THE EFFECT OF THE LYMPHOKINE(S) MIF/MAF ON MURINE MACROPHAGE BEHAVIOUR

by

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To my parents
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STATEMENT

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled "The Effect Of The Lymphokine(s) MIF/MAF On Murine Macrophage Behaviour" is based on work conducted by the author in the Department Of Zoology, of the University of Leicester, during the period between October 1982 and June 1985.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by reference. None of the work has been submitted for another degree in this or any other University.

Signed. . . . . . . . . . . . . . Date. . . . . . . . . . . . . .
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ABBREVIATIONS

BCG = Bacillus Calmette-Guérin
BSA = Bovine serum albumin
CFU-G,M = colony forming unit-granulocyte, monocyte
cpm = counts per minute
CSF = colony stimulating factor
d.f = degrees of freedom
DMEM = Dulbecco's Modified Eagle's Medium
IFN = interferon
IgG = immunoglobulin G
i.p = intraperitoneal
LPS = lipopolysaccharide
MAF = macrophage activating factor
MGF = macrophage growth factor
MIF = migration inhibition factor
PECs = peritoneal exudate cells
PMNs = polymorphonuclear leucocytes
SEM = scanning electron microscope
±SEM = ± standard error of the mean
SDS-PAGE = NaDodSO₄/polyacrylamide gel electrophoresis
SMAF = specific macrophage arming factor
TEM = transmission electron microscopy
TSN = transformed cell supernatant
**INTRODUCTION**

In his lecture of 1891, the Russian zoologist Metchnikoff first used the term *macrophage*, which means "large eater", to describe those cells which engulf foreign materials and to distinguish them from the smaller circulating leucocytes or microphages (Metchnikoff, 1891) now known as polymorphs. Metchnikoff had found that amoeboid cells from invertebrates could actively ingest and digest foreign material. He then found that functionally identical cells were present over a wide range of organisms in the animal kingdom, from starfish to mammals. Metchnikoff proposed that these phagocytic cells might be important in body defence by disposing of foreign material in wounds, where they were found to accumulate.

Since Kiyono (1914) and Aschoff (1924), macrophages have been recognised as belonging to the reticuloendothelial system of Aschoff. This system includes all those cells which were found to ingest acid vital dyes injected into the body (reviewed by Jaffe, 1938). The reticuloendothelial system encompasses a heterogenous group of connective tissue cells, including fibroblasts and endothelial cells as well as macrophages. Although anatomically associated in reticuloendothelial tissues, these cells are not developmentally closely related, so it was suggested that this ambiguous term be dropped and macrophages as a group be referred to as the mononuclear phagocyte system, on the basis of their common origin, morphology and function (van Furth et al., 1972). The mononuclear phagocyte system consists of mature macrophages in the
tissues, and blood monocytes and their precursors in the bone marrow. The reticulum cell, endothelial cell and the fibroblast cannot be included in the mononuclear phagocyte system because of differences in morphology and function and because they are endodermal and mesodermal in origin (Maximow, 1927) whereas mononuclear phagocytes descend from haemopoietic stem cells (Metcalf and Moore, 1971).

Distinguishing mononuclear phagocytes from their neighbours, the mesenchymal cells, can be difficult since the latter form the environment in which the mononuclear phagocytes function. Apart from their lineage, mononuclear phagocytes can be characterised on the basis of their morphology, cytochemistry, immunology and function (extensively discussed by van Furth, 1978). These criteria can be used, for instance, to distinguish mononuclear phagocytes from fibroblasts, which is important since the normal functioning of each of these cell types is dependent on the presence of the other. Leibovich and Ross (1975a) distinguished these two cells from one another using ultrastructural criteria. They described differences in appearance of their nuclei, veil-like and microvillus-like folds in their peripheral cytoplasm, and the extent and development of the rough endoplasmic reticulum. Immature macrophages, or monocytes, can be distinguished from another important phagocyte, namely the neutrophil, or polymorphonuclear leucocyte, using morphological and histochemical criteria. The irregularly lobed nucleus is a characteristic feature of the neutrophil which sets it apart from the monocyte, as do the different cytoplasmic enzymes (Braunsteiner and Schmalzl,
Briefly, the mature macrophage is an avidly phagocytic, large, mononuclear, metazoan cell able to engulf and enzymatically digest cell debris and foreign materials. It is usually larger than its immature form, but varies greatly in size and shape. It is a motile cell and can assume polarity. The surface of the macrophage is covered with microvilli, its nucleus is oval, indented or irregularly shaped and surrounded by abundant cytoplasm, rich in lysosomes.

Cells of the mononuclear phagocytic system are widely dispersed throughout the body, either as wandering, or "free" macrophages or "fixed" macrophages in particular organs and tissues. The latter are found most highly concentrated in the lymphoreticular organs of the body, namely the spleen, lymph nodes, bone marrow and the liver. There is a general overall similarity in the structure and behaviour of these cells; they are actively phagocytic and therefore contain ingested material, and they have the cytoplasmic apparatus with which to synthesise hydrolytic enzymes to digest this material.

Fixed macrophages are often found lining tissues or blood vessels where they can monitor streams of fluid which flow past them. Kupffer cells, first described by von Kupffer in 1876, line the blood vessels and hepatic sinusoids of the liver (Carr, 1973). Their cytochemistry is reviewed by Wachstein (1963), and their ultrastructure by Rouiller and Jezequel (1963) and Alterman (1963).
Kupffer cells can be distinguished by the presence of densely packed lysosomes which give a positive acid phosphatase reaction, and the presence of fenestrated flanges of cytoplasm which form part of the wall of the sinusoid (Wisse, 1972). Kupffer cells may be involved in the production of anaphylactic shock; when antigen–antibody complexes are injected intravenously into an animal they are taken up by Kupffer cells and cause the breakdown of lysosomes (Treadwell and Santos-Buch, 1968).

The spleen contains a large proportion of the body macrophages, mainly located around some arterial capillaries (Solnitzky, 1937), in the lining of the sinusoids and in the parenchyma of the spleen (Klemperer, 1938). The fine structure of the splenic macrophage has been studied in detail, for example by Simon and Burke (1970). These studies show the splenic macrophage has rather more processes, and is therefore more phagocytic, than those in many other sites. The most striking point, however, about the splenic macrophage is the presence of numerous prominent lysosomes. Splenic macrophages are important in the breakdown of old or damaged red corpuscles, the storage of iron from these cells and its recycling in erythropoiesis (Carr and Toner, 1982). Macrophages in the red bone marrow may also be important in the breakdown of red blood cells; the remains of these are often found in the large pleomorphic secondary lysosomes of these macrophages, and ferritin is found in large quantities in their cytoplasm, ready for recycling (Carr, 1973).

Fixed macrophages are also found lining lymph node sinusoids or
outside them. The literature on their ultrastructure has been reviewed by Carr (1970). They are characterised by their irregular surface with numerous flap-like ruffles, have a well developed microfibrillar apparatus and abundant dense bodies, probably lysosomal, in the cytoplasm (Carr, 1973).

There is also a large population of macrophages scattered throughout the nervous system. They are called microglial cells, are usually multipolar, with highly branched processes and are phagocytic (Carr, 1973). Macrophages in large numbers are found, too, in the mesodermal core of the placental villi where they phagocytose the meconium, ie. the contents of the fetal intestine. They may also be involved in the removal of bacteria from the placenta (Carr, 1973), and Enders and King (1970) suggest they may sequester fetal serum proteins in the stroma of the chorionic villus. Enders and King also give a good review of the literature on the ultrastructure of these macrophages.

Free macrophages are those which exist outside the major lymphoreticular organs and are the macrophages of the connective tissue (sometimes called histiocytes), of serosal sacs and of inflammatory exudates, and finally the pulmonary alveolar macrophages. The first three of these share similar cytochemistry and ultrastructure.

The histiocyte is a flat, rounded, oval or branched cell with a kidney shaped nucleus, smaller and denser than that of the fibroblast, a cell which is otherwise morphologically very similar.
The histiocyte can be distinguished from the fibroblast, which also exists in the connective tissue, by its avid phagocytosis (Maximow and Bloom, 1931).

The cells used in work reported in this thesis are peritoneal macrophages which are conveniently obtained by washing out the peritoneal cavity with saline or culture medium, a technique first described by Cappell (1929). He detected the lysosomal enzyme acid phosphatase in these macrophages. Since Cappell, a wide range of enzymes have been described in macrophages. These have been reviewed by Braunsteiner and Schmalzl (1970) and include lipase, esterases of various types, β-galactosidase, lysozyme, cytochrome oxidase, peroxidase, naphthylamidase, acetyl glucosamidase, ATPase, 5-nucleotidase and a variety of oxidative enzymes including succinate, lactate and malate dehydrogenases and NADP diaphorase.

The enzyme content of macrophages varies with their physiological state and anatomical location. For instance, monocytes stimulated in vivo with attenuated tubercle bacilli (BCG), show an increase in such enzymes as β-glucuronidase, lysozyme and acid phosphatase (Myrvik et al., 1962). Intravenous injection of BCG induces an increase in acid phosphatase, β-glucuronidase and cathepsin in peritoneal macrophages, liver homogenates and in plasma (Saito and Suter, 1965). Stimulation of macrophages with particles also causes an increase in O2 uptake, glucose consumption, CO2 and lactic acid output, lipid turnover and phagocytic ability. Anatomical location is also important in determining the enzyme content of macrophages. For instance, many of those enzymes given
above have been shown to be present in larger quantities in alveolar cells than in peritoneal cells (Cohn and Wiener, 1963a,b).

Dumond (1969) gives a detailed account of the ultrastructure of peritoneal macrophages. The surface membrane has the usual three layered structure and is high in nucleoside phosphatase (North, 1966). Peritoneal macrophages have a prominent cell coat composed of acid mucosubstances, which may be important in phagocytosis, adhesion to glass surfaces and recognition of foreign material (Carr, 1973). The membrane systems of the macrophage are well developed (literature reviewed by Carr, 1973). This is important for production and secretion of enzymes and for the lysosome system. Lysosomes give the macrophage cytoplasm a granular appearance. The larger heterogeneous granules are secondary or mixed lysosomes and the smaller homogeneous granules are probably primary lysosomes (Carr, 1973). These granules contain either acid phosphatase or peroxidase. Peritoneal macrophages stimulated with Listeria monocytogenes have more lysosomes than normal cells and are more active in destroying bacteria (Blanden, 1968); this may be related to the higher content of hydrolytic enzymes.

Alveolar macrophages are larger than the peritoneal macrophages of the same species, with more lysosomes, but less endoplasmic reticulum and Golgi membranes (Carr, 1973).

It is only recently that the origin of the macrophage has been elucidated. Numerous investigations have been undertaken since the beginning of this century to determine whether macrophages arise
locally from connective tissue or endothelial cell precursors or from white blood cell precursors (reviewed by van Furth, 1970). It is now well established that ontogenically the macrophage has its origin in the yolk sac (Moore and Metcalf, 1970), and that adult mononuclear phagocytes are mainly derived from haematopoietic stem cells in the bone marrow (Cline et al., 1978), where a stem cell is defined as a cell with extensive capacity for self-renewal (production of identical daughter cells) and the ability to give rise to more differentiated cells of several lineages. The route from pluripotent stem cell in the bone marrow to mature macrophage in the tissue is schematically represented in Fig. 1, with its relationship to other cells of bone marrow origin shown.

Figure 1 (taken from Golde and Hocking (1982)). Schematic representation of haematopoiesis and lymphopoiesis. Monocytes and macrophages share a common pluripotent myeloid stem cell with other cells of bone marrow origin.
The stem cells early in this series cannot be identified morphologically \textit{in vivo}. When cultured in soft-gels infused with the glycoprotein colony stimulating factor (CSF), a product of mononuclear phagocytes and activated lymphocytes (Cline and Golde, 1979; Golde and Cline, 1972; Golde \textit{et al.}, 1972), myeloid stem cells are found to give rise to \textit{committed} precursor cells, with extensive capacity for replication. The precise stage at which a precursor cell becomes committed remains unresolved although in the case of the mononuclear phagocyte lineage, most of the evidence points to this decision being made at the level of the colony forming unit-granulocyte, monocyte (CFU-G,M) (Metcalf, 1977). The CFU-G,M cells give rise to colonies of differentiated monocytes and granulocytes (Golde and Cline, 1974).

Chimeric and \textit{in vitro} and \textit{in vivo} labelling studies have also been used to determine the origin and kinetics of macrophages (reviewed by van Furth, 1981). These studies confirm the bone marrow origin of these cells; only a small percentage were found to be derived by division in tissues. Those that do divide in the tissues were found to be recent arrivals from the bone marrow and not the resident population of macrophages.

The promonocyte is the first recognisable cell in the monocytic series. It has a generation time of 16 to 19 hours and a cell cycle time of approximately two days (Golde and Hocking, 1982). Promonocytes probably undergo three or more generations before emerging as mature monocytes. Within 24 hours of their formation.
monocytes leave the bone marrow for the circulation, where they remain for a relatively long period (mouse: half-life 17.4h; man: half-life 71.0h) compared with granulocytes (van Furth, 1978, 1980). They are not extensive proliferators so the pool of cells for the mononuclear phagocyte system is probably in the bone marrow.

Much of the work described above has been concerned with exudate macrophages, elicited in the peritoneal cavity by some inflammatory stimulus. Other studies however show that unlike exudate macrophages, up to 50% of the resident macrophages of the unperturbed cavity are derived from division in situ and the rest from blood monocytes. Under steady-state conditions, blood monocytes migrate into the peritoneal cavity to replace dying macrophages and undergo one division on average during a period of about 7 days. The renewal rate is 0.18%/hour, giving a half-life time of 16 days and a renewal time of 23 days. Inflammatory stimuli accelerate both the death of resident peritoneal macrophages and the efflux of blood monocytes; they also enhance the appearance of exudate macrophages. Once they have removed the irritant, these macrophages evolve into resident macrophages (Parwaresch and Wacker, 1984).

As already mentioned, macrophages are important in inflammation, which is primarily a response against bacterial or other microscopic invaders. The mechanisms involved in inflammatory reactions are awesomely complex and remain to be fully analysed. This complexity is mainly due to the large range of interrelated
components involved and because these relationships fluctuate with time and degree of injury.

Local injuries induce an immediate, nonspecific, acute inflammatory response, and a delayed and highly specific chronic response. Within seconds of tissue injury a variety of chemical "mediators" appear, which act primarily on the microcirculation causing exudation of fluid and white blood cells, mainly polymorphonuclear leucocytes (PMNs). This leakage lasts approximately 15 to 30 minutes and it occurs both within and around the traumatized area, depending on the extent of damage. At the direct site of injury, the leakage is most prolonged, and ends only when damaged vessels are repaired or plugged. These vascular events, although not fully understood, involve changes in flow, calibre and in permeability (Ryan and Majno, 1977).

Leucocytes, predominantly the neutrophil in early stages, migrate across blood vessel walls via the inter-endothelial junctions (Marchesi, 1961), into the tissues where they crawl about phagocytosing bacteria and cellular debris. Later, how much later depending on the antigenicity and nature of the invasion and the severity of the wound, mononuclear phagocytes become the predominant cell involved in these processes. By this time the reaction has generally evolved into chronic inflammation.

Fruhman (1964) and Hurley et al. (1966) measured the cells migrating into inflammatory exudates following the injection of phlogistic agents into the peritoneal and pleural cavities.
Fruhman found that with relatively mild irritants such as glycogen solution and serum, neutrophil accumulation reaches a peak at about 4 hours then rapidly decreases. It is only after the onset of this decline that the total number of mononuclear cells begins to rise, peaking at 18 to 24 hours. In their study, Hurley and his colleagues found that the height of the neutrophil peak was dependent on the inflammatory agent whereas that of the mononuclear phagocyte was not.

The most important task for mononuclear phagocytes and neutrophils in inflammatory sites is the ingestion and disposal of foreign or effete particulate material. This is an energy-requiring process; in monocytes and macrophages the energy is obtained by respiration and glycolysis (Steinman and Cohn, 1974). The literature on phagocytosis has been reviewed by Stossel (1974), and the process has been divided into 3 phases:-

i) attachment of particles to the cell surface.

ii) ingestion of particles by the cell and

iii) breakdown of particles within the cells.

In the first of these phases, recognition of the particle as foreign material is necessary. In the case of many foreign bodies, for instance microorganisms, this is assisted by opsonization, where the particle is coated by serum factors. So far two main groups of opsonic serum factors have been characterized. They are:-

1) heat-stable IgG₁ and IgG₃ specific antibodies directed against surface components of the particles; and
2) heat-labile opsonic fragments of C3 which can become firmly bound to particles when the complement system is triggered, as it is during inflammation (Steinman and Cohn, 1974).

Once the phagocyte has recognised the foreign body, the two come into very close contact and engulfment of the foreign material ensues. The cell extends small cellular expansions or pseudopodia which become closely applied to the surface of the attached particle; these processes join together and extend around the particle. Once completely enclosed inside the phagocyte, the resulting phagosome is drawn into the cell (Hirsch, 1974). Ingestion is dependent on the presence of divalent cations, Ca$^{2+}$ and Mg$^{2+}$ (Stossel, 1973), is energy-requiring (Karnovsky, 1962) and is inhibited by cytochalasin B, which disrupts microfilaments (Allison, 1973). Hirsch (1974) used electron microscopy and cinemicrophotography to show that cytoplasmic granules (lysosomes) converge and fuse with the forming phagosome whereupon they discharge their complex enzyme arsenal into the vacuolar lumen around the particle. Reactions within the lysosome result in the death and breakdown of foreign bodies. Antimicrobial activity, for instance, has been attributed to an array of factors including acid pH, cationic proteins, lysozyme, lactoferrin, superoxide anion, hydrogen peroxide and the hydrogen peroxide-myeloperoxidase-halide system (Ryan and Majno, 1977).

Once extensive endocytosis has occurred, the phagocyte is unable to ingest particles. This suggests that receptor sites are lost from the surface by internalisation within phagocytic vacuoles.
Mononuclear phagocytes (not neutrophils) regain their endocytic capacity, probably because they can synthesize new surface receptors (Werb and Cohn, 1972).

The problem still remains as to how phagocytes accumulate at sites of inflammation. One mechanism that could account for this local accumulation of wandering cells, is chemotaxis, the unidirectional migration of cells along a concentration gradient of a chemoattractant. During tissue damage, chemotactic factors are released. When damaged tissue comes into contact with serum complement substrate(s) a reaction follows which culminates in the production of endogenous chemotactic factors; these include C3 and C5 fragments, which are components of complement (reviewed by Ward, 1974). Listed in Table 1 are candidates potentially responsible for mononuclear phagocyte chemotaxis during inflammation.

Table 1. Mediators Potentially Responsible For Chemotaxis Of Mononuclear Phagocytes.

<table>
<thead>
<tr>
<th>CHEMOTACTIC FACTOR</th>
<th>References</th>
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<tbody>
<tr>
<td>Bacterial products</td>
<td>Ward (1974)</td>
</tr>
<tr>
<td>Complement system by-products</td>
<td></td>
</tr>
<tr>
<td>C5 fragments</td>
<td>Hausman et al. (1972)</td>
</tr>
<tr>
<td>C3 fragments</td>
<td>Ward (1974)</td>
</tr>
<tr>
<td>A normal serum factor</td>
<td>Hausman et al. (1972)</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>Gallin and Kaplan (1974)</td>
</tr>
<tr>
<td>Plasminogen activator</td>
<td>Gallin and Kaplan (1974)</td>
</tr>
<tr>
<td>M.tuberculosis-treated serum</td>
<td>Symon et al. (1972)</td>
</tr>
<tr>
<td>Neutrophil lysosomal cationic protein</td>
<td>Ward (1968)</td>
</tr>
<tr>
<td>Lymphocyte-derived factor</td>
<td>Ward (1971)</td>
</tr>
<tr>
<td>Transfer factor</td>
<td>Kirkpatrick and Gallin (1975)</td>
</tr>
</tbody>
</table>

(derived from Ryan and Majno, 1977).

Injured cells also release serum-independent agents shown to be
chemotactic to leucocytes using the Boyden system, which consists of a chamber with two compartments separated by a horizontal filter membrane. Leucocytes placed in the upper chamber crawl through the pores of the filter when a chemotactic solution is placed in the lower compartment and chemotactic activity of the fluid can be evaluated by counting the number of cells on the lower side of the filter (Boyden, 1962). This system has been used to demonstrate the chemotactic activity of many substances. Recently chemotactic activity has been associated with several components of the extracellular matrix including fibronectin (Albino et al., 1983; Gauss-Müller et al., 1980; Postlethwaite et al., 1981), collagen (Postlethwaite, 1978), peptides of insoluble elastin (Hunninghake et al., 1981; Senior et al., 1980, 1982), tropoelastin (Senior et al., 1982) and laminin (Terranova et al., 1983). The sites of chemotactic activity have been partially localised on fibronectin (Albini et al., 1983; Seppä et al., 1981) and collagen (Postlethwaite, 1983). Cellular recognition of these molecules may involve very short amino acid sequences (Pierschbacher et al., 1983). A small hydrophobic repeating peptide in elastin has been found to be chemotactic for fibroblasts and monocytes (Senior et al., 1984).

Chemoreceptors for several chemotactic chemicals have been identified on bacterial cell surfaces. These have been shown to detect the gradient by a temporal mechanism, where the cell samples the milieu at separate time intervals and compares the concentrations of the attractant (Adler, 1969). Zigman (1974), however, found that leucocytes use a spatial mechanism to detect
chemical gradients, whereby the cell compares the concentration of the attractant at two or more locations on its surface at the same time.

Once attracted to inflammatory sites, migration of macrophages is inhibited by MIF (macrophage Migration Inhibition Factor), a glycoprotein derived from appropriately stimulated lymphocytes. MIF belongs to a group of factors, called lymphokines, produced by these cells. Others include chemotactic factors, lymphotoxin, skin reactive factors and mitogenic factors (Dumonde et al., 1969). The biology of these lymphokines (especially MIF) will be discussed more fully in Chapter 1.

The macrophage is therefore a normal adult cell type, which is motile, unlike most adult cells, and this motility can be precisely controlled. This would therefore seem to be an ideal model system with which to study the relationship between adhesion and motility which may prove useful in understanding and controlling the property of metastasis in malignant cells. Any comparisons, however, between normal and malignant cells must be approached with caution since malignant cells exhibit a range of properties which distinguish them from their normal counterparts.

Tumour metastasis is a complex phenomenon probably requiring numerous conditions for its successful outcome (Fidler et al., 1978; Roos and Dingemans, 1979; Weiss, 1976). One of these requirements might be the ability of tumour cells to degrade the surrounding connective tissue barriers of the host through the
action of proteolytic enzymes (Jones and DeClerck, 1980; Sträuli et al., 1980). A more fundamental requirement might be a reduction in the adhesiveness of the malignant cell (Ruoslahi, 1984).

Cells lay down and are surrounded by extracellular matrix which consists of three main types of macromolecules: collagens, proteoglycans and glycoproteins (Cunningham and Fredericksen, 1982). Fibronectin is a major glycoprotein of connective tissue (Ruoslahi et al., 1981) and has been found to be important in making the extracellular matrix adhesive to cells.

Several reviews have been written recently describing the structure and biological activities of fibronectin (Ruoslahi et al., 1981; Mosher, 1980; Hynes and Yamada, 1982). It exists in two forms, as an insoluble protein in tissues and as a soluble protein in body fluids. Fibronectin has various binding domains within its structure. One of these causes disulphide bonding, which may be important for insolubilising matrix fibronectin (Keski-Oja, 1976; Wagner and Hynes, 1980), another binds cells to the matrix. Several binding domains are important for anchoring fibronectin to the extracellular matrix. The decrease in adhesiveness of transformed cells is unlikely to be attributable to a difference in structure of fibronectin which malignant cells remain capable of producing after transformation. The only difference between fibronectins produced by normal and transformed cells is in the degree of phosphorylation. (Ali and Hunter, 1981). This may be important in inactivation of the cell attachment site.
Embryonic neural crest cell migration might provide clues to this problem. These cells are highly motile, migrating ventrally from their origin to give rise to sensory and sympathetic neurons, glial cells, and chromaffin cells in the gut area. If other cells, or indeed protein-coated beads, are injected into this pathway it is found that their ability to translocate along the pathway depends on the presence or absence of fibronectin deposited around the cell or bead. When present, fibronectin halts migration (Erickson et al., 1980; Bronner-Fraser, 1982).

Malignantly transformed cells resemble neural crest cells in that generally they do not deposit fibronectin in a matrix around themselves. Nevertheless, also like neural crest cells, transformed cells adhere to insoluble fibronectin matrices elaborated by other cells (Kramer et al., 1980; Vlodavsk et al., 1982). There is not, however, a straightforward correlation between lack of cell surface fibronectin and tumourigenicity since many malignant cell lines retain a fibronectin matrix (Der and Standbridge, 1978; Neri et al., 1981). Ruoslahti (1984) has reviewed the situation and found that the degree of expression of matrix fibronectin varies with different malignant cells. He speculates that where fibronectin is absent, the metastatic capacity of cells is affected.

If fibronectin is added to oncogenically transformed cells originally deficient in fibronectin, normal cell shape, adhesiveness, cell surface morphology and actin microfilament organisation can be restored (Yamada et al., 1976). Another example
illustrating the importance of components of the extracellular matrix (ECM) in the regulation of cell morphology, cytoskeletal organisation, and even cell type is given by Greenburg and Hay (1982) who found that when fully differentiated epithelial cells were cultured within a gel of purified collagen, the cells underwent a fundamental alteration in phenotype from epithelial to mesenchymal. The cells migrated away from epithelial sheets to form isolated bipolar cells that could invade the collagen gel and display altered biosynthetic activity. It is now well established that extracellular macromolecules affect many processes including cell differentiation, motility and growth (reviewed by Grinnell, 1978; Kleinman et al., 1981).

Many of the molecules involved in cell-extracellular material interactions have now been purified, and structural and functional domains important for these interactions have been isolated. The most intensively studied of these proteins are fibronectin, already discussed, and laminin. Laminin, also an adhesive glycoprotein, differs from fibronectin in structure and in its specificity for type IV collagen and epithelial cells. Laminin consists of two subunits linked by disulphide bonds and, like fibronectin, it functions by the combined action of independent cell-, collagen-, and heparin-binding domains on each molecule. Other adhesion-promoting molecules include chondronectin, which mediates adhesion of chondrocytes to type II collagen, and vitronectin, only recently characterised (Hayman et al., 1983). Yamada (1983) comprehensively reviews the mechanisms by which these extracellular molecules interact with cells.
Interference reflection microscopy (IRM) of living cells has been used to identify different types of cell-substrate contact sites; focal contacts, which appear as punctate dark areas, close contacts which appear as broader grey areas and extracellular matrix contacts (Abercrombie and Dunn, 1975; Bereiter-Hahn et al., 1979; Izzard and Lochner, 1976, 1980; Curtis, 1964). Many of the molecular species associated with these sites have been identified using immunofluorescent microscopy (Avnur and Geiger, 1981; Birchmeier et al., 1980; Couchman and Rees, 1979; Fox et al., 1980; Geiger, 1979; Grinnell, 1980; Hynes et al., 1981; Singer and Paradiso, 1981; Wehland et al., 1979), and electron microscopy has been used to investigate the ultrastructural morphology of contact sites (Abercrombie et al., 1971; Bereiter-Hahn et al., 1979; Brunk et al., 1971; Heath and Dunn, 1978; Heaysman and Pegrum, 1973a,b; Revel and Wolken, 1973).

A distance of only 10-20nm separates the ventral cell surface from the substratum in focal contact sites, making these the narrowest (Abercrombie et al., 1971; Bereiter-Hahn et al., 1979; Brunk et al., 1971), and incidentally also the strongest of the cell-substratum adhesion sites (Abercrombie and Dunn, 1975; Chen, 1981; Harris, 1973; Izzard and Lochner, 1980). Focal contact sites are often found at the perimeter of cells, and are associated with microfilaments (Abercrombie et al., 1971; Heath and Dunn, 1978). Close contacts are broader areas with larger gaps (30-50nm) between the cell surface and the substratum (Bereiter-Hahn et al., 1979; Couchman and Rees, 1979; Izzard and Lochner, 1976, 1980). They are
often found surrounding close contacts and are also associated with microfilaments (Couchman and Rees, 1979; Heath and Dunn, 1978). Extracellular matrix contacts (ECM) are sites where strands of ECM, including fibronectin, connect the ventral cell surface to the substratum, separated by up to 100nm (Furcht et al., 1978). Using double immunoelectron-microscopic labelling studies on cross-sections through fibroblastic contact sites, Chen and Singer (1982) confirmed previous observations about the morphology of these sites and found they could characterise them also on the basis of their labelling patterns for vinculin, α-actinin and fibronectin. From these, they proposed a tentative model for the molecular ultrastructure of these contact sites.

One area of cell adhesion so far little studied because of the technical problems involved, is that of quantifying the traction forces exerted by cells on their substrate. Harris et al. (1980) describe an inventive approach to this problem whereby cells are cultured on thin layers of cross-linked silicone rubber which is very elastic and weak enough to be visibly distorted by the small traction forces by which cells crawl. This method was used in the work reported here to determine whether the addition of the lymphokine MIF (migration inhibition factor) causes macrophages to exert more traction on their substrate. The outcome of such an experiment might lead to an understanding of how MIF affects macrophage motility. Another method used to quantify cellular traction is described by Allen and Schor (1983). They incubated given numbers of human fibroblasts for a certain length of time in 3-D floating collagen gels to study the traction forces exerted by
these cells. In this thesis, this approach is linked to scanning electron microscope (SEM) studies on how the interaction of macrophages with collagen substrates is affected by the addition of MIF. The effect of MIF on the cytoskeleton was also investigated using immunochemistry and transmission electron microscopy (TEM).

Since macrophages from different tissues, in varying physiological states or stages of maturation, show functional and structural differences, they have been described as a heterogenous group of cells. This concept has been extensively discussed in a group of reports from a conference on the subject (Förster and Landy, 1981). There is evidence showing that within any population, macrophages vary greatly in, for example, size and density (Morahan et al., 1982), and in phagocytic capacity (Peters, 1981; Mörland, 1981). Less obvious is the heterogeneity in surface receptors and antigens. As already mentioned, macrophages are motile cells able to detect and migrate along gradients of chemoattractants; Snyderman et al., (1981) found that inflammation-elicited macrophages are more responsive to chemotactic factors than are resident cells. Zolla-Pazner et al., (1981) showed that macrophages of the spleen and bone marrow can be distinguished from those in the peritoneum or blood of normal mice, on the basis of their ability to bind and ingest uncoated sheep erythrocytes or erythrocytes coated with rabbit anti-E IgM. This receptor was also used to distinguish previously undescribed subpopulations of macrophages or macrophages at various stages of differentiation.

