. 18, $P > 0.05$). The remaining three types of experimental well all showed an inhibition of invasion, as shown by a decreased measurement for the leading front ($t = 4.66, 6.8, 8.7$; d.f. 18 in all cases, $P < 0.001$ in all cases. Figure 5.3). As this assay result would seem to indicate that there was insufficient antigen at 10 μg ml^{-1} to modify neutrophil invasion (compared to conalbumin at 100 μg ml^{-1} with 10 μl of serum resulting in an inhibition of invasion), the amount of conalbumin used was increased in the following assays.



*** Significance compared with control

Key:

Control wells: no conalbumin, no serum

10 µg/ml conalbumin + 10 µl serum

100 µg/ml conalbumin + 10 µl serum

10 µg/ml conalbumin + 10 µl heat-inactivated serum

100 µg/ml conalbumin + 10 µl heat-inactivated serum

Fig. 5.3 Leading front scores from assay 3

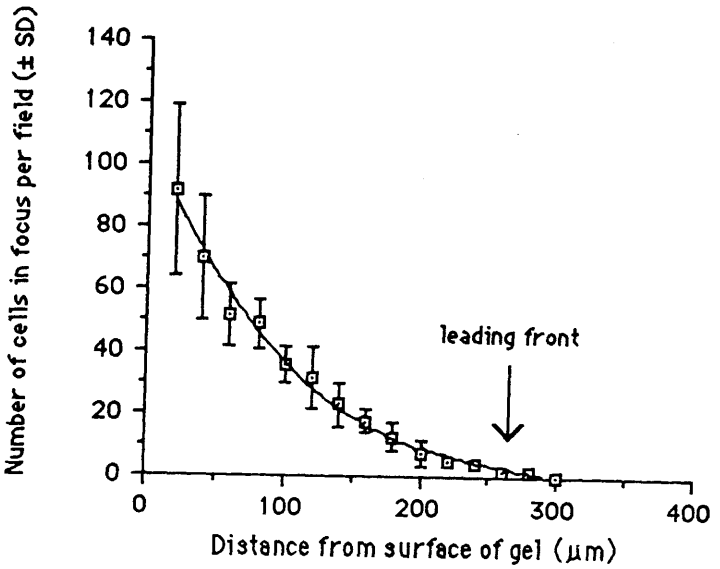
The inhibition of invasion found in wells containing 100 μg ml^{-1} conalbumin plus 10 μl serum (i.e. an antigen excess of about 2.5 times equivalence) might be expected if neutrophils are being attracted to an area not far from the surface of the gel, which would be predicted if the interaction of conalbumin and antibody in the serum were to form an IC perhaps also leading to some degree of complement activation. Results from the two wells containing heat-inactivated serum, also indicate a significant inhibition of invasion. In wells containing 10 μg ml^{-1} conalbumin plus 10 μl heat-treated serum, there should be antibody excess, with the formation of a soluble IC. In wells containing 100 μg ml^{-1} conalbumin plus 10 μl heat-treated serum, there should be near equivalence, with a very small antigen excess. It is difficult to predict the situation present in these wells, but again there was a significant inhibition of invasion, possibly due to the presence of both soluble and insoluble IC.

Assay 3 was also scored for cell distribution; triplicate counts being made from two wells each from both control and wells containing 100 μg ml^{-1} conalbumin plus normal serum. Depths of penetration are larger in this measurement as single cells in focus are counted, in contrast to the leading front method, where the two deepest cells in focus are taken to mark the leading front for that population of cells (Figures 5.4, 5.5). Cell distributions in the experimental wells containing conalbumin plus heat-inactivated serum were similar to distributions shown in the control wells, and are not shown.

The distribution as shown in Figure 5.4, of control wells, was plotted with $Log_{10}N$ versus d^2, where N is the number of cells in focus per field at a depth d μm. The resulting graph gave a straight line, suggestive of that to be expected of random locomotion by the invading cells (Zigmond & Hirsch, 1973; Islam et al., 1985). The cell distribution shown in Figure 5.5, however, clearly demonstrates a chemotactic response, as cells show a peak concentration within the collagen gels at a depth of about 100 μm below the surface of the gel.

Assay 4 was scored for the leading front, four control wells and three from each experimental well, each well having five duplicate measurements. In addition to increasing the amount of conalbumin present in experimental wells, some wells contained conalbumin incorporated in the collagen but no serum in the medium. All experimental wells were significantly different from the control, wells containing complex showing a marked inhibition of invasion, while wells containing conalbumin only showed an enhancement of invasion, as indicated by the leading front measurements. (Data given in Figure 5.6). Wells containing conalbumin and serum were expected to shown an inhibition of invasion, as neutrophils would be attracted to an area near the surface of the gel, where antigen and antibody would be interacting. (In the one case with antibody excess, in the other with antigen excess).

The finding that the conalbumin-only wells showed an enhancement of invasion was unexpected, contamination of the conalbumin being one possible explanation. As other



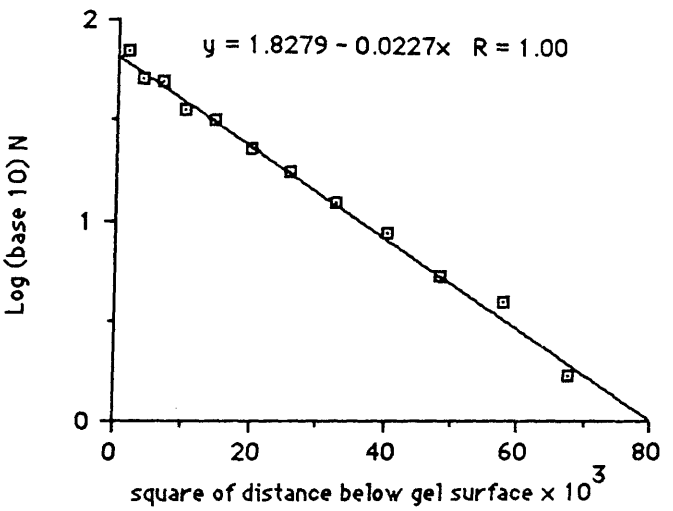
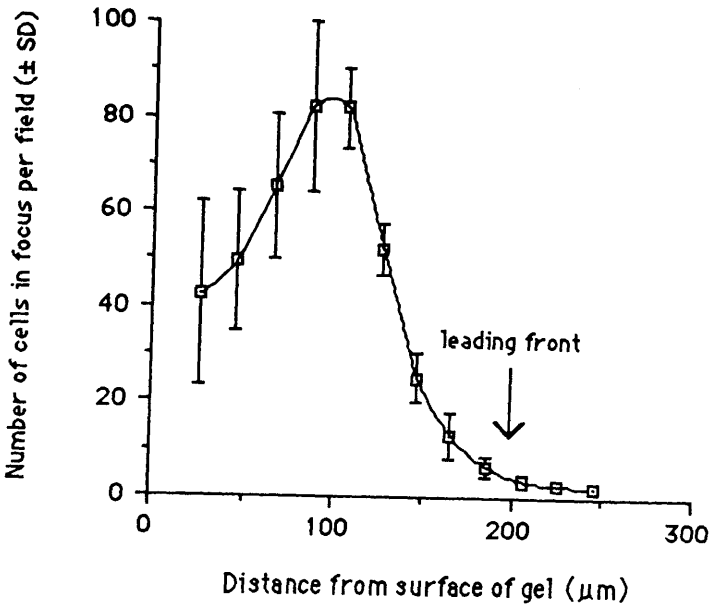


Fig. 5.4 Normal distribution of invading neutrophils in collagen gel with no chemoattractant present

(From assay 3)

Graph of LogN (base 10) versus square of distance below gel surface is also given; this is a straight line indicating cell movement by a random walk



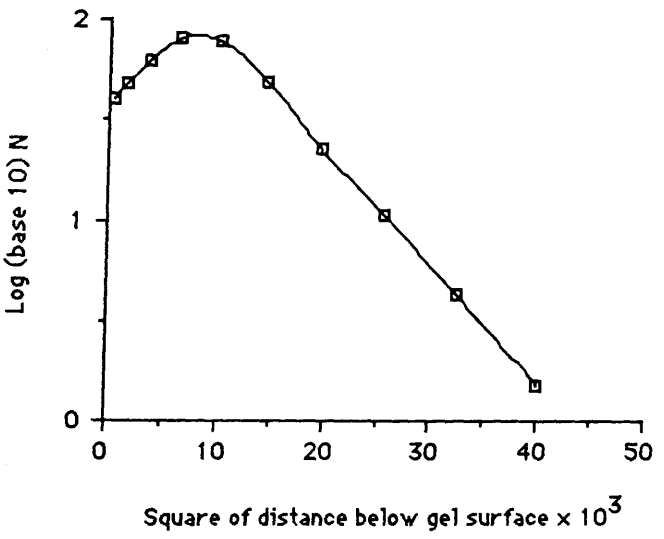
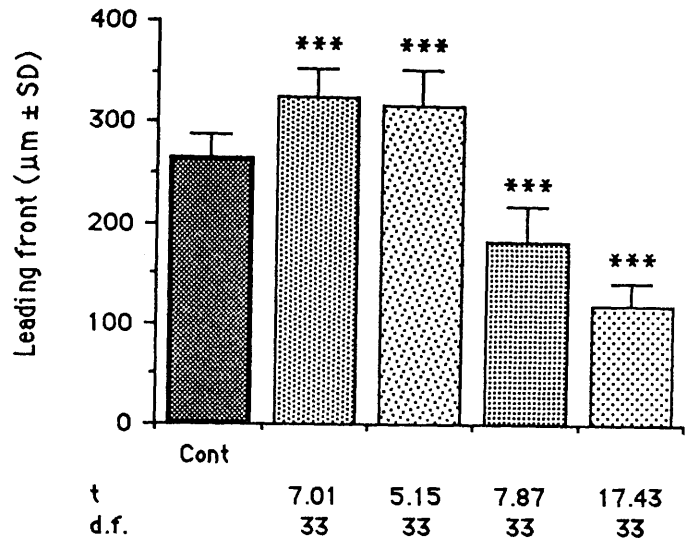


Fig. 5.5 Experimental cell distribution in collagen gel incorporating immune complex (from assay 3) (100 μg/ml conalbumin + 10 μl serum)

Also shown is plot of LogN (base 10) versus square of distance below gel surface. This is not a straight line, indicating that the cell population has undergone a chemotactic response



Key:

Control wells: no conalbumin, no serum

100 µg/ml conalbumin, no serum

500 µg/ml conalbumin, no serum

100 µg/ml conalbumin + 50 µl serum

500 µg/ml conalbumin + 50 µl serum

Fig. 5.6 Leading front scores from assay 4

assays (see results from assays 5 and 6, for example) contradicted this, and conalbumin was used from the same frozen stock, this finding cannot be explained, unless caused by contamination in the gel or well. In a series of time-lapse sequences (see section 5.3), conalbumin at concentrations of 100 $\mu g\ ml^{-1}$ and 500 $\mu g\ ml^{-1}$ was found to have no effect on the speed or persistence of neutrophils locomoting over protein-coated glass. Cells used in assays 3 and 4 were taken from different animals from those used in assays immediately before and after, and it may be worth pointing out that results from assays 3 and 4 would indicate particularly active cells, as indicated by large leading front scores.

Assay 5 was scored for the leading front. Control wells contained neither conalbumin nor serum, experimental wells contained conalbumin, serum, or conalbumin plus serum (500 $\mu g\ ml^{-1}$ conalbumin, 50 μl serum). Measurements were made from three wells of each type, five duplicates per well. Wells containing conalbumin only showed no effect on invasion. Wells containing 50 μl serum showed a small but significant increase in invasion compared to control wells. This might indicate a possible chemokinetic effect of the serum on the neutrophils. In the wells containing conalbumin plus serum, there was no apparent invasion of the collagen gels, the neutrophils having degranulated on the surface of the collagen gels. (Data given in Figure 5.7).

Results here were consistent with the activation of complement in wells containing conalbumin at 500 $\mu g\ ml^{-1}$

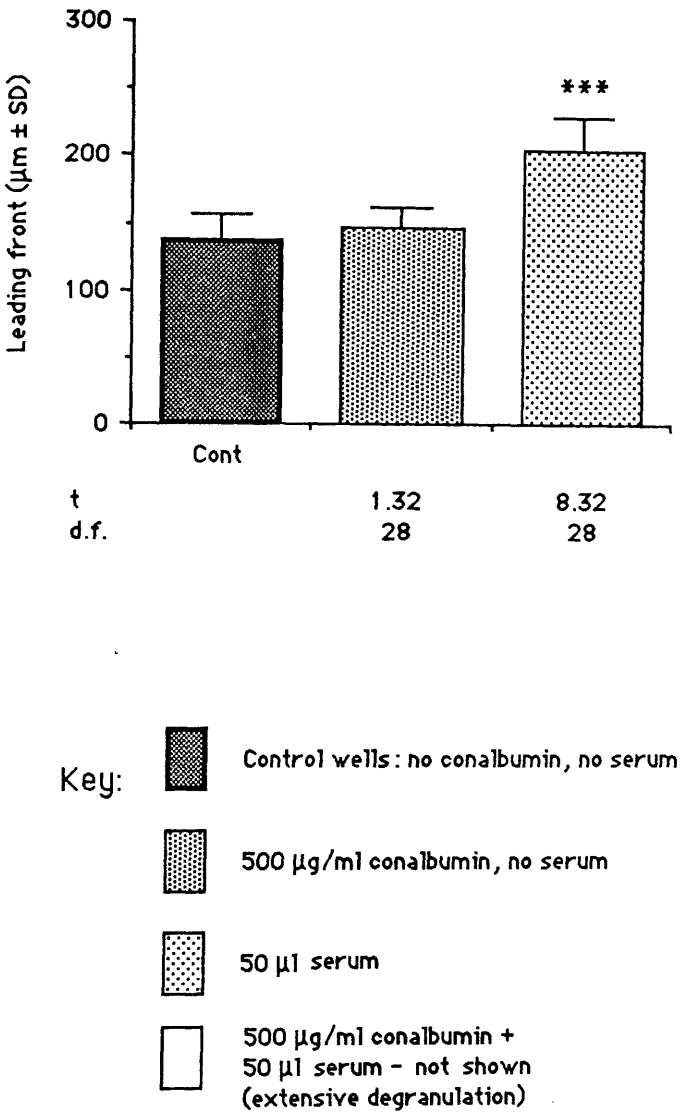


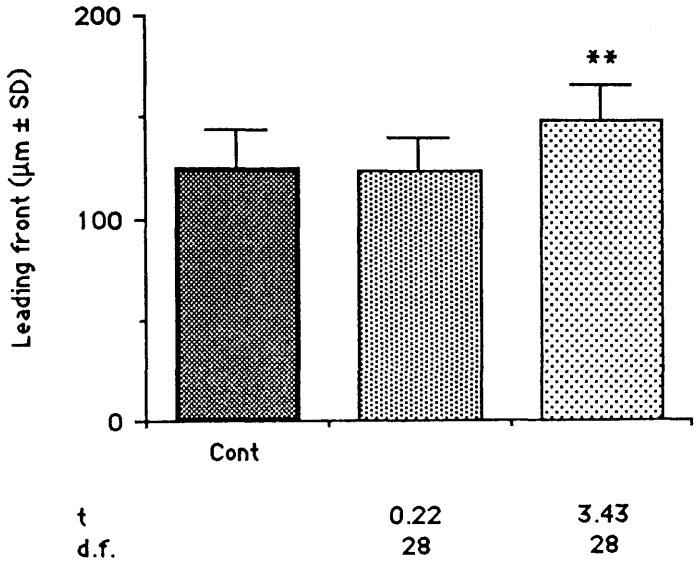
Fig. 5.7 Leading front scores from assay 5

plus 50 μl serum, as cells had degranulated on the gel surface. In contradiction to the two previous assays, wells containing conalbumin only showed no effect on invasion. Neutrophils undergo phagocytosis and release lysosomes on encountering a large number of microorganisms or immune complexes accumulated in tissue. Degranulation occurred in assay 5 but not in assay 4, in wells with the same mix of antigen and serum. This difference may be attributed to different batches of cells, as all other conditions are identical in these wells. There may be some threshold of activation required before lysosomal release, a drastic and damaging response, occurs.

Assay 6 was scored for the leading front. This assay was identical to assay 5 and results were similar, with no difference between control wells and conalbumin wells, an enhancement of invasion in serum wells, and degranulation with no invasion in complex wells. (Data given in Figure 5.8).

Assays 7 - 11 Conalbumin incorporated in all gels

In this series of assays, conalbumin was incorporated in all collagen gels, as this simplified the production of the gels. Control gels therefore contained conalbumin, though results from assays 5 and 6 indicated that it had no effect on invasion. Assay 7 used single layers of collagen gel, containing either 50 or 500 $\mu g\ ml^{-1}$ of conalbumin. Control wells contained no serum, experimental wells had 5 μl of serum in the medium. Gels were incubated for 2 h at 37°C,



Key:

Control wells: no conalbumin, no serum

500 µg/ml conalbumin, no serum

50 µl serum only

500 µg/ml conalbumin +
50 µl serum - not shown
(extensive degranulation)

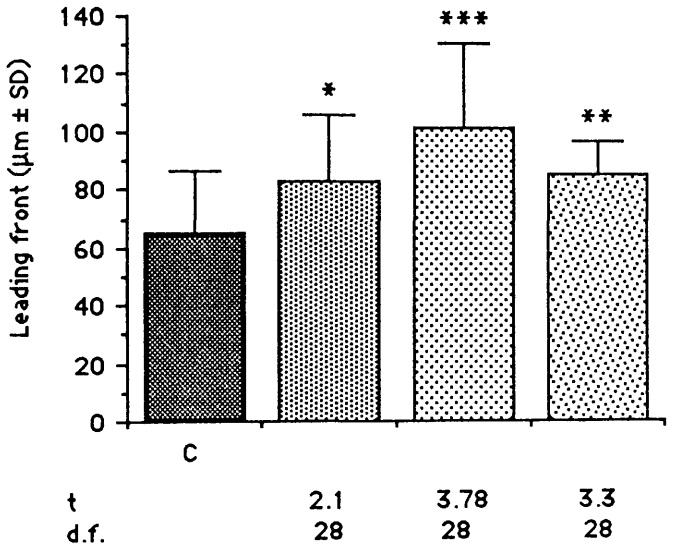
Fig. 5.8 Leading front scores from assay 6

** significance compared with control

washed several times with H2, then washed overnight at 4°C to remove free conalbumin and serum, as well as soluble IC. Cells were added and the assay incubated for 2 h at 37°C before fixing and scoring for leading fronts and for distribution. There was a small increase in invasion in the wells which had contained 500 $\mu g\ ml^{-1}$ conalbumin, compared with the wells which had contained 50 $\mu g\ ml^{-1}$ conalbumin, with a similar increase in invasion in the wells which had contained conalbumin at 500 $\mu g\ ml^{-1}$ plus 5 μl of serum. (Data given in Figure 5.9).