When antibodies bind to antigens, present for instance on the
surface of bacteria, the Fc region of antibodies are exposed and available for complexing with Fc receptors on the surface of macrophages. This process initiates phagocytosis by macrophages of bacteria, or other items recognised by the immune system. Ia antigens, determined by the immune response or I region of the major histocompatibility complex, and Fc receptors, both show heterogeneity in their expression on the surface of macrophages (Vogel and Rosenstreich, 1979; Lonai and Steinman, 1977).

Macrophages are important in the regulation of many functions largely because of the range of products they secrete. Not all macrophages, however, secrete the same range or quantity of products (Walker, 1976). This is described, for instance, with respect to the production of plasminogen activator (Unkeless et al., 1974), and Neuman and Sorg (1977) have shown that when exposed to lymphokines, various macrophage populations differ in the amount of interferon they produce.

A study by Bärlin et al. (1981) reports that macrophages which have been induced by different immunostimulants to act cytotoxically against tumour cells express important differences in other functions which can only be attributed to the different modes of stimulation used. The authors speculate that the population of macrophages used was divided into subpopulations of cells and that different macrophage functions are expressed by these different subpopulations. However, as discussed above, all macrophages have been shown to have a common origin in the bone marrow. Perhaps if the question of heterogeneity were regarded from the standpoint of
cell commitment, the picture might not be so complex. Environmental stimuli to which macrophages are subjected seem to play a pivotal role in determining the functional capabilities of the cell. Perhaps it is these stimuli, rather than some inherent differences in the cells themselves, which leads to the heterogeneity observed. This being the case, once presented with certain stimuli, do macrophages become committed along a particular pathway or do they remain capable of responding to other stimuli? Also, do macrophages show different levels of commitment and respond differently to different stimuli at different stages of their development or life span?

The specific question asked here is whether one macrophage can be activated or primed for only one or several functions at any one time, and once activated for a particular function is the cell then capable of performing others? Also, is the type or degree of activation determined by the nature, number or sequence of activating signals? As will be discussed later this whole question might be important in the pathology of malignant diseases and in the possible use of macrophages in the treatment of these diseases.

Their role in the pathology of malignant, inflammatory and many other diseases has lead to the acknowledgement of mononuclear phagocytes as secretory cells. A list of secretory products of these cells has been compiled by Nathan et al. (1980) and is given in Table 2 below. The aim of presenting the information in this table is to convey the extent of involvement macrophages have in diverse pathologic events and homeostatic mechanisms.
flavour of which can be presented here. The regulation processes involved in the production and release of these products remains far from clear, particularly in vivo.

Table 2. Secretory Products Of Mononuclear Phagocytes

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Bioactive Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>Arachidonate metabolites</td>
</tr>
<tr>
<td>Neutral proteases</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>Plasminogen activator</td>
<td>6-keto-Prostaglandin F₁α</td>
</tr>
<tr>
<td>Collagense</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>Elastase</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>Angiotensin-convertase</td>
<td>Hydroxy-eicosatetraenoic acids</td>
</tr>
<tr>
<td>Acid hydrolases</td>
<td>Platelet-activating factors</td>
</tr>
<tr>
<td>Prostacyclin</td>
<td>Factor Chemotactic For Neutrophils</td>
</tr>
<tr>
<td>(deoxy)Ribonucleases</td>
<td>Factors Regulating Synthesis of</td>
</tr>
<tr>
<td>Proteases</td>
<td>Proteins by Other Cells</td>
</tr>
<tr>
<td>Lipases</td>
<td>Hepatocytes</td>
</tr>
<tr>
<td>Phosphatases</td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td>Glycosidases</td>
<td>Haptoglobin</td>
</tr>
<tr>
<td>Sulphatases</td>
<td>Synovial-lining cells</td>
</tr>
<tr>
<td>Arginase</td>
<td>Collagenase</td>
</tr>
<tr>
<td>Complement Components</td>
<td>Factors Promoting Replication Of</td>
</tr>
<tr>
<td>C₁,C₄,C₂,C₃,C₅</td>
<td>Lymphocytes (LAF)</td>
</tr>
<tr>
<td>Factor B</td>
<td>Myeloid precursors (CSF)</td>
</tr>
<tr>
<td>Factor D</td>
<td>Erythroid precursors</td>
</tr>
<tr>
<td>Properdin</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>C₃b inactivator</td>
<td>Microvasculature</td>
</tr>
<tr>
<td>βIH</td>
<td>Factors Inhibiting Replication Of</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Enzyme Inhibitors</td>
<td>Tumour cells</td>
</tr>
<tr>
<td>Plasmin inhibitors</td>
<td>Viruses (interferon)</td>
</tr>
<tr>
<td>α₂-Macroglobulin</td>
<td>Listeria monocytogenes</td>
</tr>
<tr>
<td>Binding Proteins</td>
<td>Reactive Metabolites of Oxygen</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Superoxide</td>
</tr>
<tr>
<td>Transcobalamin II</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>Nucleosides and Metabolites</td>
<td>Singlet oxygen(?)</td>
</tr>
<tr>
<td>Thymidine</td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td></td>
</tr>
<tr>
<td>Endogenous Pyrogens</td>
<td></td>
</tr>
</tbody>
</table>

As already pointed out there is considerable variation in the repertoire of secretory products released by mononuclear phagocyte populations obtained from different sources and environments.
These variations may reflect the important functional differences which underlie the distinct differences in the roles played by mononuclear phagocytes in successive stages of host defence responses and inflammatory processes. Macrophages exert both pro-inflammatory and anti-inflammatory effects. They interact with other cell types, such as lymphocytes, granulocytes and blood platelets, and with kinin, complement and coagulation cascades.

The bulk of macrophage secretions are produced during inflammatory responses (reviewed by Nathan et al., 1980; Davies and Bonney, 1979; Davies and Allison, 1976). Of these, lysozyme is secreted in the largest quantity. Other enzymes involved include the acid hydrolases. These are present in mononuclear phagocyte lysosomes: their secretion is constitutive and triggered by particulate stimuli such as immune complexes, bacterial cell walls, mineral particles and soluble stimuli such as C3b, dextran sulphates and lymphocyte activation products (reviewed by Page et al., 1978). Lysosomal acid hydrolases are intracellular digestive enzymes but also degrade extracellular components of connective tissue including collagen and basement membrane. They may influence their own release by hydrolysing complement, kinins and immunoglobulin (Nathan et al., 1980).

Neutral proteinases are secreted by certain populations of mononuclear phagocytes elicited in response to agents such as endotoxin, foreign serum proteins, immune complex microbes or other ingestible particles. Plasminogen activator is one of these neutral proteases, it catalyses the formation of plasmin from
plasminogen. Plasmin is involved in at least three enzyme cascades. It induces the release of clotting factors through fibrin degradation, activates complement components C1 and C3, and is involved in the formation of kallikrein from prekallikrein. Other neutral proteases include collagenase and elastase, which degrade components of vessel walls and perivascular tissue. Several of these products are chemotactic for macrophages and therefore lead to more macrophage accumulation at sites of inflammation. As can be seen in Table 2, macrophages also produce enzyme inhibitors involved in the control of these processes. For example, macrophages produce enzymes which inhibit the formation of plasmin. Controlling feedback loops is also important in the interaction between macrophages and the complement system. Macrophages secrete, bind and degrade complement components which in turn both stimulate and suppress macrophage migration, endocytosis and secretory behaviour.

Although macrophages cause tissue degradation by releasing hydrolytic enzymes, for instance during chronic inflammation, they are also involved in rebuilding tissue. Macrophages enhance both the proliferation of, and the secretion of collagen by, fibroblasts (Wahl et al., 1979; Diegelman et al., 1982). From this springs the essential role for macrophages in wound repair (Leibovich and Ross, 1975b, 1976), and in growth and development (van Furth, 1980). However they also inhibit the proliferation of rapidly-dividing fibroblasts (Keller, 1974a), and stimulate the secretion of collagenases by rheumatoid synovial fibroblasts. Macrophages have been shown to modulate the behaviour of other cell types besides
fibroblasts. For instance Evéquoz et al. (1984) have shown that chondrocytes cultured in medium conditioned by macrophages stop secreting plasminogen activator, which they do under normal conditions, and switch to the secretion of large quantities of collagenases and E-type prostaglandins.

Mononuclear phagocytes also release mediators which stimulate the proliferation of bone marrow stem cells. These will be discussed more fully later in this thesis in relation to growing clones of these cells in vitro, and is also reviewed by Page et al. (1978). Polverini et al. (1977) show that under some conditions macrophages secrete a substance which stimulates angiogenesis.

The proliferation of another cell type, the lymphocyte, is also regulated by a secretion product of macrophages. Gery et al. (1972) found that following stimulation with endotoxin, human monocytes secrete lymphocyte-activating factor (LAF). LAF is now known to have a wide range of biological effects on lymphocytes ranging from the promotion of secretion of mediators by T-cells, to the stimulation of B-cell differentiation. These processes are reviewed by Unanue (1981) who also discusses the regulation of LAF production through events involved in interactions of macrophages with antigens and with immune T-cells.

As accessory cells, macrophages are essential in the induction and expression of the immune response since T-cells will only respond to antigens presented at the surface of macrophages. Effective presentation induces T-cell proliferation to antigens (Kammer and
Two features are required for effective T-cell - macrophage collaboration:

i) the proliferating T-cell and the macrophage must share part of the I region of the major histocompatibility complex (MHC) and so be genetically compatible; and

ii) the macrophage must express the I-region-associated antigen (Ia) on its surface (Rosenthal and Shevach, 1973).

The interaction between the Ia products of macrophages and the antigen is important in T-cell - macrophage stimulation (Thomas and Shevach, 1976).

Ia-bearing cells are responsible for most macrophage-lymphocyte interactions, but only a certain percentage of phagocytes bear Ia molecules on their membrane, and it is an unstable phenotypic character. Ia-negative and Ia-positive macrophage subpopulations may be interconvertible depending on in vitro culture conditions or in vivo infection. Oppenheim et al., (1981) found that a mediator(s) produced by Concanavalin A-stimulated spleen cells induce both the phenotypic and functional conversion of Ia-negative to Ia-positive macrophages by murine macrophages in vitro. This suggests that nonspecific mediators can enhance both genetically restricted and antigen-specific immunological responses.

The antigen presentation function of macrophages is also important in antibody formation, in addition, macrophages participate in T-cell - B-cell collaboration during antibody production (reviewed by Basten and Mitchell, 1976).
As well as inducing and enhancing immune responses, macrophages are also involved in suppressing these responses in self regulation systems. For instance, when macrophages present antigens to T-cells, they induce the T-cells to secrete lymphokines. These, in turn, cause macrophages to release prostaglandins which inhibit proliferation as well as T-cell secretion of mediators. Macrophages may also be involved in the regulation of immune processes by suppressor T-cells (Ptak et al., 1978).

Macrophages are more commonly associated with their role in cell-mediated immune responses. These are antigen-specific responses of T-cells that do not involve antibody secretion. On antigen stimulation T-cells divide and differentiate into activated effector cells responsible for at least three different immune reactions:

i) they specifically kill foreign or virus-infected vertebrate cells (cytotoxic T-cells);
ii) they help specific T- or B-lymphocytes respond to antigen and can activate some non-lymphocyte cells, such as macrophages (helper T-cells); and
iii) they suppress the responses of specific T- or B-lymphocytes (suppressor T cells).

Much of our understanding of the mechanisms which underlie the cell-mediated immune response comes from work done in the 1930's and 40's on tuberculosis and other infectious diseases. It was observed that in tuberculosis, the bacterium *Tubercle bacillus* becomes an intracellular parasite of macrophages which accumulate at infective foci forming tubercles. On secondary infection these
tubercles form at a greatly accelerated rate and the bacteria within the tubercles are eliminated much more quickly than during the primary infection. These events are not accompanied by a humoral antibody response. It was Lurie (1942) who first provided conclusive evidence that macrophages from vaccinated animals and macrophages infected with tubercle bacilli in vitro display enhanced capacity for inhibiting the intracellular growth of bacilli in contrast to normal macrophages which soon become overwhelmed by unrestricted multiplication of intracellular bacteria. Macrophages are non-specific in their response. If harvested from animals immunised in response to infection with one bacterium, macrophages display increased microbicidal activity to a range of unrelated bacteria (Pullinger, 1936; Nyka, 1956; Howard et al., 1959). Mackaness (1969) showed that underlying this non-specific macrophage response there is a specific immune response. In the presence of specific antigen, specifically sensitised T-cells elaborate soluble factors or lymphokines which mediate the adaptive changes in macrophages, allowing them to express acquired cell-mediated immunity. Mackaness introduced the term "activated macrophages" to describe the changes that enable macrophages to do this.

Pronounced ruffling of the plasma membrane distinguishes these activated macrophages as do their increased capacity for phagocytosis and for adhering to and spreading on a substrate (David and Remold, 1976). The increased rate of secretion, and the production of new products by activated macrophages have already been described, along with the involvement of macrophages in many
processes besides cell-mediated immunity. The process of activation or enhanced function has been associated with most of these processes but this has usually involved ignoring the original definition of the term. It has not been shown that increased microbicidal activity is generated concurrently with an increase in any of these other activities.

Tumouricidal function has become regarded by some as the ultimate indicator of macrophage activation, but there is evidence both for and against the required correlation with increased microbicidal activity. Wing et al. (1977) reported that it need not follow that characterisation by one functional criterion implies the possession of other functional characteristics of activated macrophages. They introduced the concept that differences in functional capacity may be determined by the method used to activate macrophages. The presence of one form of macrophage activation in the absence of others may indicate that activation occurs in steps, the number of steps determining which functions the macrophages will perform. This theory was substantiated by Gordon et al. (1974) who found that the secretion of plasminogen activator by macrophages might depend on a number of successional metabolic changes.

It is now evident that in vivo, the functional expression of macrophage activation is very complex. Lymphokines attract macrophages to sites of inflammation, prevent them from leaving and activate them to enhanced functional levels. As already pointed out, macrophages are involved in further recruitment to inflammatory sites, in elevating the total response and in
anti-inflammatory mechanisms. Ruco and Meltzer (1978a) were the first to point out that it seems to be the blood monocytes which migrate into the inflammatory site that are susceptible to activation, to the exclusion of resident macrophages. This implies that macrophages are not universally susceptible to activation and that it may only be possible to activate macrophages under defined conditions.

The definition of "activation" has become obscured in the literature and should be used with caution. There is a need for uniformity of nomenclature of cells in the mononuclear phagocyte series and their varying physiological states. The nomenclature adopted in this thesis is that recommended by a committee of the Reticuloendothelial Society (Morahan, 1980):

Resident macrophages are those macrophages obtained from a particular anatomic site without experimental elicitation and in the absence of any endogenous known inflammatory (phlogogenic) agent. The term "elicited macrophage" is reserved for the experimental procedure that results in accumulation of macrophages at a particular anatomic site. The use of this term does not necessarily imply a functional alteration in the cells. The committee recommended that the term "activated" be avoided and instead each system be described in detail using the statement "Macrophages from......were obtained by.......for......function".

When using the term "activated" in this thesis, it is meant in a general sense, implying that the macrophages referred to show enhanced levels of some functions perhaps not fully defined.
It now appears that the biochemical, morphological and functional state of macrophages is dynamic and depends on environmental chemical signals (David and Remold, 1979; Hibbs et al., 1977). Wing et al. (1977) however propose that the different effector functions observed are due to the presence of subpopulations of effector macrophages with different functional capacities. The concept of macrophage heterogeneity has already been discussed.

There is mounting evidence to suggest that activation is a multistage process. Hibbs et al. (1977) and Ruco and Meltzer (1978a) found that at least two signals are required to induce tumouricidal activity in macrophages. Ruco and Meltzer (1978a) showed that macrophages from in vivo immune reactions, induced for example by BCG infection, could develop full cytotoxic capability following in vitro exposure to LPS (endotoxin) or lymphokines, whereas those cells from irritant-induced exudates showed considerably less tumouricidal activity. If, however, these irritant-induced cells were exposed to both LPS and lymphokines, tumouricidal activity was greatly enhanced. They found that LPS and lymphokines were synergistic in their action on macrophage cytotoxicity. This depends, however, on the method of treatment. LPS lost its effect if presented before the lymphokine and if there was a long interval between presenting treatments. They concluded that the functional state of macrophages is dependent on a sequence of short-lived intermediary reactions.

There are two schools of thought regarding the ultimate consequence...
of macrophage activation. Ruco and Meltzer (1978b) assume that tumouricidal activity is the end product of a uni-directional flow of a series of intermediary reactions. Here it is proposed that depending on the form of stimulation macrophages become committed along separate pathways which ultimately activate the cells either for tumouricidal, microbicidal or other functions. In view of the vast array of homeostatic functions macrophages have been found to be involved in, this seems a more tenable line to follow. Evidence supporting this theory is sought in this thesis.

Schultz (1982) demonstrated that antibody-purified L-cell interferon (IFN) rendered macrophages nonspecifically tumouricidal and postulated a central role for IFN in potentiation of macrophage cytotoxic function. He found that macrophages already "primed" with lymphokine responded to IFN or LPS by becoming cytotoxic. It has since been found that there is functional identity between murine IFNγ and the lymphokine MAF (Schultz and Kleinschmidt, 1983). The situation is therefore in need of clarification. One aim of this thesis is to define the role played by the lymphokine MAF in phagocytic and tumouricidal activities. The methods used by macrophages to kill malignantly transformed cells or slow down their proliferation rates is far from clear and some of the hypotheses being tested will be discussed later. Macrophages do not phagocytose neoplastic cells, but close contact with the target cells seems to be necessary. Macrophages may secrete cytolytic factors or factors causing lysis of the target cell.

The degree and kind of activation of macrophages determine the
effector capacities of these cells. It was the intention here to investigate the effect of functional state and effector:target cell ratio on the ability of macrophages to kill, or affect the proliferation of, syngeneic transformed cells. The results suggest that macrophages may be important in host tumour resistance. If suitably primed and present at the appropriate effector:target cell ratio, macrophages may also become important in the clinical treatment of metastasis in the future.
CHAPTER 1
Occurrence and Specificity of the Lymphokine MIF (Migration Inhibition Factor) in Different Vertebrate Groups

Introduction

The delayed hypersensitivity reaction is the simplest and best studied manifestation of cell-mediated immunity. This reaction is generally recognised by a slowly evolving inflammatory lesion for instance at the site of antigen injection in suitably sensitised individuals; it usually takes between 24 and 48 hours to reach maximal size and intensity. Macroscopically a delayed hypersensitivity reaction, following cutaneous infection, appears as a raised, erythematous, indurated lump. Microscopically the reaction is characterised by a mononuclear cell infiltration. These non-specific inflammatory cells form the bulk of cells at sites of delayed hypersensitivity reactions, initiated by only a small number of specifically sensitised lymphocytes. The question of how mononuclear cell infiltrates come to be at the site of an inflammatory lesion opened the door to a new area of immunology, that of the biology of the lymphokines.

The first lymphokine to be described was MIF. Its discovery can be traced back to the work of Rich and Lewis in 1932. They studied the migration of cells from tissue explants and found that antigen inhibited the migration of neutrophils and macrophages from tissue explants taken from sensitised animals. Thirty years later, George and Vaughan (1962) introduced a modification of the explant technique that utilised peritoneal exudate cells migrating out of capillary tubes, forming a fanlike shape, the border of which
consists of the leading edge of migrating cells. Bloom and Bennett (1966) showed that lymphocytes alone do not migrate out of the capillary tubes and that this is indeed a method which measures migration of macrophages. The method is supposed to assay random migration, but the design of the system is such that it in fact measures migration in one direction only, from the open end of the capillary tube towards the chamber perimeter. The advantages of the test are simplicity and rapidity of performance, but it can only be used as a test for rough quantitation and not for the detection of fine differences in motility. The results of the capillary tube assay are not a direct expression of the motility of individual cells since cell aggregation, surface adhesion, cell shape, and passive movement contribute to its outcome.

Using the capillary tube assay Bloom and Bennett (1966) and independently David (1966) demonstrated that the inhibition of migration of normal macrophages from such tubes was due to the release of a soluble material from sensitised lymphocytes following antigen stimulation. This soluble factor was called MIF.

The capillary tube assay is still the most commonly used method for assaying the motility of phagocytic cells in vitro, and is the assay used in experiments described in this thesis. Other methods of assay have been reviewed by Pick (1979) and include:

1. **Cell migration under an agarose layer** - used to measure PMN radial migration from a well punched in agarose.
2. **Migration from an agarose droplet** - for small numbers of macrophages. Cells are incorporated in an agarose droplet and
immersed in a culture medium, from which they migrate out on to the dish surface.

3. Migration in fibrin - registers the ability of PMNs to move within a solid fibrin matrix.

4. Migration of cells into a "notch" made in a monolayer - measurement of cells migrating from the edge of a previously established monolayer.

5. Assessment of random migration in the Boyden chamber.

6. Microcinematography - a qualitative method whereby the movement of isolated macrophages on an agar base is recorded by time-lapse cinematography.

Two years after the discovery of MIF, a second lymphocytic mediator, lymphotoxin (LT) was discovered by Granger and Williams (1968), and by Ruddle and Waksman (1968). Lymphotoxins cause growth inhibition and non-specific protracted lysis of selected cell types in vitro. Following the discovery of several lymphocyte-derived factors Dumonde et al., (1969) suggested the term "lymphokines" for such mediators. These do not include all the substances produced by stimulated lymphocytes; for instance antibodies are not classified as lymphokines (Meyers et al., 1972).

Lymphocytes do not hold a monopoly over the production and release of biologically active soluble factors involved in immune processes. Cohen et al., (1974), used the term "cytokines" to describe mediators with physicochemical and biological properties similar to or identical with conventional lymphokines but produced by both lymphoid and non-lymphoid cells when appropriately
stimulated. A group of cytokines are produced by mononuclear phagocytes and have been named "monokines".

Since Dumonde coined the term "lymphokines" it has become increasingly difficult to define, mainly because of the now vast array of information concerning them and because of the lack of strict biochemical criteria to characterise most of the factors called lymphokines. It remains a useful term, though if used in its broad literal sense indicating the preponderant cell of origin and that the factors they produce set in motion certain processes in their respective target cells.

Lymphokines are produced by both T- and B-lymphocytes following specific and nonspecific stimulation. The synthesis and release of lymphokines does not occur until the appropriate agents interact with membrane receptors on the lymphocyte surface. All lymphokines are proteinaceous, mostly glycoproteins with a molecular weight larger than 10,000. They have effects on a wide variety of cells and some cell-free substances (reviewed by Pick et al., 1979).

Lymphokines affecting almost all cell types involved in inflammation have been reported. At present, at least 50 lymphokines have been described and a total of up to 100 named cytokines. A list of these has been compiled by Waksman (1979). Those considered of most significance here are given in Table 3 below.
Table 3: List of Cytokines Classified According To Role.

**Helper Factors**
- LAF (lymphocyte activating factor)
- Transferrin
- Complement components

**Suppressor Factors**
- IDS (Inhibitor of DNA synthesis)
- MIFIF (MIF inhibition factor)
- SIRS (soluble immune response suppressor)
- IFN (interferon)
- AFP (α-fetoprotein, fetuin)

**Factors Acting On Inflammatory Cells**
- SMAF (specific macrophage arming factor)
- MIF (migration inhibitory factor)
- MCF (macrophage chemotactic factor)
- MSF (macrophage slowing factor)
- MEF (macrophage enhancement factor)
- MAF (macrophage aggregation factor)
- MAF (macrophage activating factor)
- MFF (macrophage fusion factor)
- LIF (leukocyte inhibition factor)
- NCF (neutrophil chemotactic factor)
- BCF (basophil chemotactic factor)
- ECF (eosinophil chemotactic factor)
- LCF (lymphocyte chemotactic factor)

**Factors Acting On Vascular Endothelium**
- SRF (skin reactive factor)

**Factors Acting On Other Cells**
- TMIF (tumour cell migration inhibition factor)

**Growth Stimulating Factors**
- MGF (macrophage growth factor)
- MF (mitogenic (blastogenic) factor)
- LIAF (lymphocyte-induced angiogenesis factor)
- CSF (colony stimulating factor)

**Direct-Acting Factors**
- Lysosomal enzymes
- CTF (cytotoxic factors)
- MTF (macrophage toxic factor)
- SMC (specific macrophage cytotoxin)
- MCF (macrophage cytolytic factor)
- LT (lymphotoxin)

(LK=lymphokine; MK=monokine)
Following purification and full characterisation many of these cytokines may become important in clinical medicine for the regulation of the immune response. Cytokines could be used as immunosuppressors or immunostimulants and they may become important in tumour therapy and in the treatment of chronic infectious processes caused by parasitic, bacterial, viral and fungal agents. Cytokines may also become clinically important in some non-immunological processes such as the enhancement of angiogenesis, the stimulation of haematopoiesis, wound healing and tissue regeneration, repair of certain clotting defects, inhibition of tumour spread by direct inhibitory effect on tumour cell multiplication, motility and metastatic capacity, and the inhibition of viral multiplication. Specific anti-lymphokine antibodies might be used in the future as immunosuppressors, acting at the effector end of the immune response. In the future it may be possible to develop synthetic lymphokine analogues with specific functions, or drugs preventing or enhancing lymphokine production and/or action in vivo.

Although lymphokine biology has come a long way since the discovery of MIF twenty years ago, work on the purification and characterisation of the vast array of factors, some of which are listed in Table 3, is still in its infancy. Most studies of mediators to date have utilised supernatants from antigen- or mitogen-stimulated lymphocyte cultures as the source of the lymphokine and have defined mediators by their effect on target cells in vitro. Obviously such supernatants are far from pure, leading to the possibility that mediators identified by their
behaviour in different in vitro assays are actually the same molecular species, but are expressing different activities depending on the nature of the assay.

Over the past five years, as effort has been put on the molecular identification and characterisation of MIF, its molecular association with MAF activities have been hotly debated in the literature. Until recently MIF was generally regarded as being the same molecule as MAF because of similarities in their physicochemical properties (Nathan et al., 1973). It is now recognised that MAF is not a single molecule, rather there may be several, each showing different activities (Nacy et al., 1980). Since both MIF and an anti-tumour MAF fall in the same molecular weight range (35,000-55,000) on filtration over a Sephadex G-100 column, and since both activities are enhanced by preincubating macrophages with diazotized sulfanilic acid which reacts with a number of amino acids, Remold and his colleagues proposed molecular similarity between the two mediators, or that they at least share a similar mechanism of interaction with the macrophage (Remold, 1977; Plessens et al., 1976).

There is increasing awareness that both MIF and MAF represent families of molecules with wide ranging molecular weights. McCarthy et al., (1980) distinguished two distinct substances with different isoelectric points and apparent molecular weights both with guinea pig MIF activity. Guine pig MIF was reported by Klinkert and Sorg (1980) to be represented as a family of proteins of molecular weights 15K, 30K, 45K and 60K, each with an
isoelectric point at pH 5.2. Sorg (1980) found murine MIF activity in a similar range of glycoproteins of 14K, 28K, 42K and 56K daltons. Jacobs and Poretz (1982) reported murine MIF activity with apparent molecular weights of 87K, 47K, 37K and 19.5K. Kuhner and David (1976) also found MIF activity over a range of 87K to 37K daltons with highest activity at 48K to 67K daltons. Similar studies on human MIF activity also suggest MIF heterogeneity (Weiser et al., 1980). The same group later reported that MIF-active proteins produced early in cultures of ConA stimulated lymphocytes are distinct from those produced after 24 hours of culture (Weiser et al., 1981). Weiser and his colleagues (1985) have recently shown that different antigens induce the production of different second day MIF and IFN-gamma species.

On mitogen stimulation, human lymphocytes elaborate several related macrophage directed lymphokines, including interferon-gamma, MIF and three different MAF activities (anti-Leishmania-MAF, anti-tumour MAF and anti-viral MAF) (Buchmuller et al., 1983). All three of these lymphokines are heterogeneous. Lymphokine purification is not proving to be a straightforward task. In the mouse, MIF co-chromatographs with a MAF which induces macrophages to kill tumour cells and intracellular organisms. Other related MAFs only induce macrophages to kill intracellular organisms (Nacy et al., 1981).

Antibodies have been used to distinguish between molecules difficult to separate according to molecular weight and isoelectric point. Using an immunoabsorbant column conjugated with guinea pig
anti-MIF antibody Onozaki et al. (1981) obtained a highly purified MIF preparation. Macrophages responded to this preparation with a decrease in random migration, but also with an accelerated glucose consumption, indicating MAF activity. The preparation was assayed further and found not to contain four other lymphokine activities: skin reactive factor (SRF), chemotactic factors for neutrophils and macrophages (NCF, MCP) and vascular permeability factor (VPF). The authors therefore proposed that MIF and MAF are identical molecular species. It is suggested here that such evidence only supports some similarity between MIF and one of the reported MAF activities.

Another lymphokine which appears simultaneously with MIF in the sera of immunised mice and cannot be separated on a molecular weight basis is the anti-viral agent, interferon (IFN). Bloom (1971) concluded from this that these activities might be two different functions of the same molecule. Neuman and Sorg (1980a), however, found that the cellular requirements for the production of IFN and MIF were different; they describe situations where MIF but no IFN is produced by T-cells as well as situations where IFN but no MIF is produced by macrophages. They therefore concluded that MIF and IFN are distinct molecules.

There are three distinct types of IFNs, IFN-α, IFN-β and IFN-γ, and anti-viral activities are not the only biological function of these proteins (Gresser, 1977; Johnson and Baron, 1976; Balkwill, 1979; DeMaeyer and DeMaeyer-Guignard, 1981). For example they have been found to be important in the development of cell-mediated immunity. They also enhance natural killer cell activity (Gidlund et al., 1977).
1978; Djen et al., 1979), induce the formation of cytotoxic T-lymphocytes (Farrar et al., 1981), and activate macrophages to express tumour cell cytotoxicity (Schultz et al., 1977; Jett et al., 1980; Boraschi and Tagliabue, 1981). IFN-γ has proved to be difficult to obtain, is produced with relatively low antiviral activity, and is contaminated with other biologically active lymphokines.