Distribution scoring for assay 7 indicated a similar distribution in each type of well, with a correspondingly greater number of cells to be found in the wells which had contained serum (Figure 5.10). It is possible that the presence of serum is acting as a chemokinetic agent, increasing the speed of the neutrophils in these wells containing serum, and hence the number of cells found in the gels (an effect seen in assays 5 and 6, see Figures 5.7 and 5.8). That the distribution scores indicate no accumulation at a particular level in the collagen gels, such as was shown in assay 1, would support this, although the time course of the assay could also have allowed complement to dissolve an immune precipitate, and allow redistribution of cells. As with assays 3 and 4, wells which had contained conalbumin at 500 $\mu g\ ml^{-1}$ seemed to enhance cell invasion, though here the enhancement was only just significant ($t = 2.1$; d.f. 28, $P < 0.05$.

The wells which had contained 50 $\mu g\ ml^{-1}$ of conalbumin plus 5 μl of serum showed the greatest enhancement of invasion.



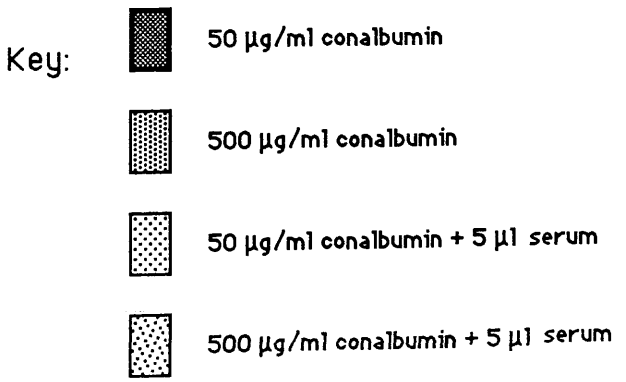
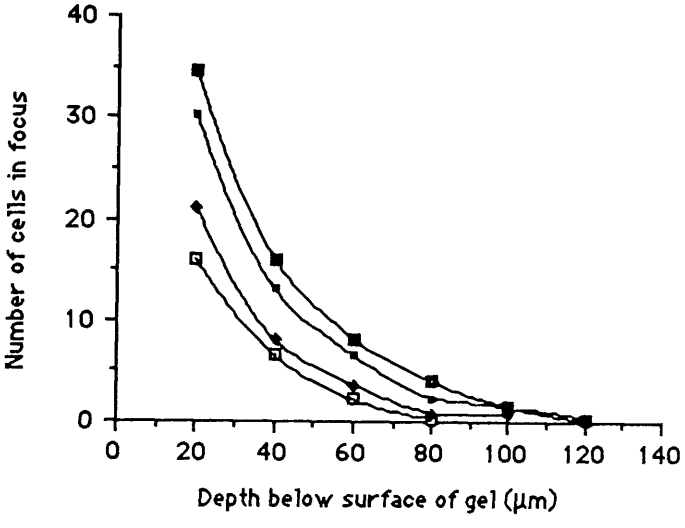


Fig. 5.9 Leading front scores from assay 7

*** Significance compared with control



t	4.98	4.38	3.99	2.15	
d.f.	22	22	22	22	
P <	0.001	0.001	0.001	0.05	†

Key:

- ▫ 50 μg/ml conalbumin
- ◆ 500 μg/ml conalbumin
- ■ 50 μg/ml conalbumin + 5 μl serum
- ▪ 500 μg/ml conalbumin + 5 μl serum

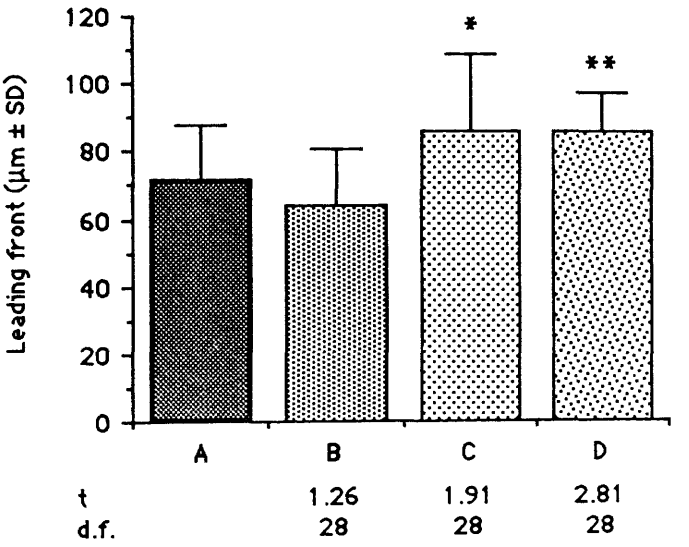
† For statistical testing, pooled conalbumin only data points were compared with pooled conalbumin plus serum data points, for each depth

Fig. 5.10 Distribution of cells in collagen gels: data from assay 7

In these wells, the ratio of antigen to antibody (2.5 times equivalence) may have been sufficient for IC to have been precipitated, leading to a limited activation of complement and enhancememt of invasion. Wells containing 500 μg ml^{-1} conalbumin plus 5 μl serum, on the other hand, giving an antigen:antibody ratio of 25 times equivalence, would contain mostly soluble IC, much of which would probably have been washed out, resulting in a smaller enhancement of invasion. The lack of degranulation of cells, as in assays 5 and 6, may indicate the existence of a threshold value, above which cells are sufficiently activated to lyse.

Assay 8 was prepared in the same way as assay 7 and scored for the leading fronts. Wells which had contained 500 μg ml^{-1} conalbumin showed no enhancement of invasion when compared with wells which had contained 50 μg ml^{-1} conalbumin. Wells which had contained conalbumin plus serum showed an enhancement of invasion over wells which had not had serum added to the medium before washing. (Data given in Figure 5.11). As in assay 7, any enhancement of invasion is likely to be due to either the presence of an insoluble immune complex, or complement activation by immune complex.

For assay 9, 10 μl per well of heat-inactivated serum was added to some of the wells, via the medium. Conalbumin was incorporated into the collagen gels at 50 or 500 μg ml^{-1}. Scoring for leading fronts, invasion was significantly reduced in the wells which had contained 500 μg ml^{-1} conalbumin, and in the wells with 50 μg ml^{-1} conalbumin plus 10 μl serum. In the wells which had contained 500 μg ml^{-1} conalbumin plus 10 μl serum, there was extensive



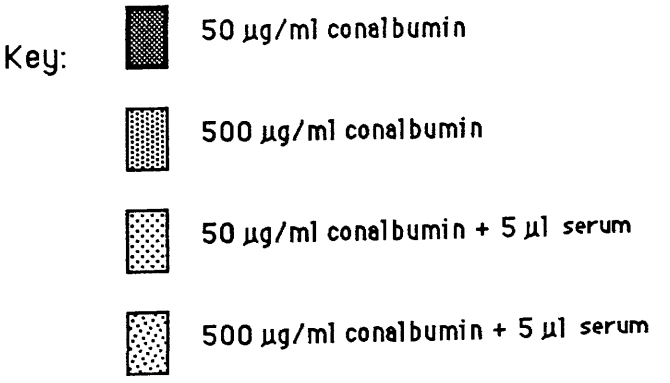


Fig. 5.11 Leading front scores from assay 8

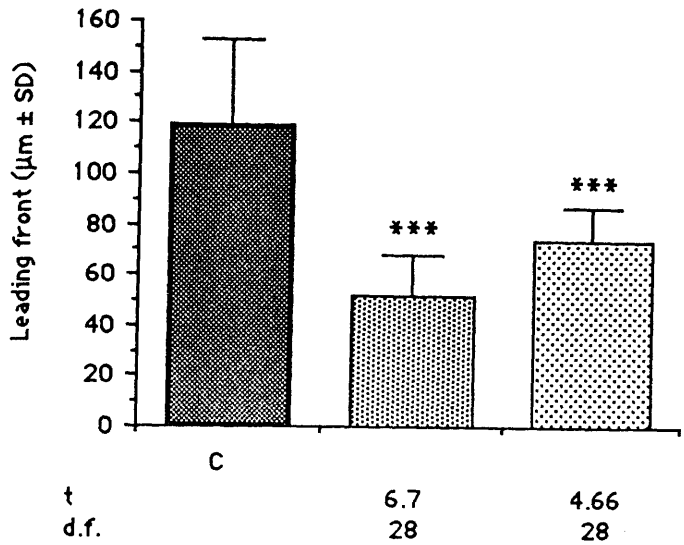
* Significance compared with A wells

** Significance compared with B wells

degranulation of the cells at the surface of the collagen gels. A few neutrophils had invaded the collagen gels in this last category of well, and though scoring these wells was impractical due to obscuring protein deposits, the maximum depth of invasion was typically about 30 μm, compared with a leading front depth of 119.3 μm in the control wells which had contained 50 μg ml^{-1} conalbumin.

The reduction of invasion in 500 μg ml^{-1} conalbumin wells cannot be explained, and is the reverse of the finding from some previous assays, where higher concentrations of conalbumin seemed to have slightly enhanced invasion. The inhibition of invasion in wells containing 50 μg ml^{-1} conalbumin plus 10 μl heat-treated serum can be explained by there being a slight antibody excess and the probable formation of insoluble IC, resistent to washing. The degranulation of cells in wells containing 500 μg ml^{-1} conalbumin plus 10 μl heat-treated serum indicates that IC must have been precipitated at or near the surface of the collagen gel, in sufficient amounts for cell lysis to occur. The ratio of antigen to antibody here was about 6 times equivalence, which is close to equivalence, but it must be pointed out, as in all of these assays, that the situation involves diffusing substances, with conditions impossible to predict, much as the in vivo situation. (Data given in Figure 5.12).

In assay 10, done on the same day as assay 9, the amount of heat-inactivated serum added to some wells was increased slightly to 15 μl. Results, scoring for the leading fronts, were similar to assay 11, with an inhibition of invasion in



Key:

Control: 50 µg/ml conalbumin

500 µg/ml conalbumin

50 µg/ml conalbumin + 10 µl heat-inactivated serum

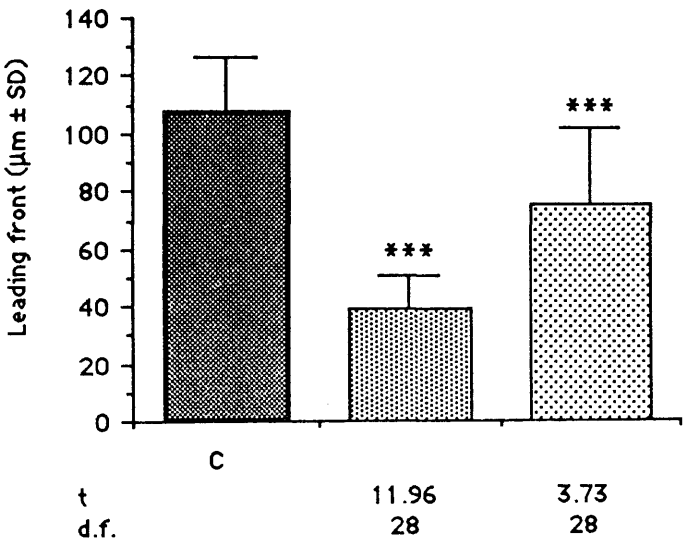
500 µg/ml conalbumin + 10 µl heat-inactivated serum - not shown (extensive degranulation)

Fig. 5.12 Leading front scores from assay 9

*** Significance compared with control wells

wells which had contained 500 μg ml^{-1} conalbumin and in the wells which had contained 50 μg conalbumin plus 15 μl serum when compared with the leading front in the control wells which had contained 50 μg ml^{-1} conalbumin. In the wells which had contained 500 μg conalbumin plus 15 μl serum, there was extensive degranulation of the neutrophils at the surface of the collagen gels. As in assay 9, deposited material made these wells difficult to score, but the few cells which had invaded the collagen gels had a maximum depth typically of about 40 μm, compared with a leading front depth of 108 μm in the control wells. (Data given in Figure 5.13). Assays 9 and 10 must be viewed with some scepticism, and perhaps as an indication of the delicate checks and balances present in such invasion assays, but the reduction of invasion in wells with 50 μg ml^{-1} conalbumin plus 15 μl of heat-inactivated serum is consistent with the formation of an IC at or near the surface of the collagen

For assay 11, one layer of collagen was used, with cells incorporated in the collagen during the mixing. The intention in this assay was to see whether it was possible to change the initially uniform distribution of cells in the collagen gel, by the formation of an IC at, or near the surface of the gel. The collagen also incorporated conalbumin, at 50 μg ml^{-1}. Two gels were fixed 30 minutes after plating, to act as starting controls, and two gels were fixed along with the other gels at 3 h after plating, to act as end controls. The remaining wells were incubated for 3 h at 37°C with either normal or heat-inactivated



Key:

50 µg/ml conalbumin

500 µg/ml conalbumin

50 µg/ml conalbumin + 15 µl heat-inactivated serum

500 µg/ml conalbumin + 15 µl heat-inactivated serum - not shown (extensive degranulation)

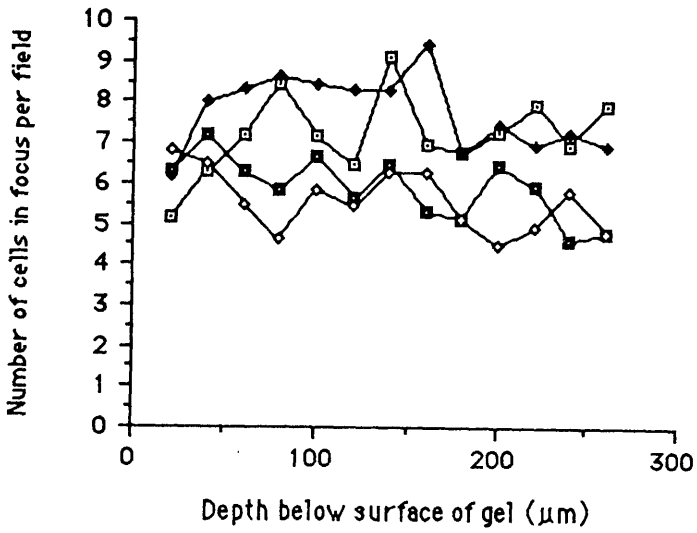
Fig. 5.13 Leading front scores from assay 10

*** Significance compared with control wells

serum in the medium. This assay was scored for distribution of cells, counting the number of cells in focus per field at 20 μm intervals. Cell distribution, tested by one way analysis of variation, was not significantly different from normal variation ($F = 0.67$; d.f. 5,30). Distribution plots are shown in Figure 5.14. Results from this assay would indicate that cells were not undergoing a chemotactic response, otherwise there would be a significant redistribution in cell count. From previous assays the formation of an IC attractive to neutrophils would be expected in this assay, so the apparent non-relocation of cells is surprising. (But see assays 16 onwards, incorporating cells in collagen gels).

Assays 12 - 15 Two layers of collagen

In an attempt to manufacture a chemotactic gradient in the gel, in assays 12 - 15 the standard visual assay system was modified slightly. For assays 12 and 13, scored for the leading fronts, the amount of conalbumin incorporated in the collagen gels was lowered from 500 μg ml^{-1} to 100 μg ml^{-1}, keeping the amount of normal serum added the same, at 50 μl. This gives an antibody excess, of about 2 times equivalence. Two layers of collagen were plated into each well; the lower one, collagen A, at a concentration of 1.5 mg ml^{-1} (to provide a better mechanical support), which contained 100 μg ml^{-1} conalbumin, or no conalbumin, and collagen B, plated over collagen A, at the standard concentration of 1.0 mg ml^{-1}. Collagen B contained either 50 μl of serum or no serum. Collagen A (0.4 ml) was



Key:

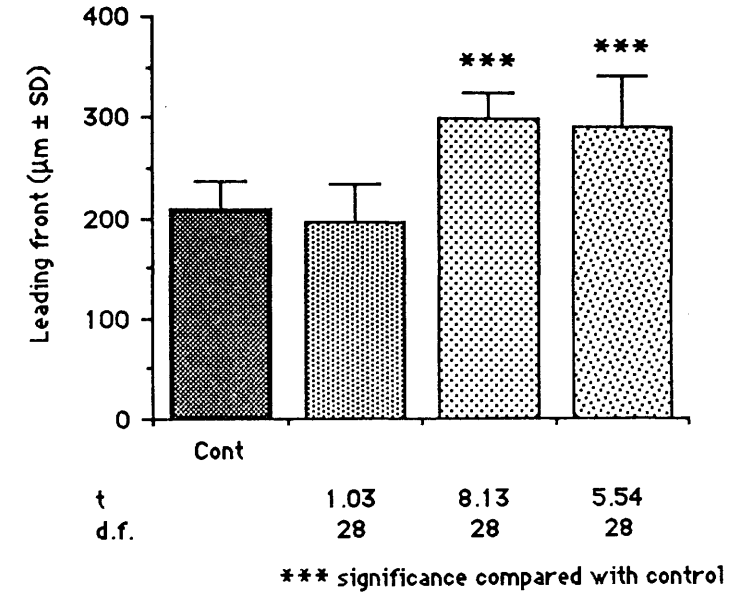
- 50 µg/ml conalbumin at 0 h
- 50 µg/ml conalbumin at 3 h
- 50 µg/ml conalbumin + 10 µl serum
- 50 µg/ml conalbumin + 10 µl heat-inactivated serum

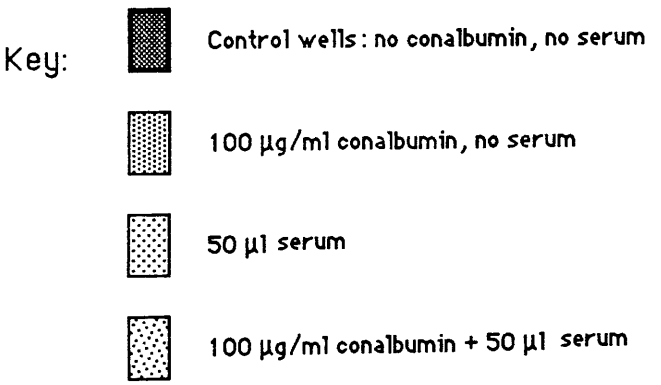
Fig. 5.14 Cell distributions from assay 11

Two gels fixed at start of incubation; two gels fixed after three hours. Cells incorporated into collagen before setting; distributions made by counting number of cells in focus per field at intervals of 20 µm

allowed to set for one hour before plating collagen B (0.3 ml). Results were similar in both assays. There was no effect on invasion seen in the well containing conalbumin alone, while invasion was significantly enhanced in the wells containing serum alone, and in the wells containing conalbumin plus serum. As wells containing serum but no conalbumin gave the same results as wells containing serum and conalbumin, this assay would indicate an enhancement of invasion due to the presence of serum in the upper layer of collagen. (Data in Figures 5.15, 5.16).