T-cell hybridoma techniques have been introduced into this field because fewer lymphokines are coincidentally produced by a cloned hybridoma than by a heterogeneous culture of lymphocytes. Le et al., (1983) used this technique to produce anti-tumour MAF and IFN-γ. They found that when interferon activity was neutralised with specific antiserum to purified IFN-γ, MAF activity was abrogated. IFN-γ from two other hybridomas, from mitogen induced peripheral blood lymphocyte cultures and Escherichia coli-derived recombinant IFN-γ were obtained by SDS-PAGE. With all three types of preparations, a close correlation was found between the presence of IFN-γ activity, demonstrable in an antiviral assay, and anti-tumour MAF activity in individual fractions. These results agree with those of Kleinschmidt and Schultz (1982) and Roberts and Vasil (1982) in suggesting the molecular identity of IFN-γ and anti-tumour MAF activity.

The present state of the literature suggests that hybridoma and antibody techniques together with defined assays must be further used to purify and characterise the various lymphokine activities. Once characterised, amino acid sequencing could be used to
establish homologies and differences between closely related lymphokines. At present it seems some lymphokines are identical to one another and others closely related, perhaps because they are derived from a common precursor molecule. In the work reported in this thesis, the lymphokines used were derived from crude preparations and no attempt was made to separate them.

In this Chapter studies investigating the occurrence and specificity of MIF in different vertebrate groups are reported. Although most of the work on MIF has been carried out using mammalian cells, this lymphokine has been shown to be produced by lymphocytes of other vertebrates. MIF is produced by fish (Jayaraman et al., 1979), amphibians (Tahan and Jurd, 1979; Rimmer and Gearing, 1980) and reptiles (Jayaraman and Muthukkaruppan, 1977) as well as mammals (Eidemiller and Bell, 1972; Friedman, 1971; Al-Askari and Lawrence, 1972; Kuramochi, 1974). Rimmer and Gearing (1980) showed that the production of MIF by sensitised T-cells is antigen specific and they postulated its association with cell-mediated immunity.

Little is known about the specificity across species or class of the effect of MIF. All that is known comes from work done on a few mammalian species. These show that for the species studied, MIFs demonstrate little absolute species specificity, but maximal reactivity by target cells from the same species as the producer of MIF is usually found. Guinea pig MIF inhibits rabbit macrophage migration (Svejcar et al., 1971). Human MIF affects macrophages of guinea pigs (Thor et al., 1968), rabbits (Svejcar et al., 1971).
mice (Gorski, 1974) and horses (Friedrich et al., 1976). Mouse MIF inhibits guinea pig macrophages (Gadol and Waldman, 1973), as does monkey MIF (Grimley and Barnes, 1973). Rabbit MIF inhibits guinea pig, hamster, and rat macrophages (Svejcar et al., 1971). This lack of species specificity is manifest also in the fact that guinea pig macrophages have been routinely used to detect MIF activity in the supernatants of nonlymphoid cells of human, monkey, mouse, rat, or hamster origin.

The present study was carried out in order to determine whether "ancient" MIFs from the non-mammalian vertebrates, Triturus (a urodele) and Xenopus (a primitive anuran), were capable of inhibiting the migration of mammalian macrophages and whether the relatively "young" mammalian MIFs had a structure sufficiently altered to change their effects on macrophages of lower vertebrates.

Materials and Methods

1.1 Animals

Mice used in these studies were the inbred strains C57BL, CBA, and Balb/c. Young adult male animals were used throughout. Sprague-Dawley rats and the mice were obtained from the animal facilities of the University of Leicester. Triturus cristatus newts were a gift from Professor H.C. Macgregor in the Zoology Department. Xenopus laevis, the African clawed toad, were obtained
from Xenopus Limited, Redhill, Surrey. All animals were housed conventionally and permitted water and food ad libitum.

1.2 Lymphokine Preparation
Spleens were removed from young adult male mice, Sprague-Dawley rats, Triturus cristatus and Xenopus laevis, and mechanically dissociated with sterile needles in a small volume of HEPES-buffered Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco), to give a cell suspension. The cells were washed three times in ice-cold medium and incubated at 37°C in DMEM containing 10% fetal calf serum (FCS; Gibco), 100mcg/ml kanamycin (Gibco) and 2mM glutamine (Gibco) (complete DMEM) to which human gamma globulin (HGG; Miles Laboratories) was added to a concentration of 2mg/ml. The presence of this antigen sensitises the T-cells which respond by producing lymphokines. In the case of amphibian cells 70% Liebovitz L15 (Flow Laboratories) medium was used instead of DMEM and the cultures were incubated at 25°C. All culturing was carried out in sterile 50ml flasks (Nunc).

After 3-5 days incubation the supernatant from the cultures was collected and dialysed overnight at 4°C against phosphate buffered saline, to decrease the salt concentration. The dialysate was lyophilised, redissolved in elutant and fractionated on a Sephadex G-100 column.

Sephadex separates molecules on the basis of their molecular size. Sephadex G-100 separates molecules within the range of 4,000 to 150,000 daltons. The column measured 65cm by 1cm and the sample
was eluted with 0.02M Tris-HCl: 0.1M NaCl buffer. The eluate was collected in 2ml fractions on an LKB fraction collector. Absorbance at 280nm by the resultant fractions was assessed on a Gilford spectrophotometer.

Before samples were run through the column, it was calibrated. Blue dextran (Sigma) was used to calculate the void volume and three proteins of known molecular weight were used for the calibration. These proteins, and their molecular weights, were as follows:

- Bovine albumin 65,000 daltons (Sigma)
- Egg albumin 45,000 daltons (Sigma)
- Bovine milk β-lactoglobulin 36,000 daltons (Sigma)

The blue dextran and these three proteins were run through the column separately, and the optical density of each fraction collected was measured at 280nm. The elutant volume was calculated using the formula:

\[ V_p = V_e - V_o \]

where

- \( V_e \) = total elutant volume
- \( V_p \) = elutant volume of protein
- \( V_o \) = void volume (ie. \( V_e \) of blue dextran)

Once calculated, the elutant volumes of the three proteins of known molecular weight were used to draw a calibration curve. Using this curve, the molecular weight of any fraction collected off the column could be determined.

The lymphokine containing samples were added to the column and
fractions then lyophilised and stored at 4°C. Capillary tube assays were performed to determine which of these fractions contained greatest MIF activity. Those fractions were then routinely used as a source of MIF.

1.3 Harvest of Peritoneal Exudate Cells (PECs)

Animals to be used were injected intraperitoneally with a 1% potato starch (Sigma) suspension (0.5ml for mice; 2.0ml for Xenopus), or 1ml New Born Calf Serum (for mice; Gibco). Three to seven days later the peritoneal exudate cells (PECs) were harvested immediately following sacrifice of the animals by cervical dislocation (for mice) or stunning and decapitation (for Xenopus).

Under sterile conditions ice cold medium without serum was injected into the peritoneal cavity, and the abdomen was gently massaged to bring the peritoneal cells into suspension. The cells, suspended in medium, were then collected into the syringe and transferred into 20ml tubes (Sterilin) on ice. Cells were washed 3 times by centrifugation at 900g for 10 minutes.

80% of elicited mouse PECs are large, vacuolated mononuclear cells with typical macrophage morphology. The remaining 20% consists of non-adherent lymphocytes and the adherent but short lived granulocytes (Fidler, 1975).

1.4 Capillary Tube Assay

2x10^7 PECs/ml in complete medium were taken up into sterile heparinised capillary tubes (75mm by 1mm bore; Bilbate), by
capillary action. The tubes were sealed at one end with dental wax, centrifuged at 900g for 10 minutes, and cut at the cell/medium interface with a diamond pencil. The segment of each cut tube containing the cells was affixed in a horizontal position in the bottom of a chamber of a Sterilin migration tray with a spot of Vaseline. The chamber was filled with complete medium (control) or medium being tested for MIF activity. It was covered with a sterile coverslip and sealed with Vaseline so that no air space was left to permit agitation of the fluid. Replicate tubes were set up for each sample. The trays were coded and incubated at 37°C (or 25°C for amphibian cells) for 18 hours. During incubation the cells migrated out from the cut end of the tube and formed a fan on the bottom of the chamber. At the end of 18 hours each tray was placed on a photographic enlarger and the image projected onto a grid below. The maximum distance migrated by the cells was measured on the grid for each chamber and the value expressed as a percentage of that of the control for each animal, as follows:

\[
\% \text{ migration} = \frac{\text{migration distance of PECs in MIF}}{\text{migration distance of PECs in control}} \times 100
\]

Therefore the lower the value recorded the greater the degree of inhibition. At least 4 animals were used in each test. Up to 5 replicate tubes were set up for each animal, depending on the number of PECs collected, which varies considerably from one individual to another.

1.5 Effect of MIF on Macrophage Locomotion In Vitro

PECs were harvested from C57BL mice as described in Section 1.3, and incubated on glass coverslips in a petri dish (Sterilin) for 1 hour at 37°C. Non-adherent cells were washed away leaving a
virtually pure culture of mononuclear phagocytes apart from a few granulocytes and contaminating fibroblasts.

Complete medium containing mouse MIF prepared as described in Section 1.2 was passed through a Millipore filter (0.45μ; Sartorius) to ensure it was cell free, and then added to the macrophage culture. Observations were made on a Nikon Diaphot inverted microscope with an attached camera. A series of photographs of individual macrophages were taken over a known time period using Pan X film (Kodak). Cultures were maintained throughout at 37°C.

Results

1.1 Capillary Tube Assay

Figure 2 shows a capillary tube containing mouse PECs at the end of 18 hours' incubation at 37°C in the absence of MIF. The cells have migrated out of the tube forming a fan shape. The white cells, including the macrophages, are located around the periphery of the fan. When incubated in the presence of MIF, the area of the fan is smaller, as shown in Figure 3. By measuring the fan size in the presence of MIF-containing cell culture supernatants, and comparing these with control fan size, the capillary tube assay can be used to quantify cell migration and determine whether MIF is indeed present in the cell culture supernatants being tested.
Figure 2:

The micrograph shows a capillary tube containing PECs after incubation for 18 hours in complete medium in the absence of MIF/MAF. The large fan of cells protruding from the cut end of the capillary tube indicates that cells have migrated out of the capillary tube during the incubation period.

Figure 3:

The micrograph shows a capillary tube containing PECs after incubation for 18 hours in complete medium in the presence of MIF/MAF. No cell migration has occurred. The cells have all remained in their original position at the cut edge of the capillary tube.
Control supernatants from cultures of T-cells not sensitised with HGG, were dialysed, lyophilised and assayed using the capillary tube method and found not to have MIF activity.

1. Calibration Curve For MIF

Blue dextran and three proteins of known molecular weight were used to calibrate the Sephadex G-100 column as described in Section 1.2 in this Chapter. The line of best fit was found to have a correlation coefficient of 0.9985 with one degree of freedom. Following elution of the lymphokine preparations over a Sephadex G-100 column, the optical density of each 2ml fraction was measured by spectrophotometry and the profile obtained by this method is shown in Figure 4 for mouse cells. Each fraction was assayed for MIF activity by the capillary tube assay and the peak found to consistently give the highest MIF activity is arrowed. The molecular weight of this fraction, assessed by column chromatography, is approximately 60,000 daltons, as shown in Figure 5, where the values for mouse, rat, Xenopus and Triturus MIF activities are shown plotted on the calibration curve. The open circles show the positions of the proteins used to calibrate the curve, and the molecular weights of the MIFs calculated using this curve are given below:

- Rat MIF = 68,400 daltons
- *Xenopus laevis* MIF = 61,000 daltons
- Mouse MIF = 60,000 daltons
- *Triturus cristatus* MIF = 59,000 daltons.
Figure 4:

This graph shows the optical density (measured at 280nm) of 2ml fractions eluted from a Sephadex G-100 column after the addition of murine MIF/MAF containing supernatant. Each fraction was assayed for MIF/MAF activity and the peak arrowed contains the fractions with the highest levels of MIF/MAF activity.
Figure 5:

Sephadex G-100 column calibration curve. Bovine albumin, egg albumin and bovine milk β-lactoglobulin were used to calibrate the curve. The molecular weights at which mouse, rat, *Xenopus* and *Triturus* MIFs elute from the column is also indicated.
In order to compare the effect of different MIFs on macrophage migration, the distance migrated by the cells in the presence of various MIFs was measured and expressed as a proportion of the distance migrated by the control cells as a migration index (see Section 1.4 in Methods). The results for mouse and Xenopus PECs and mouse, Xenopus, Triturus and rat MIFs are given in the histogram in Figure 6, which plots the mean migration index value for Xenopus and mouse PECs treated with each of the four MIFs. The bars show 95% confidence limits. The means were compared using Student's "t" test and the values obtained for "t" are given in tables 4 and 5 below:

**Table 4: Comparison Of Mouse MIF Activity With That Of Xenopus, Rat and Triturus on Mouse Macrophages**

<table>
<thead>
<tr>
<th>Origin of MIF</th>
<th>Value of &quot;t&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenopus MIF</td>
<td>0.129</td>
</tr>
<tr>
<td>Rat MIF</td>
<td>2.273</td>
</tr>
<tr>
<td>Triturus MIF</td>
<td>1.049</td>
</tr>
</tbody>
</table>

**Table 5: Comparison Of Xenopus MIF Activity With That Of Mouse, Rat and Triturus on Xenopus Macrophages**

<table>
<thead>
<tr>
<th>Origin of MIF</th>
<th>Value of &quot;t&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse MIF</td>
<td>3.652</td>
</tr>
<tr>
<td>Rat MIF</td>
<td>6.571</td>
</tr>
<tr>
<td>Triturus MIF</td>
<td>3.582</td>
</tr>
</tbody>
</table>
Figure 6:

This histogram indicates the effect of various MIFs on mouse and Xenopus PEC migration. The percent migration represents the mean distance migrated by PECs in the presence of MIF expressed as a percent of the mean distance migrated in the absence of MIF, taken as 100% migration.
MIF from both mammalian and amphibian sources had some inhibitory effect of the migration of mouse and Xenopus macrophages. The size of this effect, however, varied depending on the combination of MIF and macrophages. Mouse cells were inhibited to almost the same extent by all MIFs and there proved to be no significant difference in inhibition between these treatments at a probability of 98%. Xenopus macrophages responded differently. Their migration was most severely diminished by MIF from Xenopus itself. Xenopus macrophages show a lower response to MIF from non-Xenopus cells. This was found to be highly significant when assessed by the Student's "t" test. Mouse and Triturus MIFs did, however, reduce the migration of Xenopus macrophages by about 50%, which is considerable. Rat MIF caused a smaller inhibition of migration.

1. iv Effect of MIF on Macrophage Locomotion In Vitro

Figure 7 shows a mouse peritoneal macrophage cultured in vitro on a glass coverslip. This cell shows the familiar polarity associated with cells moving in culture. The thick arrow indicates the leading lamellipodium with the characteristic thin layer of cytoplasm and ruffled edge. The thin arrow indicates retraction fibres at the trailing end of the cell. This photograph was taken immediately before the addition of mouse MIF to the culture. Figure 8 shows the same macrophage 30 minutes after the addition of MIF to the medium. Figure 9 shows that same macrophage 17 hours later. The overall shape of the cell was dramatically altered on exposure to MIF as shown by comparing Figures 7 and 8. The shape assumed at 30 minutes exposure to MIF is maintained while MIF is present in the culture medium. That this macrophage did not
Figure 7:

Phase contrast micrograph of a mouse peritoneal macrophage cultured on a glass coverslip in vitro. The white arrow indicates the leading edge and the black arrow indicates retraction fibres at the trailing edge.

Figure 8:

This micrograph shows the same macrophage as in Figure 7 30 minutes following the addition of MIF/MAF.

Figure 9:

This micrograph shows the same macrophage as in Figures 7 and 8, 17 hours after the addition of, and still in the presence of, MIF/MAF. The presence of MIF/MAF has immobilised the macrophage completely. Scale bar = 50μm
undergo translocation during exposure to MIF was confirmed by relating the position of the cell to a reference point on the glass coverslip. Translocation was, however, resumed shortly after replacing the MIF-containing medium with fresh complete DMEM without MIF.

**Discussion**

The evolution of the host defence system is an intriguing phenomenon but its nature makes it difficult to investigate. A prerequisite requirement of any such defense system is the ability to distinguish self from non-self which, in its simplest form, is manifested in simple association or nonassociation of the cells involved. Contact recognition is well developed in lower vertebrates, even in protozoa and coelenterates, and is seen virtually unchanged in higher forms in the reassociation of like cell with like among dissociated cell suspensions of the various organs. Dissimilar cells, on the other hand, are rejected upon contact. The origins of the host defence system in the comparatively primitive defence responses of many invertebrates have now been well-documented (Tripp, 1969; Hildemann, 1972; Hostetter and Cooper, 1972; Cooper, 1973).

Only vertebrates can show humoral immunity since only they have true lymphocytes and so can produce immunoglobulins. But a form of cell-mediated immunity is found even in the lowest invertebrates in
the form of phagocytic cells, similar to macrophages. These phagocytic cells can recognise and phagocytose or encapsulate foreign matter. But this is not a true immune response since the cells involved neither show memory or specificity. A limited form of cell-mediated immunity is, however, present in higher invertebrates. Graft rejection does occur but it is much slower than in vertebrates.

The origin of lymphocytes is unclear although T- and B-cell lineages may be derived from undifferentiated primitive wandering cells. The vertebrate macrophage, on the other hand, as already mentioned, closely resembles cells found in many invertebrate groups. These highly efficient phagocytic cells probably represent the forerunners of the vertebrate macrophage.

As stated previously, the macrophage surface provides recognition sites at which T-cells can recognise and react against foreign substances that it may have taken up. It also produces and releases a vast array of factors and enzymes in response to various stimuli. Many of these monokines are similar to lymphokines perhaps indicating a link between the phylogeny of lymphocytes and macrophages.

The lymphocytes of higher vertebrates produce a wider range of immunoglobulins than do the lymphocytes of lower vertebrates. Cell-mediated immunity is also more advanced in the higher vertebrates than in the lower vertebrates. It is therefore of interest to determine whether lymphokines are made in lower
vertebrates at all and the extent of cross reactivity between lymphokines from different vertebrates.

A great deal of work has been done on the mammalian immune system. Much less has been done on that of the amphibians. Across the Class Amphibia there appears to be a microevolutionary progression in terms of the immune response. The Apoda or caecilians are the most primitive amphibians; they show a slow graft rejection response, only have a primitive thymus and produce only one immunoglobulin, IgM. The urodeles, or tailed amphibians, also have a slow graft rejection response, so their cell-mediated response is still primitive, but their lymphoid tissue is more advanced than in the Apoda. The urodeles have a 3-lobed thymus and a spleen and some secondary lymphoid sites. The Anura, or frogs and toads, can be divided into primitive and advanced forms by their immune response as well as by other characteristics. Xenopus is a member of the Pipidae, and shows primitive Anuran immune responses. Graft rejection is slower in Xenopus than in the more advanced Anurans such as Rana. All Anura, however, produce two immunoglobulins, IgM and IgG unlike the rest of the amphibians which produce only IgM. These immunoglobulins represent only two out of the five produced by the mammals. Lymphoid tissue is quite well advanced in the Anura. They have a well developed thymus, spleen and secondary lymphoid sites, although Xenopus does not have lymph nodes while the Ranidae do.

It was mentioned earlier that all major vertebrate groups produce molecules with MIF activity. Here it is confirmed that MIF is
produced in the urodeles and in Xenopus, a much more advanced amphibian. The inhibition of macrophage migration would therefore seem to be an old strategy in terms of evolution of the immune response and may be expected to have become refined in parallel with the increasing sophistication of the immune system witnessed throughout vertebrate evolution.

The results reported here show that mouse macrophages are as susceptible to inhibition by amphibian MIF as by mammalian MIF. Xenopus macrophages, however, exhibit a definite but decreased response to heterologous MIFs. At present the explanation of this remains obscure. The similarity of molecular weights, reported here and in the literature, and the efficacy of activity of amphibian MIF on mammalian cells suggests that the molecule has changed little during the course of vertebrate evolution. The crucial role fulfilled by MIF in host defence is confirmed by this apparent conservation of molecular structure. It is possible that there may have been changes in number or configuration of MIF receptors over time, rendering mammalian macrophages more susceptible to general MIF effects.

The phylogenetic emergence of various receptors on phagocytes is under examination (Sekizawa et al., 1984). The mechanism whereby MIF alters macrophage function remains largely unknown. Components of the plasma membrane have been shown to be important in the inhibition of macrophage migration caused by MIF (Remold, 1973; Remold, 1974; Remold and Rosenberg, 1975; Remold, 1977; Jacobs and Poretz, 1982). One common cell surface component, the
monosaccharide α-L-fucose was found to block the effect of MIF on macrophages, while other sugars including α-L-rhamnose, α-methyl-mannoside, α-D-glucose, β-D-galactose, and N-acetyl-β-D-glucosamine had no effect. It was shown that to block MIF, α-L-fucose had to be present throughout the assay period. Inhibition of MIF action was also obtained by treating macrophages with α-L-fucosidase, but enzyme-treated cells later regained responsiveness. These data are compatible with free α-L-fucose competing with an α-L-fucose containing macrophage binding site for MIF.

A role for glycolipids as components of the macrophage MIF receptor has been suggested. Higgins et al., (1978) show that guinea pig PECs pretreated with macrophage-derived water soluble glycolipids show an enhanced response to MIF. This enhancement of PEC responsiveness to MIF was found to be specific for glycolipids from guinea pig macrophages. Glycolipids extracted from other guinea pig cell types had no effect. L-fucose is a common component of lipid-linked oligosaccharide chains in glycolipids (Talmadge and Burger, 1975; Hakomori, 1970; Hakomori et al., 1967; Smith et al., 1973; Smith et al., 1975), and may be a necessary component of a glycolipid MIF receptor on the macrophage surface (Liu et al., 1978). Whether macrophage glycolipids serve as MIF receptors or function in some other way to enhance the responsiveness of PECs to MIF is still not clear. Several authors have, however, described experiments that indicate that certain cell surface receptors are glycolipids (Cuatrecasas, 1973; Van Heyningen, 1974; Moss et al., 1976; Hughs and Gardas, 1976; Vengris et al., 1976)
Very little is known about the relation of the MIF receptor to other macrophage receptors specific for the Fc fragment of immunoglobulins, C3, or plant lectins. Fc binding sites are resistant to trypsin and other proteolytic enzymes (LoBuglio and Reinehard, 1970), this distinguishes them from MIF receptor sites since the MIF response is abolished by trypsinisation. Further work on the characterisation of MIF receptor sites is necessary.

The issue of the action mechanism of MIF at the cellular level is as yet far from clear. In a review Pick (1979) suggested a scheme for the action mechanism of MIF which took into account the experimental data available at that time. He visualised a sequence of events composed of three main phases:

1. the interaction of MIF with membrane receptors
2. the generation of second messengers in the cytoplasm, and
3. an effect on the cytoskeleton.

Following the MIF-receptor interaction there is an increase in the level of Ca\(^{2+}\) in the cytosol, which can occur by liberation of bound intracellular Ca\(^{2+}\) from the cell membrane or mitochondria or by influx from the extracellular medium. It is not known how this increased level of Ca\(^{2+}\) is achieved. Pick postulates from circumstantial evidence that this increased level of Ca\(^{2+}\) acts as a stimulus for guanylate cyclase which in turn results in an increase in the cGMP (cyclic guanosine monophosphate) level. An elevated cGMP level might lead to increased tubulin polymerisation and microtubule assembly, although it is not known by what mechanism
cGMP influences tubulin polymerisation, and a direct effect on polymerisation in vitro has not been found. At the time of tubulin polymerisation, the Ca^{2+} concentration must return to normal otherwise the high concentration of Ca^{2+} would prevent assembly.

On exposure to cytochalasin B, a microfilament-blocking drug, macrophage motility is reversibly inhibited. Colchicine and vinblastine, which disrupt microtubules, enhance random migration and totally block MIF action. If, however, the microtubules are stabilised by treatment with deuterium oxide, spontaneous migration is inhibited and the effect of MIF enhanced. A shift in the tubulin dimer-polymer equilibrium toward the polymerised state, by whatever mechanism, probably results in the cellular rigidity and lack of motion observed in MIF-treated macrophages (Cohen et al., 1977). In Chapter 2 some work on the effect of MIF on the cytoskeleton of macrophages is reported. The results suggest that the microtubule array is affected by exposure to MIF.
CHAPTER 2

effect of MIF and Substrate on Macrophage Adhesion, Locomotion and Invasiveness.

Introduction

Macrophages are one of the few normal adult vertebrate cell types, along with white blood cells, which exhibit highly motile behaviour in the course of their normal activities.

Immature macrophages, or monocytes, in the blood stream are responsive to chemoattractants produced during inflammatory reactions. Such chemoattractants cause monocytes to extravasate by burrowing through the endothelium and basement membrane of the capillaries. During this process, monocytes mature into macrophages (Cohn and Benson, 1965). Mature macrophages retain this property of motility in response to appropriate stimuli. Motility is a part of the basic phenotype of macrophages, except where it has been secondarily lost by stationary specialised cells such as osteoclasts or brain microglia. It is therefore implied that when macrophages are required to remain at a site of infection, tumour growth or tissue damage, specific steps must be taken to halt their movement. One way such an effect can be mediated is by the presence of MIF. The mechanism by which MIF maintains macrophages at localised sites is not yet fully understood.
Mononuclear phagocytes mostly migrate on collagenous matrices in vivo. As monocytes extravasate they encounter a collagen-containing basement membrane. Mature wandering tissue macrophages are mostly located around the basement membranes of blood vessels and in the connective tissue stroma or the collagenous capsule of organs. A large population of macrophages reside on or in the mesenteries and the dense connective tissue of the body wall of the peritoneum. Other populations of naturally migratory cells also interact intimately with collagen in vivo (reviewed by Wylie et al., 1981). These include embryonic neural crest cells (Löfberg et al., 1980; Tosney, 1978), corneal fibroblasts (Bard and Hay, 1975), and primordial germ cells (Swan et al., 1983).

As well as being permissive to motility, there is growing evidence that collagenous substrates play an important role in cell differentiation. This has been shown for mouse mammary gland epithelium (Gordon and Bernfield, 1980) and chondrocytes (Nathanson and Hay, 1980; Belsky et al., 1980). Various cell types also seem to depend on a collagenous substrate to maintain their differentiated state (Toole, 1981). The functional activity of many cells may depend on the chemical composition and spatial arrangement of the components of their substrate.

Several of the components of the extracellular matrix are synthesised and secreted by fibroblasts including collagen, glycosaminoglycans, proteoglycans and fibronectin. Other cells of the connective tissue, chondroblasts and osteoblasts, also secrete
components of the extracellular matrix, as do muscle and epithelial cells. The regulatory processes governing the synthesis of matrix components by these cells remain largely unidentified. This is not the case, however, for fibroblasts. Macrophages play a major role in controlling collagen synthesis and degradation by fibroblasts (Laub and Vaes, 1982; Laub et al., 1982; Douglas, 1980). Fibroblasts secrete collagen and are primarily responsible for tissue architecture, remodelling and repair (Harris et al., 1981; Ross and Benditt, 1961; Ross, 1968). Macrophages cause fibroblasts to produce a variety of anabolic and catabolic products which affect connective tissue but also have wider roles in general tissue growth and breakdown. As already mentioned, macrophages elaborate products which control the proliferation of fibroblasts in vivo and in so doing largely determine the number of fibroblasts present at sites where the extracellular matrix is being laid down (Kurland et al., 1979), for instance at sites of wound healing (Leibovitch and Ross, 1975). They have also been shown to elaborate products which control fibroblast proliferation in vitro (Leibovitch and Ross, 1976). Macrophages also produce a variety of monokines which activate collagen, collagenase or protease synthesis by fibroblastic cells, such as wound fibroblasts, chondrocytes, and corneal cells (Dingle et al., 1979; Newsome and Gross, 1979; Ridge et al., 1980; Meats et al., 1980). Monokines also cause fibroblasts to produce plasminogen activator (Laub and Vaes, 1982) generating plasmin which in turn activates latent collagenase (Eeckhout and Vaes, 1977) and the neutral proteases involved in proteoglycan degradation (Vaes, 1972). Wahl and his colleagues (1974; 1975; 1977) gave evidence that macrophages
themselves secrete collagenase, they have also been reported to secrete neutral protease (Huybrechts-Godin et al., 1979), although this activity may be due to contaminating fibroblasts in macrophage populations (Werb et al., 1980).

Macrophage products control collagen synthesis in fibroblasts (Kulonen and Potila, 1980; Heppleston and Styles, 1967). Some evidence suggests that macrophages suppress collagen synthesis by producing arginase, which reduces the level of arginine below that permissible for collagen synthesis (Jalkanen et al., 1982). Macrophages also produce prostaglandins (Gemsa et al., 1978) which also suppress collagen synthesis by fibroblasts (Goldstein et al., 1982). Macrophages are, therefore, very important in shaping their microenvironment. They also exhibit interesting and characteristic features on interaction with their immediate substrate; they have peculiar adhesion properties, unusual spreading tendencies and highly developed locomotory abilities.

The adhesive and spreading behaviour of macrophages is of considerable importance since it is relevant to the in vivo interactions of these cells with other cells and more importantly with connective tissue, basement membrane and other extracellular materials. Mackaness (1970) suggests that there is a correlation between the way macrophages respond to these materials in vitro, with their response in vivo, and that this response largely determines the activities of the cells as a consequence.

Macrophages bind fibrin in two ways, by binding the fibrin monomer.
(Gonda and Shainoff, 1982), and secondly by a mechanism involving fibronectin, but little is known about macrophage interactions with collagen, the major component of the extracellular matrix. *In vivo* macrophages within the collagenous connective tissues are generally motile, and wander randomly until attracted to sites of tissue damage, infection or cell death. Macrophages within whole mount spreads of mesentery or omentum are usually rounded or lobular, and move by extending large blunt pseudopodial processes in an amoeboid fashion. In this Chapter this observation is compared with the situation on artificial and purified collagen substrates *in vitro*. This Chapter also explores the effect of substrate on macrophage adhesion and spreading.