Two layers of collagen were used in assay 14, 0.4 ml of collagen A at 1.5 mg ml^{-1} being plated first, allowed to set for one hour, then 0.3 ml of collagen B at 1.0 mg ml^{-1} being plated over collagen A. Collagen A contained 50 μg ml^{-1} conalbumin, collagen B none. 10 μl of serum, either heat-inactivated or not, was incubated in 1 ml of medium above the set collagen gels for 2 h at 37°C, while control wells had no serum in the medium. Wells were then washed overnight in H2 before adding cells as normal. Scoring for the leading fronts, there was a significant increase in the invasion of the wells which had contained the normal serum, with no difference found in the wells which had contained heat-inactivated serum. Conditions in the wells containing conalbumin and normal serum left a situation enhancing cell invasion, presumably due to the formation of precipitated IC and/or complement deep in the layer of collagen B, the antigen - antibody mix being close to equivalence. The wells which had contained heat-inactivated serum, on the other hand, showed no effect on invasion, presumably due to





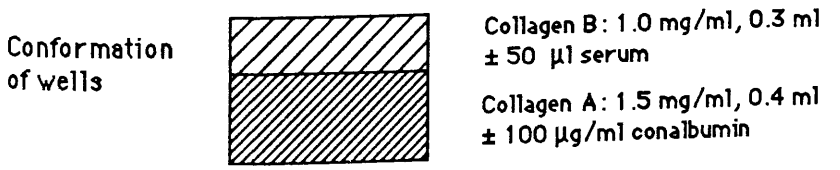
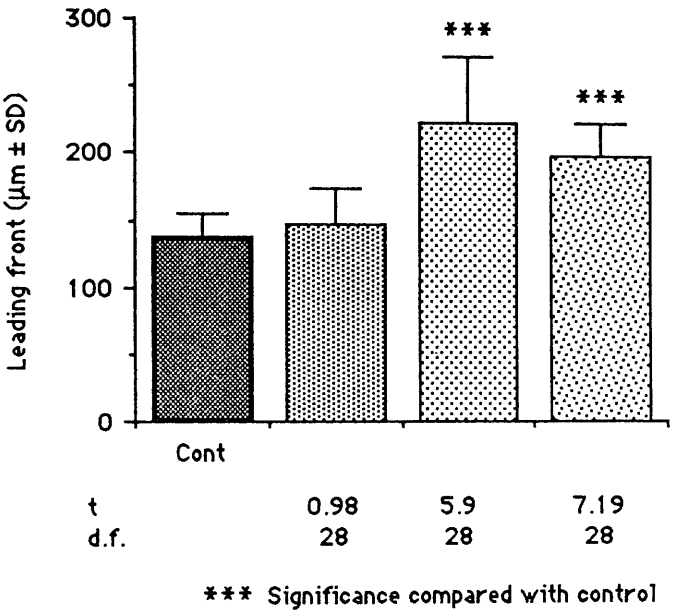
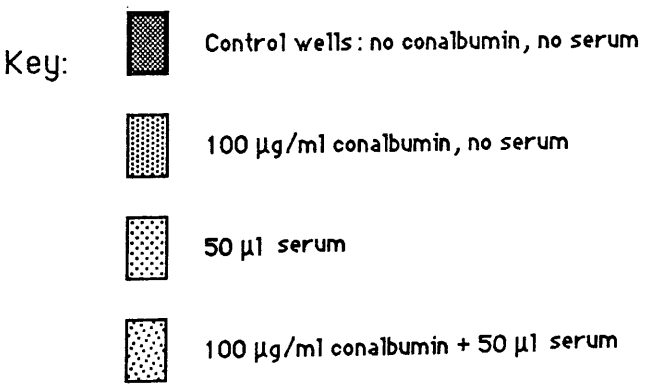


Fig. 5.15 Leading front scores from assay 12; two layers of collagen





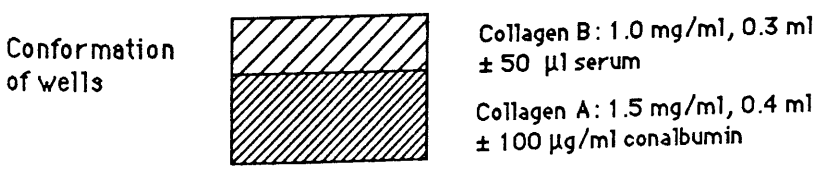


Fig. 5.16 Leading front scores from assay 13; two layers of collagen

the absence of factors chemotactic for neutrophils, the antigen - antibody mix giving a probable small antibody excess. (Data in Figure 5.17).

Assay 14 was different from the preceding one in that serum was removed by washing overnight before the addition of cells. The serum had been found to give a chemokinetic effect to invading neutrophils, and it was desirable to remove this feature from the assay.

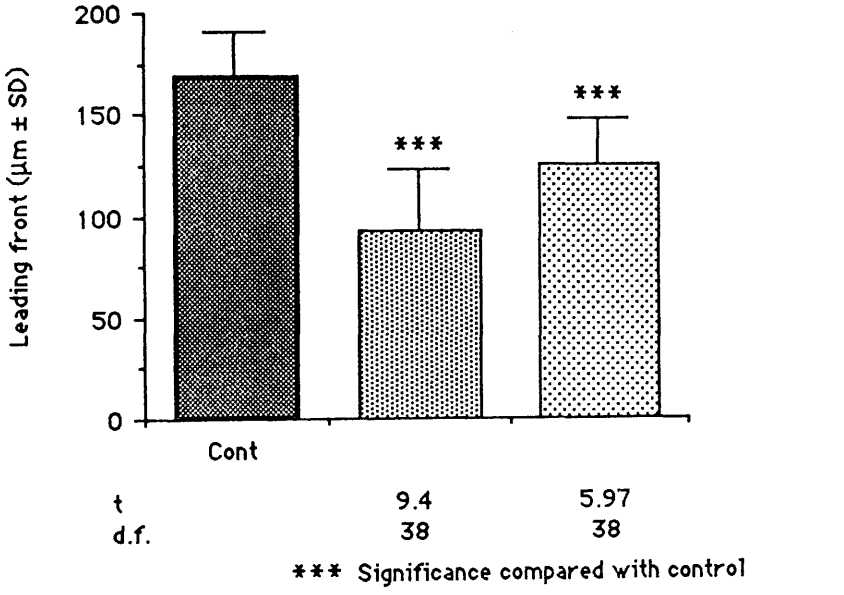
Two layers of collagen were used in assay 15, but here the volume of collagen B, the upper layer, was reduced from 0.3 ml to 0.15 ml, the smallest volume which could conveniently be plated into the 16 mm diameter culture wells and still form a layer at the viscosity used. Calculations on the depth of collagen gels indicated that a volume of 0.3 ml plated into a 16 mm tissue culture dish would give a layer of collagen some 1500 μm thick, meniscus effects making the layer thinner than this in central regions. While proteins have diffusion constants in the range 0.3 - 1.0 x 10^{-6} cm s^{-1}, which should allow for the formation of a gradient across a distance of 1 mm in a few hours (Crick, 1970), the conditions in a collagen gel are an unknown quantity, and it was felt that the use of as thin a layer as possible of collagen (ca. 1 mm) would ensure the formation of a gradient reaching to the surface of the collagen.

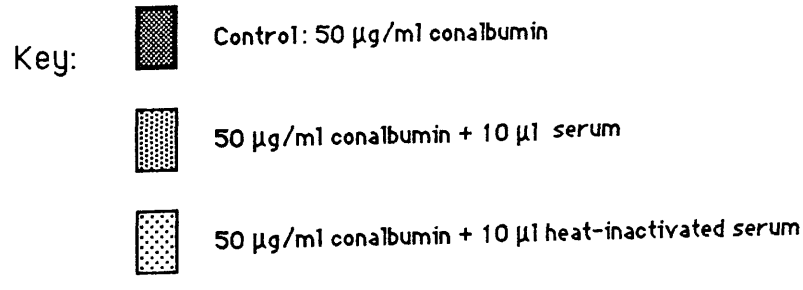
The lower layer of collagen contained 50 μg ml^{-1} conalbumin, the upper layer was collagen only, while 10 μl of serum was added to each well in one ml of H2. Serum used was either normal or heat-inactivated. The wells were

incubated for 4 h at 37°C then washed overnight before adding cells. Scoring for leading fronts, invasion was significantly inhibited in all wells which had contained serum. The reduction in invasion seen in wells which had been incubated with serum might be explained as being due to cells being trapped relatively close to the surface compared to earlier assays, a thin layer of collagen being used in this assay. Unfortunately, a distribution count was not made in this case, which might have confirmed this explanation. The smaller reduction of invasion seen in wells which had heat-inactivated serum incubated above the collagen can be explained by assuming that the small antibody excess in this case provided less IC as a chemoattractant. (Data in Figure 5.18).

5.2 Collagen slab assay

Scoring the standard visual invasion assay for distribution of cells using an inverted microscope is a technique made optically more difficult by the relatively great depth of collagen. This difficulty becomes greater when two layers of collagen are used in an invasion assay, as it is very difficult to determine the boundary between the two layers. In an attempt to circumvent this problem, and allow a more efficient collection of data, a new assay system (referred to as a slab assay) was set up (see Figure 5.19, and Materials & Methods for a description of the technique). Basically this assay allows a thin slab, or slabs, of collagen to be cast between coverslips. If the first slab is allowed to set, a second collagen can be cast adjacent,





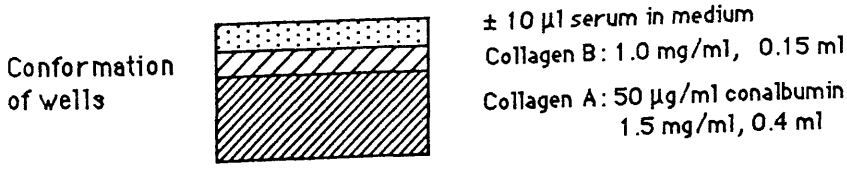


Fig. 5.18 Leading front scores from assay 15; two layers of collagen

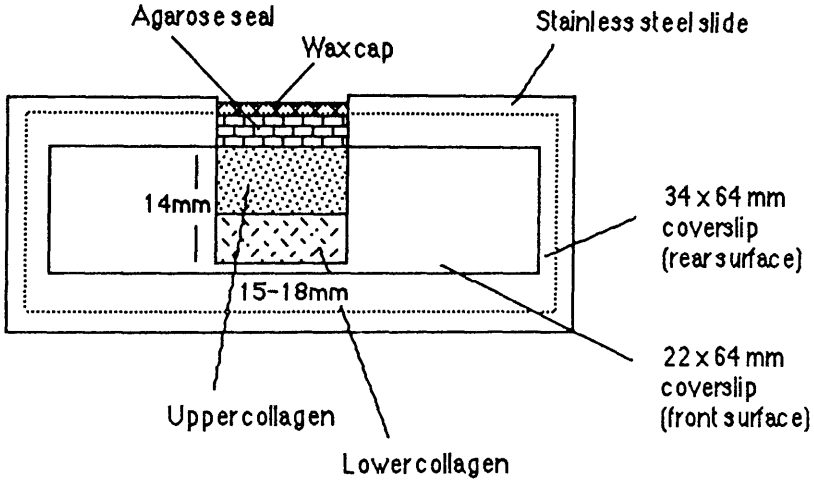


Fig. 5.19 Novel assay system for chemotaxis or distribution scoring

conditions allowing for the two collagen areas to be distinguishable only by a very fine boundary line. Cells can easily be incorporated into one or both collagens during mixing, and in theory the system should facilitate the scoring of assays for chemotaxis, with one slab containing a chemoattractant, for example, while the adjacent slab does not. This assay is optically superior to the standard invasion assay, due to the reduced thickness of collagen (Plate 5.1).

Cell distribution across the collagen boundary was tested for significant differences from the population mean using a one-way analysis of variance. Assays were scored using a x 10 or x 20 objective and an 8 x 10 grid. Cell numbers were taken from the 8 rows parallel to the collagen boundary on both sides of the boundary, rows being pooled into pairs, and the resulting areas, three or four on either side of the boundary then tested for significance. This ensured that the distribution of cells at a distance of at least 480 μm on either side of the collagen boundary was examined.

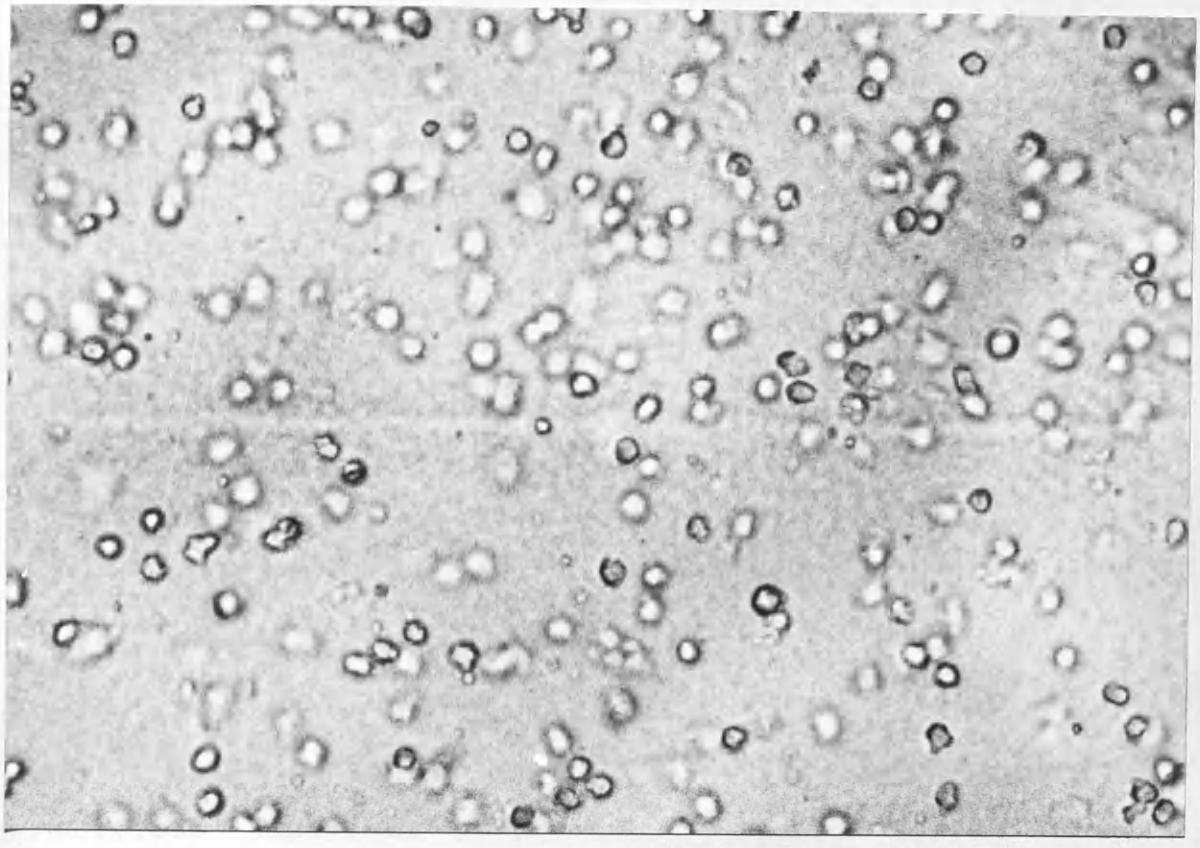
Assay 16 used two slides, each with two collagen areas. Both collagens contained cells at a concentration of 1×10^6 ml^{-1}. One slide acted as a control, and had identical collagen areas, with no chemoattractant. The other slide contained one collagen without chemoattractant, and the other collagen incorporating f-NLP at 2×10^{-8}. Both of these slides were time-lapse filmed using the automated tracking system described earlier, in areas away from, and including the boundary between the two collagens. This

filming should have indicated whether or not the cells could move through the collagen matrix in this system, and whether the boundary between the collagens was 'transparent' - allowing cells to cross in either direction. In two control, and three experimental film sequences, each of duration 30 minutes, no cells were seen to have been actively locomoting.

Assay 17 used a slide in which one collagen was with, and the other without cells. No chemoattractant was used and the assay was examined after a 3 h incubation period. No cells were seen in the area which had begun without cells.

The same method was used in assay 18, using Vitrogen instead of rat-tail collagen. Results were identical to the previous assay, no cells being seen outwith the area of collagen which originally had all the cells incorporated in it before and during plating.

The concentration of collagen was increased in assay 19, from the standard 1.0 mg ml^{-1} to 1.5 mg ml^{-1}, in order to see whether this would facilitate cell movement. Cells were incorporated into the collagen at the increased concentration of 2.0×10^{6} ml^{-1}, while collagen was also made at 1.5 mg ml^{-1} and plated without cells. A third collagen mix contained cells and f-NLP at 2×10^{-8} as a chemoattractant. The assay was run for 4 h at 37°C before scoring. A standard visual invasion assay was run in parallel, using the same batch of cells and collagen. There was no obvious displacement of cells between any of the collagen areas, whether from an area of cells to an area of



Scale bar: 50 µm

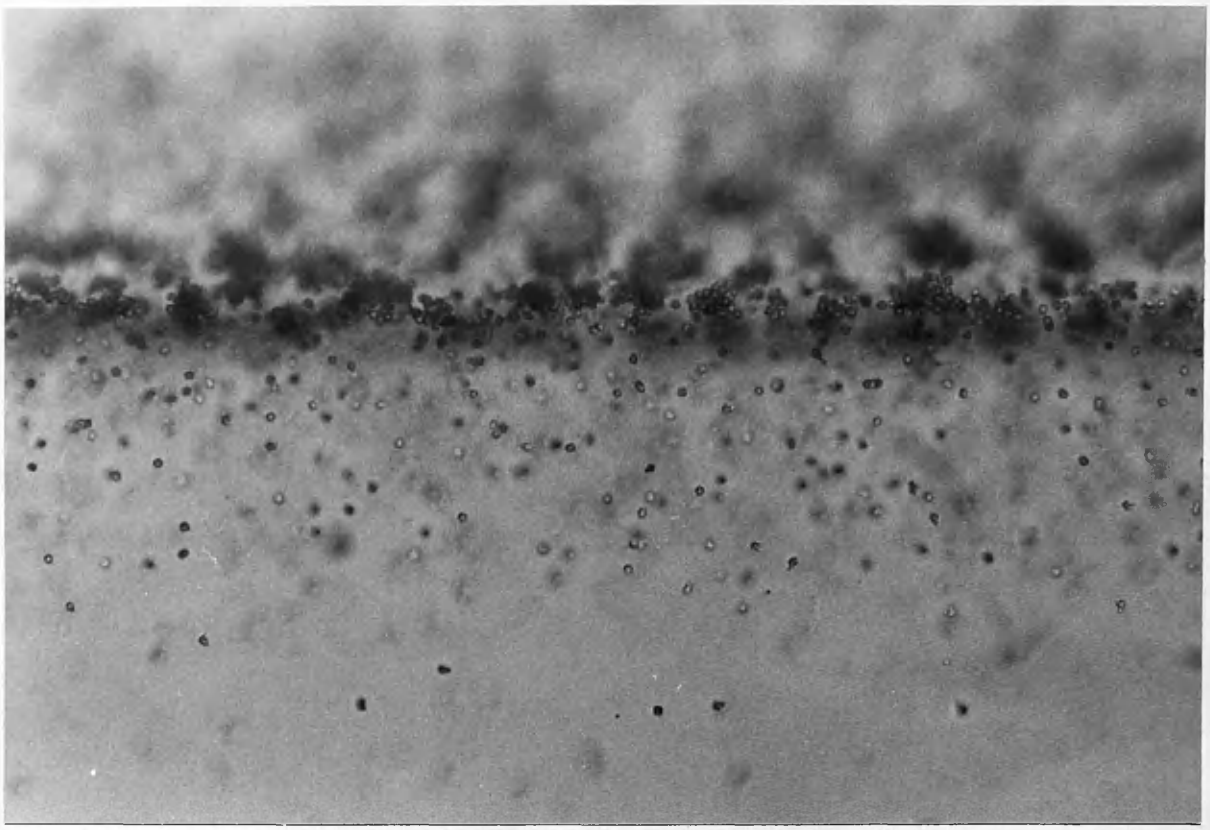
Plate 5.1 Collagen boundary assay. Boundary between two different areas of collagen is just visible, running across middle of print. Incorporated neutrophils are seen within gel matrix. Very few show locomotory morphology

no cells, or from an area without chemoattractant to an area with chemoattractant. In the standard visual invasion assay, which was stopped after 2 h, cells were seen to have invaded the collagen gels to depths of 160 μm.

From the last assay, (and earlier workers, e.g. Brown, 1982), it was known that the collagen gels were suitable for neutrophil locomotion, as using a standard invasion assay neutrophils were seen to have invaded the gels to appropriate depths. It was decided to check whether there was some factor in the geometry of the new wells which affected the gelation process, preventing neutrophils from locomoting. In assay 20, conditions were set up to determine whether cells could invade the collagen areas from medium lying above the collagen. Collagen, without cells, was prepared at a concentration of 1.0 mg ml^{-1}, either with or without f-NLP at 2×10^{-8}. The collagens were plated into the prepared wells as normal, but in place of a second collagen on top of the first, a cell suspension (1×10^{6} ml^{-1} in H2) was carefully pipetted on top of the set collagen gel and the assay incubated at 37°C for 4 h. Cells were seen to have invaded the collagen gels in both types of well, in both cases to a maximum of 230 μm below the surface of the collagen, indicating that the geometry of the new assay system had not unduly changed the formation of the collagen gels. (Plate 5.2)

Assay 21 involved three different collagen mixtures.

1. Collagen + cells
2. Collagen + cells + conalbumin (240 μg ml^{-1})
3. Collagen + cells + serum (8 μl ml^{-1}).



100 µm

Scale bar:

Plate 5.2 Invasion of collagen gel in novel collagen boundary assay. Gel contains f-NLP, cells at 1 million per ml

Cells were incorporated into the collagen gels at a concentration of 2×10^6 ml^{-1}, and the assay was incubated for 4 h at 37°C. Two slides were scored for cell distribution; collagen + cells against collagen + cells as a control (in which there should be no difference in cell distribution between the two areas), and a conalbumin-containing collagen against a serum-containing collagen as an experimental, in which we might expect the formation of an IC near or at the boundary between the two collagens. Cell distributions were scored as described earlier, using a graduated eyepiece and beginning at the collagen boundary counting the number of cells in focus per field in strips parallel to the boundary in both directions, and in five different focal planes at 20 μm intervals, using a x 10 or x 20 objective. At x 20, each strip covered an area 600 x 60 μm. Cell numbers are shown in Tables 5.2 and 5.3, while the distributions are shown graphically in Figure 5.20. There was no significant redistribution of cells found in the scored wells. (Control and experimental distributions: $F = 0.6$; d.f. 3, 16, $P > 0.05$, and $F = 2.24$: d.f. 3,16, $P > 0.05$).

Assay 22 repeated the same conditions as assay 21 using f-NLP at 2×10^{-8} as a chemoattractant. Results were the same, there being no significant redistribution of cells in any of the three wells tested for significance, one well containing cells only in both areas ($F = 3.09$: d.f. 3,16, $P > 0.05$), and two wells containing cells only in one area and cells with f-NLP in the other ($F = 1.47$; d.f. 3,20, $P > 0.05$, and $F = 1.81$; d.f. 3,20, $P > 0.05$). (Not shown as

Table 5.2

Cell distribution from assay 21 - Control well

square	no. of cells in focus					mean	SD
A	12	13	13	17	6	12.2	3.5
B	13	13	12	11	7	11.2	2.2
C	7	14	16	17	14	13.6	3.5
D	17	15	12	21	7	14.4	4.7
E	13	12	17	16	16	14.8	1.9
F	15	13	12	9	14	12.6	2.1
G	21	19	16	13	13	16.4	3.2
H	18	14	17	13	15	15.4	1.9
A	26	23	15	13	15	18.4	5.1
B	27	14	14	13	14	16.4	5.3
C	23	12	12	17	14	15.6	4.1
D	22	15	21	13	13	16.8	3.9
E	25	19	17	16	16	18.6	3.4
F	23	13	15	14	11	15.2	4.1
G	26	17	16	20	13	18.4	4.4
H	18	7	6	8	4	8.6	4.9
A	21	11	15	12	14	14.6	3.5
B	19	11	15	17	11	14.6	3.2
C	15	18	12	14	13	14.4	2.1
D	21	20	11	13	9	14.8	4.8
E	23	15	16	15	7	15.2	5.1
F	11	21	23	15	13	16.6	4.6
G	19	16	13	18	11	15.4	3.0
H	14	11	14	16	10	13.0	2.2
A	15	14	16	14	15	14.8	0.7
B	11	8	17	16	10	12.4	3.5
C	14	14	15	9	9	12.2	2.6
D	14	16	17	15	11	14.6	2.1
E	15	15	12	12	13	13.4	1.4
F	18	15	18	19	10	16.0	3.3
G	13	13	17	18	15	15.2	2.0
H	12	17	9	6	14	11.6	3.8

Table 5.2

Double line running across table indicates boundary between upper and lower areas of collagen in slab assay. Cell distributions were scored using a graduated eyepiece and beginning at the collagen boundary counting the number of cells in focus per field in strips parallel to the boundary in both directions (graticule strips indicated by letters A-H), and in five different focal planes at 20μm intervals, using a x 10 or x 20 objective. Distributions are also shown graphically in Figure 5.20.

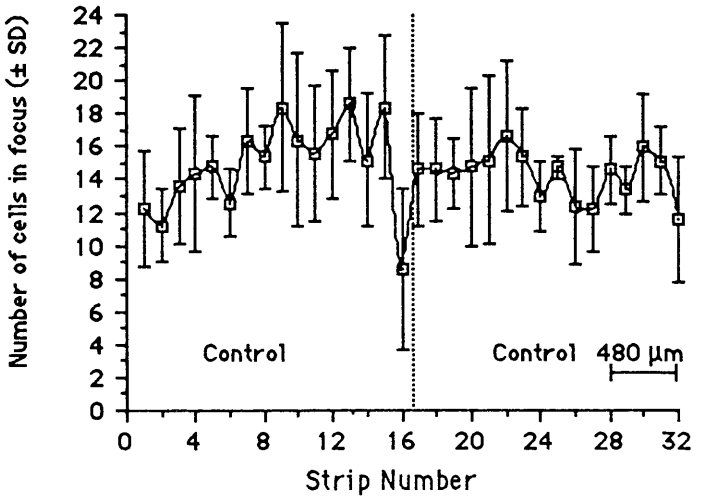
Table 5.3

Cell distribution from Assay 21 - Experimental well

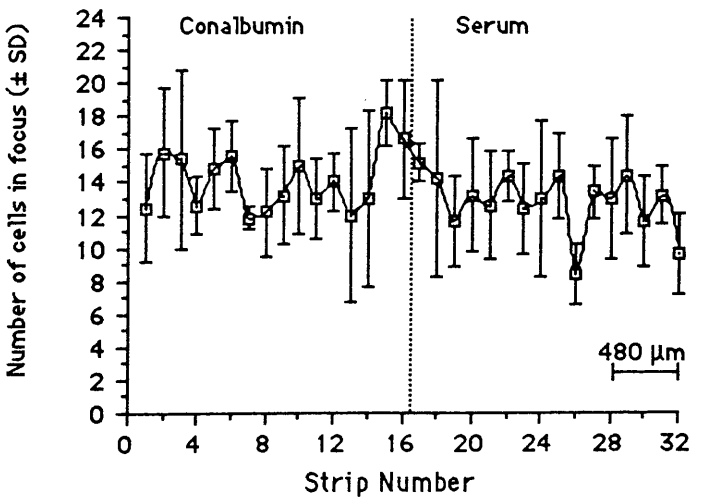
square	no. of cells in focus					mean	SD
A	14	18	9	11	10	12.4	3.3
B	20	16	13	10	20	15.8	3.9
C	20	23	8	13	13	15.4	5.4
D	16	12	11	12	12	12.6	1.7
E	17	17	11	16	13	14.8	2.4
F	18	14	17	17	12	15.6	2.2
G	12	11	13	11	12	11.8	0.7
H	11	17	11	9	13	12.2	2.7
A	17	13	15	13	8	13.2	3.0
B	21	18	14	9	13	15.0	4.1
C	12	18	12	11	12	13.0	2.5
D	16	15	12	15	12	14.0	1.7
E	21	14	10	6	9	12.0	5.2
F	17	20	13	5	10	13.0	5.3
G	17	21	19	15	19	18.2	2.0
H	19	10	20	16	18	16.6	3.6
A	15	14	14	17	16	15.2	1.2
B	19	22	6	9	15	14.2	6.0
C	9	15	9	10	15	11.6	2.8
D	19	15	11	10	11	13.2	3.4
E	11	19	12	10	11	12.6	3.3
F	13	15	14	17	13	14.4	1.5
G	15	13	8	11	15	12.4	2.7
H	17	17	5	10	16	13.0	4.8
A	16	13	16	17	10	14.1	2.6
B	8	10	11	6	7	8.4	1.9
C	15	14	15	12	11	13.4	1.6
D	7	17	17	12	12	13.0	3.7
E	10	14	14	21	13	14.4	3.6
F	11	16	13	10	8	11.6	2.7
G	10	15	13	15	13	13.2	1.8
H	11	13	6	8	10	9.6	2.4

Table 5.3

Double line running across table indicates boundary between upper and lower areas of collagen in slab assay. Cell distributions were scored using a graduated eyepiece and beginning at the collagen boundary counting the number of cells in focus per field in strips parallel to the boundary in both directions (graticule strips indicated by letters A-H), and in five different focal planes at 20μm intervals, using a x 10 or x 20 objective. Distributions are also shown graphically in Figure 5.20.



F = 0.6; d.f. 3,16, P > 0.05



F = 2.24; d.f. 3,16, P > 0.05

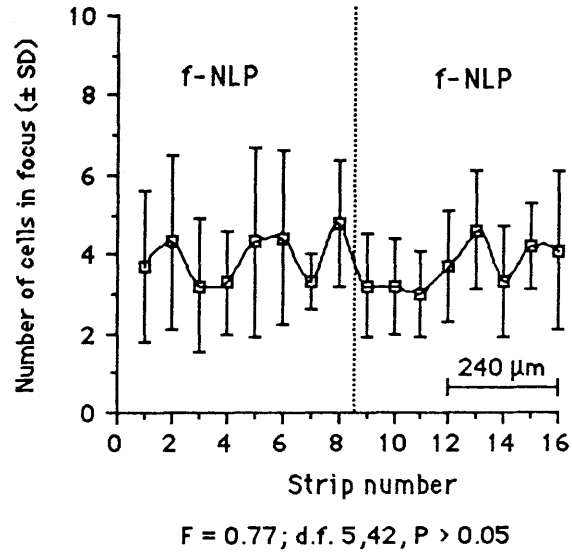
Position of boundary between collagen areas

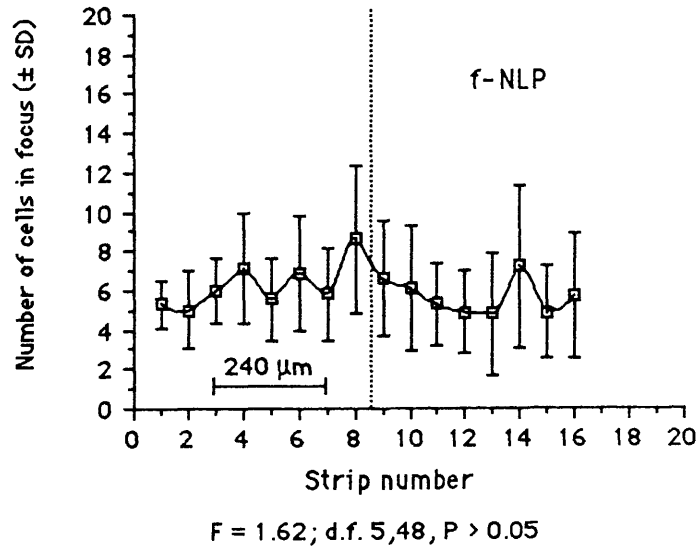
Fig. 5.20 Cell distributions across collagen boundary from assay 21; control and experimental wells

Figure).

In assay 23, f-NLP at 2 x 10^{-8} was used as a chemoattractant and cells were incorporated into the collagen gels at a concentration of 10 x 10^6. The increase in cell concentration was made as it was felt that perhaps some cell released factor may have been involved in normal situations of invasion, where cells are in close proximity at the beginning of an assay (such as in the standard invasion assay, where cells begin on the surface of the gel). With this novel assay system, cells begin their incubation dispersed throughout the collagen gel, and so are not likely to be in close proximity to a neighbouring cell. Wells were incubated for 3 h at 37°C. Distribution counts were made on two of the wells; a control well in which both collagens contained f-NLP, and an experimental well with one side containing f-NLP and the other not. Scoring was done using a x 20 objective. The distributions are shown graphically in Figure 5.21. As in previous assays, there was no significant redistribution of cells in any of the wells. ($F = 0.77$; d.f. 5,42, $P > 0.05$, and $F = 1.62$; d.f. 5,48, $P > 0.05$).

In assay 24, both f-MLP (at 10^{-8} M) and immune complex were used as chemoattractants, adding conalbumin and serum to the same collagen batch at 50 μg ml^{-1} and 16 μl ml^{-1} respectively. Wells were incubated for 3 h at 37°C. Cell concentration was increased to 15 x 10^6 ml^{-1}, the maximum at which the assays could be accurately scored. Scoring was done using a x 20 objective, counting the number of cells in focus in 8 strips parallel to the boundary in both





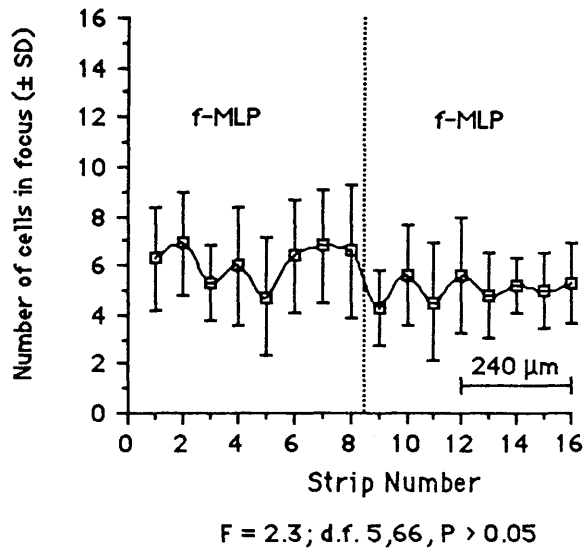
Position of boundary between collagen areas

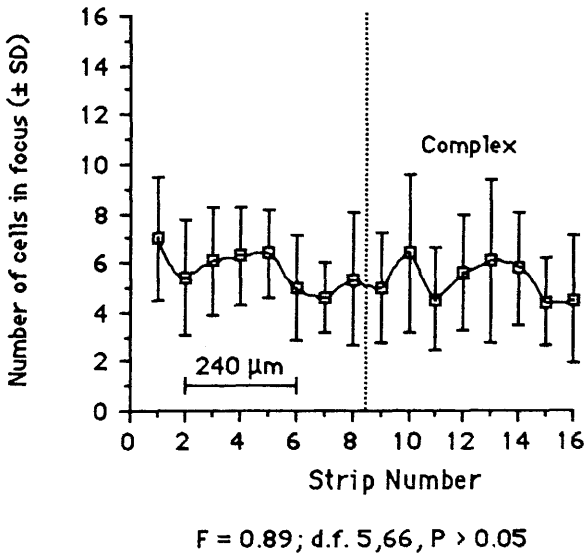
Fig. 5.21 Cell distributions across collagen boundary from assay 23; control and experimental wells

directions and in two different areas of the boundary, at six planes of focus at 20 μm intervals. Distributions are shown in Figures 5.22 to 5.24. As in previous assays, there was no significant redistribution of cells in any well.

Similar results were obtained in Assay 25, with f-MLP as a chemoattractant (at 10^{-8} M), cells at 15 x 10^6 ml^{-1}, and collagen at the increased concentration of 1.5 mg ml^{-1}. Two wells were scored, both containing cells only in one area of collagen, and cells with f-MLP in the other. There was no significant redistribution of cells in either case ($F = 0.88$; d.f. 3,44, $P > 0.05$, and $F = 2.34$; d.f. 5,66. $P > 0.05$).