Macrophages have an unusual adhesion mechanism, quite unlike that of other cell types studied. Macrophages are not removed from their substrate by trypsin; indeed trypsin and proteolytic enzymes in general actually induce spreading by these cells (Rabinovitch and DeStefano, 1974). Macrophages can be detached from their substrate by mechanical force and by lidocaine, a membrane-acting anaesthetic. A variety of surface phenomena in these cells are inhibited by cationic anaesthetics; these include cell to substrate adhesion, spreading, phagocytosis, locomotion, cell fusion, capping of surface Ig, and cell mediated-cytotoxicity (Rabinovitch and DeStefano, 1975; 1974; Cullen and Haschke, 1974; Gail and Boone, 1972; Poste and Reeve 1972; Ryan *et al.*, 1974; Kemp and Berke, 1973). Lidocaine was used to detach macrophages from substrates *in vitro* for experiments reported in this thesis. In this Chapter its effect on macrophages *in vitro* is described. Metabolic inhibitors,
membrane-active agents (anaesthetics and detergents), anti-inflammatory compounds such as indomethacin and phenylbutazone, and colchicine and vinblastine at high concentrations, all block proteolytic enzyme-induced macrophage spreading (Rabinovitch, 1975). Rabinovitch and DeStefano (1973) also found that macrophages require divalent cations for spreading on glass.

Experiments are described in this chapter which are intended to investigate the spreading process and subsequent behaviour of adherent macrophages on various substrates in vitro. The effect of MIF on the morphology and adhesive interactions of macrophages incubated on these substrates is also described.

To date all research into the spreading process of macrophages has been done on macrophages cultured on glass or plastic in vitro. Two types of spreading have been described. The first, fast spreading, requires reserves of surface membrane materials (Follett and Goldman, 1970) and has no serum requirement. It occurs within minutes of cell-substrate adhesion and can be identified by a thin flat sheet of cytoplasm protruding from beneath the bulk of the rounded cell. Slow spreading contrasts with fast spreading in having a serum requirement and involving protein synthesis. It also takes longer than fast spreading and results in the extreme flattening of the cells characteristic of cells cultured in vitro for over one hour on glass or plastic substrates.

During the spreading reaction, cultured cells adhere tightly to
their substrate. Using micromanipulation and time lapse cinemicrography, Harris (1973) identified localised points of tight adhesion over the ventral surface of several types of cells in culture. He found that cultured fibroblasts adhere to solid substrata only over a small fraction, up to 15-35%, of their lower surface. The adhesions represent tiny points distributed principally around the periphery of the cell and are the areas of closest approach between the cell and the substrate.

Other authors have reported similar distributions of adhesions in other cell types, for instance in cultured chick epidermal cells (Vaughan and Trinkaus, 1966). Macrophages, however, were reported by Chambers and Fell (1931) to differ from many other cells in this respect, and adhere more generally to the substratum.

Harris (1973) discusses the distribution of these attachments in moving fibroblasts. Since adhesion is restricted to the periphery of the cell and new attachments are continually being formed at the leading edge of a moving cell, there must be detachment of previously formed adhesions occurring a short distance inward from the margin and at the trailing edge of the cell. Harris showed that spread fibroblasts are under tension and that this tension is important for marginal retraction. As cells move in one direction the distance between adhesion zones at the rear of the cell and those at the leading edge increase, causing increased tension in the cell such that eventually the attachments at the rear of the cell give way, and there is retraction of the trailing end of the cell.
As cells move across their substrate they exert a rearward tractional force on the external substrate, believed to be generated by the cytoplasmic actinomyosin network. This is a very weak force exerted over an area too small to be studied by conventional techniques. Harris and his colleagues (1980) came up with an inventive approach to this problem. They realised that if cells are cultured on the surface of an elastic material weak enough to be visibly distorted by the small tractional forces exerted by crawling cells and if the elastic properties of the substratum can be measured, cellular tractional forces could be quantified. Silicone rubber culture substrata provide such a material; they show the elastic qualities required, are biochemically inert, non toxic and transparent.

Silicone rubber can be made by crosslinking linear polymeric chains of polydimethyl siloxane (silicone fluid) under the influence of certain free radical catalysts, heat or strong acids (Lewis, 1973). These silicone rubber sheets are very thin, only about 1μm thick, and the un-cross-linked fluid beneath it serves as a lubricant.

It was hoped that by measuring the forces exerted by macrophages in the presence and absence of MIF, it would be possible to determine whether MIF inhibits macrophage motility by causing macrophages to increase the tractional (adhesive) force they exert on their substrate.
Materials and Methods

2.1 Animals
Young adult male mice, strain C57BL, were used in these studies.

2.2 MIF
MIF was prepared as described in section 1.2.

2.3 Cells
Starch or New Born Calf serum (Gibco) elicited mouse macrophages were prepared as described in section 1.3. Resident mouse PECs were prepared as for elicited PECs but animals were given no treatment prior to sacrifice. This differentiates the two populations of cells. The injection of a suspension of potato starch causes blood monocytes to migrate into the peritoneal cavity forming a population of inflammatory induced or elicited macrophages.

3T3 cells and 3T3 cells transformed by Simian virus 40 (SV40 virally transformed fibroblasts) were kindly donated by Dr. D. R. Critchley, Biochemistry Department, University of Leicester. These cells were maintained in complete medium at 37°C. Cells were also stored frozen in liquid nitrogen in complete medium with 10% DMSO (Dimethyl sulphoxide) and 20% fetal calf serum. L-cells (NCTC Clone 929 - clone of strain L, connective tissue; Mouse. Gibco) were maintained in complete medium at 37°C, subcultured at least every 7 days with 0.25% trysin (Gibco Bio-cult).
2.4 Preparation Of Silicone Rubber Substrate

Distortable sheets of silicone rubber were made by the method devised by Harris and his colleagues (1980). A slide with a large hole through its centre was fixed to a coverslip with wax. 60,000 centistoke dimethyl polysiloxane fluid (Sigma) was spread over the bottom of the well produced. A few sheets were also made using 30,000 centistoke dimethyl polysiloxane fluid. This surface was then turned face down onto the top of a Bunsen-burner flame for 2 seconds. During flaming, a sheen of tiny wrinkles form on the fluid surface, the slide was withdrawn as soon as these were seen and they refiattened to form the desired smooth elastic surface. The Bunsen-burner must be adjusted so that the flame is low and oxygenated barely enough to prevent it being yellow and smoky at the top.

Macrophages or fibroblasts suspended in complete medium were seeded onto this silicone sheet then the chamber was sealed with a coverslip. When necessary MIF was added to the complete medium. The cultures were maintained at 37°C.

2.5 Preparation Of 3-D Floating Collagen Gels

1 volume of cold 0.1M NaOH was mixed with 1 volume of cold 0.2M Na₂HPO₄ in 1.3M NaCl plus a few drops of phenol red. This mixture was passed through a Millipore filter (0.45μm diameter), then added to 8 volumes of commercially prepared collagen, Vitrogen 100 (Flow Laboratories U.K.). It was found necessary to make this solution fresh each time it was required to prevent denaturation of the collagen occurring.
2ml of this solution was poured in 35mm tissue culture dishes (Nunc). Gels set within 10-15 minutes incubation at 37°C and were firmly attached to the walls of the culture dish. The gels were then overlaid with 1ml of complete medium and incubated at 37°C for 2 hours before casting. A Pasteur pipette was passed around the perimeter of the gel to gently dislodge it from the walls of the culture dish. The gel floats on the surface of the medium.

2.6 Measurement Of Gel Contraction

Collagen solution was prepared as described in Section 2.5. Rather than incubate cells on the surface of the gel, cells were incorporated into the gel when measurements on their ability to contract the gel were to be made.

Before pouring the 3-D collagen gels single cell suspensions of in vivo elicited macrophages, 3T3 fibroblasts or SV40 virally transformed 3T3 fibroblasts were uniformly distributed throughout the collagen solution by gentle pipetting. Elicited macrophages were plated into gels at densities ranging from $10^5$ to $10^6$ cells per gel. $10^5$ fibroblasts were plated out per gel. Gels containing elicited macrophages were cultured in the presence or absence of MIF.

Gels were incubated at 37°C for 24 hours after they were floated. When contraction occurred it was symmetrical, forming a flat circular collagen disk. Gel diameters were measured using the calibrated linear scale described by Bell et al., (1979).
replicate gels were measured for each experimental determination and the standard deviations were less than ± 0.7mm. The following formula was used to calculate the percentage contraction:

\[
\text{percentage contraction} = \frac{35 - d \times 100}{35}
\]

where \( d \) = the gel diameter at the end of the 24 hour incubation period

Gels were also incubated in the absence of cells to ensure that any contraction observed was not due to collagen shrinkage during incubation.

In order to investigate the morphology of cells on contracted gels, and their effect on the collagen matrix, cells were cultured on top of the gels and observed on a Nikon Diaphot inverted microscope. Preparations were also observed on a Cambridge Stereoscan 100 SEM following processing by the method described in section 2.8.

2.7 Preparation Of 2-D Collagen Gels

A few drops of commercially prepared collagen in solution, Vitrogen 100, was smeared onto coverslips. The collagen was allowed to dry completely in a stream of sterile air.

2.8 SEM (Scanning Electron Microscopy) Of Stimulated Macrophages + MIF On 2-D Collagen Gels

Peritoneal exudates containing elicited macrophages were seeded out onto collagen coated coverslips and incubated at 37°C for 2 hours
before fresh medium was added. MIF was also added at this stage when required.

Cultures were incubated at 37°C for varying time periods before the coverslips were prepared for SEM.

The culture medium was washed off with several changes of PBS, then the cells were fixed in Karnovsky's fixative (2.5% gluteraldehyde; 4% paraformaldehyde; 0.075% anhydrous CaCl₂) for 30 minutes. The Karnovsky-fixed preparations were then washed three times in 0.2M cacodylate buffer (42.8gms Na.cacodylate in 1 litre distilled water, pH adjusted to 7.4 with HCl). The cells were post fixed in 1% osmium tetroxide for 1 hour followed by two 10 minute washes in distilled water and dehydration in ascending concentrations of alcohol. Next the preparations were passed through a series of ascending concentrations of acetone in ethanol before drying from acetone in a Samdri 780 critical point dryer. Dried preparations were coated with gold to 10nm in a Polaron sputter coater, and observed at 40kV on an ISI 60 SEM or a Cambridge Stereoscan 100 at 25kV.

2.9 Measurement Of Cell Attachment And Spreading

Cell attachment was measured for 2 substrates, glass and 3-D collagen matrices. Washed PECs containing either resident or elicited macrophages were suspended in a known volume of complete medium at a known concentration of macrophages. Whenever macrophages were counted an Improved Neubauer haemocytometer (Griffin and George) was used. The cells were allowed to adhere to
the chamber for 30 minutes at 37°C in order that adherent macrophages could be recognised and only these were counted. Macrophages appeared as phase dense cells at varying stages of spreading.

MIF was added to some preparations in order that the following three macrophage states could be investigated:

- resident macrophages
- elicited macrophages
- elicited macrophages + MIF

The PECs were plated out on coverslips or 3-D collagen gels. After incubation for the appropriate length of time at 37°C, the cells in suspension were removed and counted.

The preparations of coverslips with attached cells were washed in PBS, fixed in 2.0% gluteraldehyde for 30 minutes, washed again in PBS then stained with Giemsa (C.I. 15510 Eastman Kodak Ltd, New York C8685). Eastman's Giemsa is made up by mixing 0.5g Giemsa powder with 33ml glycerol at 60°C for 2 hours, then adding 33ml methanol. This stock solution is diluted to 2% using phosphate buffer, pH 6.8 (31.3ml, 0.5M KH₂PO₄ mixed with 22.8ml 0.5M Na₂HPO₄ and diluting to 500ml with distilled water).

The coverslips were stained for 10 minutes in a Coplin jar then the coverslips with stained cells were washed with tap then distilled water before air drying and mounting in XAM (B. D. H. Chemicals Ltd.). Once completely hardened, the slides were observed on a
Zeiss Photomicroscope.

In contrast, following removal of medium for cell counting, cells on 3-D collagen gels were not fixed. Spreading was assessed by observing the living cells by phase contrast on a Nikon Diaphot inverted photomicroscope.

2.10 Behaviour Of Macrophages Seeded Onto Basement Membrane Collagen

Basement membrane collagen from beneath the epidermal cells of Xenopus tadpole tails was kindly prepared by Dr. Alma Swan using the method devised by Overton (1977). The tails were removed from stage 58 tadpoles of Xenopus laevis, they were washed in saline, then frozen and thawed completely in liquid nitrogen three times. The tails were then washed again to remove remnants of epidermal cells, and incubated in trypsin at 37°C for 30 minutes to remove the proteoglycan/glycosaminoglycan lamina rara layer and expose the collagen. This collagen has a much more regular pattern of fibrils than does the collagen in the gels prepared from Vitrogen 100.

In vivo elicited and resident macrophages were seeded onto the basement membrane collagen, incubated at 37°C overnight, fixed in Karnovsky's fixative for 1 hour and prepared for SEM using the method described in section 2.8.

2.11 Measurement Of Macrophage Locomotion

Cultures of elicited macrophages on glass coverslips, 3-D collagen matrices and silicone rubber were prepared, as described above.
Cultures were established 24 hours before the start of the experiment. Photographs of identified macrophages were taken in sequence over a known time period. The photographs were used to analyse the method and speed of movement on the three substrates.

2.12 Effect Of Cell State On Invasion

Resident and in vivo elicited macrophages, in the presence or absence of MIF, were incubated on 3-D collagen gels for 72 hours at 37°C. Preparations were washed in PBS, cut in half, fixed in Karnovsky's fixative for 1 hour. One half was prepared for SEM using the method described in section 2.8, the other half of each preparation was prepared for sectioning as follows.

The preparations of fixed collagen were washed in PBS then dehydrated through a graded ethanol series with 30 minutes at each step to allow the ethanol to fully penetrate the collagen matrices. The preparations were cleared in Histo-Clear for 30 minutes then given three changes of Paraplast at 60°C under vacuum to ensure total infiltration of the Paraplast into the collagen matrices. The collagen matrices were embedded in Paraplast blocks and the Paraplast allowed to set at room temperature overnight.

Each Paraplast block was trimmed and melted onto a chuck then 20μm thick transverse sections were cut off the block in ribbons using an LKB Historange microtome.

These ribbons were cut into short strips and placed onto glass slides thinly smeared with gelatin. The wax strips were floated on
a few drops of water, and the slides placed on a hot plate for a few seconds to allow the wax sections to spread out over the water on the glass slide. Excess water was carefully removed from the slides before they were incubated overnight at 37°C to melt the wax sections onto the slides.

The slides were then prepared for staining. The wax surrounding the collagen sections was dissolved off by 2 two minute changes in xylene or Histo-Clear. The sections were rehydrated by passage through a short alcohol series then distilled water for two minutes. Sections were stained in 1% aqueous crystal violet (G. T. Gurr Ltd.) for at least 15 minutes, rinsed in distilled water then placed in an iodine solution (1% in 2% aqueous potassium iodide solution) for one minute and rinsed again in distilled water. Sections were dehydrated in fresh 70% and 90% ethanol, only 10 seconds in each to prevent destaining, then stained in eosin (G. T. Gurr Ltd. prepared as a 1% solution in 90% ethanol), for one minute, washed in 90% ethanol and dehydrated completely in 100% ethanol for 1 minute. The slides were cleared in xylene or Histo-Clear for 2 minutes, then mounted in XAM and the coverslips allowed to harden onto the slides on a hot plate overnight.

Using this staining method, the collagen stains pink and the cells blue/violet. A Zeiss Photomicroscope was used for observations and an eyepiece micrometer used to measure distances.

2.13 Effect Of Lidocaine On Macrophages

Elicited macrophages in culture on glass coverslips were incubated
at 37°C for up to 30 minutes in complete medium supplemented with 1/6 volume 2% lidocaine-HCl. Lidocaine was obtained from Sigma. Cultures were observed on a Nikon Diaphot inverted Photomicroscope.

2.14 Effect Of MIF On The Cytoskeleton, Analysed Using Indirect Immunocytochemistry

In vivo elicited macrophages were plated out on three coverslips in vitro and incubated overnight in complete medium. MIF was then added to one coverslip for 4 hours. All three coverslips were fixed in 4% formaldehyde in PBS for 30 minutes then given 3 washes in PBS. Cells were permeabilised by incubation in the non-ionic detergent Triton X-100 at a concentration of 0.1% for 15 minutes. Two of the coverslips, including the one which had been incubated with MIF for 4 hours, were incubated with anti-tubulin (Miles Scientific, used as directed by the manufacturer) for 30 minutes at 37°C then given 3 washes in PBS. All three coverslips were then incubated with FITC-Anti-Rabbit IgG (Goat) (Miles Scientific, used as directed by the manufacturer), for 30 minutes, then washed three times in PBS. The coverslips were mounted in glycerol and sealed with clear nail varnish.

Fluorescent preparations were examined on a Zeiss Universal microscope equipped with a Zeiss III RS epi-fluorescence condenser. The source of UV was an HBO/50W high pressure mercury lamp. The epi-fluorescence condenser was fitted with Zeiss filter sets 48 77 02 and 48 77 09. Photographs were taken using a range of exposure times (up to 3 minutes) with Ilford Pan F film (ASA 50) or Technical Pan film 2415 (ASA 25). Phase contrast photographs were
taken on a Zeiss Universal photomicroscope. Paired phase contrast and immunofluorescence micrographs were made.

This procedure was repeated for anti-actin (Miles scientific, used as directed by the manufacturer).

2.15 Effect Of MIF On The Cytoskeleton, Analysis Using TEM (Transmission Electron Microscopy)

Elicited macrophages were seeded onto Thermolux (Lux) coverslips in complete medium. After incubation at 37°C for 1 hour, non-adherent cells were washed off and fresh complete medium added. MIF was added to some of the cultures at this stage. Cultures were incubated for 2 hours prior to preparation for TEM.

Cells were fixed in 4% glutaraldehyde buffered to 7.2-7.4 in Sørensen phosphate buffer (5ml 0.067M KH₂PO₄, 5ml 0.067M Na₂HPO₄, 90ml distilled water, pH 6.8) for 4 hours, then washed three times in buffer and postfixed in 1% osmium tetroxide in phosphate buffer for 1 hour followed by 2 rinses in distilled water and dehydration in ascending concentrations of ethanol. The coverslips were subsequently rinsed three times in propylene oxide then left overnight in 50:50 propylene oxide to Spurr resin. The coverslips were transferred into fresh Spurr for at least 3 hours, then embedded in fresh Spurr and the Spurr allowed to polymerise at 70°C overnight.

Thin (80nm) sections were cut transversely through the coverslips cut on a Cambridge Huxley Ultra-microtome, then placed on EM grids
and stained to achieve contrast in a saturated solution of uranyl acetate for 20 minutes, then rinsed in distilled water. The grids were then stained in lead citrate for 2 minutes and dipped in dilute NaOH before rinsing in distilled water and drying.

The grids were subsequently viewed in an AEI Corinth 275 electron microscope.

Results

2.1 Tractile Forces Of Macrophages And Effect Of MIF

A. Tractile Forces Exerted On A Silicone Rubber Substrate

3T3 fibroblasts were seeded onto 60,000 centistoke silicone rubber sheets to show that the sheets produced in this laboratory are elastic and thin enough to be visibly distorted by fibroblasts in the way described by Harris and his colleagues (1980).

Figure 10 is a phase contrast micrograph of 3T3 fibroblasts which have been spreading on the silicone rubber substratum for 1 hour after trypsinisation. The cells show varying degrees of spreading, from very early stages, when the cell is very rounded, to extreme flattening of the cell. At this stage there are no convincing
Figure 10:

Phase contrast micrograph of 3T3 fibroblasts 1 hour after seeding onto a silicone rubber sheet. Arrow indicates 1 extremely flattened, well spread cell.
Scale bar = 50μm
signs of substrate wrinkling. Following 18 hours' incubation, however, fibroblasts cause pronounced distortion of the silicone sheet, as shown in Figures 11 and 12. Figure 11 shows an individual 3T3 cell which has compressed the rubber layer beneath it into numerous folds. The folds, or distortions, are produced by the cell slowly pulling the rubber sheet centripetally pasts its lower surface and stretching the rubber sheet below. A more complex pattern of substrate distortion is produced when the cells are in groups, as in Figure 12.

Numerous PEC cultures were established on silicone rubber substrates of various thickness. In the presence or absence of MIF, distortion of the substrate was not, or only barely, visible, even on the thinnest sheets and on sheets made with 30,000 centistoke siloxane. This was the case even when large numbers of cells were incubated on the rubber sheet as is shown in Figure 13. Clearly this method is not suitable for measuring tractile forces produced by macrophages.

Following 3 or 4 days incubation of the rubber sheets, macrophages show phase dense lines across their cytoplasm, as shown in Figure 14. These lines may be indicative of distortion of the substrate beneath the cell. The lines are not visible throughout the cell's cytoplasm; they disappear on changing the depth of focus on the microscope as shown in Figure 15.
Figure 11:

Phase contrast micrograph of a 3T3 cell after 18 hours' incubation on a silicone rubber sheet. Arrow indicates compression wrinkles on the substrate produced by the cell.
Scale bar = 50µm

Figure 12:

Phase contrast micrograph of a small group of 3T3 cells. The arrow indicates compression wrinkles on the substrate produced by these cells. Some of the cells are out of the plane of focus.
Scale bar = 50µm
Figure 13:

Phase contrast micrograph of PECs cultured for 18 hours on a silicone rubber substrate. The substrate does not appear to be wrinkled in the way seen in Figures 11 and 12.
Scale bar = 50μm

Figure 14:

Phase contrast micrograph of macrophages incubated on a silicone rubber substrate for 4 days. The arrow indicates lines referred to in the text.
Scale bar = 50μm
Figure 15a:

Phase contrast micrograph of macrophages following incubation for 4 days on a silicone rubber substrate. The arrow indicates the lines referred to in the text.
Scale bar = 50μm

Figure 15b:

Micrograph of the same macrophage as in Figure 15a, taken at a different depth of focus illustrating that the lines shown in Figure 15a do not extend throughout the cell and may be on the substrate beneath the cell.
Scale bar = 50μm
The Contraction Of Collagen 3-D Matrices By Fibroblasts And Macrophages

The starting diameter of gels was that of the dish in which they were cast, 35mm. No gel contraction was observed in control gels containing no cells. When contraction of the 3-D collagen matrix occurred only the radius of the gel was altered, there was no change in the overall shape of the gel. Exclusion of water during lattice contraction causes the disk to lose its transparency and become opaque making it easier to measure.

After 24 hours incubation, gels containing 3T3 cells contracted by 23% and those containing SV40-virally transformed 3T3 cells contracted by 9%. That transformed fibroblasts exert a weaker tractional force relative to normal fibroblasts was also found by Harris et al., (1981).

Gels containing macrophages showed no contraction whether in the presence or absence of MIF, even after prolonged culture of 5 days or more.

When cultured on glass or plastic in vitro, 3T3 cells divide to give monolayers of cells. When cultured on 3-D collagen matrices, 3T3 cells were found to form aggregates of cells. The tractional force exerted by these aggregates of cells is sufficient to cause gel contraction, as described above, and to align the randomly-oriented collagen fibrils in the gel on which they are growing. This is shown in Figures 16 and 17. Clumps of 3T3 cells and the cells emerging from the clumps stress the gel causing
Figure 16:

Phase contrast micrograph of 3T3 cells grown on a 3-D collagen gel which they have caused to shrink. The arrow indicates cells moving along the circumferentially aligned collagen fibrils.
Scale bar = 50\(\mu m\)

Figure 17:

SEM micrograph of 3T3 cells grown on a 3-D collagen gel. The arrow indicates disruption of the collagen gel caused by 3T3 cell traction.
Scale bar = 50\(\mu m\)
radial alignment of many of the collagen fibrils in the area surrounding the clumps and obvious distortion of the substrate. Stopak and Harris (1982) describe this as *tractional structuring* of the gel. 3T3 cells which move out from the cell aggregates are usually monopolar, extending pseudopodia from only one end of the cell. They move along the aligned fibrils away from the clump of cells from which they emerged.

SV40-transformed 3T3 cells form a greater number of much smaller cell aggregates which also align the collagen fibrils, but to a lesser extent as shown in Figure 18. In contrast, Figure 19 shows that macrophages do not cause such obvious alignment of collagen fibrils when they are cultured on a 3-D collagen gel. This may be partly due to macrophages forming much smaller aggregates when cultured on collagen gels than do fibroblasts, as well as each individual cell exerting less traction than do individual fibroblasts.

### 2.1 C Effect Of MIF On Macrophages Cultured On 2-D Collagen Gels

In *vivo* elicited macrophages were incubated for 18-24 hours in complete medium on 2-D collagen gels then fixed and prepared for SEM.

Some macrophages are rounded (Figure 20), presumably stationary, and exhibit small membranous processes or microvilli on their surface.

Observations of live cells have indicated that elongated bipolar
Figure 18:

Phase contrast micrograph of SV40-transformed 3T3 cells on the surface of a contracted collagen gel. The arrow indicates ridges of aligned collagen fibrils along which some cells seem to be moving.
Scale bar = 50µm

Figure 19:

Phase contrast micrograph of elicited macrophages on the surface of a 3-D collagen gel. No alignment of collagen fibres is observed.
Scale bar = 50µm
Figure 20:

SEM micrograph of a rounded macrophage on a 2-D collagen matrix in vitro. The arrow indicates a small cellular process extending towards the substrate.
Scale bar = 10μm

Figure 21:

SEM micrograph of an elicited bipolar macrophage on 2-D collagen. The preparation is tilted by 30° to show that the leading and trailing edges are not raised as far from the substrate as is the middle portion of the cell.
Scale bar = 10μm
cells are migratory cells with a leading and trailing edge. Figure 21 shows that at these points, the cell has closest apposition with the substrate. It has previously been shown that areas of closest contact are also the areas of greatest adhesive strength (Harris, 1973). The surface appearance of the motile macrophage is very similar to that of the non-motile cell as shown by comparing Figure 20 with Figure 21.

Elicited PECs were incubated in complete medium on 2-D collagen for 2 hours then non-adherent cells were washed off and cells were incubated in complete medium plus MIF for periods ranging from 2 hours to 48 hours before they were fixed and prepared for SEM. In agreement with the results of Homma et al., (1982), a large proportion of macrophages treated with MIF for at least 2 hours were found to have enlarged flat and petal-like ruffles on their surfaces (Figure 22), indicating that these structures are formed when macrophages treated with MIF are cultured on collagenous and glass substrates. Homma reports that the proportion of macrophages bearing these large ruffles was highest when macrophages were cultured with MIF for 12-24 hours.

Rounded macrophages in the presence of MIF often appeared to differ from those in the absence of MIF by having more exploratory processes extending to the substrate. This is illustrated by comparing Figure 20 with Figure 23.

The effect of MIF on more spread cells was very interesting, as is illustrated by comparing Figure 21 with Figure 24. In the presence
Figure 22:

SEM micrograph of a rounded, elicited macrophage cultured on a glass coverslip in vitro in the presence of MIF/MAF for 8 hours. The arrow indicates the petal-like ruffles referred to in the text. Scale bar = 10μm

Figure 23:

SEM micrograph of a rounded macrophage, presumably stationary at the time of fixation. It was cultured in the presence of MIF/MAF on 2-D collagen for 4 hours prior to fixation. The arrow indicates the small exploratory processes extending from the cell referred to in the text. Scale bar = 10μm
Figure 24:

SEM micrograph of an elongate macrophage cultured on a 2-D collagen mat in the presence of MIF/MAF for 4 hours prior to fixation. The arrow indicates numerous exploratory processes extending from the cell.

Scale bar = 10μm
of MIF, macrophages extend many more processes towards the substrate. Macrophages treated with MIF also exert some noticeable traction on their substrate, as is illustrated by the aligning of collagen fibres shown in Figure 25. These results imply that MIF may indeed cause increased adherence of macrophages to the substrate. The very small forces involved, however, make it extremely difficult to correlate these observations with quantitative results.

2.11 Effect Of Substrate And Cell State On Adhesion

A known number of resident and elicited macrophages were seeded onto glass coverslips and incubated in complete medium in the presence or absence of MIF. At given times the medium was removed and the number of macrophages in the medium counted to give a crude indication of the percentage of macrophages which remain unattached to the substrate at a given time. It was found that most macrophages adhere very quickly to glass substrates in vitro. At 10 minutes after plating out, between 75% and 100% of in vivo elicited macrophages in the presence or absence of MIF, are attached to the glass and by 30 minutes all elicited macrophages have adhered to the glass. 2% of resident macrophages were found to remain unattached at 30 minutes but by 120 minutes all macrophages have attached to the substrate.

Macrophages were found to take much longer to adhere to the substrate when seeded onto 3-D collagen matrices. If collagen gels were flipped over or new medium added, even after incubation for up to 24 hours between 70% and 90% of macrophages fell off the gel or
Figure 25:

Micrograph showing a macrophage on a 2-D collagen substrate following a 4 hour exposure to MIF/MAF. The cell is rounded and has begun to exert traction on the substrate (see text), apparent by the aligning of collagen fibres in the immediate vicinity of the cell (arrows).

Scale bar = 10μm
were pipetted off with the old medium. This result was regardless of cell state. This result contrasts with that reported for fibroblasts by Allen and Schor (1983) and confirmed here. Fibroblasts attach to collagenous substrates within a 2 hour incubation period.

2.iii Effect Of Substrate And Cell State On Macrophage Spreading

Analogous with the staging of other workers in the field (Rabinovitch and DeStefano, 1973), cells were classified as in stage 1 of spreading when the veil of cytoplasm protruding from beneath the cell body was not wider than \( \frac{1}{4} \) to \( \frac{1}{3} \) of the cell diameter (Figure 26). Cells in stage 2 were more flattened, with a bulging central area giving them a fried egg appearance; veils extending \( \frac{1}{2} \) to 1 of the initial cell diameter (Figure 27). Stage 3 macrophages were maximally spread; their diameter was 2 to 4 times that of unspread cells and their wide thin cytoplasm was often irregular in shape (Figure 27). Nuclei could clearly be seen in stage 2 and 3 cells using phase contrast microscopy.