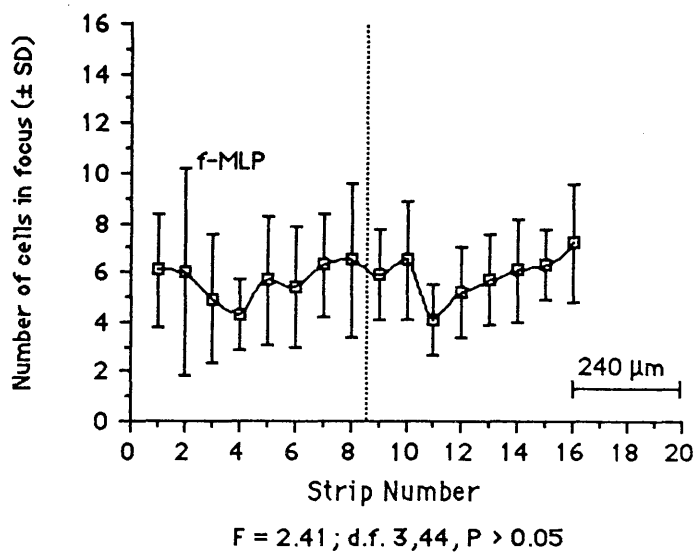
As a last attempt to address the problem of lack of movement of cells in this assay system, a low density of collagen was used in assay 26, with gels at 0.5 mg ml^{-1}, the lowest concentration at which collagen can be made to gel. The reasoning here was that perhaps cells were being trapped or bound by collagen fibres during gelling, and that a low density of collagen would then allow at least a significant number of cells to move. Cells were incorporated into the collagen at a concentration of 10 x 10^6 ml^{-1}, and f-MLP at 10^{-8} M was used as a chemoattractant. The wells were incubated for 3 h at 37°C before scoring the well containing f-MLP on one side only. As in previous assays, no significant redistribution of cells was seen in counting the number of cells in focus in 8 strips parallel to the boundary and in 6 different planes of focus at 20 μm intervals. ($F = 0.84$; d.f. 5,30, $P > 0.05$, Figure 5.25).

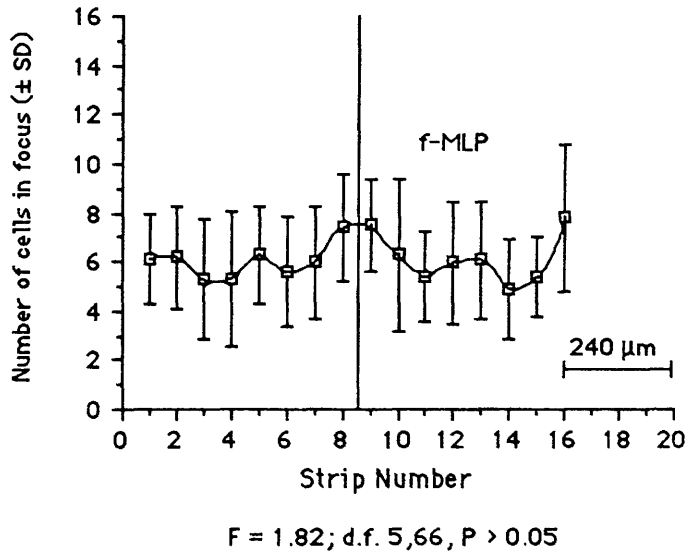




Position of boundary between collagen areas

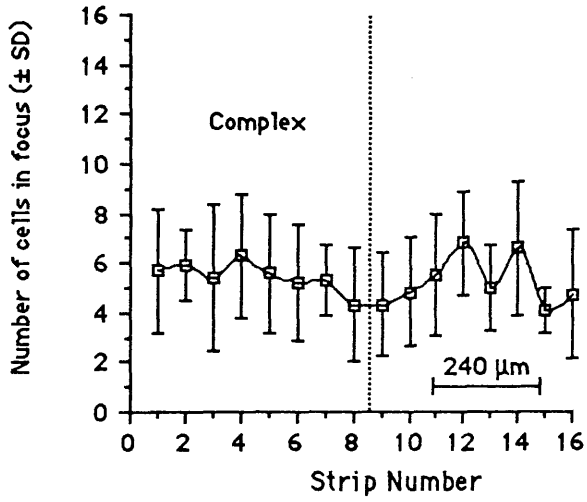
Fig. 5.22 Cell distribution across collagen boundary from assay 24; control and experimental wells





Position of boundary between collagen areas

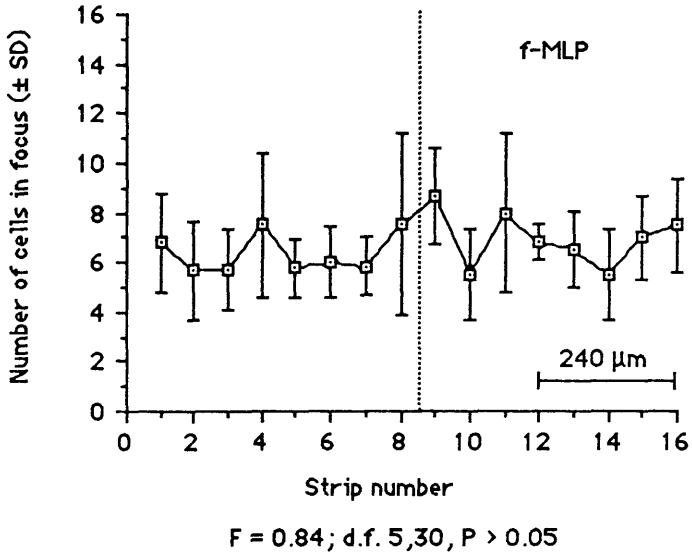
Fig. 5.23 Cell distributions across collagen boundary from assay 24; experimental wells



F = 1.31 ; d.f. 5,66, P > 0.05

Position of boundary between collagen areas

Fig. 5.24 Cell distributions across collagen boundary from assay 24; experimental well



F = 0.84; d.f. 5,30, P > 0.05

Position of boundary between collagen areas

Fig. 5.25 Cell distributions across collagen boundary from assay 26; experimental well

5.3 Effect of conalbumin, serum, and immune complexes on cell locomotion over a 2-D substratum

As an associated series of experiments, the effect of conalbumin, antiserum, and immune complexes on the locomotion of neutrophils, was examined using the electronic cell tracking system. Three assays were carried out, each consisting of six, 30-minute sequences. Cells were filmed at a concentration of 0.5×10^6 ml^{-1}, at 37°C, allowing filming chambers 10-15 minutes to warm up. Cells were in a 50% peritoneal exudate, 50% H2 medium in end control sequences. Chambers used in experimental sequences contained either 100 mg ml^{-1} conalbumin, 500 mg ml^{-1} conalbumin, 10 μl complement-free serum, or an immune complex mix with 100 mg ml^{-1} conalbumin plus 10 μl heat-treated serum, with all chambers containing 50% peritoneal exudate. End controls were compared using the Mann-Whitney U-test and as no significant differences were found they were pooled for comparison against experimental sequences. No significant inter-assay variation was found, and like sequences were then pooled for further statistical treatment.

Basic population data are shown in Figures 5.26 and 5.27. Conalbumin, at either 100 or 500 μg ml^{-1}, had no effect on speed or persistence ($U = 0.41$, $N1 = 191$, $N2 = 102$, $P > 0.05$; $U = 1.47$, $N1 = 191$, $N2 = 93$, $P > 0.05$). The presence of immune complex resulted in a significant reduction in speed ($U = 2.61$, $N1 = 191$, $N2 = 86$, $P < 0.01$), but no effect on persistence ($U = 0.81$, $N1 = 191$, $N2 = 86$, $P > 0.05$). The converse was true with sequences containing

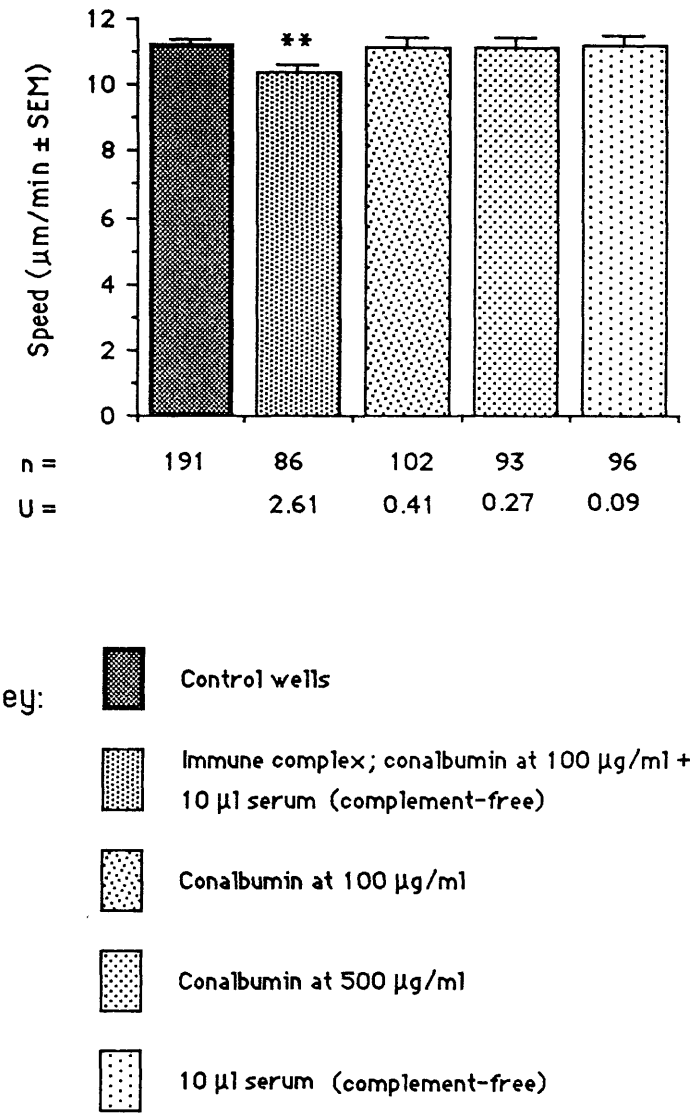


Fig. 5.26 Effect of conalbumin , serum and immune complex on speed over a substratum

** significance compared with controls

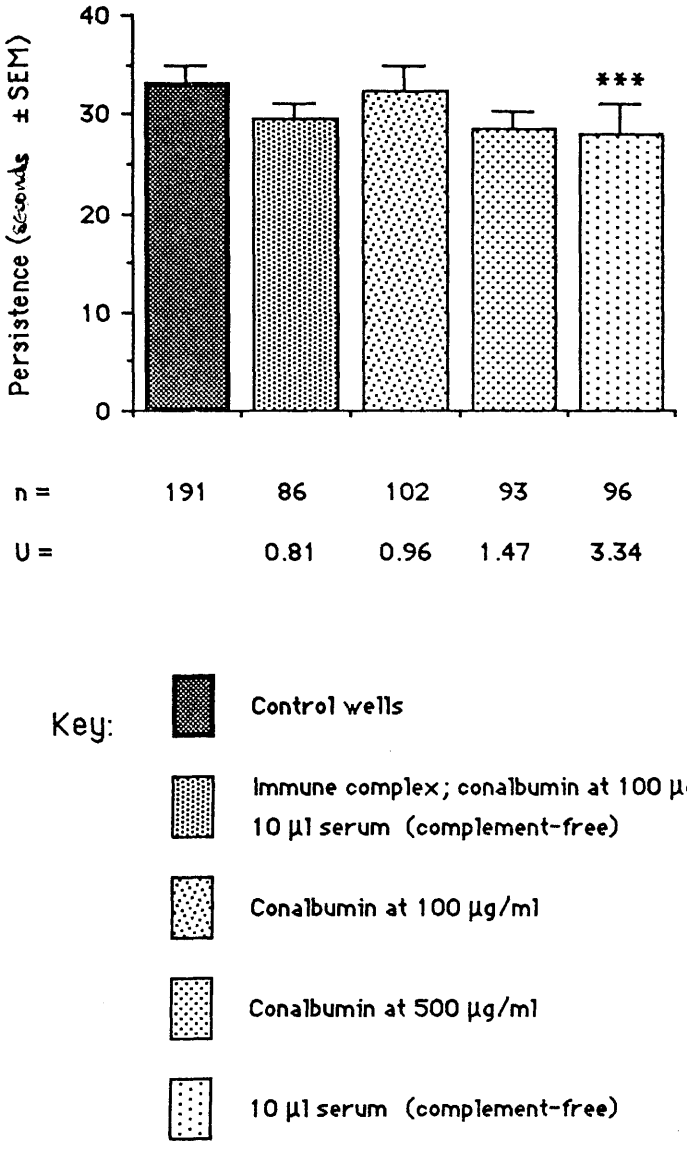


Fig. 5.27 Effect of conalbumin, serum and immune complex on persistence

*** significance compared with controls

serum, where there was no effect on speed ($U = 0.09$, $N1 = 191$, $N2 = 96$, $P > 0.05$), but a highly significant reduction in persistence, when compared with controls ($U = 3.34$, $N1 = 191$, $N2 = 96$, $P < 0.001$).

Frequency distribution histograms were drawn for the cell speeds taken from control and immune complex sequences (Fig. 5.28), and also for the cell persistences taken from control and serum sequences (Fig. 5.29).

The reduction in speed in the presence of immune complex is in accord with recent work using a flow chamber and slides coated with immune complex, where neutrophils show a dramatic adhesion effect in conditions of flow (Wilkinson et al., 1984; Lackie et al., 1987). In flow chamber assays from the latter work, for example, slides are precoated with conalbumin, while neutrophils are suspended in medium containing 10% rabbit serum. Interestingly, if heat-treated serum is used, as here with the time-lapse sequences, the adhesion of neutrophils to the conalbumin strip is considerably slower and less marked. This rapid adhesion effect under flow conditions is in line with the cells' function, where on receipt of a chemotactic signal during their passage through blood vessels, they marginate and adhere to the endothelial wall, before migrating through the endothelial wall towards the focus of the signal.

The significance of this effect, a reduction in speed over a 2-D substratum in the presence of immune complex, may be difficult to quantify with respect to the invasion of

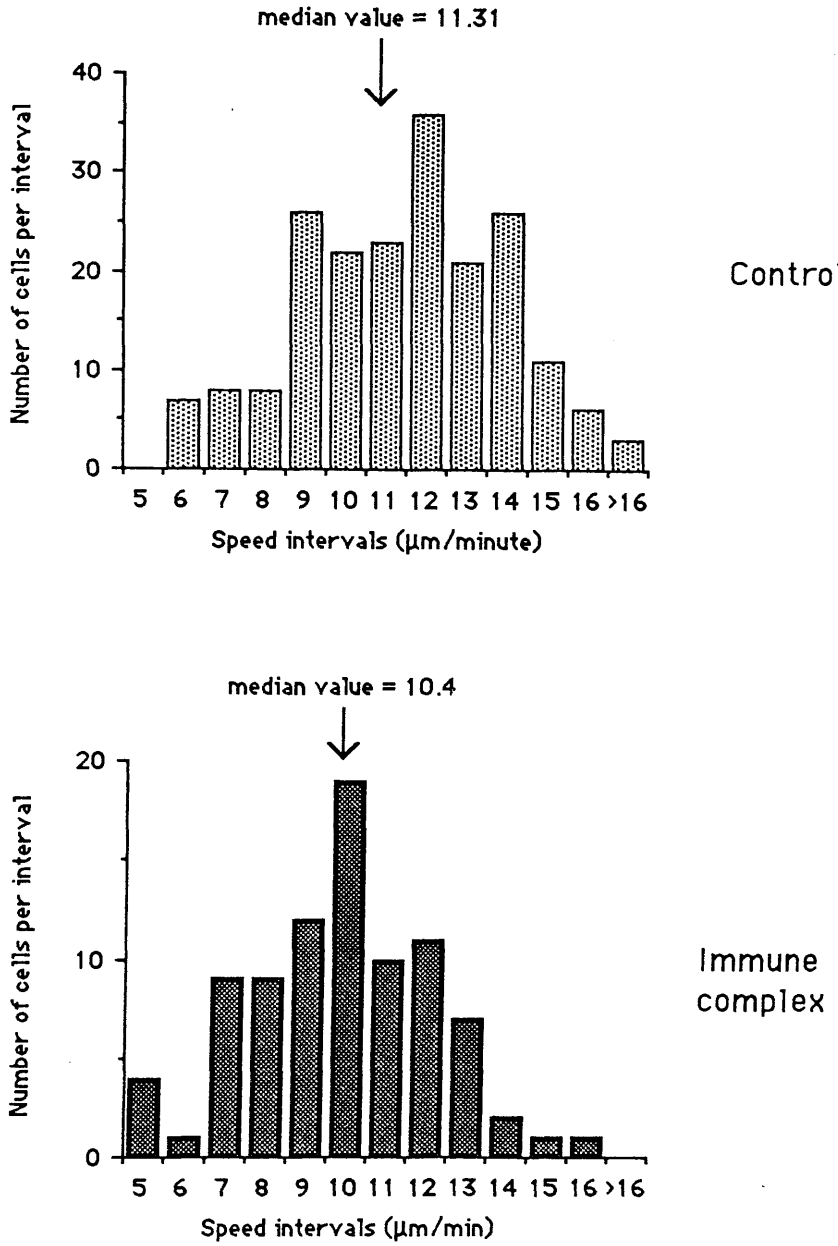


Fig. 5.28 Frequency distributions for cell speeds taken from control and immune complex sequences. Intervals taken in steps of one (Mann-Whitney U-test; U = 2.61, N1 = 191, N2 = 86, P < 0.01)

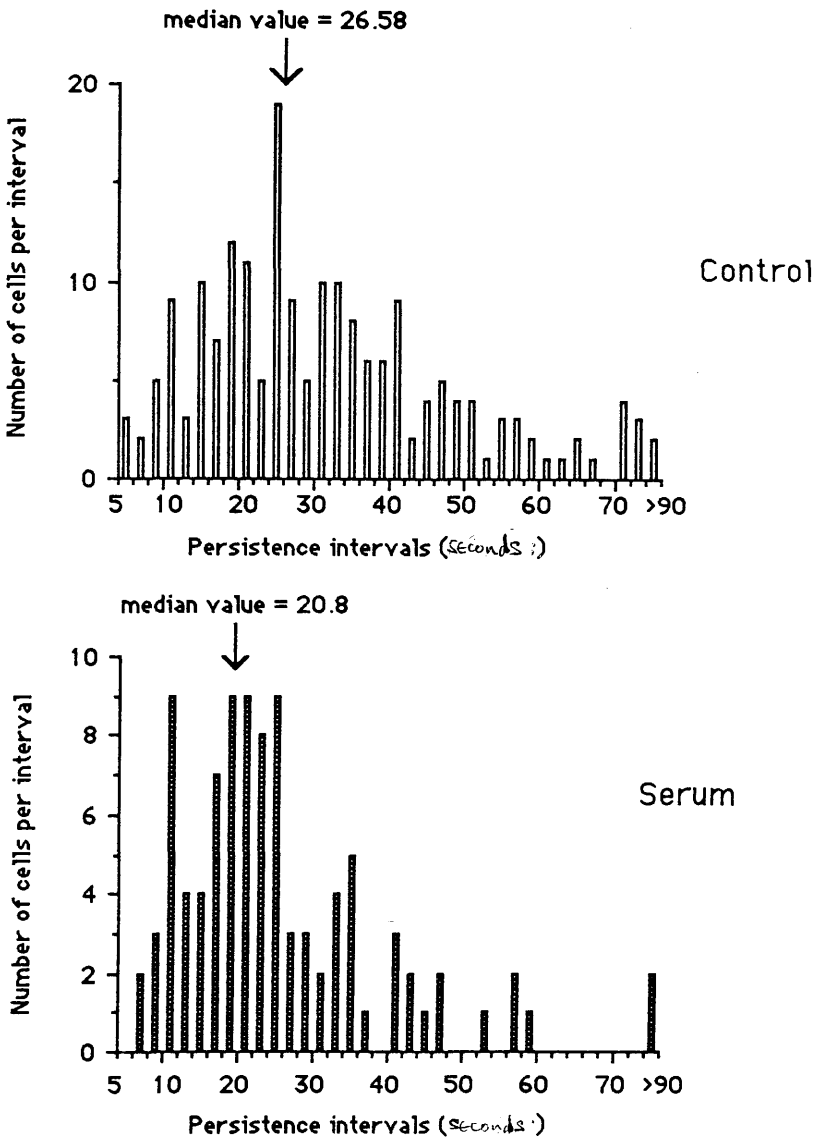


Fig. 5.29 Frequency distribution for persistence; from Control and serum sequences

(Mann-Whitney U-test; U = 3.34, N1 = 191, N2 = 96, P < 0.001)

collagen gels, as the methods of locomotion in the two environments are almost certainly quite different. Neutrophils, for example, do not move over collagen-coated glass, though they readily invade gels made from reconstituted collagen. If the effect of the immune complexes is on the strength of adhesive bonds only, which seems likely from the flow chamber evidence, it may have little effect on the locomotion of cells through the ECM, where movement is likely to be by a process involving the expansion, contraction and elongation of pseudopods. The part played by adhesion in this mode of locomotion is likely to be a minor one.

Once at the site of an inflammatory locus, where deposits of immune complex may be present, neutrophils will adhere strongly and aggregate, undergoing cell lysis if sufficiently stimulated. On the other hand, it may be that small immune complexes, such as those formed in conditions of antigen excess, act principally as chemotactic signals for neutrophils. These small complexes have a fairly long life in the circulation, where they are not readily phagocytosed (Anderson, 1985), and there is evidence that neutrophils are probably not involved in the physiological clearance of IC if these are much smaller than opsonised microorganisms (Hofstaetter & Brammsen, 1984).

5.4 Effect of supernatant from collapsed collagen gels on neutrophil locomotion over a 2-D substratum

Neutrophils invade and move through collagen gels, yet seem incapable of locomotion if incorporated in a collagen gel before gelling occurs. There would seem to be two likely explanations for this inability to move if a cell is within a gel solution as it gels.

a) There is a substance associated with the gelation process, inhibiting thereafter the locomotion of cells which have been exposed to it within the gel, or some cell-released factor, triggered during gelation.

b) There is mechanical trapping of incorporated cells by collagen fibrils, which could nucleate round cells during gelation, effectively binding them in the gel matrix.

The first explanation is perhaps unlikely. Neutrophils are exposed *in vivo* to collagen-producing fibroblasts, and it is difficult to suggest any benefit accruing from immobilisation of this type. More obviously, neutrophils have been shown capable of normal invasion and movement through such gels, though admittedly after gelation has taken place. It is difficult to explain how neutrophils could have an effect on, or be affected by the gelation process, which renders them immobile.

There is as yet little evidence for the second explanation, though SEM (which was not done in this study), may help show whether binding by collagen fibrils during gelation is a significant factor. Grinnell (1982) thought that collagen

fibres observed in collagen gels seemed to be more phase-dense in the region of moving neutrophils. SEM, however, showed no changes in the gel matrices adjacent to cells, which were not incorporated in the collagen before gelling. If the second explanation is indeed the case, it is a surprisingly universal one, as there seem to be no free cells in these assays, and a very efficient one, as there seem to be no cells showing obvious locomotory morphology, even when in a milieu containing amounts of chemotactic peptide calculated to provide maximum stimulation.

It was felt that it was possible to test for the first possibility, by going through the procedure for making the collagen gels containing incorporated cells, allowing the gels to set in microcentrifuge tubes, then centrifuging the gels (13500 rpm, 10 min) so as to obtain the supernatants on the consequent collapse of the gels. In a preliminary assay, using supernatant from one centrifugation, cells were seen to begin to locomote actively then round up within minutes of the start of the assay. On examination of the chambers however, it was seen that a weak gel had formed, indicating the presence of sufficient collagen to form a gel at 37°C, despite centrifugation for 10 min at 13500 rpm.

For this reason, the subsequent assay used supernatant from a second centrifugation, and even here, it is suggested, sufficient collagen was carried over to form a weak layer on the glass coverslip of the film chamber and slow the cells. Neutrophils were then tracked moving over protein-coated glass, with 50% peritoneal exudate, in chambers

containing, as medium, the supernatants obtained from these spins. Gels were made using x 10 H2 as an indicator while control sequences contained the same medium as was used to make the collagen gels. The Mann-Whitney U-test was used for statistical comparisons.

Four 30-min sequences were used for this experiment; two control sequences, one at the beginning and one at the end, and two experimental sequences. The first experimental sequence used normal supernatant, while the second experimental sequence used supernatant which had been heat-treated, 30 min at 56°C. The two end controls were compared and as there was no statistical difference they were pooled. The two experimental sequences were similarly compared and as there was no statistical difference between them they were also pooled.

The pooled control and pooled experimental data were then compared using the Mann-Whitney U-test. Data are shown in Figure 5.30. Looking at the speed of the two populations, there was a significant drop in speed in the experimental population, 11.05 ± 2.24 $\mu m\ min^{-1}$ compared to 14.07 ± 4.88 $\mu m\ min^{-1}$ in the control population (N1 = 51, N2 = 30, U = 2.68, $P < 0.01$). There was no significant difference in persistence between control and experimental populations (control persistence 46.83 ± 44.8 S., experimental persistence 53.7 ± 46.3 S., U = 1.63, $P > 0.5$).

While this assay does show a significant drop in speed in chambers using supernatant from centrifuged collagen gels, the cells were nonetheless moving actively, and it is

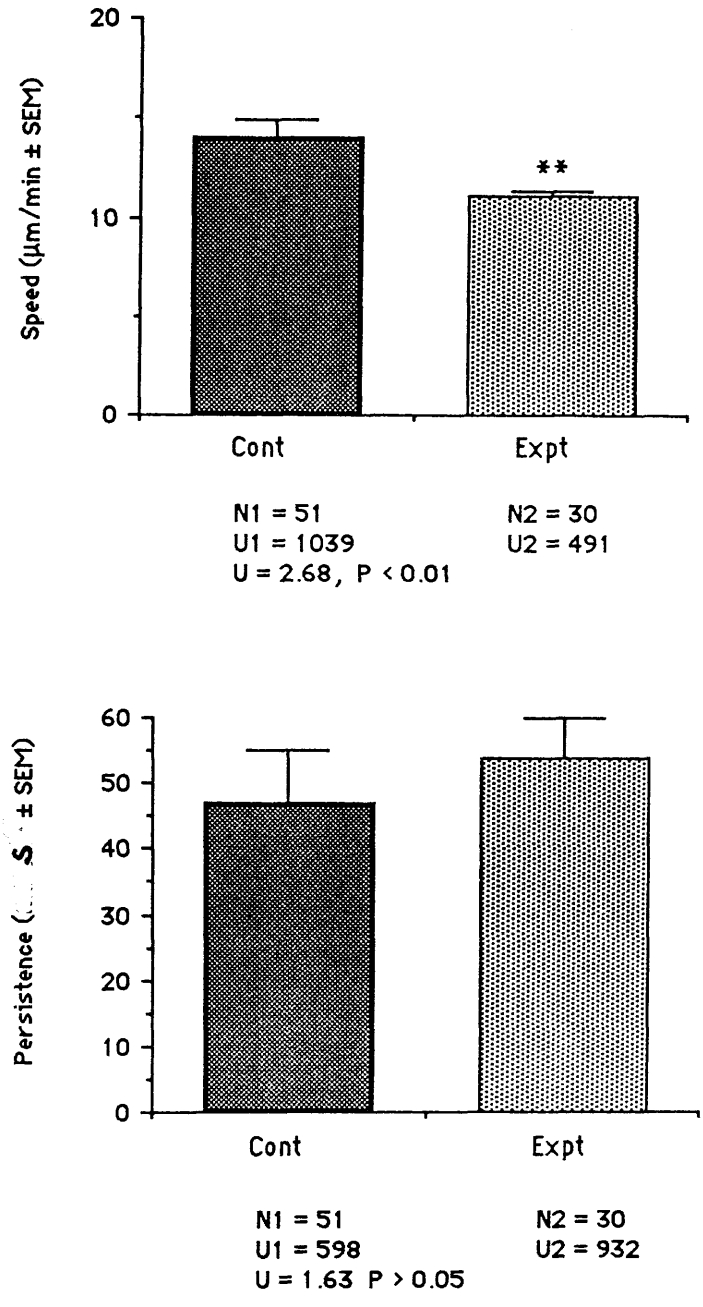


Fig. 5.30 Effect of supernatant from centrifuged collagen gels on neutrophil speed and persistence over protein-coated glass

doubtful whether this decrease in speed shown in the time-lapse assays could be extrapolated to a complete inhibition of movement as seen in the collagen boundary assays.

Heating the supernatant was tried to see whether there was a heat-labile factor in the supernatant. As there was no difference between experimental sequences, it was concluded that there was no heat-labile factor present. This was not unexpected, as other work has shown that cell-released factor, capable of stimulating neutrophil migration, is heat stable (Cramer et al., 1986).

The immobilisation of neutrophils incorporated within a collagen gel remains unexplained, though the most likely explanation is mechanical trapping by collagen fibres. As one last tantalising piece of evidence, time-lapse filming was done using chambers in which neutrophils had been allowed to settle in the standard 50% peritoneal exudate, 50% H2 mix. Excess medium was gently poured off, and the top coverslip was placed over the lower coverslip containing the adherent cells. The top coverslip contained an attached, hanging drop of collagen, made at a concentration of 2.0 mg ml^{-1} for extra strength and allowed to gel for 20 min at 37°C. The size of the collagen drop was calculated so as to completely cover the attached cells on the exposed circle of the lower coverslip. The well was sealed with paraffin wax as normal then placed on the microscope stage for filming.

In several sequences set up as described, and containing a total of over 100 cells, no cell was seen to locomote under

the 'ceiling' of collagen. When observed during the warming period, some neutrophils were seen to extend very long, thin pseudopods, snake-like and about 15 -20 μm in length. This behaviour soon ceased however, and all cells rounded up. This result is very surprising, as neutrophils, though very flattened, move well under a 'ceiling' of agarose (Vicker et al., 1986). While it is known that neutrophils move poorly over a collagen substratum, their immobilisation when sandwiched between collagen and protein, the latter over which they move very well, is unexpected. One explanation is that collagen is leaching from the hanging drop and coating the protein, thus immobilising the cells.

Summary

1. Visual migration assays were done to examine the effects of immune complexes and chemotactic peptides on neutrophil invasion of collagen gels.

2. The presence, in medium above a collagen gel, of either immune complex or complement, inhibits neutrophil invasion of the gel, while invasion of a collagen gel is modified by immune complex in the gel, with enhancement or inhibition depending on the design. Different ratios of antigen to antibody have different modifying effects on neutrophil invasion of collagen gels. Immune complexes reduce neutrophil speed over a 2-D substratum, with no effect on persistence.

3. The presence of serum enhances neutrophil invasion of collagen gels, probably by a chemokinetic effect.

4. Neutrophils incorporated in collagen gel while gelation occurs are immobilised. This immobilisation is not caused by a cell released factor. Neutrophils attached to protein-coated glass under a collagen roof are immobilised.

Chapter 6

Discussion

Introduction

This thesis reports further studies (Brown, 1982) on the locomotory behaviour of neutrophil leucocytes in 3-D collagen gels, modifying the movements of cell populations by several means, including the use of methyl xanthines and immune complexes. In addition, the interaction of invading neutrophils and collagen gels has been looked at, to see whether there was cooperation between invading cells, and whether proteolytic activity was involved.

It has been shown that neutrophils readily invade and move through collagen gels made from reconstituted rat tail tendon (Senda et al., 1975; Grinnell, 1982; Brown, 1982; Islam et al., 1985). Brown (1982) found that neutrophils adhered very poorly to, and were unable to move over, collagen-coated glass, though they invaded and moved through three-dimensional gels of collagen; he considered that movement through a collagen gel was largely independent of adhesion to collagen fibres. Increasing the collagen concentration reduced neutrophil invasion, as did the incorporation of hyaluronic acid into gels of high collagen concentration. This inhibition of invasion, Brown (1982) suggested, was due to physical obstruction. Tucker & Erickson (1984) found an inhibition of invasion into collagen gels by quail neural crest cells both when the collagen concentration was increased, and when hyaluronate was incorporated into gels of high collagen concentration.

a finding in agreement with that of Brown (1982). Schmalstieg et al., (1986) proposed that neutrophils undergo two different kinds of motion; one over surfaces which is adherence dependent, and motion through a three-dimensional matrix, which is largely adherence independent, and may involve hydraulic or pushing motions. If this is fundamentally correct, then deformability will be an important factor in neutrophil movement through a 3-D matrix.

The use of collagen gels as an in vitro model for cell invasion and migration is more akin to the in vivo situation than is a model using a rigid filter, as the matrix of a collagen gel, like much of the extracellular matrix, is non-rigid. Neutrophils in such a collagen gel have been shown by SEM to be entangled in a meshwork of collagen fibres, with shape changes suggestive of active locomotion within the gel by a process involving pushing and squeezing (Brown, 1984).

Pentoxifylline, a methyl xanthine, has been shown to increase erythrocyte deformability (Ehrly, 1978), and is extensively used in the treatment of peripheral vascular disorders, where it improves circulation without causing vasodilation. Sheetz et al., (1984) have demonstrated that pentoxifylline increases the movement of rabbit peritoneal neutrophils through a rigid matrix, a micropore filter in a standard Boyden chamber system. The enhancement of neutrophil invasion of collagen gels with similar concentrations of pentoxifylline was in agreement with Sheetz et al., (1984). It is known that pentoxifylline has

no effect on neutrophil adhesion (Crocket et al., 1987), and adhesion is probably, in any case, of minor importance in a collagen gel model. The increase in invasion with pentoxifylline might then be explained in two ways; firstly, by an increase in the speed of locomotion, or secondly by a change in cell deformability. Pentoxifylline is not chemotactic for neutrophils (Sheetz et al., 1984), eliminating that possibility.

Taking the second possibility, a marked change in cell deformability would seem of doubtful benefit for a neutrophil moving through a collagen gel. In such a non-rigid matrix, consisting of a randomly arrayed meshwork of collagen fibrils, displacement of the fibrils by a locomoting cell would be necessary for movement, with a certain degree of cell stiffness a prerequisite, in order to push aside parts of the matrix. Schmid-Schönbein et al., (1982) have shown that during active motion, leucocytes project pseudopods which are stiffer than the passive cytoplasm. If pentoxifylline made a neutrophil more deformable, as it does erythrocytes, the neutrophil would be less able to push against the matrix, thus making it harder to locomote.

Approaching the problem of neutrophil locomotion from the other direction, an assay was done using collagen gels stiffened by fixation with glutaraldehyde. The expected inhibition of invasion seen is in accord with the argument above, that a neutrophil has to be able to push aside part of the matrix through which it is locomoting. The effect of stiffening the matrix should be the same as increasing the

deformability of the cell, making it more difficult for the cell to move through the small gaps in the matrix.

In collagen gels of different concentrations, invasion was enhanced by pentoxifylline, though to a lesser extent at the highest collagen concentration used. If an increase in deformability helped cell movement through a collagen gel matrix, then one might expect the enhancement to be more marked in the highest concentration gel; but it is not so.

The time-lapse filming showed that both rabbit and human neutrophils increased their speed of locomotion over protein-coated glass, with little effect on persistence. As there is no effect by pentoxifylline on neutrophil adhesion to Nuclepore filters (Schmalzer & Chien, 1984), and flow-chamber and aggregation assays have also shown a lack of adhesive effects (Crocket et al., 1987), then direct activation of the cell motor would seem to explain best the increase in speed. The lack of sensitivity to pentoxifylline by neutrophils pre-stimulated with f-MLP would support this; if their motors are already maximally stimulated by the f-MLP, pentoxifylline would be ineffective. An increase in speed would be sufficient to explain the enhancement of invasion into collagen gels with pentoxifylline, since the population diffusion coefficient is proportional to $2S^2P$ (Wilkinson et al., 1984).

Stefanovich (1978) examined the biochemical mechanisms of action of pentoxifylline, finding that one of its actions was to increase ATP levels in rat erythrocytes. Under normal circumstances, calcium is chelated with ATP. As

high calcium levels in the erythrocyte membrane have been observed to reduce deformability (Weed et al., 1969), this might suggest that a reduction in ATP levels due to the binding of excess calcium to the membrane protein contributes to a sol-gel transformation, i.e. greater rigidity. Pentoxifylline could then act by increasing ATP and reducing rigidity, at least in erythrocytes.

In neutrophils, dramatic increases in polyphosphoinositide levels, of up to 8 times, were observed by Sheetz et al., (1984), using pentoxifylline at a dose which stimulated migration (0.02 mg ml^{-1}). In the luminometry experiments, pentoxifylline-treated cells showed a faster response to f-MLP than control cells. This may indicate a membrane disruption effect, allowing luminol to cross the cell membrane more quickly. Chemiluminescence of intracellular origin is limited by diffusion of luminol into the cells (Briheim et al., 1984). Little is known of the role of the membrane skeleton in the locomotion of neutrophils however, and until a definitive deformability assay is developed, an increase in speed with pentoxifylline would appear to explain the enhancement of invasion into collagen gels. 3-D tracking of cells would also help confirm this conclusion, though this is not yet a standard technique (Noble, 1987).