The effect of cell state on spreading was measured by seeding resident and elicited macrophages onto glass coverslips, incubating the cells in complete medium in the presence or absence of MIF then fixing them at given times and measuring the number of cells in the three stages of spreading described above. Between 250 and 400 cells were measured for each group, that is for each cell state at each of the chosen times. The number of cells in stages 2 and 3 were added together and expressed as a percentage of the total number of cells counted per group. The results are shown in Figure
Figure 26:

SEM micrograph of macrophages on glass *in vitro* in stages 1 and 3 of spreading (see text for definition). Arrow indicates small veil of cytoplasm protruding from beneath the cell body.
Scale bar = 10μm

Figure 27:

SEM micrograph of macrophages in stages 2 and 3 of spreading. The arrow indicates a cell in stage 3 of spreading which has lost the fried-egg appearance characteristic of early spreading and has become bipolar.
Scale bar = 10μm
Figure 28: The effect of cell state on macrophage spreading on glass. The graph shows how the percentage of macrophages in stages 2 and 3 of spreading changes with time. Cells were plated out at zero time. • = percentage of elicited cells in stages 2 or 3 of spreading. ■ = percentage of resident cells in stages 2 or 3 of spreading. ▲ = percentage of elicited cells incubated with MIF in stages 2 or 3 of spreading.
Chi-squared tests performed on the raw data show that at p=0.95 there is a statistically significant effect of cell state on spreading at 30 minutes after plating the cells out. More elicited macrophages are in the later stages of spreading by this time than are either resident macrophages or elicited macrophages cultured in the presence of MIF.

The presence of MIF in the culture medium has reduced by 30% the proportion of cells which achieve stages 2 or 3 of spreading following incubation for 30 minutes.

It is interesting to note that at 120 minutes the percentage of resident or elicited cells in the later stages of spreading is the same as it was at 30 minutes. This is not the case, however, for elicited macrophages in the presence of MIF which continue to spread on glass beyond 30 minutes. At 120 minutes there is no statistically significant difference between the extent of spreading of resident macrophages and elicited macrophages in the presence of MIF.

The experiment was terminated after 120 minutes so it is not known whether in the fullness of time the percentage of elicited macrophages cultured in the presence of MIF in stages 2 or 3 of spreading could reach that of elicited macrophages in the absence of MIF. Clearly MIF reduces the rate at which macrophages spread in vitro on glass substrates.

Regardless of cell state, spreading was much faster and resulted in
much more extreme flattening of the cells when glass rather than collagen was used as the substrate. Figure 29 shows elicited macrophages which have been cultured for 40 hours on a 2-D collagen mat which does not completely cover the glass surface below. The edge of the mat is shown. The macrophages on the glass are generally well spread and bipolar, typical of locomotory cells on glass in vitro. In contrast those macrophages on the collagen mat are rounded or extending only small lamellipodia.

Resident and elicited macrophages were seeded onto 3-D collagen gels. Figure 30 shows a typical culture 10 minutes after seeding the cells onto a gel. The macrophages appear as rounded phase dense cells. No thin veils of cytoplasm can be seen extending from cell bodies. A few of the cells do, however, protrude and retract small lobular processes. The same culture of cells is shown in Figure 31, taken 5 hours later. There is little change in the culture, only there are more cells with protruding lobular processes. After 18 hours' incubation (Figure 32) many of the cells are no longer rounded up but bipolar or multipolar with several regions of pseudopodial extension. Following incubation for 3 days on a 3-D collagen gel many more macrophages are bipolar or multipolar and translocating over the gel (Figure 33). Results were similar for resident and elicited macrophages. Not only do cells plated onto collagenous matrices take longer to spread, they also never spread as extensively as they do on glass. Indeed spreading in the sense described by Rabinovitch and DeStefano (1973) may be an artifact of in vitro culture conditions when glass or plastic is used as a substrate.
Figure 29:

Phase contrast micrograph of PECs on the edge of a 2-D collagen mat (C) and on the glass coverslip (G) surrounding the collagen mat. The cells on the glass coverslip have spread and adopted bipolar and multipolar morphologies, whereas those on the collagen mat, seeded out at the same time and cultured under identical conditions apart from the substrate, have remained rounded up. Scale bar = 50μm
Figure 30:

Phase contrast micrograph of elicited macrophages 9 minutes after seeding onto a 3-D collagen gel. The arrow indicates a cell with extended lobular processes. These processes are extended and retracted rapidly and most cells have a generally rounded morphology.
Scale bar = 10μm

Figure 31:

Phase contrast micrograph of elicited macrophages 5 hours and 16 minutes since seeding onto a 3-D collagen gel. The arrow indicates a cell extending a lobular process. A higher proportion of macrophages appear to have extended lobular processes after a longer incubation period. These processes also seem to be more stable perhaps indicating the formation of cell-substrate attachment sites.
Scale bar = 10μm
Figure 32:

Elicited macrophages 18 hours after seeding onto a collagen gel. Cells are now seen moving over and through the gel. The arrow indicates a multipolar macrophage.
Scale bar = 50μm

Figure 33:

Elicited macrophages 3 days since seeding on a collagen gel. Only a few cells are in the plane of focus.
Scale bar = 50μm
Heath and Hedlund (1984) describe similar behaviour for fibroblastic cells on collagen gels but they report that some fibroblasts adopt a monopolar shape within 2 hours. Here macrophages were found to take much longer to adopt that shape.

2 iv Effect Of Substrate On Macrophage Locomotion

From serial photographs the speed of locomotion of elicited macrophages on glass, silicone rubber and collagen gels was measured. Cells were incubated on collagen gels for at least three days before measurements were begun.

The speed of locomotion was in the range 30-50μm/hour for cells on glass and silicone rubber, and 5-20μm/hour for cells on collagen gels. Macrophages therefore crawl a little slower over collagen gels than over planar glass coverslips or silicone rubber sheets. The results of Heath and Hedlund (1984) show that fibroblasts also move more slowly over collagen gels than on glass. The amount by which speed is reduced by having a collagen rather than glass or silicone substrate is much greater for fibroblasts than was found here for macrophages. Generally, however, fibroblast locomotion is a little faster than macrophage locomotion, whatever the substrate.

The mechanism of locomotion was found to be quite different on the two substrates. On glass and silicone, macrophages extend very broad, flat lamellipodia at the anterior end, bordered by ruffled membrane. The cytoplasm flows forward into this lamellipodium, leaving retraction fibres attached to the substrate at the
posterior end. Eventually these lose their adhesion to the substrate, probably because of tension generated in the cell (Harris, 1973), and the whole cell moves forward.

In contrast, on a collagenous substrate, broad, flat lamellipodia are never seen, instead locomotory processes are pseudopodial or filopodial. But, like fibroblasts, these pseudopodia are blunt ended (Figures 31 and 32). Heath and Hedlund (1984) used TEM to show that similar blunt ended pseudopodia on fibroblasts were lamellar indicating that the leading edges of these pseudopodia retain some of the characteristics seen on glass, where the lamellar leading edges are much broader than in cells cultured on collagen. The major differences observed between cells cultured on glass and collagenous substrates can probably be attributed to a difference in the proportion of the cells surface which is attached to the substrate. In the case of collagenous substrates, the mass of the cell remains raised off the substrate, whereas the cell surface remains in much closer contact with a glass or silicone rubber substrate.

2.5 Behaviour Of Macrophages Seeded Onto Basement Membrane Collagen

Macrophages seeded onto basement membrane collagen did not flatten appreciably, translocate, or show signs of invading beneath the surface of the substrate to the extent observed on 2-D substrates in vitro (Figure 34). This may be due to fixing the cells before the necessary cell-substrate adhesions had formed. An incubation period of 18 hours was sufficient for only a small proportion of macrophages to form the necessary adhesions for macrophage motility.
Figure 34:

SEM micrograph of elicited macrophages (arrow) on basement membrane collagen.
Scale bar = 5μm
on the collagen gels described above, perhaps this is also the case for cells seeded onto basement membrane collagen. Any future investigation should take this into account and allow sufficient time for cells to adhere to such a substrate.

2. vi Effect Of Lidocaine On Macrophages

Since trypsin has no effect on macrophage adhesion, lidocaine, a membrane-acting anaesthetic, is used to detach macrophages from their substrate.

Within a few minutes of exchanging complete medium for lidocaine-containing medium, macrophages stop putting out large pseudopodia, reduce their ruffling activity, and recoil the cell margins leaving retraction fibres along their circumference. Within 10 minutes most large cell processes are withdrawn and numerous retraction fibrils are apparent around the circumference of the cells. Cell membranes become highly blebbled (Figure 35). Rabinovitch and DeStefano (1976) describe these as small petal-like processes. Blebs are preferred here since Rabinovitch and DeStefano's term has been used elsewhere in this thesis to describe a separate process. These processes or blebs are extended and withdrawn during exposure to lidocaine, indicating membrane activity.

Within 10-20 minutes of exposure to the drug, most macrophages are rounded and can be readily detached from the substrate. When replated in medium without lidocaine they readily respread. Rabinovitch and DeStefano report that repeated cycling with
Figure 35:

10 minutes following the addition of lidocaine-HCl to a culture of macrophages. The arrow indicates the blebs referred to in the text.
Scale bar = 50μm
lidocaine has no detrimental effect on the macrophages in culture. The mechanism of action of these drugs is as yet unknown; it is possible that they influence divalent ion flux or some component of the contractile cells' machinery, or the target of action may be metabolic.

2.vi Effect Of Cell State On Invasion

Elicited and resident macrophages were incubated in the presence or absence of MIF on 3-D collagen gels before fixing. Part of each preparation was embedded in Paraplast then thick transverse sections were cut and the cells which invaded the gels were counted and the distance migrated by these cells measured.

A. Qualitative Results

Cells on the surface of the gels in varying stages of invasion were observed by SEM. In the presence of MIF the surfaces of many macrophages are thrown into the petal-like ruffles described earlier (Figure 36), indicating that these structures persist for long periods under culture conditions and when the substrate is collagenous. Previously this effect of MIF has only been described for cells cultured on glass or plastic substrates. Figure 37 shows that these cells also interact closely with their substrate, aligning the collagen fibrils in a 3-D collagen gel in the way already described for a 2-D collagen mat (Figure 25).

In the absence of MIF, elicited macrophages readily invade collagenous matrices (see below). When incubated on collagenous substrates macrophages maintain a rounded morphology and locomote
Figure 36:

A resident macrophage cultured on a 3-D collagen gel in the presence of MIF/MAF. The arrow indicates petal-like ruffles (see text).
Scale bar = 5μm

Figure 37:

Resident macrophages on the surface of a 3-D collagen gel following 72 hours incubation in complete medium plus MIF/MAF. The arrow indicates fibrils which appear to be aligned by the macrophage.
Scale bar = 5μm
by extending large rounded processes (Figure 38) as described in section 2. iv. During invasion, these processes are extended into the collagen gel (Figure 39). The main body of the cell, still on the surface of the collagen (Figure 40), is eventually drawn into the matrix (Figure 41). As macrophages burrow, they disrupt the structure of the matrix, sometimes quite extensively (Figure 42). The collagen matrix, however, appears to regain its structure as the cell burrows deeper into the gel (Figure 43).

Figure 39 shows that macrophages well within the gel still retain a generally rounded morphology and extend filopodia, which are often highly branched, into the gel. It is possible, in most cases, to differentiate between macrophages and any contaminating fibroblasts. The stellate appearance of fibroblasts contrasts with the rounded morphology of macrophages within collagenous gels.

Fibroblasts, both virally-transformed and non-transformed, were also incubated for varying periods of time on 3-D collagen gels to compare their invasive properties with those described above for macrophages. Fibroblasts were found to show many of the same characteristics as macrophages (Figures 44 and 45).

B. **Quantitative Results**

Resident and *in vivo* elicited macrophages were incubated on 3-D collagen matrices for 72 hours in the presence and absence of MIF, then the gels were fixed, embedded in Paraplast, sectioned, and stained sections observed under a light microscope. Between 200 and 560 cells were counted and analysed for each of the 4 groups
Figure 38:

20µm thick transverse section cut through a 3-D collagen gel on which resident macrophages were incubated for 72 hours prior to fixation. Sections were stained with crystal violet and eosin. The arrow indicates a macrophage on the surface of the collagen extending a long process as described in the text.
Scale bar = 10µm

Figure 39:

20µm thick transverse section cut through a 3-D collagen gel on which resident macrophages were incubated for 72 hours prior to fixation. Sections were stained with crystal violet and eosin. The arrow indicates a macrophage within the gel with a long extended cellular processes.
Scale bar = 10µm
Figure 40:

SEM micrograph of an elicited macrophage on the surface of a 3-D collagen gel. The cell (arrow) which appears to be *sinking* into the gel has aligned the surrounding collagen fibrils.
Scale bar = 10\(\mu m\)

Figure 41:

Macrophage (arrow) invading a 3-D collagen gel. The body of the cell remains rounded even after it has been drawn into the gel.
Scale bar = 10\(\mu m\)
Figure 42:

A macrophage incubated on a 3-D collagen gel for 24 hours before fixing. The cell has burrowed into the matrix and is completely covered with collagen fibrils. Arrows indicate areas where the collagen fibrils have been damaged.
Scale bar = 10μm

Figure 43:

Macrophages incubated for 72 hours on a 3-D collagen gel. The arrow indicates a macrophage which has burrowed beneath the surface of the gel. The collagen matrix appears undisrupted.
Scale bar = 10μm
Figure 44:

Section through a 3-D collagen gel on which 3T3 cells were incubated for 48 hours prior to fixation. Arrow indicates a 3T3 cell which has extended a long process into the gel.
Scale bar = 10\mu m

Figure 45:

Section through a 3-D collagen gel. Arrow indicates a 3T3 cell which has burrowed through the gel.
Scale bar = 10\mu m
tested. The percentage of cells which invaded the collagen gels and the average distance migrated by those cells was recorded. The results are given in Table 6 below.

Table 6: Effect of MIF and Cell State on Macrophage Invasiveness

<table>
<thead>
<tr>
<th>Cell state and treatment</th>
<th>Z of total cells invaded ± SEM</th>
<th>Average distance invaded by invasive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resident macrophages-MIF</td>
<td>17.07% ± 1.96%</td>
<td>33.75µm</td>
</tr>
<tr>
<td>Elicited macrophages-MIF</td>
<td>49.40% ± 2.73%</td>
<td>58.95µm</td>
</tr>
<tr>
<td>Elicited macrophages+MIF</td>
<td>5.92% ± 1.00%</td>
<td>73.31µm</td>
</tr>
<tr>
<td>Resident macrophages+MIF</td>
<td>5.18% ± 1.60%</td>
<td>28.10µm</td>
</tr>
</tbody>
</table>

A greater proportion of in vivo elicited cells invade collagen matrices than of resident macrophages. On average elicited macrophages also invade further into the gel than do invasive resident cells. The addition of MIF results in a reduction in the proportion of invasive resident and elicited macrophages, to a level of between 5% and 6% in both cases. Although most cells are prevented from migrating by the presence of MIF, those cells not affected show levels of migration as great or greater than those shown by cells incubated in the absence of MIF. A proportion of those cells which are invasive in the presence of MIF may be fibroblasts with could not be differentiated from macrophages on the basis of morphology.

2. viii Effect of MIF on the Cytoskeleton, Analysed Using Indirect Immunochemistry

Elicited macrophages were incubated overnight in vitro on glass
coverslips. MIF was added to one culture for 4 hours before the cells were fixed and anti-tubulin followed by a fluorescent tag were added. Following preparation, phase contrast/fluorescent paired photographs were taken of the preparations, to investigate the effect of MIF on the microtubule component of the cytoskeleton.

In the absence of MIF, microtubules form networks throughout the cytoplasm (Figure 46). These networks are not as obvious in the cells which have been incubated for 4 hours in the presence of MIF (Figure 47). Short microtubules do, however, appear to be radiating out from the perimeter of the nucleus, and there is a concentration of fluorescent labelling around the periphery of the cytoplasm.

Preparations were also made using anti-actin antibody to investigate the effect of MIF on the arrangement of macrophage microfilaments. Fluorescence was mainly confined to the area around the cell nucleus and a fine network throughout the cytoplasm which extends to the margins of the cell (Figure 48). Fluorescence was less obvious in cells treated with MIF (Figure 49). MIF-treated cells fluoresce much more weakly around the nucleus and networks in the cytoplasm are less obvious. These cells do still fluoresce around their periphery and around vacuoles.

Figure 50 shows a control situation where cells were incubated only in the fluorescent anti-rabbit IgG second antibody.

The normal situation of networks of microtubules and microfilaments
Figure 46a:

Permeabilised macrophages incubated with anti-tubulin then with FITC-anti-rabbit IgG. The fluorescence indicates the complex network of microtubules in the cytoplasm of the macrophage. Exposure time was 30 seconds.

Scale bar = 10μm

Figure 46b:

Phase contrast micrograph of the cells in Figure 46a.

Scale bar = 10μm
Figure 47a:

Macrophages were incubated for 4 hours in MIF/MAF prior to fixation. The cell membranes were permeabilised with Triton X-100 then the cells were incubated with anti-tubulin then with FITC-anti-rabbit IgG. There is a concentration of fluorescence around the periphery of the cells and around the cell nuclei indicating areas of high tubulin concentration. The complex cytoplasmic microtubule network observed in Figure 46a is not apparent. Exposure time was 25 seconds.
Scale bar = 10μm

Figure 47b:

Phase contrast micrograph of the cells shown in Figure 47a.
Scale bar = 10μm
Figure 48a:

Macrophage membranes were permeabilised with Triton X-100, then the cells were incubated with anti-actin and FITC-anti-rabbit IgG. There are bright fluorescent spots in the perinuclear region. The fluorescence also indicates a network of microfilaments in the cytoplasm. Exposure time was 150 seconds.
Scale bar = 10\mu m

Figure 48b:

Phase contrast micrograph of the cells shown in Figure 48a.
Scale bar = 10\mu m
Figure 49a:

Macrophages were pretreated with MIF/MAF for 4 hours then fixed and the cell membranes permeabilised with Triton X-100. The cells were then incubated with anti-actin followed by FITC-anti-rabbit IgG. The fluorescence indicates a concentration of actin around the cell periphery and around cell vacuoles. Exposure time was 120 seconds. Scale bar = 10μm

Figure 49b:

Phase contrast micrograph of the cells shown in Figure 49a. Scale bar = 10μm
Figure 50a:

The membranes of cultured macrophages were permeabilised with Triton X-100 then cells were incubated with FITC-anti rabbit IgG only, as a control. There is uniform low level of fluorescence over the cells. Exposure time was 180 seconds.

Scale bar = 10µm

Figure 50b:

Phase contrast micrograph of the cells shown in Figure 50a.

Scale bar = 10µm
does appear to be disrupted by the presence of MIF. Rather than concentrate in networks within the cytoplasm of the cell, these cytoskeletal elements become concentrated around the periphery of MIF treated cells.

2. ix Effect of MIF on the Cytoskeleton, Analysed Using TEM

Macrophages were incubated on Thermolux coverslips in the presence or absence of MIF then fixed and prepared for TEM.

Figure 51 shows a TEM micrograph of a macrophage incubated in the absence of MIF. The cell is rounded and most of its volume is taken up by the nucleus. Short stubby pseudopodia are present over its surface. An enlargement of the boxed area is shown in Figure 52. It shows the granular cytoplasm of the macrophage, part of the nucleus and a mitochondrion. Cytoskeletal structures were difficult to visualise because of the granular nature of the cytoplasm.

Figure 53 is a montage of TEM micrographs showing a macrophage which has been incubated with MIF prior to fixation. The surface of the cell is thrown into folds and the cytoplasm is full of endoplasmic reticulum. Again, however, the cytoskeleton is not an obvious feature of the cell.
Figure 51:

TEM micrograph of a macrophage cultured on a Thermolux coverslip (C). The arrow indicates the nuclear envelope. The boxed area is shown in Figure 52. (N) = nucleus. The cytoplasm surrounding the nucleus is highly granular and no cytoskeletal elements can be distinguished.
Scale bar = 10μm

Figure 52:

TEM micrograph of boxed area in Figure 51. Even at this higher magnification the granular cytoplasm precludes the identification of cytoskeletal components.
Scale bar = 10μm
Figure 53:

Montage showing a macrophage incubated on a Thermolux coverslip. The cell was incubated in the presence of MIF/MAF prior to fixation. The cytoplasm is rich in endoplasmic reticulum (ER) and vacuoles (V). The arrow indicates the folding of the plasma membrane (seen as petals by SEM).

Scale bar = 10μm
Discussion

Collagen gels do not contract when incubated in the absence of cells. Contraction of gels containing fibroblasts is a mechanical effect of cell contractility and not due to the collagen shrinking (Bell et al., 1979; Stopak and Harris, 1982). As yet the mechanism by which this tension is generated is unclear Bell et al., (1979) found that the cytoskeleton must be intact and functional for gel contraction to take place. Allen and Schor (1983) describe two possible mechanisms by which contraction might occur. Tension might be generated by a change in cell shape, in a manner analogous to muscle contraction. Alternatively cells may exert a tractional, or shearing force on their substrate, which is equivalent to the force by which they spread and propel themselves.

Fibroblasts align themselves by contact guidance along the predominant axis of extracellular fibres. Harris and Stopak (1982) cite evidence to suggest that migratory cells in vivo rearrange and align extracellular matrices, by a mechanism they describe as tractional structuring. By exerting tractonal forces on their substrate, Harris and Stopak suggest that cells are capable of generating a variety of anatomical structures, especially those of connective tissues. They propose that ligaments, muscles and tendons are organised during development by tractional structuring. This mechanism may also be important during tooth eruption and wound healing (Steinberg et al., 1980; Bellows et al., 1981; Bell et al., 1979).
Tractional forces exerted by macrophages are much weaker than those exerted by fibroblasts and increased only slightly by the addition of MIF, manifested by the alignment of fibres immediately surrounding the cell. Neutrophils have also been shown to adhere very poorly to collagen-coated surfaces (Brown and Lackie, 1981), as have lymphocytes (Haston et al., 1982).

In the presence of MIF, macrophages spread at a reduced rate, do not invade collagenous matrices and extend numerous extracellular processes from their surfaces. The surface of macrophages not treated with MIF is much smoother. The lymphokine/s used here also cause extensive ruffling of the surface membranes. This may result from a lymphokine with MAP activity since ruffling is known to be correlated with macrophage activation. Macrophages treated with bacterial lipopolysaccharide or muramyl dipeptide, show the same feature (Homma et al., 1982). Ruffling is abolished by vinblastine, which causes macrophages to round up, and by cytochalasin B, which causes them to flatten, indicating that the cytoskeleton is important in determining the shape and appearance of the cell. The lymphokines used here probably also affect the cytoskeleton of macrophages. Some evidence for the disruption of microtubules and microfilaments was cited, but more work is necessary to determine the exact effect MIF has on the cytoskeleton. Purified MIF should be used in future studies since some of the surface effects may be caused by lymphokines other than MIF which are contaminating the crude preparations used here.
Cell state was found to affect macrophage behaviour quantitatively. Elicited macrophages spread on glass substrates more extensively than do resident macrophages. Also, a much higher proportion of elicited macrophages show invasive properties, and invade further into collagen gels than do resident cells. This should not be surprising since macrophages which accumulate at sites of tissue damage are predominantly elicited cells, newly emigrated from the blood and differentiated from monocytes. They are not the product of proliferation of resident cells. By definition, therefore, elicited macrophages are cells capable of responding to chemoattractants and invading through connective tissue.

Interestingly, it was shown here that the mechanism by which macrophages burrow into collagenous matrices is very similar to the mechanism used to migrate over the surface of such substrates. It would be interesting to investigate the effect of incorporating a chemoattractant into the experimental system. This might increase the proportion of invasive elicited, and perhaps also resident, cells. Chemoattractants have been shown to cause a change in cell shape (Smith et al., 1979; Cianciolo and Snyderman, 1981), and to enhance locomotion (Snyderman and Goetzl, 1981) of leucocytes.

Heath and Hedlund (1984) describe the differences between the locomotory behaviour of chick embryo fibroblasts moving on glass coverslips and in fibrillar collagen gels as rather trivial, although superficially, fibroblasts appear very different on 2-D and 3-D substrata. The broad flattened leading lamella characteristic of fibroblasts moving on glass are not seen in cells
moving in gels. Indeed the term leading lamella was introduced by Ingram (1969) to describe the appearance of fibroblasts on glass substrates in vitro. Instead several long narrow pseudopodia with only small lamellae at their ends extend from fibroblasts in collagen gels. Since lamellae do occur, and fibroblasts retain a dorso-ventral flattening in gels, Heath and Hedlund believe that the structural differences are only ones of scale. They attribute the reduced rate of locomotion observed on collagen gels to extending processes failing to form adhesions with the collagen and not due to any fundamental difference in the way cells on the two substrates extend pseudopodial processes.

Here it is suggested that although there are fundamental characteristics shared by motile cells on all substrates the study of cells on a plane inert substrate is prone to artifactual conclusions (Haemmerli et al., 1983). The evidence presented here show that the morphology, adhesion and locomotion of macrophages is affected by substrate. The phenomenon of cell spreading was found to have no meaning when cells were plated on collagen gels in vitro.

More important is the effect of substrate on cell function. Macrophages have been ascribed numerous functions. Many of these have been deduced from studies undertaken at least partly in vitro on glass or plastic substrates, but how is cell behaviour affected by the substrate? When monocytes are cultured on glass or plastic substrates in vitro, their behaviour and morphology remains the same throughout the incubation period. If, however, they are
cultured on a collagenous substrate in vitro they differentiate into macrophage-like-cells, a very fundamental effect of substrate.

Although fraught with problems, the effect of substrate on cellular functions must be fully investigated since behaviour observed on glass or plastic may have little bearing on events in vivo.

Interactions of the macrophage with its substrate may profoundly alter protein synthesis and secretion and the expression of some cell surface components. Arend and Ragsdale (1981) found that human monocytes plated onto a plain fibrin substrate slowly synthesise and secrete plasminogen activator. In contrast, monocytes plated onto fibrin substrates with adherent immune complexes were inhibited from the secretion of plasminogen activator and exhibited a burst of release of plasminogen-independent fibrinolytic enzymes. Arend and Ragsdale also report that the expression of Fc and C3 receptors is affected by substrate. Monocytes were plated on substrates containing different amounts of adherent immune complexes. A greater degree of Fc receptor loss was observed after plating on the higher amounts of complexes. This effect was most marked when younger monocytes were used; monocytes maintained in culture for three to seven days required more complexes to reach the reduction in Fc receptor observed for younger monocytes. A loss of C3 receptors was also observed when monocytes were plated onto complexes and complement.

An investigation into the effect of substrate on macrophage
cytotoxicity was carried out here and its outcome is reported later in this thesis. Macrophages have recently been implicated as effector cells in an immune surveillance theory, so it is important that macrophage cytotoxicity is fully understood and that results obtained in vitro reflect the in vivo situation as closely as possible.
CHAPTER 3

Functional Differentiation Of Peritoneal And Bone Marrow Derived Macrophages.

Introduction

Several fundamental questions are raised by the heterogeneity described in numerous studies of the diverse biological properties of cells belonging to the mononuclear phagocyte system (Unanue, 1972; Forster and Landy, 1981; Walker, 1976). The mononuclear phagocyte system is marked by both vertical heterogeneity related to organ site and horizontal heterogeneity within organ site (Lee, 1980; Hopper et al., 1979). There is much debate as to the origin of this diversity. Are macrophages one population or are there a number of developmentally distinct lineages? Are functional differences related to different macrophage sublines or to different differentiation stages? Can one macrophage be activated, or stimulated, to perform only a very limited range of functions? Or can one macrophage be activated for secretory, anti-tumour, anti-microbial and other functions at the same time? To answer these questions it is essential that there is further understanding of the degree of commitment of cells of the mononuclear lineage at different stages of their developmental or life span, and of the environmental influences to which they are susceptible during their differentiation from promonocytes in the bone marrow to mature tissue macrophages.
There are numerous examples of horizontal heterogeneity, or differences in the properties of cells that have followed different pathways of development. Macrophages are present in every tissue in the body and exhibit regional differences in their morphology, biochemistry and function. Notably comparisons have been made between alveolar and peritoneal macrophages because of the ease with which large numbers of such "free" macrophages can be obtained from the lungs and the peritoneum. Metabolic differences between these two populations of cells have been clearly defined. Alveolar macrophages rely primarily on oxidative metabolism, peritoneal macrophages on glycolysis (Leake et al., 1964; Karnovsky et al., 1970). There is a clear distinction between these two populations morphologically (Leake and Heise, 1967; Bennett, 1966), and in general, the lysosomal enzyme content of alveolar macrophages is higher than that of peritoneal macrophages (Pavillard and Rowley, 1962; Cohn and Wiener, 1963a). Despite their higher content of lysosomal enzymes, however, alveolar macrophages do not destroy bacteria as readily as do peritoneal macrophages (Pavillard and Rowley, 1962; Pavillard, 1963). Results presented in this chapter confirm this finding, that a high level of lysosomal enzyme activity does not necessarily correlate with a high level of phagocytic activity.

Cohn (1964) found that alveolar macrophages are more active in the breakdown of bacterial antigens but less able to cause antibody formation from such antigens than are peritoneal macrophages. Following antigen ingestion and "processing", peritoneal, but not
alveolar macrophages, make RNA preparations with immunogenic activity (Gottlieb, 1968).

Peri et al., (1982) found considerable heterogeneity in the capacity of mononuclear phagocytes from diverse anatomical sites to mediate direct or antibody-dependent cytotoxicity. Human macrophage populations were also found to have a heterogeneous effect on natural killer activity. Bronchoalveolar macrophages inhibit natural killer activity at a much lower suppressor:effector ratio than do blood monocytes or peritoneal macrophages. Pulmonary alveolar macrophages share many properties with mononuclear phagocytes from other tissues, but have distinctive structural, metabolic and functional characteristics.

Functional heterogeneity of macrophages from several different tissues is reviewed by Walker (1976).

Functional variation has also been described within macrophage populations. For instance, McIntyre et al., (1967) found that within a population of macrophages some cells were less bactericidal than others. The proportion of the population capable of antibacterial activity can be increased by specific immunisation or by nonspecifically stimulating the host with agents such as BCG (Bacillus Calmette-Guérin), which result in marked morphological and enzymatic changes within the population of cells (Blanden, 1968).

Resident and inflammatory-elicited macrophages may represent two
separate subpopulations of cells. They can be distinguished on the basis of their responsiveness to a serum protein, macrophage stimulating protein (MSP). Addition of MSP to the culture medium enhances mouse resident macrophage responsiveness to chemotacticants but has no effect on the chemotactic response of inflammatory exudate macrophages. Resident cells are however, affected during peritoneal inflammation since no MSP responsive cells are recovered during peritoneal inflammation (Leonard and Skeel, 1981).

Resident and inflammatory-elicited macrophages can also be distinguished by the pattern of ecto-enzyme expression on the cell surface. Ecto-enzymes are studded over the outer face of the plasma membrane of all cells. They are protein receptors, binding sites, recognition structures, and surface antigens and are found among the various general classes of cell surface components. Recently the pattern of ecto-enzyme expression on the cell surface has been used to define cells in different stages of mononuclear phagocyte differentiation and to illustrate the heterogeneity of phagocyte populations (reviewed by Edelson, 1981). Wachsmuth (1975) suggested from data on enzyme activity of resident and inflammatory-elicited macrophages, that the inflammatory response involves cellular maturation or differentiation.

The biochemical and functional responses of mononuclear phagocytes vary according to the nature of the stimulus to which they are exposed and are influenced by the kind of stimuli they have been exposed to previously. Peritoneal macrophages maintained in
culture media containing high concentrations of newborn calf serum show large increases in lysosomal enzyme activity (Cohn and Fedorko, 1969), accompanied by increased rates of pinocytosis (Con and Parks, 1967; Davis et al., 1973), in the absence of any DNA synthesis (Cohn and Fedorko, 1969). Taylor et al., (1971) suggest these responses may be triggered by antibody present in the newborn calf serum.

Morphological and functional responses of macrophages depend to a large extent on the previous stimuli to which these cells have been exposed. Mononuclear phagocytes mediate many of their diverse functions through secretory products which are not all secreted in response to the same stimuli. The nature of the stimulus presented to the mononuclear phagocytes often dictates the nature of the response of the cell (reviewed by Davies et al., 1977). Neumann and Sorg (1981) found that on exposure to lymphokines, macrophages secrete interferon but that various macrophages differ considerably in their response. Macrophages are also heterogeneous with respect to plasminogen activator production. Neumann and Sorg found that LPS (bacterial lipopolysaccharide) induces the production of interferon, whereas ConA and PMA stimulate plasminogen activator secretion.

Karnovsky and Lazdins (1978) suggest that macrophages are composed of different subpopulations which differ in tissue distribution, morphology, enzymatic content, phagocytic capacity, bactericidal activity and the expression of membrane receptors (Ia, Fc, C3). Mononuclear phagocyte heterogeneity exists in density as well as
function (Walker, 1976; Weinberg et al., 1978). Large, but not small, peripheral blood monocytes are Fc receptor positive and capable of lysing human and chicken erythrocyte targets in antibody dependent cell cytotoxicity assays (Norris et al., 1979). Normann and Weiner (1981) used elutriation centrifugation to separate peripheral blood monocytes and found that the small monocytes caused all of the natural tumour cytotoxicity of the unfractionated population. These cells were also responsive to activation to enhanced levels of cytotoxicity.

Segal et al., (1981) fractionated thioglycollate elicited peritoneal exudate cells on discontinuous BSA gradients and found two morphologically distinct major subpopulations of macrophages. One of these populations was found to contain heavily granulated cells with a large cytoplasm, a relatively small and dense nucleus and a ruffled plasma membrane. This population was also enriched for Ia+ cells. The second population comprised cells which contained few if any granulated elements and had a large, distinct nucleus and a relatively small cytoplasm. This population did not contain Ia+ cells and was found to display much higher endocytic activity in a SRBC (sheep red blood cell) assay than did the other population which was more potent in antigen presentation.

These data suggest there is functional heterogeneity among macrophage subpopulations.

Domzig and Lohmann-Matthes (1979) separated macrophages of different stages in their life history and found that cell function
depends on maturation stage. They found that the promonocyte, a nonadherent, nonphagocytic macrophage precursor cell, is a highly potent cytotoxic effector cell against antibody-coated tumour targets, but is totally inactive as an effector cell in lymphokine-induced macrophage-mediated cytotoxicity.

The evidence suggests, therefore, that macrophages are a highly heterogenous group of cells, located in various organs and tissues. As already discussed, however, macrophages originate from a common bone marrow derived precursor (Blussé van Oud Alblas and van Furth, 1979). This contradictory evidence is meaningful if environmental influences are found to regulate the properties of this cell series. The evidence given points to this being the case. For instance, the metabolic pathways involved in respiration and glycolysis are apparently influenced by the level of oxygen in the environment and serve to explain the properties of the peritoneal versus the alveolar macrophage.

The specific question asked here is whether one macrophage can be activated for a wide range or only a limited number of functions? In other words, how diverse are macrophages?

A very simple experimental approach was devised to answer this question. A heterogenous population of macrophages was presented with a set of environmental conditions demanding a particular response, then the same population was tested for its response to a second stimulus which demanded a different form of response. The two responses tested were cytotoxicity toward transformed cells and
phagocytosis of yeast cells. These were chosen because they are readily quantified and require very different activities as a response by the macrophage. The method by which macrophages kill or prevent transformed cells from proliferating are as yet not fully understood and will be discussed in Chapter 4, but cytotoxic behaviour results in target cell lysis and is not due to phagocytosis.

Macrophages were tested for acid phosphatase activity at the beginning and end of each test as an independent histochemical assay of macrophage activation state. Acid phosphatase is a lysosomal enzyme and a high level of acid phosphatase activity correlates with the enhanced activity shown by macrophages from immunised hosts. For example it correlates with enhanced phagocytic capacity of macrophages from mice immunised with BCG (Saito and Suter, 1965). Here it is shown that high levels of this enzyme do not necessarily indicate that the cell is capable of a high level of phagocytosis.

In order that the results obtained by this approach can be collaborated, methods must be developed to study homogenous populations of cells. Separation of populations on the basis of cell size or density yield populations with a considerable degree of residual heterogeneity. It is necessary, therefore, to devise methods of establishing clones of macrophages.

Mononuclear phagocytes from bone marrow (Buhles, 1979; Goud and van Furth, 1975; van der Meer et al., 1979), blood (Lin, 1977). lung
(Lin et al., 1975; Reppun et al., 1979), thymus (MacVittie and McCarthy, 1977), spleen (MacVittie and Provaznik, 1978; Stewart, 1979), lymph node (MacVittie and McCarthy, 1977), liver (Chen et al., 1979) and the peritoneal (Lin and Stewart, 1974; MacVittie and Provaznik, 1978; Stewart et al., 1975; van der Zeijst et al., 1978) and pleural (Chu and Lin, 1976) cavities form colonies in liquid and agar media. It is necessary to supplement culture media with macrophage growth factor (MGF), fetal calf serum and/or horse serum.

MGF was first described by Virolainin and Defendi (1967) and its properties have been reviewed by Stewart and Lin (1978). It is an acidic glycoprotein containing sialic acid and has a molecular weight of approximately 70,000 (Tsuneoka and Shikita, 1977). MGF is identical to a subclass of the colony-stimulating factors (Stanley, 1979).

L-cell conditioned medium is a potent source of MGF (Stanley and Guilbert, 1979), and continuous presence of MGF is required to maintain mononuclear phagocyte proliferation (van der Zeijst et al., 1978).

Several of the methods described in the literature for growing mononuclear phagocytes and their precursors in vitro were attempted here, and the resulting clones were morphologically and functionally characterised. The outcome of these experiments is reported here.
Materials and Methods

3.1 Animals
Young adult male mice, strain Balb/c, were used in these studies.

3.2 Fibroblasts
SV40-virally transformed fibroblasts and L-cells were obtained and maintained as described in Section 2.3.

3.3 Preparation of Transformed Cell Supernatant (TSN)
Culture medium in which SV40-virally transformed 3T3 fibroblasts were grown for 4 or more days was collected and stored at -20°C until required.

3.4 Phagocytosis Assay
Baker's yeast cells, Saccharomyces oviformis, were suspended in phosphate buffered saline (PBS). Heat-killed yeast cells were obtained by boiling the yeast cell suspension in PBS for 30 minutes. The cells were then washed in PBS, suspended in PBS at $10^7$ cells/ml and autoclaved at 115°C for 10 minutes. This suspension was used to assay phagocytosis or the yeast cells were washed in PBS and resuspended at $10^7$ cells/ml in complete medium and stored at 4°C until required for an assay.

The medium around macrophage cultures was washed off and macrophages were incubated for 20 minutes at 37°C with 3ml of yeast cell suspension. Cultures were then washed thoroughly with PBS and
fixed in 2% glutaraldehyde or 4% formalin, in PBS for 30 minutes at 4°C or room temperature respectively. Cells were then washed in PBS and stained for 10 minutes at room temperature in a 2% solution of Giemsa prepared as described in Section 2.9.

The coverslips with stained cells were washed with tap water and then distilled water before air drying and mounting in XAM. Once completely hardened, the slides were observed on a Zeiss Photomicroscope and macrophages scored as having phagocytosed 0, 1, 2, 3 or >3 yeast cells.

3.5 $[^3]H$-Thymidine Incorporation Into Target Cells

Exponentially growing SV40-virally transformed 3T3 fibroblasts were subcultured by incubation with 0.5% trypsin in PBS at 37°C for approximately 10 minutes or until the cells detached from the bottom of the culture vessel. The cell suspension was washed with complete medium then resuspended and plated at 5x10^4 cells/ml. To a culture containing 4ml of the cell suspension, 15μl of 37.0 MBq/ml [methyl-$^3$H]-thymidine, specific activity 1.52 TBq/mmol. (Amersham International plc, Buckinghamshire, England) was added. Cells were incubated for 4-7 days at 37°C to allow incorporation of the label into replicating cells.

At the end of the incubation period, the cell culture was washed thoroughly to remove any label not incorporated into cells. Cells were detached from the bottom of the culture vessel by incubation in 0.5% trypsin in PBS at 37°C, washed in medium then suspended at a known concentration in complete medium.
3.6 Cytotoxicity Assay

PECs were obtained from Balb/c mice as described in Section 1.3. Washed PECs containing elicited macrophages were suspended in a known volume of complete medium at a known concentration of macrophages, as described in Section 2.9.

Cells were plated onto glass coverslips and maintained at 37°C for 90 minutes then all non-adherent cells were washed away. Macrophages were then incubated for a given time (8 hours unless stated otherwise) in one of the following treatments.

1. MIF/MAF in complete medium
2. 30% TSN in complete medium
3. 30% TSN + MIF/MAF in complete medium
4. complete medium

MIF/MAF was prepared as described in Section 1.2.

TSN was prepared as described in Section 3.3.

At the end of the incubation period macrophage cultures were washed with complete medium then a known and equal number of \([\text{methyl}^{3}\text{H}]\)-thymidine labelled SV40-transformed 3T3 cells, prepared as described in Section 3.5, were added to each culture. The ratio of effector:target cells was therefore recorded and equal in each case.

Cultures of \([\text{methyl}^{3}\text{H}]\)-thymidine labelled SV40-transformed 3T3 cells in the absence of macrophages were set up at the same initial
concentration as in the co-cultivation cultures described above. These cultures served as controls.

After a given time (72 hours unless stated otherwise), the culture medium was removed, the volume measured and a 10μl sample assessed for specific radioisotope release in 2ml FisoFluor "1" fluid (Fisons) in a Pickard Tri-care liquid scintillation spectrometer. From these two pieces of information it is possible to determine the total number of counts released from the labelled cells. Release of label indicates cell lysis.

The experiment was either terminated at this stage or fresh medium was added and the amount of label released into the culture medium assessed at a later time.

In order that the total releasable label could be measured all the cells in the culture were lysed and the radioisotope released measured at the end of the experiment. Cells were lysed by removing all the culture medium and replacing it with 1ml (sterile) distilled water then incubating the culture at 65°C for 30 minutes, allowing it to cool and counting 10μl samples in the liquid scintillation counter. The total releasable label could then be calculated.

All cultures were established in duplicate or triplicate.

3.7 Assay Of Acid Phosphatase Activity

Enzyme assays were performed on macrophages cultured on glass
A dilute citrate solution was prepared by adding distilled water to citrate concentrate (Sigma, Stock No. 386-1) to give a final concentration of 0.383 mol/litre. pH 5.4. This was stored at -0.5°C until required. The fixative was made by adding 30ml of acetone to 20ml of this dilute citrate solution. Coverslips with attached macrophages were incubated in the fixative for 30 seconds at room temperature, rinsed in deionised water then air dried.

2.0ml of substrate, Naphthol AS-B1 Phosphoric Acid solution (Sigma, Stock No. 386-4), which contains Naphthol AS-B1 Phosphoric Acid, 12.5mg/ml in N,N'-Dimethyl Formamide, was added to a buffer solution. The buffer consisted of 2.0ml acetate solution (Sigma, Stock No. 386-3), in 46.0ml distilled water prewarmed to 37°C, giving a final concentration of 2.5 mol/litre at pH 5.2. The contents of a capsule containing 15mg of purified Fast Garnet GBC salt (Sigma, Stock No. 386-15) was added to this mixture then it was stirred using a magnetic mixer for 30-60 seconds. Immediately following mixing, the solution was filtered through Whatman 54 paper. The coverslips with attached fixed macrophages were incubated in the filtrate for 1 hour at 37°C in the dark. Coverslips were washed in distilled water for 3 minutes, stained in Acid Hematoxylin solution (a solution of Mayer's Hematoxylin, pH 3.3; Sigma, Stock No. 285-2) for 5 minutes, rinsed in distilled water for 3 minutes then air dried. Coverslips were mounted in XAM and allowed to harden onto slides overnight. Preparations were observed on a Zeiss photomicroscope. For each experiment between
300 and 900 macrophages were assessed for acid phosphatase activity.

Prepared macrophages were evaluated subjectively for level of acid phosphatase activity. A scoring system was used whereby cells were graded from 3+ (intense granulation) to 0 (no discernible activity).

3.8 Conditioned Medium

L-cell-conditioned medium was used as a source of MGF for most experiments. L cells were obtained and maintained as described in Section 2.3. 15ml of L cells at 5x10^4 cells/ml were plated in replicate 80cm^2 tissue culture flasks (Nunclon). After 4 days the culture medium was collected and ready for use as conditioned medium.

Fibroblast-conditioned medium was also used as a source of MGF. Embryo fibroblasts were obtained from 14- to 19-day old mouse fetuses. The tissue was teased apart and single cell suspensions at 5x10^5 cells/ml were used to establish cultures. 6ml of the cell suspension was added to replicate 25cm^2 tissue culture flasks (Nunclon). The fibroblasts were cultured in complete DMEM and conditioned medium harvested 7 days later. Conditioned medium was stored at -20°C until required. The fibroblast cultures were subcultured as required and maintained until an adequate stock of conditioned medium was made.
Resident macrophages rarely synthesise DNA or divide \textit{in vivo} or \textit{in vitro}. Macrophages induced by phlogogenic stimuli, however, synthesise DNA and show limited proliferation both \textit{in vivo} and \textit{in vitro}. Proliferation \textit{in vitro} only occurs, however, in the presence of conditioned medium containing MGF (van der Zeijst \textit{et al.}, 1978).

Elicited macrophages were obtained from Balb/c mice by the method described in Section 1.3. Cells were washed then resuspended in 3ml of growth medium to a final concentration of $10^5$ cells/ml. Growth medium consists of DMEM with 20% fetal calf serum, 100mcg/ml kanamycin, 2mM glutamine and 10% conditioned medium. Macrophages were grown on glass coverslips.

Macrophages were incubated for 14 days, during which time the growth medium was replaced every 3-4 days. At 14 days the macrophages were approaching monolayers. Some coverslips were pulse labelled with $[^3]H$-thymidine and autoradiographed to test for DNA synthesis. Other coverslips were assayed for phagocytosis, to ensure that the cells in culture were macrophages. The remaining cells were subcultured and replated at $10^4$ cell/ml in 3ml growth medium. Thereafter macrophages were subcultured before they achieved monolayers.

\section*{3.10 Subculturing Of Macrophages}

Cells were incubated at 37°C for 30 minutes in medium supplemented with $1/6$th volume of 2% lidocaine-HCl. 2ml of this medium was used
for each 35mm dish containing a glass coverslip of proliferating macrophages. The cells rounded up during this incubation period and were removed by a jet of medium from a Pasteur pipette. The suspension of cells was collected by centrifugation (200g for 5 minutes), resuspended in growth medium, counted, appropriately diluted and replated.

3.11 Pulse Labelling With [³H]-Thymidine And Autoradiography

Macrophages grown on coverslips in growth medium were supplemented with 5μCi/mmol [methyl-³H]-thymidine at 41Ci/mmol (Amersham). The macrophages were incubated for 2 hours at 37°C then the coverslips were removed, washed once with phosphate-buffered saline, twice with 5% trichloroacetic acid, and once with ethanol:ether 1:1 by volume. The dry coverslips were mounted onto microscope slides, leaving the cell covered surface of the coverslip exposed.

The slides to be dipped were placed in a glass slide rack together with 2 clean slides. The rack was covered with foil and placed on a hot plate in the dark room. This prewarmed the slides and prevented them from lowering the temperature of the emulsion during dipping. The whole procedure must take place in total darkness. NTB2 emulsion (Eastman Kodak Company, Rochester, New York 14650, U.S.A) was used diluted 1:1 with water. A vial of emulsion was taken from the stock box and immersed in the water bath, preset at 45°C. The emulsion was allowed to melt for 30 minutes, then poured into the dipping chamber, a glass or perspex chamber measuring approximately 8cm × 3cm × 0.7cm. One of the clean, prewarmed slides was lowered into the dipping chamber, held for 10 seconds
then removed, allowed to drain for a few seconds against the edge of the chamber and stood in the drying rack. The remaining slides were dipped then the drying rack, full of slides was placed in front of a small bench fan. The emulsion takes about 1 hour to dry, then the slides were placed into lightight storage boxes.

The slides were exposed for 3 weeks before developing.

In total darkness the box of slides was opened and the slides placed in an empty Coplin jar. Kodak D19 developer made up freshly to half-normal strength, adjusted to 20°C was poured into the Coplin jar with the slides. After 140 seconds the developer was poured off and replaced with distilled water at 20°C. The distilled water was immediately poured off and fixative (Kodak Photostat fixer no. 4) at 20°C was added for 10 minutes. The slides were washed thoroughly with PBS at pH7 then stained with Giemsa for 20 minutes. The stain was washed off with tap then distilled water, the slides air dried and observed under a Zeiss photomicroscope. Photographs were taken using Panatomic X.

3.12 Growth Of Blood Monocytes In Vitro

Immediately following sacrifice by cervical dislocation, Balb/c mice were bled from cardiac punctures and the blood taken up into heparinised (Boots Company Ltd) sterile syringes. An equal volume of PBS was added to the blood collected and the mixture was layered onto 3ml of Ficoll-Paque (Ficoll-Paque comprises a mixture of Ficoll 400, 5.7% w/v and sodium diatrizoate, 9.0% w/v and has a density of 1.077g/ml; Pharmacia Fine Chemicals) in siliconised
Sterilin centrifuge tubes, without mixing the diluted blood sample with the Ficoll-Paque. White blood cells were then separated by centrifugation, 400g for 30 to 40 minutes at 18-20°C. The upper layer of plasma was carefully removed by pipette and discarded. Using a siliconised pipette the white blood cell layer was removed and added to 3 volumes of Ca\(^{2+}\) and Mg\(^{2+}\) free buffer (NaCl, 8.0g/litre; KCl, 0.2g/litre; Na\(_2\)HPO\(_4\), 1.15g/litre; KH\(_2\)PO\(_4\), 0.2g/litre) and mixed gently. The suspension was centrifuged at 60-100g for 10 minutes at 18-20°C, the supernatant removed and the white blood cells resuspended in 6-8ml Ca\(^{2+}\), Mg\(^{2+}\) free buffer. Cells were washed twice then suspended in complete medium in culture dishes pretreated by incubation with fetal calf serum at 37°C overnight. The cultures were incubated for 2 hours then refed with macrophage growth medium, this removes all non-adherent cells leaving only monocytes in culture.

Cultures were refed with growth medium every three to four days.

3.13 Cloning Bone Marrow Cells In Soft Agar

Cloning of bone marrow cells was performed by an adaptation of the method described by Johnson et al., (1983a). Balb/c mice were sacrificed by cervical dislocation and the bone marrow was flushed from each femoral shaft with complete medium. Bone marrow cells were gently pipetted into suspension then washed twice and resuspended in 0.4ml complete medium to which the following mixture was added. 5ml 20% fetal calf serum plus 10% conditioned medium in DMEM containing 100mcg/ml kanamycin and 2mM glutamine, mixed with 1.2ml of 0.3% freshly autoclaved Bacto-agar (Difco Laboratories).
both at 42°C. The agar and culture medium were mixed thoroughly with the cell suspension to give a final concentration of approximately $10^3$ bone marrow cells/ml. 3.5ml of the cell suspension was dispersed into $2 \times 35$mm tissue culture dishes (Sterilin).

After 15 days incubation at 37°C large (for example 2mm in diameter) bone marrow cell colonies were visible. These were picked out, dissociated into single cell suspensions and resuspended in the medium plus agar mixture and incubation continued.

3.14 Cloning Bone Marrow Cells In Liquid Culture

Bone marrow cells were prepared as described in Section 3.13 and plated out at $10^5$ cells/ml in macrophage growth medium with 20% conditioned medium. 3ml of the cell suspension was dispensed into 35mm tissue culture dishes.

By day two of incubation at 37°C some cells were spread over the bottom of the tissue culture flask and some clones of cells were obvious. These cells were believed to be mature macrophages from their morphology and readiness to adhere to and spread over the tissue culture dish. After 3 days' incubation non-adherent cells were removed, washed and resuspended at approximately $10^4$ cells/ml in the growth medium described above. This procedure was repeated 4 days later. At the end of a further 4 day incubation period cultures with adherent clones of cells were refed and the spent medium containing non-adherent cells discarded. Cultures were
refed 3 to 4 days later and then clones were assayed for phagocytic activity. One culture was incubated with MIF for 2 hours then clones tested for acid phosphatase activity and compared with clones not incubated with MIF. Other cultures were incubated with anti-macrophage antibody to show that the mononuclear phagocyte-like-clones were indeed mononuclear phagocytes.

3.15 Identification Of Macrophage Clones Using Indirect Immunocytochemistry

Cultures were washed with PBS then fixed in 4% formaldehyde in PBS for 30 minutes then given 3 washes in PBS. Cultures were incubated with a monoclonal antibody against mouse macrophages raised in the rat. The antibody was purchased from Sera-lab, and diluted 1 in 5 with buffer before use. It reacts with polypeptides of molecular weight 190,000 daltons and 10,500 daltons found specifically on the surface of macrophages and their precursors.

Cultures were incubated with this antibody for 40 minutes at 37°C then washed 3 times in PBS and incubated with FITC-goat anti-rat IgG (kindly donated by Dr. D. R. Critchley) for 40 minutes. The cells were washed three times in PBS, mounted in glycerol, a coverslip placed on top, sealed with clear nail varnish and observed and photographed on a Zeiss photomicroscope fitted with epifluorescence as described in Section 2.14. Paired phase contrast and immunofluorescence micrographs were made.
3.i Effect Of Incubation With SV40-Transformed Cells On Ability Of Macrophages To Phagocytose Yeast Cells

Elicited macrophages were obtained from Balb/c mice and incubated in one of the four treatments described in Section 3.6. At this stage macrophage cultures from each of the treatments were assayed for phagocytic activity as described in Section 3.4, others for levels of acid phosphatase activity as described in Section 3.9. Two further sets of macrophage cultures were presented with unlabelled SV40-transformed 3T3 cells at an effector:target cell ratio known to be cytotoxic from experiments described in Chapter 4. At the end of 64, 72 or 86 hours, the fibroblasts were removed from cultures by trypsin, which is known to leave macrophages attached to the culture vessel, and these macrophages were assayed for phagocytic capacity or acid phosphatase activity.

The effect of a period of co-cultivation with SV40-transformed cells on the ability of elicited macrophages to phagocytose yeast cells is shown in Table 7 below. Figure 54 shows a Nomarski micrograph of elicited macrophages which have been incubated with yeast cells for 20 minutes.
Figure 54:

Nomarski micrograph of elicited macrophages which have been incubated for 20 minutes with yeast cells. Cells which have phagocytosed 0, 1 and several (S) yeast cells are shown. Scale bar = 10μm
Table 7: Effect of Co-cultivation With SV40 transformed Cells on Phagocytosis of Yeast by Macrophages Pretreated With Medium Containing MIF/MAF, TSN, TSN-MIF/MAF or Medium Only

<table>
<thead>
<tr>
<th>Treatment of macrophages</th>
<th>MIF/MAF (±SEM)</th>
<th>TSN (±SEM)</th>
<th>TSN+MIF/MAF (±SEM)</th>
<th>Medium only (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before co-cultivation</td>
<td>72.4 (±4.1)</td>
<td>72.3 (±4.1)</td>
<td>78.1 (±4.1)</td>
<td>64.8 (±4.5)</td>
</tr>
<tr>
<td>After co-cultivation</td>
<td>6.6 (±1.3)</td>
<td>11.7 (±1.8)</td>
<td>10.1 (±1.5)</td>
<td>9.3 (±1.5)</td>
</tr>
<tr>
<td>Control (incubation alone for 3 days)</td>
<td></td>
<td></td>
<td></td>
<td>42.2 (±4.5)</td>
</tr>
</tbody>
</table>

Student's t tests were performed to compare the groups shown in Table 7. There is a highly significant effect of co-cultivation with SV40-transformed 3T3 cells on the ability of macrophages to phagocytose yeast cells. The pretreatment of macrophages with MIF/MAF and/or TSN has no statistically significant effect on this result.

A high proportion, ranging between 60.3% and 82.2% (taking standard error into account), of elicited macrophages phagocytose one or more yeast cells following incubation in vitro in complete medium for 90 minutes then in one of the four treatments described for 60 or 90 minutes. This ability to phagocytose yeast cells decreases dramatically when elicited macrophages are incubated with SV40-transformed 3T3 cells, at an effector:target cell ratio at which macrophages are known to be cytotoxic to transformed cells.
The results shown are for elicited macrophages incubated in vitro for 8 hours in MIF/MAF and/or TSN or in complete medium only before co-cultivation with SV40-transformed 3T3 cells for 64, 74 or 86 hours (results were grouped together since this amount of variation in the length of the co-cultivation period was found to have no bearing on the results obtained), before assessment for phagocytic ability. The proportion of elicited macrophages now capable of phagocytosing one or more yeast cells ranges between 5.3% and 13.5%, taking error into account.

As a control, elicited macrophages were incubated alone in complete medium for 72 hours then assayed for phagocytic ability. The results show that a significantly higher proportion of these macrophages phagocytose yeast cells than do macrophages incubated for the same period of time in vitro in the presence of SV40-transformed 3T3 cells for most of that time. There is also a significant difference between the proportion of macrophages which are phagocytic following 72 hours incubation in vitro as compared to those cells cultured in vitro for only a few hours. As time spent in vitro increases, the proportion of macrophages capable of phagocytosing yeast cells decreases.

These results suggest that the ability of elicited macrophages to phagocytose yeast cells is affected by co-cultivation with SV40-transformed 3T3 cells and by the length of time spent in vitro, to the greatest extent by the former.
Figure 56: The effect of incubation with MIF/MAF and co-cultivation with SV40-transformed 3T3 cells on acid phosphatase activity. The histogram shows the percentage of macrophages which score 2+ or 3+, which indicates a high level of acid phosphatase activity. The results are expressed as the mean number of elicited macrophages which score 2+ or 3+ ± the standard error of the mean (p<0.01). Treatment of elicited macrophages prior to assessment of acid phosphatase activity were as follows:

A=MIF/MAF (8 hours)
B=complete medium only (8 hours)
C=MIF/MAF (8 hours) then co-cultivation with SV40-transformed 3T3 cells (20 or 72 hours)
D=complete medium only (8 hours) then co-cultivation with SV40-transformed 3T3 cells (20 or 72 hours)
E=complete medium only (26 or 72 hours).
In order to determine whether this reduced ability of macrophages to phagocytose yeast cells is due to a decrease in the level of activation for phagocytosis, the level of acid phosphatase activity within macrophages was measured before and after co-cultivation with SV40-transformed cells. In Figure 55 the criteria used to score the level of acid phosphatase activity in each cell are illustrated. The results are shown in Figure 56 above.

The results given in Figure 56 show that rather than causing reduced acid phosphatase activity, co-cultivation with SV40-transformed 3T3 cells increases significantly the level of acid phosphatase activity in elicited macrophages as determined by the Student's t test. The histogram also shows that incubation with MIF/MAF has a statistically significant effect on the level of acid phosphatase activity. Elicited macrophages pretreated with MIF/MAF have a significantly higher level of acid phosphatase activity than those incubated in the absence of MIF/MAF, irrespective of whether or not the macrophages were incubated with SV40-transformed 3T3 cells. Bar E in the histogram shown in Figure 56 shows the control situation, when elicited macrophages are incubated in complete medium for 26 or 72 hours in the absence of MIF/MAF without co-cultivation with SV40-transformed cells. These macrophages show significantly less acid phosphatase activity than any of the other groups, indicating that prolonged culturing in vitro without stimulation results in a loss of acid phosphatase activity.
Figures 55a and b:

Elicited macrophages stained by the method described in Section 3.7 which stains for acid phosphatase activity within the cell. Cells are graded from 3+ (intense granulation) to 0 (no discernible activity).

Scale bar = 10μm
Elicited macrophages were obtained from Balb/c mice as described in Section 1.3, plated at $4 \times 10^5$ macrophages per glass coverslip, maintained at 37°C for 90 minutes and all non-adherent cells washed off. Cultures were then incubated with MIF/MAF for 15 hours. One macrophage culture was assayed for yeast phagocytosis as described in Section 3.4, using yeast cells suspended in complete medium. 83.4% ± 5.6% of the elicited macrophages phagocyted one or more yeast cells indicating that under the conditions described macrophages are capable of a high level of phagocytosis.

Other cultures of elicited macrophages incubated with MIF/MAF then yeast were assayed for acid phosphatase activity before and after a 24 hour co-cultivation period with SV40-virally transformed 3T3 cells. 84.2% ± 7.4% of macrophages scored 2+ or 3+ acid phosphatase activity levels before and 82.0% ± 5.0% after co-cultivation with SV40-transformed 3T3 cells. There was therefore no statistically significant effect of this short co-cultivation period on the level of acid phosphatase activity. The levels of acid phosphatase activity found here cannot be differentiated statistically from that given in bar C of the histogram shown in Figure 56, substantiating the finding that presentation of some forms of stimulation in the micro-environment is correlated with a high level of acid phosphatase activity in the macrophage.
MIF/MAF for 15 hours at 37°C. To one triplicate set of cultures 3ml of a suspension of yeast in complete medium at $10^7$ cells/ml was then added for 20 minutes at 37°C. Both sets of cultures were washed thoroughly with medium and then labelled or unlabelled SV40-transformed 3T3 cells were added, at the same concentration to each culture. Cultures were incubated at 37°C for 70 hours, then the medium was removed, its volume determined and a 10μl sample assessed for specific radioisotope release as described in Section 3.6. Cells in the cultures were lysed, and a sample of the lysate measured on a scintillation counter so that the total releasable label could be calculated.