In the acute inflammatory response, neutrophil leucocytes must attach to, and invade across, the endothelium of postcapillary venules and the underlying basal lamina (Lackie & Smith, 1980; Harlan, 1985). The mechanism by which neutrophils penetrate these barriers is poorly

understood. Although it is clear that active movement by the neutrophils is involved, and that the cells move through endothelial cell junctions (Schoefl, 1972), it is not clear what effect this cell traffic has on the vessel wall. Since there is a constant slow traffic of neutrophils across the endothelium, even in non-inflamed tissue (Harlan, 1985), it seems unlikely that they would damage the endothelium under normal circumstances. Neutrophils must often have to traverse collagenous tissues en route to a site of inflammation, and the *in vitro* model using collagen gels therefore makes it possible to look for any effects that invading neutrophils may have on a 3-D matrix.

As reported in Chapter 4, a first wave of neutrophils invading a collagen gel conferred no benefits on a later, second set. If the first set had made some permanent changes to the gel matrix, perhaps leaving a set of pathways or tunnels behind, either by mechanical or enzymic means, it would facilitate the invasion of following cells, reflected by an increase in the number of cells invading the gel. Brown (1984), in an SEM study, found no sign of damage or changes to the collagen matrix following neutrophil invasion. Neutrophils moving through such a matrix probably depend on contact with the collagen fibres for locomotion, and it is doubtful whether any advantage would be gained by dissolution activity. Increasing the collagen concentration inhibits invasion, as does stiffening the collagen matrix, both situations in which enzymic activity might assist neutrophil locomotion.

The effect of initial neutrophil concentration on invasion

is similar to the invasion of Syrian hamster melanoma (tumour) cells described by Schor et al., (1982), with the number of cells entering a collagen gel being directly proportional to initial cell number. In contrast, Schor et al., (1982) also describe a human fibroblast line, whose invasion showed an inverse relationship to cell density.

The increased invasion of neutrophils does not result from a lack of available area at the gel surface, which can be seen between cells in different spots throughout the wells. Additionally, a straightforward calculation of areas confirms the availability of surface area (Abercrombie & Heaysman, 1954). It may be that the more densely plated cells produce a factor which stimulates cell invasion. Cell migratory factors have previously been shown to be produced by a variety of transformed cells (Burk, 1973), and there is evidence that neutrophils do release factors which stimulate the movement of other neutrophils (Zigmond & Hirsch, 1973). Venge (1979) has shown that migration of neutrophils, both directed and random, was increased by increased cell concentration, and there was a suggestion that this increase in migration correlated with the release of a chymotrypsin-like cationic protein.

Time-lapse filming on different concentrations of neutrophils moving over protein-coated glass showed an effect on speed only at the highest concentration used, 2×10^6 ml^{-1}. At this concentration the population speed was significantly reduced, when compared with the other sequences. It might be expected that if

cells at a higher concentration were activated to some degree, then this would be reflected by a higher speed. On the other hand, cells at this concentration are probably colliding more often with other cells, which would tend to lower the average speed as calculated by the electronic tracking system. The effect of cell density on persistence is more consistent, with a graded response, persistence decreasing as cell concentration increases. This decrease in persistence, it is suggested, is again due to an increase in the number of cell collisions at the higher concentration.

It is difficult to see how invasion could be enhanced without an increase in speed or persistence. It may be possible that neutrophils, moving at high densities over protein-coated glass, are activated, but because of collisions with other cells this increased activity is not being reflected in a measured increase in speed. On invasion into the collagen gel however, the considerable reduction in interaction with neighbouring cells could allow the increase in speed to show, by an increase in the leading front. The development of a good automated tracking system, capable of measuring the locomotory characteristics of cells in a 3-D matrix might help confirm this; such systems are being developed (Noble & Levine, 1986; Noble, 1987).

One possible mechanism by which neutrophils invade and move through collagen matrices is by proteolytic activity; there is evidence that endothelial permeability is modified by neutrophils in sites of inflammation (Williamson & Grisham,

1961; Wedmore & Williams, 1981; Issekutz, 1981; Meyrick et al., 1984). Proteases are probably important for local invasion from a primary tumour and at secondary sites (Levine et al., 1984; Matzner et al., 1985). Wilkinson & Bradley (1981) have shown that proteins which stimulate neutrophil movement induce protease secretion, while it is known that normal human neutrophils contain a specific collagenase, capable of degrading fibrillar collagen (Lazarus et al., 1968). Human neutrophils can release 25-30% of their neutral proteases (collagenase and elastase) in response to damaged tissue and immune complexes (Ohlsson & Olsson, 1977).

As reported in Chapter 4, there was no measurable degradation of radiolabelled collagen gels by stimulated, invading neutrophils. Labelled collagen gels, on the other hand, when treated with collagenase in the absence of cells, showed a graded fragmentation of the collagen, using concentrations of collagenase at levels approaching 1% of that expected to be released by the rabbit cells. Neutrophil leucocyte collagenase, from human cells, has been shown to attack type I collagen preferentially (Horwitz et al., 1977). This lack of proteolytic activity by neutrophils invading a collagen gel agrees with studies using other cell types. Radiolabelled collagen gels have been used to study the migratory behaviour of Chinese hamster ovary cells (a fibroblastoid cell line), and RPMI-3460 melanoma cells (Schor et al., 1980). After 3 days of culture more than 40% of the melanoma cells were within the gel, yet there was no apparent increase in the release of

degraded collagen, nor was there any obvious disruption of the collagen fibrils in SEM.

The tritium-labelled collagen was suitable for neutrophil invasion and migration, as shown by a series of standard invasion assays done in parallel. Additionally, neutrophils were seen in the thin layers of collagen.

Another approach was used to look at the possibility of proteolytic activity by invading neutrophils. This was the use of protease inhibitors. Neutrophil invasion of collagen gels was not inhibited in the presence of two known metallo-protease inhibitors, EDTA and o-phenanthroline, indicating a lack of proteolytic activity. Robertson et al., (1972) showed that EDTA inhibited collagenase in human neutrophils. Forrester et al., (1983) found that the plasma antiproteases α-2-macroglobulin, α-1-antitrypsin (actually an anti-elastase, Carrell, 1986), and α-2-antiplasmin had no effect on neutrophils invading a collagen gel, though the high molecular weight protease inhibitors used may not have been able to access surface-associated proteases.

It may be that rabbit neutrophils do not engage in much specific collagenase activity: Werb & Reynolds (1975) found little such activity in rabbit neutrophil granules. They thought it possible that much of the activity might be due to either the lysosomal elastase, or to the neutral proteases. This does not negate the conclusion from assays using tritium-labelled collagen, where no protease inhibitors were used, and no cellular degradation of the collagen was observed.

In some of the o-phenanthroline assays there was a paradoxical increase in invasion. Goetzl (1975) produced a brief stimulation of random locomotion with α-2-macroglobulin and α-1-antitrypsin, confirmed by Czarnetzki & Schultz (1980). The enhancement with o-phenanthroline may be a similar event. A slight enhancement was also seen using EDTA at 10^{-5} M. Both of these enhancements remain unexplained.

It is accepted that a neutrophil may, in certain circumstances, release proteases. However, the findings using ^{51}Cr-labelled cells, radiolabelled collagen gels and protease inhibitors, reported in Chapter 4, suggest that migration through a collagenous matrix in normal situations does not require mechanical changes to, or proteolysis of, the gel by the cell. This is in agreement with the model of locomotion put forward by Brown (1982) for neutrophils, and Haston et al. (1982) for lymphocytes. In both cases, it is suggested that locomotion through a 3-D matrix such as a collagen gel does not depend on adhesions to the substratum. Traction in such a matrix is likely to be by the extension and expansion of pseudopods between the fibres of the matrix, the expanded pseudopods acting as attachment points for anchorage. Such a locomotory mechanism would allow neutrophils to locomote without causing damage to healthy tissues.

Neutrophil leucocytes are of prime importance in the defense against infectious agents. Initiating their defense strategy, neutrophils migrate toward the site of infection or inflammation, having recognised a chemotactic

substance. It has been shown that both soluble and insoluble immune complexes (IC) can attract neutrophils (Hawkins & Peeters, 1971; Wilkinson, 1982; Leung-Tack et al., 1977). The smaller, soluble IC, such as those formed at 100 times equivalence antigen excess, are virtually ineffective at releasing neutrophil intracellular enzymes. In contrast, homologous antigen-antibody complexes in precipitate form and soluble antigen-antibody complexes made in antigen excess of 20 times equivalence are equally effective at releasing intracellular enzymes (Hawkins & Peeters, 1971).

In generating active locomotion of neutrophils from the blood vessels to an inflammatory locus a complement-generated chemotactic gradient probably provides the most effective means of neutrophil accumulation (Wilkinson et al., 1984). Haptotaxis, or cell trapping by differences in the adhesive properties of substrates is another probable mechanism by which cells arriving at an area of increased adhesion, such as a site of IC deposition, might accumulate (Carter, 1967).

Wilkinson et al., (1984) showed that neutrophils move more slowly over IC-coated glass than over BSA-coated glass. There was no consistent difference in turning behaviour between cells on the two surfaces, therefore the IC were negatively chemokinetic in comparison with BSA, resulting in the gradual accumulation of cells on the IC. This finding was confirmed using HSA and IgG anti-HSA surfaces (Wilkinson & Lackie, 1985). Dahlgren & Elwing (1983) had shown, using a modification of the agarose

method described by Nelson *et al.*, (1975), that neutrophils moving over an IC-coated surface were inhibited.

The adhesive effect by IC-coated glass was also confirmed in the present study, using time-lapse filming and a conalbumin-anticonalbumin immune complex. The antiserum used was heat-treated. Lackie *et al.*, (1987) have shown in flow-chamber studies that neutrophils are capable of reacting almost instantly to an immune complex-coated surface, with the adhesion being slower and less marked in the absence of complement. This rapid adhesion effect under flow conditions is in line with the cells' function, when on receipt of a chemotactic signal during their passage through blood vessels, they marginate and adhere to the endothelial wall, before migrating through the endothelial wall towards the focus of the signal.

Wilkinson *et al.*, (1984) showed that neutrophils on IC-coated surfaces redistributed their Fc receptors to the under surface, and that the lowered speed of locomotion is due to tethering of neutrophils by substratum-bound IgG-Fc. While this trapping mechanism would appear to be true for cell movement over a substratum, where adhesion is important, the effect on movement through an extracellular matrix is more difficult to predict, as here adhesion may not be as important (Brown, 1982). Collagen gels, in conjunction with IC, should provide a fairly realistic *in vitro* model for an inflammatory response, where neutrophils moving through a three-dimensional matrix encounter diffusing substances. The outcome of

these encounters, however, are not as readily predictable as those on plane substrates, and it may be that it is not possible to study any selected single property of IC, since they are probably always present as heterogeneous mixtures (Hofstaetter & Brammsen, 1984).

Preliminary assays reported in Chapter 5, using sheep antiserum and antigen in the medium above the collagen gels, gave a predicted inhibition of invasion. The remaining IC assays, discussed below, used a high titre anti-conalbumin serum, and in many cases incorporated antiserum, antigen, or both in the collagen gel.

Examining the cell distribution in a well in which conalbumin had been incorporated into the collagen and antiserum was present in the medium above the collagen, there was a marked accumulation of cells at depth in the collagen gel. in this instance at about 100 μm below the gel surface, with the leading front being just under 200 μm. The antiserum was not heat treated. and though there appeared to be no cell lysis, complement activation was probably responsible for at least part of the accumulation of cells at depth. IC would have been formed in the gel by the diffusion inwards of antiserum from the medium combining with a suitable amount of conalbumin in the collagen matrix (Figures 5.4, 5.5). This deviation from the normal distribution of invading cells is evidence for a true chemotaxis, as random locomotion alone, whether stimulated or not, would have given a peak of cell concentration at the origin, in this case the gel surface. (Zigmond & Hirsch, 1973; Dunn, 1983; Islam et al., 1985).

Adhesion has been shown to have little effect on neutrophil movement through a collagen gel (Brown, 1982). The formation of an immune complex within a collagen gel has not been described before now, however, and the mechanism of cell accumulation within a collagen gel is unknown. It is possible that deposited complex between the collagen fibrils is being adhered to by neutrophils, and that haptotaxis is taking place (Carter, 1967). If so, the accumulation may be short-lived, as this cell distribution was not seen again in wells in which distribution counts were done.

It is just as likely, and the two are not mutually exclusive, that complement is attracting neutrophils to the site of IC deposition. Wells containing complement free serum in the same assay as that described in Figure 5.5 showed a distribution similar to control wells. Cell-release factors, also chemotactic for neutrophils, may be present (Henson, 1971). As chemotactic factors will also be diffusing, the situation in these assays is obviously highly unpredictable. This result nonetheless shows that the formation of an immune complex within a collagenous, 3-D matrix, is capable of causing accumulation of invading neutrophils, a result in full accord with the function of these cells *in vivo*.

Some assays indicated an enhancement of invasion due to the presence of serum. This is probably a chemokinetic effect. Cramer *et al.*, (1986) described the effect of 10% human serum as increasing the migration of neutrophils across an epithelial monolayer, though they suggested that this might

be due to a permeability-increasing, heat-stable factor in serum. Investigating the effects of various antigen-antibody ratios, Hawkings & Peeters (1971) found that soluble complexes made in antigen excess of 20 times equivalence were virtually equally effective as complexes in precipitate form as releasing agents for neutrophil enzymes. This was confirmed, with neutrophils undergoing cell lysis in the presence of soluble complexes without complement.

In invasion assays using a single layer of collagen, conditions were dependent on the inward diffusion of reagents into the collagen, and the outward diffusion of reagents into the medium above the collagen. The formation of an IC, a somewhat unpredictable event, might then tend to happen at or near the surface of the collagen gel, inhibiting invasion of the gel. The obvious modification to this assay was to use two layers of collagen, thereby making it possible to produce IC at greater depths, and perhaps leading to the converse effect on neutrophil invasion of gels, an enhancement. Islam *et al.*, (1985) had investigated the chemotactic responses of human neutrophils by incorporating, within a collagen gel, blocks of agarose containing the peptide f-MLP, chemoattractive for neutrophils.

Crick (1970) has calculated that proteins have diffusion constants in the range 0.3 - 1.0 x 10^{-6} cm^2 s^{-1}, which should allow for the formation of a linear- gradient of

concentration across a distance of 1 mm in a few hours (assuming a constant source at one end, and a sink at the other). Zigmond (1980) has also furnished calculations, showing, for example, that a large molecule such as BSA, with a diffusion constant of 6×10^{-7} cm^2 s^{-1}, will set up a concentration gradient across a filter in $\leq$ 1.5 minutes, and across an agarose assay in ca. 5 h.

Several collagen gel assays, using two layers of collagen with the upper layer about 1500 μm thick, showed no modification of neutrophil invasion, the conclusion being that the layer of collagen was too thick to allow a chemotactic substance to reach invading cells. The upper layer of collagen was therefore reduced to about 750 μm in thickness, with results indicating an accumulation of cells in the upper layer, presumably due to the formation of an immune complex and/or complement (Figure 5.18).

These visual migration assays employing immune complexes, while indicating that IC were modifying neutrophil invasion of the collagen gels, either enhancing or inhibiting invasion according to the conditions set up, were to some extent limited by the relatively great depths of collagen necessarily used. It was impractical to stain for protein deposits, for example, without staining the entire gel, and hence determination of the boundary between the collagen layers, and the approximate location of deposited IC, was not feasible. Additionally, data collection on cell distribution was time-consuming, moving vertically down through the gel for each replicate. Accordingly, a

novel assay system was devised, allowing both for an increased speed of data collection, and for the rapid, visual determination of the precise position of the boundary between different areas of collagen, without staining. The assay method, referred to as the slab assay, is described in Chapter 2 and shown in Figure 5.19.

The unexpected finding from the slab assay was the immobilisation of neutrophils incorporated in the gels. The immobilisation was verified by video time-lapse filming, electronic tracking, and distribution analyses. One noticeable difference from standard invasion assays was in the morphology of the cells, which in the slab assay did not seem to be showing shape changes to any marked degree (Plate 5.1), in contrast to neutrophils invading a collagen gel which usually exhibit locomotory morphology, with polarisation (Senda et al., 1975; Grinnell, 1982). This immobilisation of neutrophils has not so far been reported elsewhere, and it raises some interesting questions. Cells incorporated into a collagen gel, which they would normally invade and migrate readily through, could become immobile for a number of reasons. The cells might be attempting to move, but be mechanically trapped by collagen fibrils, or they may have become rounded up due to some change in the structure of the collagen gel caused by the presence of the cells, or to some cell-release factor.

The collagen slab without incorporated cells was suitable for neutrophil locomotion, as shown by the ability of neutrophils plated on top of such a slab to invade and

migrate into the collagen. To test whether slabs with incorporated cells are suitable for neutrophil locomotion by invading cells, radiolabelled cells could be plated on top of the collagen slab; unfortunately, insufficient time precluded this experiment. Schor et al., (1983) incorporated lymphocytes into collagen gels, finding that although active cell movement was observed, the cells maintained their original, homogeneous distribution, this being expected in the absence of any chemotactic cue. About 2% of the cells were reclaimed from the medium after each 24 h incubation period, indicating the establishment of a regular traffic of cells. When plated back onto collagen gels, these cells invaded normally. Islam et al., (1985) incorporated neutrophils into fibrin gels, finding that they could locomote towards a source of f-MLP.

Schaack & Persellin (1981) found, in an under-agarose assay, that there was a threshold density for neutrophils of 1.8×10^{2} mm^{-2}, below which chemotaxis was not seen, while Takeuchi & Persellin (1979) had reported finding a cytoplasmic fraction from disrupted neutrophils which was chemokinetic. They suggested that a soluble, chemokinetic factor is released by densely packed neutrophils. Cell released factors have been suggested by others (Henson, 1971; Venge, 1979). Increasing the concentration of neutrophils in the slab assay made no difference to the results however, cells remaining immobile.

While it cannot be ruled out that neutrophils incorporated in a gel become immobilised due to their isolation from each other, the assay using cells incorporated at the very

high density of 15 x 10^6 ml^{-1} would make this possibility unlikely. Neutrophils have been shown to release an inhibitor, known as NIF (neutrophil-immobilising factor). This is a small peptide, 4-5 Kdaltons, which has an irreversible inhibitory effect on neutrophil locomotion in the presence of chemotactic factors (Goetzl & Austen, 1972; Goetzl et al., 1973). It is possible that neutrophils incorporated in a collagen solution are stimulated to produce superoxide, which could then inhibit locomotion. Boxer et al., (1978) have shown that 2,3, dihydroxybenzoic acid, which is an antioxidant and H_2O_2-Scavenger, enhances neutrophil locomotion to chemotactic factors. Such an inhibition would be independent of cell density, and hence cell contact.