Control cultures of SV40-virally transformed 3T3 cells at the same concentration as in the experimental situation were established and incubated for 70 hours then the total counts released and remaining determined.

The results are shown in Table 8 below:
Figure 57:

Elicited macrophages incubated for 14 days in MGM.
Scale bar = 50\mu m
Table 8: Effect of Yeast Phagocytosis on Cytotoxicity of $[^2H]$-labelled SV40 Transformed Cells by Macrophages Pretreated With MIF/MAF at 10:1 Macrophages:3T3 Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean cpm ± SEM</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages not incubated with yeast before presentation of 3T3 cells</td>
<td>303,661 ± 50,650</td>
<td>(91%)</td>
</tr>
<tr>
<td>Macrophages incubated with yeast before presentation of 3T3 cells</td>
<td>233,817 ± 10,128</td>
<td>(70%)</td>
</tr>
<tr>
<td>Control 3T3 cells only</td>
<td>249,744 ± 42,108</td>
<td>(75%)</td>
</tr>
</tbody>
</table>

Mean total releasable counts: 333,669 ± 25,030

The results shown in Table 8 suggest that incubation with a suspension of yeast cells reduces macrophage cytotoxicity towards SV40-transformed 3T3 cells. Once macrophages have phagocytosed yeast cells they are less efficient at lysing SV40-virally transformed 3T3 cells, although they still exhibit a high level of acid phosphatase activity.

3.iii Cloning Peritoneal Macrophages

Figure 57 shows elicited peritoneal macrophages which have been incubated in macrophage growth medium (MGM) for 14 days. Clearly these cells are still alive. In contrast, macrophages cultured in complete medium not containing MGF (macrophage growth factor) but otherwise maintained under identical conditions do not normally
survive for 14 days in culture.

Figure 58 shows a culture of elicited macrophages incubated for 14 days in MGM then incubated with yeast at $10^7$ yeast cells/ml for 30 minutes immediately before the micrograph was made. It shows that the cells in culture are highly phagocytic. This, together with cell morphology and the finding that the cells could not be removed from the culture vessel by trypsin, was taken as evidence that the cultured cells were macrophages and that the culture had not been overtaken by contaminating fibroblasts.

Figure 59 shows elicited macrophages which have been grown in MGM for 14 days then pulse labelled with [methyl-3H]-thymidine and autoradiographed, then stained with Giemsa. It shows that a number of cells in the culture synthesised DNA during the 2 hour exposure to [methyl-3H]-thymidine. The conditions described therefore stimulate mitosis of peritoneal macrophages.

Even in the continued presence of MGF, however, it was not found possible to establish clones of peritoneal macrophages in numbers sufficient to test for functional heterogeneity. It was decided, therefore, to attempt to grow clones of blood monocytes for this purpose.

3.iv Cloning Blood Monocytes

Blood monocytes were obtained from Balb/c mice as described in Section 3.12 and grown in MGM. As for peritoneal macrophages, in the presence of MGF, monocytes divide in culture but the number of
Figure 58:

Elicited macrophages incubated for 14 days in MGM then for 30 minutes with $10^7$ yeast cells/ml. Arrows indicate cells containing yeast cells.
Scale bar = 50μm

Figure 59:

Elicited macrophages cultured in MGM for 14 days prior to pulse labelling with [methyl-$^3$H]-thymidine and autoradiography. Arrow points to a heavily labelled cell indicating thymidine incorporation hence DNA synthesis.
Scale bar = 50μm
Unfortunately the small clones grew very slowly in culture so it was decided to compare the phagocytic response of cells within clones and to study the effect of MIF on phagocytosis and acid phosphatase activity by looking at cells from different clones.

One culture was incubated in the presence of MIF/MAF for 2 hours then with yeast for 20 minutes followed by assessment for acid phosphatase activity. The results were compared with those for small clones treated in the same way but not incubated with MIF/MAF. Clones either phagocytosed large numbers of yeast cells (> 8 per macrophage) or none. No clones contained cells which phagocytosed a wide range of numbers of yeast cells. Similarly for acid phosphatase activity, all cells in the small clones studied showed high levels of acid phosphatase activity. No difference, however, in either response was observed between clones incubated in the presence or absence of MIF/MAF, both showed equally high levels of each activity. Perhaps cells were activated to high levels of acid phosphatase activity and phagocytosis by coming into contact and adhering to the glass substrate or yeast cells. Kaplan (1983) found that this causes activation of cultured monocytes.

Figure 60 shows cells belonging to a typical non-phagocytic, non-monomonuclear phagocyte-like clone of cells. The cells shown in Figure 61 belong to small mononuclear phagocyte-like, phagocytic clones. To determine whether the small phagocytic, mononuclear phagocyte-like clones of cells did indeed consist of mononuclear phagocytes, cultures were incubated with anti-macrophage antibody. Figure 62 shows paired phase contrast and immunofluorescent
cloned cells produced was not sufficient for experiments on functional heterogeneity. It was therefore decided to attempt to grow clones of mononuclear phagocytes from their precursors in the bone marrow.

3. y Cloning Mononuclear Phagocytes From Bone Marrow Cells

Two methods were used to grow mononuclear phagocytes from bone marrow precursor cells. These methods are described in Sections 3.13 and 3.14. Both methods yield clones of cells, the latter most successfully.

In liquid culture macrophage-like cells spread over the bottom of the culture flask and form clones of cells within days of establishing the culture. These were believed to be mature bone marrow macrophages since macrophage precursors in the bone marrow have a generally rounded morphology and do not readily adhere to glass or tissue culture plastic (van Furth and Cohn, 1968). In order to remove these from bone marrow cultures only non-adherent cells were subcultured until none of these mature-macrophage-like cells were left. The more poorly adhering bone marrow cells were then allowed to adhere to plates preincubated with fetal calf serum overnight, and clones grown.

Two types of clones were formed, one much faster growing than the other. From their morphology the smaller clones were believed to consist of promonocytes or monocytes from the description given by Domzig and Lohmann-Matthes (1979).
Figure 60:
Clone of non-phagocytic, non-mononuclear-phagocyte-like cells derived from a culture of bone marrow cells.
Scale bar = 50μm

Figure 61:
Small clone of phagocytic, mononuclear-phagocyte-like cells.
Scale bar = 50μm
Figure 62a:

A clone of phagocytic, mononuclear-phagocyte-like cells incubated with anti-macrophage antibody then with FITC-goat anti-rat IgG. The surface membranes of the cells within the clone fluoresce brightly indicating the presence of this antibody.
Scale bar = 10μm

Figure 62b:

Phase contrast micrograph of cells belonging to the clone shown in Figure 62a.
Scale bar = 10μm
micrographs of a small phagocytic, mononuclear phagocyte-like clone of cells which was incubated with yeast cells then exposed to the anti-macrophage antibody. In Figure 63 a cell from a large clone of non-phagocytic cells is shown. The use of this antibody confirms the view that the small, but not the large, clones consist of mononuclear phagocyte cells.

The large clones may be contaminating fibroblasts but are more likely to be non-phagocytic hematopoietic cells, probably granulocytes.

Discussion

The results given in Sections 3.i and 3.ii suggest that mature macrophages become committed along particular pathways depending on external stimuli from the micro-environment surrounding the cell and that the performance of particular functions precludes the cell from performing certain other functions. That macrophages are responsive to their micro-environment is hardly surprising considering all hemopoietic cells differentiate in response to signals from the environment (Golub, 1982).

There are two possible explanations for the functional
Figure 63a:

Cells belonging to a clone of non-phagocytic, non-mononuclear phagocyte-like cells incubated with anti-macrophage antibody then with FITC-goat anti-rat IgG. The complete lack of any fluorescence indicates the absence of this antibody on the surface of cells in this clone of cells.

Figure 63b:

Phase contrast micrograph of a cell belonging to the clone of cells referred to in Figure 63a. Scale bar = 10 \( \mu \text{m} \)
heterogeneity of macrophages described in the literature. One is that there are subpopulations of mononuclear phagocytes as is known for the T-cell/B-cell lineages (Sorg and Neumann, 1981). Another possibility is that bone marrow derived macrophages pass through different maturation stages or intermediate relatively stable phenotypes on their way to maturity and senescence, expressing characteristic functions in each maturation stage (Neuman and Sorg, 1980a). In a review of earlier work, Sorg (1982) concludes that experimental data to date is insufficient to determine whether functional heterogeneity of macrophages is due to subpopulations or phenotypes of different maturation stages. He does, however, postulate the role of the cell cycle in observed macrophage heterogeneity. The model he describes is based on earlier work (Neuman and Sorg, 1980b). He starts with the knowledge that macrophages differentiate from precursors in the presence of colony-stimulating factor. The precursors proliferate intensively and keep on cycling after differentiation into macrophages. Cells therefore accumulate in late $G_1$, lose a series of constitutive functions and pass on to a $G_0$-like state, characterised by the production of fibrinolysis inhibitors. (The functional state of normal resident macrophages should be compatible with $G_0$ in this model, and that of elicited macrophages with either early or late $G_1$, depending on the eliciting stimulus.) The model also proposes that macrophage differentiation in $G_1$ is reversible and that macrophages of various functional states have to go through the bottleneck of late $G_1$ before entering $S$. The model proposes that the functional response of macrophages is determined by where it is in the cell cycle. Thus, macrophages not
responsive to, for example, activation by lipopolysaccharides to kill tumour cells (Hibbs et al., 1977) can be made responsive by preincubation with lymphokines which push macrophages from $G_0$ or early $G_0$ or early $G_1$ into late $G_1$, where they are inducible to kill tumour cells.

This model suggests, as is proposed here, that the micro-environment is extremely important in determining the functional capabilities of the macrophage. It was found here that all cells within a clone of cells all at the same state of differentiation, respond in the same way to particular stimuli. The results given in Sections 3.1 and 3.11 suggest that the response produced is determined by the stimulus presented, indicating that macrophage behaviour is precisely modulated by the micro-environment. This might indicate that the functional capabilities of mononuclear phagocytes is not determined early during their differentiation but that macrophages remain labile until the cells are quite differentiated.

To answer these questions it is necessary to look at the response of single cells, but growing mature macrophages beyond a few passages has been found to be very difficult.

As already described, mouse peritoneal macrophages are normally blocked in the $G_0$ phase of the cell cycle but can be stimulated to initiate DNA synthesis by exposure to conditioned medium containing MGF. Unlike most cell types (Epifanova and Terskikh, 1969), once DNA synthesis has been initiated it is not necessarily followed by
mitosis in the mature macrophage. This has led to difficulties in producing continuous lines of macrophages. Although treatment with, for example, SV40 or polyoma virus can stimulate macrophage DNA synthesis, it is often not followed by mitosis and many tetraploid cells are produced. Only after unusually long latent periods of several weeks are continuous lines of SV40-transformed macrophages isolated and little is known about how similar these lines are to primary macrophages in properties and functions (Gordon and Cohn, 1973).

Abnormalities in macrophage division have also been described in vivo. Spector and Mariano (1975) studied macrophage behaviour in granulomas and describe macrophage division within lesions. Karyotyping proliferating macrophages reveals a remarkably high proportion of chromosome abnormalities, including gaps, breaks, minute pairs and ring forms. The authors suggest that these deformities limit the success of macrophage proliferation. They also linked the formation of multinucleate giant cells (macrophage polykaryons) with the formation of chromosomal abnormalities. They suggest that giant cell formation results from the recognition of the abnormal surface of ageing macrophages arising as a result of the chromosomal abnormalities. Although their hypothesis for macrophage fusion in vivo has since been proven wrong (Chambers, 1977; Chambers and Spector, 1982), the chromosomal abnormalities they observed are not disputed. After formation of macrophage polykaryons the nuclei enter into the mitotic cycle and the cycle is occasionally completed without cytoplasmic division, but more usually by the formation of polyploid nuclei with chromosome
pooling. Once formed, macrophage polykaryons are only viable for a day or so.

Here there was more success with cloning macrophage precursor cells from the bone marrow. When these cells were tested for their response to a variety of stimuli (MIF/MAF, yeast, acid phosphatase reactivity and the presence of anti-macrophage antibody on their cell surface), all the cells within each clone responded in an identical fashion. It is suggested that this indicates that within a clone of macrophage precursor cells, the cells are homogeneous as regards their response to the stimuli tested. Taken together with the results given in Sections 3.i and 3.ii, these results suggest that the functions performed by macrophages are determined to a large extent by environmental factors.

These results should be borne in mind when considering the use of macrophages as effector cells in clinical situations, for instance in the treatment of spontaneous metastasis. The pathogenesis of cancer metastases will be discussed in more detail in Chapter 4; suffice it to say here that they are responsible for most deaths from cancer (Fidler et al., 1978; Sugarbaker, 1979a; Poste and Fidler, 1980), and that macrophages activated to destroy tumourigenic cells are under consideration as potential effector cells in the eradication of metastases.

Macrophages can be activated for tumouricidal behaviour through their interaction with microorganisms and/or their products (Allison, 1979; Hibbs, 1974a). These biologic agents, however.
often cause undesirable side effects, such as granuloma formation and allergic reactions (Allison, 1979) so synthetic compounds are best used instead. One such compound is N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP). MDP is relatively nontoxic yet possesses immune-potentiating activity to activate macrophages in vivo for many functions including cytotoxicity (Chedid et al., 1979; Matter, 1979; Parant et al., 1979). Following parenteral administration, the water-soluble synthetic MDP is unfortunately rapidly cleared (in less than 60 minutes) from the body and excreted in the urine (Parant et al., 1979). In order to overcome this problem an inventive approach was developed involving the use of liposomes (concentric phospholipid vesicles separated by aqueous compartments containing entrapped MDP) (Sone and Fidler, 1981; Fidler et al., 1981). These liposomes can be injected IV whereupon they are phagocytosed by mononuclear phagocytes and the MDP released directly into these cells. In theory the MDP then stimulates macrophage cytotoxic activity. Some success has been reported for this approach in the regression of established spontaneous metastases originating from a subcutaneous murine melanoma, but the results given here suggest that once macrophages have phagocytosed particles they are less capable of cytotoxic activity. It is suggested therefore that future studies of this type should consider that by phagocytosing liposomes, macrophages may become less cytotoxic. It may be, however, that they remain responsive to MDP and that this compensates for any such reduction in the cytotoxicity response. Macrophage tumouricidal behaviour is discussed more fully in Chapter 4, which deals with the effect of
macrophage tumouricidal and cytostatic behaviour.
CHAPTER 4

Effect Of MAP, Effector:Target Cell Ratio and Substrate On Macrophage Cytotoxicity

Introduction

Metastases rather than primary neoplasms cause most deaths in cancer patients. This is largely due to the inaccessibility of metastases to the surgeon and their resistance in the majority of cases to the most aggressive adjuvant therapies available to date.

A metastasis is a neoplastic lesion that arises from a primary tumour at another site with which it is no longer in contiguity. The ability to produce metastases is a property used to characterise tumour cells as malignant rather than benign.

It is now well established that the process of metastasis involves a series of sequential steps in which malignant cells detach from the primary tumour and disseminate to new sites where they proliferate to form new tumour foci (Trinkaus, 1976). Early in metastasis, cells from the primary tumour invade the tissue surrounding the tumour. The mechanism by which these cells become invasive is unknown, although it may involve the release of tissue-destructive enzymes, such as lysosomal hydrolases and collagenolytic enzymes (Sylvén, 1973; Kleinerman and Liotta, 1977; Weiss and Poste, 1976; Poste, 1977; Willis, 1973). Not all
malignant cells, however, release tissue-destructive enzymes or the same range of enzymes (Sylvén, 1973; Kleinerman and Liotta, 1977; Weiss and Poste, 1976; Poste, 1977).

Although some distant tumour foci are established by cells spreading from the primary lesion along preformed mechanical pathways, such as fascia planes, basement membranes and nerve sheaths (Willis, 1973), most tumour spread is via blood vessels and/or lymphatics. Following invasion into either of these systems, tumour cells are either carried passively to distant organs in the blood or lymph, or remain at the site of vessel penetration where they proliferate and continue to shed emboli into the circulation.

Certain tumours consistently metastasise to particular organs, the site of arrest not necessarily due to mechanical factors alone (Willis, 1973; Tarin, 1976). It is not yet known why circulating cells arrest in a wide variety of organs but metastases only arise in a few of these (Sugarbaker, 1979b). Most tumour cells that enter the circulation do not survive to produce metastases. Those that do must arrest, implant in the vessel wall, then extravasate into the surrounding tissue(s), where they form micrometastases. Further growth into clinically important macroscopic metastases requires vascularisation of the lesion to provide adequate nutrition for the proliferating cells. Tumour cells capable of producing angiogenic factors may be necessary for the formation of these lesions. However, following interaction with metastatic cells, host lymphocytes and macrophages produce angiogenic factors
(Folkman and Greenspan, 1975). On interaction with metastatic cells, other host cells, the platelets, release cell growth stimulating factors so that the normal host's homeostatic mechanisms aid the occurrence of métastasés (Poste and Fidler, 1980).

The process of metastasis is highly selective. Only a small proportion of cells from the primary tumour can penetrate the circulation and of these only a few may survive dissemination and arrest. Once extravasated their viability also depends on nutrient availability. The primary tumour has been shown to consist of subpopulations of cells of widely differing metastatic potential (Fidler and Kripke, 1980). Nowell (1976) proposed that only very few of the most highly malignant cells which arise during tumour progression overcome the selective pressures imposed by the host.

Besides heterogeneity in the metastatic potential of cells in the primary tumour, these cells, and those of metastatic lesions, differ in their antigenic properties, biochemical characteristics and response to cytotoxic drugs (Poste and Fidler, 1980). This heterogeneity has important implications for the treatment of metastatic disease, particularly with regard to chemotherapy. Many neoplasms contain subpopulations of cells with differing sensitivities to cytotoxic drugs. Subpopulations sensitive to the treatment masking its true effect. A treatment which limits the growth of a given tumour to a certain extent does not give any insight into the pharmacological susceptibility of the remainder of the tumour load. It is necessary therefore to screen for
therapeutic agents which act against subpopulations with defined metastatic capabilities.

Treatment by specific immunotherapy is hampered by the similar problem of immunological heterogeneity of tumour cells. The search, therefore, for suitable means of eradicating metastases continues.

The role of the immune system in the recognition and destruction of tumour cells remains unclear. Host resistance to tumours was for a long time attributed to the process of **immune surveillance**, whereby susceptible tumour cells are recognised and destroyed by mature immune T-lymphocytes. In order to test the immune surveillance theory, groups of people with an abnormal immune system have been investigated as to whether they show a changed incidence of cancer. People with immune deficiency, whether due to age, inherited defects or the administration of immunosuppressive drugs during transplant operations, are not more susceptible to most of the common cancers, only those cancers affecting the immune system itself. Nor are the common cancers less common in people with an overreactive immune system (Doll and Kinlen, 1970). Also, the incidence of spontaneous tumours in T-cell-deficient nude mice is no higher than in immunocompetent animals (Herberman and Holden, 1978).

It is now generally accepted that apart from T lymphocytes, which were assigned the central role for so long, different broadly based non-induced mechanisms responding promptly to transformed cells are
more directly involved in surveillance against tumours (Herberman, 1980; 1982; James et al., 1977; Levy and Wheelock, 1974; Normann and Sorkin, 1982). Among these effectors, mononuclear phagocytes, natural killer (NK) cells and even polymorphonuclear leukocytes are presently viewed as operationally potent. It still remains exceedingly difficult, however, to evaluate the nature and relative contribution of each of these and other natural effector mechanisms to host tumour resistance. It has become apparent that by cell-to-cell contact or via their secretory products, mononuclear phagocytes have inherent potential to interfere with tumour growth in multiple ways and with varying consequences (reviewed by Keller, 1985).

That the host can recognise tumour cells as foreign has been shown by Klein et al., (1961). Macrophages have been implicated as being involved in this process. It is well known that macrophages are a common infiltrate of tumours and that macrophages isolated from solid tumours can be cytotoxic to tumour cells (van Loveren and den Otter, 1974b). There is also recent evidence suggesting that natural killer (NK) cells and macrophages are closely related (Lohmann-Matthes et al., 1982), and that cells with macrophage surface antigens can exert natural killer activity (Ault and Springer, 1981; Breard et al., 1980; Lohmann-Matthes et al., 1979). It would seem therefore that macrophages may serve as useful cytotoxic effector cells against metastatic cells and as such deserve to be fully investigated with respect to their potential use on a therapeutic basis.
Five separate functional states have been described for macrophages regarding their cytotoxic behaviour towards tumour cells.

**Normal macrophages** - Macrophages from normal, untreated and disease-free mice. These macrophages are not cytotoxic towards tumour cells in vitro whether in a syngeneic (Evans and Alexander, 1970), or an allogeneic (den Otter et al., 1972), situation.

**Immune macrophages** - Peritoneal macrophages isolated from immunised mice. These cells are cytotoxic towards tumour cells, both in an allogeneic (Granger and Weiser, 1964; 1966) and syngeneic situation (Evans and Alexander, 1970).

**Armed macrophages** - Macrophages from non-immunised mice but incubated with SMAF (specific macrophage arming factor). SMAF is a factor released by immune lymphocytes on incubation with specific target tumour cells (Evans and Alexander, 1972a; 1972b; den Otter et al., 1977). Rather than lyse target cells, armed macrophages inhibit their growth (den Otter, 1981).

**Activated-armed macrophages** - Armed macrophages incubated with immune lymphoid cells and the target cells. These cells are lytic towards the tumour-target cells (van Loveren and den Otter, 1974a).

**Activated macrophage** - Macrophages elicited from mice by many substances such as bacterial products (Basic et al., 1972;
Keller, 1973; 1974b), systemic adjuvants (Bruley-Rosset et al., 1976), BCG (Cleveland et al., 1974; Nathan et al., 1976), chronic infections with intracellular parasites (Hibbs et al., 1972a; 1972b; Hibbs, 1974a; Krahlenbuhl and Remmington, 1974; Krahlenbuhl and Lambert, 1975; Krahlenbuhl et al., 1976), viruses (Stott et al., 1975; Rodda and White, 1976), endotoxin and double stranded RNA (Alexander and Evans, 1971), pyrancopolyraer (Kaplan et al., 1974) and other products. Activated macrophages are cytostatic or cytolytic towards tumour cells nonspecifically.

Activated macrophages are cytostatic or cytolytic towards tumour cells nonspecifically.

That cytotoxic macrophages discriminate between normal and neoplastic cells in that they are cytotoxic only towards the latter, has been shown (Hibbs et al., 1972a; 1972b; Hibbs, 1974a; Cleveland et al., 1974; Meltzer et al., 1975; Piessens et al., 1975; Mansell and DiLuzio, 1975). This, together with the knowledge that macrophages are found within the tumour mass (Evans, 1973) has led to numerous investigations on the role of macrophages within solid tumours (Evans, 1977; den Otter, 1981).

Evaluating the information available on macrophage infiltration and cytotoxicity within solid tumours is not straightforward. Numerous investigators have attempted to correlate the proportions of macrophages within a solid tumour with its immunogenicity, the incidence of distant metastases (Eccles and Alexander, 1974; Gaucia and Alexander, 1975), and whether a tumour is progressing or regressing (Moore and Moore, 1973; Russell et al., 1976). Most of these fail to take into account cases which do not fit their
prediction, for instance the finding by Fidler (1975) of non-cytotoxic macrophages within a progressively growing tumour. Another aspect rarely taken into account is that as a result of an immune reaction, tumours can be infiltrated with large numbers of cells other than macrophages and that as the tumour grows the composition of the infiltrate can change (Holden et al., 1976; Wood and Gollahon, 1978). The rejection of a tumour depends on the interplay of many mechanisms, so it should not be surprising that animals may die from tumours which contain a high percentage of cytotoxic macrophages (den Otter, 1981).

As mentioned previously, immune macrophages, for instance peritoneal macrophages from allogeneic mice immunised either with living or irradiated tumour cells, are potent cytotoxic cells (den Otter, 1981). Also, lymphocytes from mice immunised with tumour cells release a specific macrophage arming factor (SMAF) which renders macrophages cytotoxic to the tumour cells (Evans et al., 1972). There is, however, still debate as to whether the specificity of a lymphocyte can be transferred to macrophages by factors. T-cell factors with the capacity to render macrophages cytotoxic have been described as specific (Evans and Alexander, 1972c; Lohmann-Matthes et al., 1973; Pels and den Otter, 1974) or nonspecific (Fidler et al., 1976a; Piessens et al., 1981). The type of sensitisation or the phase of the immune reaction might, however, determine which type of factor is produced (Pels et al., 1984). T-cells immunised against allogeneic tumour cells were shown by Pels et al., (1984) to produce a factor capable of rendering macrophages cytotoxic. They isolated the subpopulation
of T-cells which produced this factor and found it was only produced when these T-cells are triggered by cells bearing H-2d antigens and that macrophages armed with the factor only kill tumour cells bearing H-2d antigens. They also showed that the factor has an affinity for macrophages and the specific sensitising antigen, so they concluded that the factor and the factor-armed macrophages have the same specificity as the sensitised T-cells which produce the factor. If these factors are indeed mainly antigen specific, their use therapeutically is limited as they are only useful against tumours which are immunogeneic.

Antibody-dependent macrophage-mediated cytotoxicity has recently been shown to operate synergistically with a non-specific means of macrophage-mediated cytotoxicity, whereby macrophages are activated by lymphokines to affect the replication and/or viability of tumour cells (Lohmann-Matthes et al., 1982).

The role of mononuclear phagocytes in the control of primary and secondary tumour growth has been reviewed recently (Fidler and Poste, 1982; Fidler, 1982; Keller, 1980a). There is evidence for the presence and efficient operation of spontaneous host antitumour mechanisms in which mononuclear cells are the principal effectors, for instance from work done on a D-12 rat fibrosarcoma model (Keller, 1980b; 1981; 1982a; 1982b; 1983; Keller and Hess, 1982; Keller et al., 1983). That macrophages are the effector cells in this system is substantiated, for instance by finding that killing of the fibrosarcoma cells is abolished by antimacrophage agents such as silica particles, carrageenan, hydrocortisone or tumour
promoters (Keller, 1980b). Stimulation of the host's antitumour reactivity by adjuvants of immunity such as Bacillus Calmette-Guérin (BCG) or by adoptive transfer of C. parvum-induced mononuclear cells, has been shown to cause marked inhibition, and often complete regression, of a variety of experimental tumours (Sadler and Castro, 1978; Keller, 1985). The direct, in vivo, application of lymphokines to experimental tumour model systems has given analogous results (Fidler, 1982).

Lymphocytes stimulated by specific antigen (Churchill et al., 1975), or mitogen (Fidler et al., 1976b), produce a lymphokine, macrophage activating factor (MAF) that activates macrophages for nonspecific tumour cytotoxicity. Resident peritoneal macrophages, however, are relatively resistant to this factor, whereas macrophages from sites of sterile inflammation respond well to such lymphokines (Johnson et al., 1983). Ruco and Meltzer (1978a) carried out in vitro experiments which pointed to the distinct possibility that the importance of inflammation to macrophage activation is that it ensures the local accumulation of blood monocytes which are susceptible to activation unlike resident macrophages. The mechanism by which lymphokines activate such inflammatory-elicited macrophages to nonspecific tumour cytotoxicity remains to be elucidated, and it is this macrophage response that is of interest in this chapter.

Activation of murine mononuclear phagocytes for spontaneous cytolysis of tumour cells results from a complex chain of events as shown by several investigators (Hibbs et al., 1977; Hibbs et al.,
1978; Meltzer et al., 1979; Meltzer, 1981a; Ruco and Meltzer, 1978b; Johnson et al., 1983b; Schultz, 1982). Subthreshold levels of lymphokine preparations have been found to prime inflammatory-elicited macrophages so that they can be triggered by subthreshold levels of a second activation stimulus (Meltzer et al., 1981; Ruco and Meltzer, 1978b; Meltzer, 1981b). Several investigators have described sequences of events whereby MAF-primed macrophages can be activated for cytotoxicity by incubation with lipopolysaccharide (LPS) or muramyl dipeptide (MDP), substances normally present in the cell walls of bacteria, or L-cell interferon (IFN) (Ruco and Meltzer, 1978b; Sone and Fidler, 1980; Schultz, 1982). Since cell culture reagents are frequently contaminated by LPS (Weinberg et al., 1978), the cytotoxicity demonstrated by macrophages incubated with lymphokines in vitro may be due to the action of MAF plus any contaminating LPS. In systems free of detectable endotoxin, lymphokines alone have been shown not to activate macrophages for cytotoxicity (Weinberg et al., 1978; Taramelli et al., 1980; Taramelli and Varesio, 1981; Pace and Russell, 1981).

Ruco and Meltzer (1978b) make the assumption that tumouricidal activity is the ultimate consequence of macrophage activation and that there is a uni-directional temporal sequence of events leading to that ultimate activated state. In order to test this assumption here, the sequence of events leading to optimal activation for yeast phagocytosis was compared with that required for optimal activation for tumour cytotoxicity. Threshold and sub-threshold levels of LPS were used as defined by Schultz (1982) in his
investigation into the synergistic activation of macrophages by lymphokine and LPS for cytotoxicity against P815 mastocytoma cells.

Depending on the functional activity of the effector cells, the actual ratio of effectors to targets, and an array of poorly understood characteristics of target cells, macrophages have the potential to either suppress (Fidler, 1982; Fidler and Poste, 1982; Herberman, 1980), or promote tumour growth (Evans and Eidlen, 1982; Keller, 1980a). Such enhancement of tumour growth can be achieved via various mechanisms such as direct stimulation of growth and/or functional activity and the possibility of the adaptive potential of the tumour cell, the stimulation of angiogenesis in the tumour bed, or indirectly by suppressing the effector functions of other tumour defence mechanisms such as lymphocytes or NK cells. The question of whether products secreted by the virally-transformed target cells used here affects the response of macrophages in a cytotoxicity assay, was investigated.