The presence of some cell-released factor, inhibiting neutrophil locomotion, was not verified, as shown by the results given in Chapter 5, tracking neutrophils on protein-coated glass, and using supernatant from centrifuged collagen gels containing incorporated cells. The slight decrease in speed seen in some of these assays is probably due to the deposition of collagen onto the protein-coated glass, a substrate on which neutrophils are unable to gain traction. In a preliminary assay using supernatant from the first centrifugation, a very weak gel was formed in the filming chamber, and even after a second centrifugation, it is suggested, sufficient collagen will be present in the medium to affect cell locomotion over the glass.

As evidence that collagen fragments are being removed from a collagen gel in the absence of collagenase or cells, sequential samples taken from the medium above a tritium-labelled collagen gel without stirring, showed a release of label (Figure 2.2). This confirms the finding of Schor et al., (1983), who looked for the degradation of collagen during lymphocyte migration, finding none that was cell-associated. They did, however, obtain counts in the medium above collagen gels whether cells were present or not.

A final explanation for the immobilising effect of neutrophils during collagen gelation is that mechanical trapping of the cells by collagen fibrils takes place, each cell acting as a nucleation centre for the formation of collagen fibrils. Neutrophils certainly are inhibited at higher densities of collagen (Brown, 1982). The assay done with low collagen concentration and high cell density gave the same result, with immobilised cells. An SEM study of neutrophils incorporated in a collagen gel might show such a trapping effect, though this was not done.

Harris et al., (1981) looked at the various traction forces exerted by individual cells, culturing them on thin, distortable sheets of silicone rubber. They found that the weakest traction was found with cells which were the most mobile, including leucocytes, whereas fibroblasts from several sources exerted forces several orders of magnitude stronger than that required for locomotion. In collagen gels, such fibroblasts were able to distort the gel. It may be that neutrophils, mechanically trapped by collagen fibres during gelation, have insufficient force, or

inappropriate movements, to free themselves for locomotion. From the work of Schor et al., (1983), who showed that incorporated lymphocytes could move through collagen gels, it would then be predicted that lymphocytes would possess stronger tractive forces than neutrophils, or have a subtly different locomotory behaviour.

Concluding Remarks

The results presented in this thesis, using collagen gels and other techniques, extend earlier work on the invasive behaviour of neutrophil leucocytes. The effect of a methyl xanthine, pentoxifylline, which enhances neutrophil invasion, is shown to be that of an increase in cell mobility.

No cooperative effects between neutrophils invading a collagen gel could be found. The collagen matrix is not degraded, nor do metallo-protease inhibitors affect invasion. This is probably true for invading neutrophils in vivo, at least in the early stages of migration, where healthy tissue is traversed and damage by migrating cells is inappropriate. Time-lapse filming of cells on an immune complex-coated substratum shows an inhibitory effect on cell locomotion, as does a high concentration of cells on a protein-coated surface. The effect in vivo would be to accumulate cells at an inflammatory site, where there may be deposits of immune complex, with accumulation then being reinforced by the concentration effect.

The incorporation of immune complexes within the matrices of collagen gels was shown to modify neutrophil invasion. This *in vitro* system closely models an inflammatory process, where neutrophils are induced to migrate through an extracellular matrix and accumulate at the inflammatory locus. Incorporation of neutrophils in collagen solutions before and during gelation has a consequent immobilising effect on the cells. This remains unexplained, though a cell released factor is unlikely.

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Appendices

A. Media preparations

1. Calcium- and Magnesium-free Salts Solution (HS)

	Amount ($litre^{-1}$)
NaCl	8.0 g
KCl	0.4 g
Glucose	1.0 g
HEPES	2.39 g
Phenol Red (1% w/v)	10.0 ml

pH adjusted to 7.4 with 1N NaOH and preparation autoclaved.

2 Hepes-buffered Salts Solution (H2)

	Amount ($litre^{-1}$)
NaCl	8.0 g
KCl	0.4 g
$MgCl_2.6H_2O$	0.2 g
$CaCl_2$	0.14 g
Glucose	1.0 g
HEPES	2.39 g
Phenol Red (1% w/v)	10.0 ml

pH adjusted to 7.4 with 1N NaOH and preparation autoclaved.

BHK21 growth medium was Glasgow-modified Eagles Medium (Gibco, Paisley).

3 Borate buffer

	Amount ($litre^{-1}$)
Boric acid (H_3BO_3)	6.18 g
Sodium tetraborate ($Na_2B_4O_7$)	9.53 g
NaCl	4.38 g

Borate saline

Borate buffer	50 ml
saline (0.9% w/v NaCl)	950 ml

4. Kenacid Blue staining for Ouchterlony gels:

Kenacid Blue	1.25 g	
Acetic acid	50 ml	
distilled H_2O	185 ml	(gives 0.5% stain)

Procedure:

1. Remove free protein with PBS, 24 h, several changes.
2. Stain 5 m.
3. Destain, several changes over several days, as necessary.

Stock solutions:

30% acrylamide acrylamide 28.5 g
bis-acrylamide 1.5 g H_2O to 100 ml

Running gel buffer Tris 18.15 g
SDS 0.4 g

Dissolve in ca. 50 ml, pH to 8.9 with 5N HCl, bring volume to 100 ml with H_2O.

Stacking gel buffer Tris 5.9 g

SDS 0.4 g

Dissolve in ca. 90 ml, pH to 6.7, bring to 100 ml

x5 upper tank buffer Tris 31.6 g To 1 l distilled H_2O

glycine 20.0 g

SDS 5.0 g

x5 lower tank buffer Tris 60.5 g

SDS 5.0 g

Dissolve in 900 ml, pH to 8.1, bring to 1 l.

Boiling mix: Stacking gel buffer 2.5 ml

mercaptoethanol 2.5 ml

SDS 1 g

Warm until SDS dissolves, add a few drops of glycerol (1 ml), and 1 ml of 1% Bromophenol Blue and make up to 10 ml with H_2O.

5. Stain for gels:

0.1% Kenacid Blue (Coomasie Blue) 250 ml H_2O

Methanol: Water: Acetic acid (50:50:7) 250 ml Methanol

35 ml Acetic acid

0.5 g Kenacid Blue

Destainer:

Methanol: Water: Acetic acid (50:880:70) 3520 ml H_2O

200 ml Methanol

280 ml Acetic acid

Acrylamide solutions were made up as above. The solutions were added to a gradient maker, and the solution pumped between the glas plates of a standard PAGE system. The surface of the acrylamide solution was overlaid with 0.1% (w/v) SDS in H_2O to produce a smooth surface on the gel as it set. Following polymerisation the overlay was removed, a comb inserted between the plates, and a 5% stacking gel poured. Removal of the comb after polymerisation of the stacking gel provided wells for samples. Slab gels are run at 40 mA for 2-3 h.

Sample boiled for 3 min in boiling mixture, in ratio 5 parts sample: 1 part boiling mix, or dissolved directly in it, depending on amount of protein present (if dissolved directly, boiling mix should be made 1:5 with stacking gel buffer.

B. Development of automated cell tracking

Neutrophil leucocytes are highly motile cells, capable of locomoting over a substratum at speeds of over 20 $\mu m\ min^{-1}$. Sensitive to certain chemotactic and/or chemokinetic substances, they change their speed and persistence accordingly, though it must be noted here that a true negative chemotaxis has so far not been documented. Time-lapse recording of moving cells *in vitro* has been a useful laboratory tool for several decades, with the subject of cell locomotion playing a key role in morphogenesis, wound healing, inflammation and oncology (see Bellairs *et al.*, 1982, for a review).

Until recently, the analysis of cine films involved hours of laborious manual work; normally involving the tracing of cell tracks onto paper, then calculating the displacements, speeds and angles. The introduction of microcomputers into the lab allowed the tracings of tracks (see Plate 2.1) to be transferred to computer via a bit pad, with subsequent calculations being performed by computer. Though cell tracks still had to be transferred one by one to paper or bit pad, this period of cell tracking, designated computer-assisted, was much less laborious than an all manual method, though there remained still the taking, developing and projecting of the cine film.

While engaged in cell tracking as part of this thesis, I collaborated in the development of a fully automated cell tracking system. The details of the development of the system is more fully described in a recent publication (Dow, Lackie & Crocket, 1987). While engaged in cine film work, sequences of cell movements were also recorded electronically for direct comparison with the traditionally acquired data. Time-lapse films, previously analysed by computer-assisted methods, were projected onto a wall, where cell images were viewed with a video camera aligned close to the axis of projection, so as to minimise parallax effects.

The cell population locomotory parameters were then calculated using the same programs as devised for use with cine films, and the two sets of data compared. In all, over a dozen assays were employed in such a manner, with the technical details of the electronic method being improved

and customised as necessary. Coincidentally, the electronic tracking program became fully functional not long before the cine film processing laboratory ceased operation.

The section of the thesis dealing with time-lapse filming of pentoxifylline-treated cells used the computer-assisted method. The new system was used to examine the effect of cell concentration on locomotory parameters, as well as the effect of antigen, antiserum, and immune complex on cell locomotion over protein-coated glass.

C. Rheological tests on collagen

Testing carried out by Dr Brownsey at the Agricultural, Food and Research Establishment, Norwich, though not original work by me, is briefly presented here as being relevant to the use of collagen gels for the study of cell invasion. Additionally, results confirm practical experience in the laboratory.

Methods: Some of the measurements were made using a Rank Pulse Shearometer, constructed in such a way that a torsional shear wave can be propagated between a pair of parallel discs, each mounted on a piezoelectric transducer. One disc initiates the shear wave, the other detects its arrival shortly thereafter. Propagation time is determined for various disc separations and a plot of the two will then provide the wave velocity. The value of the shear modulus can then be calculated from the approximation:

$$G = \sigma V^2$$

where: G = shear modulus in Nm^{-2}

σ = density of material in kg m^{-3}

V = wave propagation velocity in m s^{-1}

Initial viscosity measurements were made on collagen stock solution at 4 mg ml^{-1}, under conditions of constant rotation. A strain of 0.1 (10%) was chosen, so as not to affect the structure of the gel. A frequency dependent modulus was determined, showing that for frequencies of below about 0.1 Hz the collagen solution behaves like a liquid; above, more like an elastic solid. This is in accord with what we know about the structure of gels, which consist of polymers, or long-chain molecules, cross-linked to create a tangled network and immersed in a liquid medium. The liquid prevents the polymer network from collapsing into a compact mass, while the network prevents the liquid from flowing away (Tanaka, 1978).

Measurements on gels, of 1% concentration (i.e. 1 mg ml^{-1}), were made with the gel <u>in situ</u>, in the instrument. The dependence of gel strength on age was made in this manner, showing that while gels were very weak, gel strength reached 90% of its maximum value within about 60 min., having a measureable gel structure within 10 min. (Figure 2.3). These measurements agree with empirical findings, with collagen gels setting within 5 - 10 minutes at 37°C, and reaching a functional strength after 1 h incubation. If used before this there is an increased risk of the gel collapsing or detaching from the wall of the culture well.

The value of G, the shear modulus, was 120 Pa, from which Dr Brownsey calculated (workings not given) that the density of crosslinks is 0.048 mole m^{-3}.

Using the pulse shearometer with a fixed frequency of 100 Hz and a deformation of 0.001% (a very low twist test), gels aged for 2.5 h gave gel strengths of 134 ± 8 Pa.

Finally, density measurements of collagen stock solutions were made. As in the making of collagen gels, air in the collagen gel proved to be a problem, overcome here by preparation of fluids under a partial vacuum. In the making of collagen gels for the collagen boundary assays, media were degassed before use, thus minimising the formation of bubbles in the gels.

Densities were given as:

Temp (°C)	Density	Sample
25	1.00043	4 mg ml^{-1} collagen stock
37	0.99640	4 mg ml^{-1} collagen stock

D. Neutrophil speed and persistence

The method used in this thesis to analyse time-lapse cine films of locomoting neutrophils was based on determining the position of the cell centre every ten frames, a time interval of 60s. The straight-line distance between these points was then measured and totalled for each cell track. This track is an approximation; locomoting neutrophils take a more smoothly curving path, but for the purposes of analysis in this work the segmented track is used. The electronic cell tracking procedure uses basically the same method, with a time interval of 30s between positions of the centroid of a digitised image (Dow et al., 1987).

The length of sampling time intervals can be a source of bias when attempting to measure the two parameters of speed and persistence. At shorter time intervals the speed rises; probably due to the increased difficulty in estimating the position of the cell centre. If time intervals are taken more than 20s apart, speed measurement is relatively insensitive to interval. The turning behaviour however, is more difficult to estimate, and shows a positive correlation with the sampling interval.

In the absence of external cues which might give vectorial information, the path of the cell can be treated as a random walk with internal bias. This internal bias has been referred to as the tendency for the cell to persist in moving in a particular direction over a short time interval. If the movement is random then $d^2 \propto t$, and the vector sum of displacements of cells in the population will

probably be below predictable limits (Dunn, 1981).

The cell path can then be described in terms of two parameters, speed (S), and persistence (P), which together contribute to a single parameter R (which approximates to a diffusion coefficient). In the analysis done here,

$$\langle d^2 \rangle = 2S^2[Pt - P^2(1 - e^{-t/P})].$$

Following the suggestion of Dunn (1983), we have characterised the movement of neutrophils using a simplification of the full analysis, which we use to estimate S & P from the equation:

$$1/\langle d^2 \rangle^* \approx 1/S\ [1/t + 1/6P].$$

In practice, we plot $1/\langle d^2 \rangle^*$ versus 1/t, and obtain the slope (1/S) and intercept on y-axis ($-1/6P$). Using a least squares regression we obtain S and P. Estimates of the variances of S, P and R were obtained using the 'Jackknife' technique (Mosteller & Tukey, 1977).

This analytical approach has been used in previous work (Wilkinson et al., 1984), and reviewed in Lackie et al., 1987.

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E. Effects of methyl xanthines, including pentoxifylline, on cells.

Pentoxifylline, one of a member of a group of related compounds which includes caffeine, theophylline and aminophylline, is known to improve the flow properties of blood (Müller, 1983), increase the deformability of erythrocytes (Seiffge & Kiesewetter, 1981), and increase the concentration of adenosine triphosphate in erythrocytes (Buchanan & Moodley, 1977, Stefanovich, 1978). Although the precise mechanism of action of pentoxifylline on cells is unknown, like other methyl xanthines it raises the intracellular cAMP levels by inhibition of cAMP-phosphodiesterase, and may disturb membrane fluidity (Rahmani-Jourdheuil et al., 1987).

Membrane fluidity, as discussed here, refers to the physical state of the fatty acyl chains of the membrane bilayer structure. The behaviour of these acyl chains can be influenced by polyphosphoinositides (Sheetz et al., 1983). It is possible that pentoxifylline disturbs polyphosphoinositide metabolism, and thus membrane fluidity. In support of this is the evidence that erythrocyte membrane shape and deformability depend on the amount of polyphosphoinositides in the membrane (Quist & Powell, 1985).

Looking at the effect of pentoxifylline on monocytes and polymorphonuclear cells, Bessler et al. (1986) found the drug to inhibit the phagocytosis of latex particles. Superoxide anion production during the phagocytic process

was also reduced following incubation of the cells with pentoxifylline, and Bessler et al. (1986) suggested that this inhibitory effect is due to increased intracellular levels of cAMP induced by pentoxifylline.

It is known that pentoxifylline reduces the stiffness of leucocytes (Skalak et al., 1987), which could explain the increased movement of leucocytes through a rigid matrix (Sheetz et al., 1984). This thesis has shown that pentoxifylline also increases neutrophil penetration of a deformable matrix, a collagen gel, and in this case it may be that an increase in cellular motility is being seen. The effect is obviously a complex one, and may be related to the activity of protein kinase, known to be affected by pentoxifylline (Bikle et al., 1984).

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F. Plasminogen Activators

The urokinase-type plasminogen activator is one of two activators which convert the extracellular zymogen plasminogen into plasmin, an active protease. This activator, released from a variety of mammalian cells including cultured human monocytes, may be an important factor in the regulation of cell migration (Dano et al., 1985, reviewed by Blasi et al., 1987). Endothelial cells have been shown to produce a latent inhibitor of plasminogen activators (Hekman & Loskutoff, 1985). A distinct protease inhibitor specific for plasminogen activator is released from cultured monocytes-macrophages (Wohlwend et al., 1987). Werb et al., (1977) have indicated that plasminogen activator may play a role in the

activation of latent collagenase secreted by rheumatoid synovial cells in culture. Wünschmann-Henderson et al. (1972) have suggested that plasminogen activator is secreted by neutrophils.

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G. Haemopoiesis

Blood cell formation, or haemopoiesis, is initiated by a population of multipotential haemopoietic stem cells that arises in the yolk sac and migrates to the foetal liver. Subsequent populations of differentiating haemopoietic cells develop in the bone marrow (Metcalf & Moore, 1971).

Haemopoietic stem cells are similar to two other stem cell populations, the primordial germ cells and primordial melanoblasts, in that all three populations arise outside the body, later migrating into the embryo proper.

Haemopoiesis is entirely sustained by the capacity for self-replication of the initial stem cells, and their capacity to generate more differentiated progenitor cells that are the specific committed precursor cells for the erythroid, granulocyte (i.e. neutrophilic), macrophage, eosinophil, megakaryocyte and lymphocyte populations.

Progenitor cells cannot dedifferentiate or transform to other progenitors, but are able to generate clones of maturing progeny cells, the most mature of which appear in the blood. Many granulocyte-macrophage progenitors are bipotential, and are able to form either granulocytes or macrophages.

In mouse bone marrow, ca. 1 cell in 400 is a progenitor cell apparently committed irreversibly to the formation of granulocyte and/or macrophage progeny (GM-CFC, granulocyte-macrophage colony-forming cells). These GM-CFC have been shown to be the progeny of multipotential haemopoietic stem cells (CFU-S), to have essentially no capacity for genuine self-replication, to be unable to become progenitor cells of any other cell lineage, or to revert to multipotential, self-replicative, CFU-S. In the normal adult marrow, these granulocyte and/or macrophage progenitors are in active cell cycle, continually being generated by CFU-S, and continually being expended by generating more granulocytes and macrophages. The proliferation of GM colonies <u>in vitro</u> has been shown to require continuous stimulation by a specific macromolecule, granulocyte-macrophage-colony stimulating factor (GM-CSF). (Metcalf, 1981. See Metcalf, 1980, for a review.)

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