There is very little consistency in the literature concerning the ratio at which effector cells optimally kill or suppress proliferation of targets of syngeneic, allogeneic and xenogeneic origin. Hibbs (1976) and Keller (1976) found that cytotoxically activated macrophages are able to kill effectively a large array of such target cells at a final ratio of 10 effectors per target. Fidler (1975), however, describes optimal cytotoxicity at a ratio of 100:1 effector:target cells in a syngeneic situation, and 50:1 when the macrophages and target cells are allogeneic. Indeed effective ratios of between 1:1 and 100:1 have been reported (James
et al., 1977). The effect of effector:target cell ratio on both the cytotoxic and cytostatic response of macrophages was investigated here within a syngeneic situation, and the outcome reported in this chapter.

Another aspect of macrophage and transformed cells' interaction investigated here was that of chemoattraction. Inflammatory-elicited macrophages were incubated on one side of a layer of collagen and the proportion of invasive cells and the distance migrated by these cells determined in the presence and absence of transformed fibroblasts on the other side of the collagen layer.

The effect of substrate on cytotoxicity is also reported here, as is the much-debated requirement for direct cellular contact for effective cytotoxicity. Recent studies have reported both that cytolysis requires intimate contact between the effector and target cells (Piessens, 1978; Marino and Adams, 1980a; 1980b; Adams and Marino, 1981; Hamilton and Fishman, 1982), and that lymphokine-activated macrophages can lyse tumour cells at a distance, for example through a filter membrane (Philippeaux and Mauel, 1984). That these findings, and those reported here, indicate that macrophages may have several means of affecting tumour growth will be discussed later.
4.1 *Animals*

Young adult male mice, strain Balb/c, were used in these studies.

4.2 *Fibroblasts*

SV40 virally-transformed and non-transformed 3T3 fibroblasts were obtained and maintained as described in Section 2.3.

4.3 *Lipopolysaccharide (LPS)*

Lipopolysaccharide (phenol extracted from *E. coli*, 026:B6; Sigma), was used either at a concentration of 5µg/ml or 10µg/ml as required. Schultz (1982) found a concentration of 10µg/ml activates murine macrophages to develop cytotoxicity against P815 mastocytoma tumour cells.

4.4 *[methyl-³H]-Thymidine Incorporation Into Target Cells*

SV40 virally-transformed 3T3 fibroblasts were labelled with [methyl-³H]-thymidine as described in Section 3.5. Non-transformed 3T3 fibroblasts were labelled by the same procedure.

4.5 *Macrophages*

Resident and elicited macrophages were obtained from Balb/c mice by the procedure outlined in Sections 2.3 and 1.3 respectively.
4.6 Cytotoxicity Assay

This assay was performed as outlined in Section 3.6.

4.7 Cytostatic Assay

The method used to measure cytostatic activity was adapted from that described by Ball and his colleagues (1972).

PECs elicited by i.p. administration of 0.5ml of a potato starch suspension were obtained as described in Section 1.3. 10⁵ elicited macrophages were plated onto glass coverslips and maintained at 37°C for 90 minutes. All non-adherent cells were washed away, and the macrophages were incubated for a further 8 hours in the presence or absence of MIF/MAF, prepared as described in Section 1.2.

Macrophage cultures were then washed with complete medium and 10⁴ SV40 virally-transformed 3T3 cells added to all but one set of macrophage cultures. A set of cultures containing only the SV40 virally-transformed 3T3 cells was also established.

These cultures were maintained at 37°C and fresh complete medium was added every 3 to 4 days. After 11 days the coverslips were removed and transferred to a sterile glass coverslip holder, each coverslip to a known position in the holder.

The cells were incubated at 37°C in 7ml complete medium to which 10µl of 37.0 MBq/ml [methyl-³H]-thymidine with a specific activity of 1.52 TBq/mmol. (Amersham International plc., Buckinghamshire,
England) was added. Cells which replicate DNA while the [methyl-³H]-thymidine is present will incorporate label into their DNA.

After 60 minutes the coverslips were transferred to a glass coverslip holder containing ice-cold isotonic saline, to quench any unincorporated radioisotope. The saline was then replaced with a 1.5% v/v ice-cold perchloric acid solution which fixes the cells without cell loss and solubilizes most of the acid-soluble radioactivity. Following two further washes with cold perchloric acid, 30% alcohol was added for 10 minutes, to remove lipids and any remaining unincorporated label.

The coverslips were transferred to individual glass Petri dishes in order to measure all the [methyl-³H]-thymidine which was incorporated into cells which replicated during the 60 minute incubation period when radioisotope label was present. 1ml of a 5% v/v perchloric acid solution was placed over each coverslip then all the Petri dishes were placed in an oven set at 80°C for 40 minutes. This heats the perchloric acid to a level which causes the cells to solubilise and so release all incorporated label into the perchloric acid. After a cooling period, 10µl samples of the perchloric acid were assessed for specific radioisotope content in 2ml Fisodfluor "1" fluid (Fisons), in a Pickard Tri-care liquid scintillation spectrometer.

Cultures were established in duplicate.
Using this procedure it is possible to determine the level of cell proliferation over a given time period.

4.8 Phagocytosis Assay
This assay was performed as outlined in Section 3.4 using killed yeast cells suspended at $10^7$ cells/ml in complete medium. Cells were fixed in 4% formalin at the end of the 20 minute assay period and coverslips processed as described in Section 3.4. Prepared slides were observed on a Zeiss Photomicroscope and the number of yeast cells phagocytosed by each macrophage in the test sample were determined. From this the mean number of yeast cells phagocytosed by each cell was calculated.

4.9 Cytotoxicity On A 3-D Collagen Gel
6 collagen gels were prepared as described in Section 2.5. Once the collagen was completely set, $5 \times 10^4$ elicited macrophages were seeded onto 4 of the gels. Macrophages were incubated in complete medium for 30 minutes then in the presence or absence of MIF/MAF at 37°C. At the end of 8 hours the medium was replaced with fresh complete medium containing $10^4$ SV40-transformed 3T3 cells which had been prelabelled with [methyl-$^3$H]-thymidine as described in Section 3.5.

After an incubation period of 64 hours, the culture medium was removed from each culture, the volume measured, and a 10ul sample assessed for specific radioisotope release in 2ml FisoFluor "1" fluid (Fisons) in a Pickard Tri-care liquid scintillation spectrometer. The total number of counts released from the
labelled cells during the incubation period was then determined, release indicating cell lysis.

The total releasable label was determined by measuring the amount of label released during incubation by the prelabelled SV40-transformed 3T3 cells cultured on glass coverslips and adding this value to the number of counts released following cell lysis at the end of the incubation period by the method described in Section 3.6.

4.10 Effect Of Physical Separation By Millipore Filters On Cytotoxicity

Sterile glass rings were placed on top of Millipore filters (pore size 0.45μm; Millipore Corp., France) in sterile culture dishes. PECs containing $2.7 \times 10^5$ elicited macrophages in a small volume of complete medium were placed on top of each Millipore filter inside each glass ring. The cells were incubated for 90 minutes to allow the macrophages to adhere to the Millipore filter, then nonadherent cells were carefully removed with all the medium on the filter and replaced with medium containing MIF/MAF. Incubation was continued for 8 hours and then all medium was removed from the cultures.

To two of the cultures $1.4 \times 10^4$ prelabelled SV40-transformed 3T3 cells in complete medium were added directly on top of the macrophages so that the two cell types were in direct contact. The other filters were flipped over and supported by broken glass coverslips. Glass rings were placed directly on top of two of these filters, and $1.4 \times 10^4$ prelabelled SV40-transformed 3T3 cells
placed inside the ring. Two extra Millipore filters were placed on top of two filters which had been flipped over, such that the glass ring rested on top of a total of 3 Millipore filters. Again 1.4 x 10^4 prelabelled SV40-transformed 3T3 cells were placed inside the ring. Following incubation for 2 hours to allow the 3T3 cells to adhere to the filters, complete medium was added, but not above the level of the glass ring.

Using this procedure the target and effector cells were either in direct physical contact or separated by 2 different distances.

After 72 hours the culture medium was assessed for radioisotope release as described in Section 3.6.

4.11 Effect Of Physical Separation By Collagen On Cytotoxicity

3-D floating collagen gels were prepared as described in Section 2.5. Elicited macrophages were seeded onto collagen gels once they had completely set and were incubated for 24 hours at 37°C, 8 hours of which was in the presence or absence of MIF/MAF. 24 hours was chosen because it was found that if the gels were flipped over in less than 24 hours the macrophages did not have enough time to form attachments firm enough to prevent them from falling off the collagen.

After flipping the gels over, prelabelled SV40-transformed 3T3 cells were placed on top of the gels. Cultures containing gels onto which only prelabelled SV40-transformed 3T3 cells were placed were also established as controls. All cultures were incubated for
64 hours before the culture medium was assessed for radioisotope release as described in Section 3.6.

4.12 Chemoattraction Of Elicited Macrophages Through Collagen Gels Towards Transformed Or Non-Transformed 3T3 Fibroblasts

3-D floating collagen gels were prepared as described in Section 2.5. Elicited macrophages were seeded onto collagen gels once they had completely set and were incubated at 37°C. At the end of 24 hours, gels were flipped over and either SV40 virally-transformed, or non-transformed 3T3 cells were placed on top of the gel. Other gels with only SV40-transformed 3T3 cells were set up simultaneously. All gels were then incubated for 64 hours at 37°C in complete medium, then washed in PBS, fixed in Karnovsky’s fixative for 1 hour and thick transverse sections embedded in Paraplast, sectioned and stained as described in Section 2.12.

Using a Zeiss Photomicroscope, the proportion of each cell type which invaded the collagen gels could be determined. An eyepiece micrometer was used to determine the distances invaded by those cells which penetrated the gels.
Results

4.i Cytotoxicity Towards 3T3 (non-transformed) Cells By Elicited Macrophages Treated With MIF/MAF

Elicited macrophages were incubated for 8 hours with MIF/MAF then for 72 hours with prelabelled 3T3 fibroblasts at a ratio of 10:1 macrophages to 3T3 cells. The experiment was set up in triplicate and the results are presented in Table 9 below.

Table 9: Cytotoxicity Towards Normal 3T3 Cells By Elicited Macrophages Treated With MIF/MAF

<table>
<thead>
<tr>
<th></th>
<th>Mean cpm ± SEM released by labelled 3T3 cells in the presence or absence of MIF/MAF treated macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elicited macrophages</td>
<td>120,161 ± 12,408</td>
</tr>
<tr>
<td>+ MIF/MAF</td>
<td></td>
</tr>
<tr>
<td>No macrophages</td>
<td>126,658 ± 1,497</td>
</tr>
</tbody>
</table>

Using Student's *t*-test there was found to be no significant effect of the presence of activated macrophages on release of label from 3T3 cells. It was concluded, therefore, that under the experimental conditions described here, macrophages activated with MIF/MAF do not destroy non-transformed 3T3 fibroblasts.
Cytotoxicity Towards SV40—Transformed Cells By Resident Macrophages Treated In Vitro With MIF/MAF

Resident macrophages incubated either in the presence or absence of MIF/MAF for 8 hours in vitro were incubated with prelabelled SV40-transformed 3T3 cells at an effector:target cell ratio of 15:1. Cultures of SV40-transformed 3T3 cells alone were set up simultaneously. After 15.5 hours, 41 hours and 64 hours the total number of counts released from the prelabelled SV40-transformed 3T3 cells was determined and the results obtained are presented in Table 10 below. All cultures were established in triplicate.

Table 10: Cytotoxicity Towards SV40—Transformed 3T3 Cells By Resident Macrophages Treated In Vitro With MIF/MAF

<table>
<thead>
<tr>
<th>Pretreatment of macrophages</th>
<th>Mean cpm ±SEM released by prelabelled SV40-transformed 3T3 cells cultured with resident macrophages for 15.5 hours</th>
<th>41 hours</th>
<th>64 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF</td>
<td>35,664±11,244</td>
<td>14,739±1,134</td>
<td>9,672±2,557</td>
</tr>
<tr>
<td>No treatment</td>
<td>15,733±2,834</td>
<td>19,792±3,264</td>
<td>7,033±848</td>
</tr>
<tr>
<td>No macrophages</td>
<td>24,497±1,345</td>
<td>16,173±3,178</td>
<td>12,377±7,400</td>
</tr>
<tr>
<td>Total releasable counts</td>
<td>61,824±5,666</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Using Student's t-test each of the treatments was compared with each of the other two for all three times. There was found to be
no significant difference between any of the treatments at 15.5 hours, 41 hours and 64 hours. From this it can be concluded that resident macrophages are not effective killers of transformed cells, even when preincubated with MIF/MAF.

4.iii Effect Of Pretreatment Of Elicited Macrophages With MIF/MAF, TSN Or MIF/MAF + TSN For 1 Hour On Cytotoxicity

Elicited macrophages were treated with MIF/MAF and/or TSN or incubated in medium only for 1 hour before prelabelled SV40-transformed or non-transformed 3T3 cells were added at an effector:target cell ratio of 10:1. At the end of 72 hours the total number of counts released from lysed fibroblasts was determined and the results presented in Tables 11 and 12 below. The results given in Table 11 are from triplicate cultures. For Table 12 the results for macrophages pretreated with either MIF/MAF and/or TSN were taken together and the mean value for the three treatments is shown.
Table 11: Cytotoxicity Towards 3T3 Cells By Elicited Macrophages

Pretreated For 1 Hour With MIF/MAF and/or TSN

<table>
<thead>
<tr>
<th>Pretreatment of macrophages</th>
<th>Mean cpm ± SEM released by 3T3 cells cultured with elicited macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF</td>
<td>131,104 ± 17,968</td>
</tr>
<tr>
<td>TSN</td>
<td>104,790 ± 10,685</td>
</tr>
<tr>
<td>MIF/MAF + TSN</td>
<td>124,976 ± 10,303</td>
</tr>
<tr>
<td>No treatment</td>
<td>165,848 ± 23,046</td>
</tr>
<tr>
<td>No elicited macrophages</td>
<td>99,111 ± 5,918</td>
</tr>
</tbody>
</table>

Using Student's t-test each of the treatments was compared with each of the other treatments and there was found to be no effect of pretreating the elicited macrophages with any of the treatments for only 1 hour on cytotoxicity towards 3T3 cells.

Table 12: Cytotoxicity Towards SV40-Transformed 3T3 Cells By Elicited Macrophages Pretreated For 1 Hour

With MIF/MAF And/Or TSN

<table>
<thead>
<tr>
<th>Pretreatment of macrophages</th>
<th>Mean cpm ± SEM released by SV40 transformed 3T3 cells cultured with elicited macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>245,568 ± 55,381</td>
</tr>
<tr>
<td>Untreated</td>
<td>216,162 ± 14,908</td>
</tr>
</tbody>
</table>

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There was found to be no significant difference between the number of counts released by SV40-transformed 3T3 cells incubated with elicited macrophages treated for 1 hour or not treated at all (Student's t-test, \( p > 0.05 \)).

4.iv Effect Of Elicited Macrophages Pretreated With MIF/MAF On The Proliferation Of SV40-Transformed 3T3 Cells

Elicited macrophages were incubated in the presence or absence of MIF/MAF for 8 hours then SV40-transformed 3T3 cells were added to give an effector:target cell ratio of 5:1. Cultures of SV40-transformed 3T3 cells were also established alone though at the same initial concentration of cells as for the co-culture experiments. Similar cultures were set up for elicited macrophages.

At the end of an 11 day incubation period macrophages were tested for their cytostatic effect on SV40-transformed 3T3 cell proliferation using the method described in Section 4.7. The effect of co-culture with macrophages on SV40-transformed 3T3 cell proliferation is shown below in Table 13, for 2 experiments.
Table 13: Cytostatic Effect Of Elicited Macrophages Pretreated With MIF/MAF On SV40-Transformed 3T3 Cells

<table>
<thead>
<tr>
<th>Cells in culture</th>
<th>Mean cpm ± SEM incorporated into cells during a 1 hour incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elicited macrophages treated with MIF/MAF + SV40-transformed 3T3 cells</td>
<td>355 ± 51</td>
</tr>
<tr>
<td>Elicited macrophages + SV40-transformed 3T3 cells</td>
<td>405 ± 69</td>
</tr>
<tr>
<td>SV40-transformed 3T3 cells</td>
<td>1,848 ± 120</td>
</tr>
<tr>
<td>Elicited macrophages</td>
<td>15 ± 13</td>
</tr>
</tbody>
</table>

The results presented in Table 13 are from 2 experiments with an effector:target cell ratio of 5:1. The results show that after 11 days in culture elicited macrophages synthesise very little DNA whereas SV40-transformed 3T3 cell cultured alone synthesise a great deal. The results show that at an effector:target ratio of 5:1 both MIF/MAF treated and untreated macrophages inhibit SV40-transformed 3T3 cell DNA synthesis (p < 0.01, Student's t-test). The effect of macrophages on DNA synthesis by SV40-transformed 3T3 cell is therefore a long-term effect and occurs at a relatively low effector:target cell ratio.
4. Effect of Effector:Target Cell Ratio on SV40-Transformed 3T3 Cell Lysis by Elicited Macrophages Pretreated With MIF/MAF

Elicited macrophages were incubated in the presence or absence of MIF/MAF for 8 hours then with prelabelled SV40-transformed 3T3 cells at an effector:target cell ratio of either 5:1 or 40:1 for 72 hours. Cultures of SV40-transformed 3T3 cells at the same initial concentration were established simultaneously. At the end of the 72 hour incubation period the total number of counts released was determined and any cytotoxic effect calculated. The results for 3 experiments are presented in Table 14 below.

Table 14: Effect of Effector:Target Cell Ratio on SV40 Transformed 3T3 Cell Lysis by Elicited Macrophages Treated With MIF/MAF

<table>
<thead>
<tr>
<th>Treatment of macrophages</th>
<th>Mean cpm ± SEM released by prelabelled SV40-Transformed 3T3 cells cultured with elicited macrophages (cytotoxicity expressed as a percent of mean total releasable counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Ratio 5:1</strong></td>
</tr>
<tr>
<td>MIF/MAF</td>
<td>430,659 ± 40,566</td>
</tr>
<tr>
<td></td>
<td>(84.8%)</td>
</tr>
<tr>
<td>No treatment</td>
<td>419,439 ± 60,994</td>
</tr>
<tr>
<td></td>
<td>(71.3%)</td>
</tr>
<tr>
<td>No macrophages</td>
<td>298,040 ± 7,163</td>
</tr>
<tr>
<td></td>
<td>(53.0%)</td>
</tr>
<tr>
<td>Total releasable counts</td>
<td>166,177 ± 2,458</td>
</tr>
</tbody>
</table>

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The results presented in Table 14 were analysed for statistical significance using Student's $t$-test; the $t$-scores are shown below in Tables 15 and 16.

Table 15: Analysis By Student's $t$-test Of The Effect Of Incubating SV40-Transformed 3T3 Cells With MIF/MAF Treated Macrophages At 5:1 Effector:Target Cells (comparison of mean cpm released by each group)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>score (t)</th>
<th>significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF treated v.s. untreated macrophages</td>
<td>0.153 4 d.f</td>
<td>p &gt; 0.005</td>
</tr>
<tr>
<td>MIF/MAF treated macrophages v.s. no macrophages</td>
<td>3.219 4 d.f</td>
<td>0.05 &lt; p &lt; 0.01</td>
</tr>
<tr>
<td>Untreated macrophages v.s. no macrophages</td>
<td>1.977 4 d.f</td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
</table>
Table 16: Analysis By Student's t-test Of The Effect Of Incubating SV40-Transformed 3T3 Cells With MIF/MAF Treated Macrophages At 40:1 Effector:Target Cells (comparison of mean cpm released by each group)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>score (t)</th>
<th>significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF treated v.s. untreated macrophages</td>
<td>3.536 4 d.f</td>
<td>0.05 &lt; p &lt; 0.01</td>
</tr>
<tr>
<td>MIF/MAF treated macrophages v.s. no macrophages</td>
<td>11.274 4 d.f</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Untreated macrophages v.s. no macrophages</td>
<td>5.540 4 d.f</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

At an effector:target cell ratio of 5:1, the t-scores in Table 15 indicate that the amount of label released by prelabelled SV40-transformed 3T3 cells is only slightly increased when MIF/MAF-treated macrophages are incubated with the transformed cells, as compared to the amount of label released by SV40-transformed cells cultured alone. There was, however, found to be no significant effect of culturing SV40-transformed 3T3 cells alone or with untreated macrophages. Nor was any significant difference found between MIF/MAF-treated and untreated macrophages regarding the amount of radioisotope released from the prelabelled SV40-transformed cells with which they were cultured.

The t-scores given in Table 16, however, show that when the
effector:target cell ratio is increased to 40:1 there are significant differences between each of the groups studied. Macrophages are cytotoxic towards SV40-transformed 3T3 cells at this ratio, irrespective of whether or not they are treated with MIF/MAF. Pretreatment of the macrophages does, however, significantly increase the level of cytotoxicity. Cytotoxicity measurements shown in Table 14 were estimated as the mean c.p.m released by three experiments and are expressed as a percent of the mean total releasable counts.

4.vi Effect Of Effector:Target Cell Ratio On Time At Which MIF/MAF Has Its Effect

Elicited macrophages were incubated in the presence or absence of MIF/MAF and/or TSN for 8 hours then with prelabelled SV40-transformed 3T3 cells at an effector:target cell ratio of 10:1, 25:1 or 50:1. After defined intervals, the culture medium was removed and the counts released into it measured. Complete medium was replaced and incubation continued for up to 160 hours. Using this method it is possible to determine whether and when MIF/MAF has its effect at the effector:target cell ratios tested. The results are shown in Tables 17, 18 and 19 below. The amount of radioisotope measured at any given time represents the amount released into the medium since the previous measurement was made.
The results given in Table 17 are from duplicate cultures, and by using Student's $t$-test the results obtained for each macrophage treatment can be compared at each time. After 15.5 hours' and 39.5 hours' co-culture, the amount of radioisotope released from prelabelled SV40-transformed 3T3 cells is significantly greater ($p < 0.01$) when they are cultured with macrophages rather than alone. The pretreatment of macrophages with MIF/MAF, however, has no significant effect ($p > 0.05$). The amount of radioisotope released between 39.5 hours and 63.5 hours by SV40-transformed 3T3 cells is,
however, affected by the pretreatment of macrophages. Macrophages pretreated with MIF/MAF lyse significantly more ($0.05 < p < 0.01$) SV40-transformed 3T3 cells than do untreated macrophages. After 63.5 hours the presence of MIF/MAF treated, or untreated macrophages has no significant effect on SV40-transformed 3T3 cell lysis.

Throughout the co-culture period it is during the first 40 hours that most cytotoxicity occurs so it is not surprising that any increase in cytotoxicity between 40 and 63.5 hours caused by the pretreatment of macrophages with MIF/MAF has little overall effect on cytotoxicity by macrophages.
Table 18: Effect Of Hours Of Incubation On Cytotoxicity By MIF/MAF And/Or TSN Treated Macrophages At An Effector:Target Ratio Of 25:1

<table>
<thead>
<tr>
<th>Treatment of macrophages</th>
<th>Time at which the radioisotope release was measured (since start of co-culture period)</th>
<th>17 hrs.</th>
<th>41 hrs.</th>
<th>112 hrs.</th>
<th>Total at 112 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean cpm±SEM released by prelabelled SV40-transformed JT3 cells cultured with elicited macrophages (cytotoxicity expressed as a percent of the mean total releasable counts)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIF/MAF</td>
<td></td>
<td>115,879±9,279 (30.9%)</td>
<td>134,225±8,742 (30.9%)</td>
<td>158,397±8,531 (30.9%)</td>
<td>408,501±3,006 (94.2%)</td>
</tr>
<tr>
<td>TSN</td>
<td></td>
<td>119,603±8,452 (35.5%)</td>
<td>154,212±3,503 (39.9%)</td>
<td>161,184±5,745 (94.2%)</td>
<td>435,000±12,965 (98.0%)</td>
</tr>
<tr>
<td>MIF/MAF + TSN</td>
<td></td>
<td>128,202±18,960 (29.5%)</td>
<td>128,049±6,527 (29.5%)</td>
<td>179,161±13,517 (94.2%)</td>
<td>435,413±2,499 (98.0%)</td>
</tr>
<tr>
<td>No treatment</td>
<td></td>
<td>97,094±12,455 (33.3%)</td>
<td>144,431±19,270 (33.3%)</td>
<td>125,454±8,127 (33.3%)</td>
<td>366,878±48,716 (84.6%)</td>
</tr>
<tr>
<td>No macrophages</td>
<td></td>
<td>82,232±1,170 (14.3%)</td>
<td>62,179±4,872 (14.3%)</td>
<td>140,962±7,631 (65.8%)</td>
<td>285,372±1,946 (65.8%)</td>
</tr>
</tbody>
</table>

Total releasable counts 433,855 ± 15,544

The results presented in Table 18 for macrophages treated with MIF/MAF and/or TSN were obtained from cultures set up in triplicate. The results for untreated macrophages and SV40-transformed 3T3 cells cultured in the absence of macrophages are from duplicate cultures.

At each time shown in Table 18 the total amount of radioisotope
released was measured and using Student's t-test the results obtained for each macrophage treatment were compared. At 17 hours since the start of co-culture, there is no significant difference between the amount of radioisotope released by prelabelled SV40-transformed 3T3 cells cultured in the presence or absence of treated or untreated macrophages (p < 0.01). The amount of radioisotope released between 17 and 41 hours since the start of co-culture is, however, affected by the presence or absence of treated or untreated macrophages. There is significantly more radioisotope released when prelabelled SV40-transformed 3T3 cells are co-cultured with macrophages treated with MIF/MAF and/or TSN as compared with SV40-transformed 3T3 cells cultured alone (p < 0.01). There is, however, no significant difference between the amount of label released due to lysis of SV40-transformed 3T3 cells in the presence or absence of untreated macrophages. The counts released after 41 hours until the end of the experiment at 112 hours were also compared using Student's t-test and there was found to be no significant difference in the amount of SV40-transformed 3T3 cells lysed by any of the groups tested. The results presented in Table 18 therefore suggest that at an effector:target ratio of 25:1, pretreatment of macrophages with substances activating them for cytotoxicity against transformed cells is most effective between 17 and 41 hours. The results presented in Table 18 show that the effect on cytotoxicity observed at 41 hours is carried through to the end of the experiment at 112 hours indicating that cytotoxicity between 17 and 41 hours is crucial to overall cytotoxicity. When the total amount of radioisotope released from lysed SV40-transformed 3T3 cells is compared for the different macrophage
treatments using Student's t-test there is found to be a significant difference between the amount of label released when SV40-transformed cells are cultured with macrophages treated with MIF/MAF and/or TSN, as compared to the amount released by prelabelled SV40-transformed cells cultured alone (p < 0.01).

Table 19: Effect Of Hours Of Incubation On Cytotoxicity By MIF/MAF Treated Macrophages At An Effector:Target Cell Ratio Of 50:1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean cpm±SEM released by prelabelled SV40-transformed 3T3 cells cultured with elicited macrophages (cytotoxicity expressed as a percent of the mean total releasable counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time at which the amount of radioisotope released was measured (since start of co-culture)</td>
<td>21 hrs.</td>
</tr>
<tr>
<td>MIF/MAF</td>
<td>64,959 ± 3,289 (42.3%)</td>
</tr>
<tr>
<td>No treatment</td>
<td>69,911 ± 8,716 (45.5%)</td>
</tr>
<tr>
<td>No macrophages</td>
<td>42,097 ± 4,203 (27.4%)</td>
</tr>
<tr>
<td>Total releasable counts</td>
<td>153,628 ± 4,738</td>
</tr>
</tbody>
</table>

The results presented above in Table 19 were from duplicate cultures. The results for each of the different macrophage treatments were compared at each of the times given using Student's t-test. It was found that after 21 hours, 39 hours and 65 hours more SV40-transformed 3T3 cells were lysed when either MIF/MAF treated or untreated macrophages were cultured together with the
transformed cells than if the transformed cells were incubated alone (p < 0.01). Only in the interval between 39 hours' and 65 hours' incubation was there a significant increase (p < 0.01) in the amount of cells lysed by MIF/MAF-treated as compared to untreated macrophages.

At an effector:target cell ratio of 50:1, therefore, macrophages show significant cytotoxicity towards SV40-transformed 3T3 cells throughout the co-culture period, irrespective of whether they are pretreated with MIF/MAF. Those macrophages which are pretreated with MIF/MAF, however, show a high level of cytotoxicity towards SV40-transformed cells for a longer period than do untreated macrophages.

The results presented in Tables 17, 18 and 19 suggest that macrophages are most effective as cytolytic cells towards transformed 3T3 cells when the effector:target cell ratio is high (here tested at 50:1). In addition, when treated with MIF/MAF, macrophages can maintain a high level of cytolysis for longer than when untreated.

4.vii Synergistic Activation Of Macrophages By Lymphokine And Lipopolysaccharide For Phagocytosis Of Yeast And Effect Of Length Of Exposure To MIF/MAF And LPS

In order to evaluate any synergistic effects of MIF/MAF and LPS, and the treatment sequence necessary to demonstrate synergism on macrophage activation for phagocytosis of yeast, LPS at 5μg/ml and MAF were added in varying combinations and sequences for varying
times to elicited macrophages before they were assayed for yeast phagocytosis. Between 500 and 1640 macrophages were analysed per group. The number of yeast cells phagocytosed in 20 minutes by each macrophage was recorded and from this the mean number phagocytosed by each macrophage calculated. The results are presented in Tables 20 and 21 below.

Table 20: Effect Of Sequence Of Treatment With MIF/MAF Plus LPS For A Total Of 4 Hours On Activation Of Macrophages For Yeast Phagocytosis

<table>
<thead>
<tr>
<th>Treatment of elicited macrophages (time of exposure)</th>
<th>Mean ± SEM no. of yeast cells phagocytosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF (4 hrs.)</td>
<td>6.208 ± 0.227</td>
</tr>
<tr>
<td>MIF/MAF(2 hrs.) → LPS(2hrs.)</td>
<td>6.992 ± 0.238</td>
</tr>
<tr>
<td>LPS (4 hrs.)</td>
<td>5.694 ± 0.214</td>
</tr>
<tr>
<td>LPS(2hrs.) → MAF(2hrs.)</td>
<td>6.240 ± 0.227</td>
</tr>
<tr>
<td>LPS + MAF (4 hrs.)</td>
<td>8.717 ± 0.270</td>
</tr>
<tr>
<td>No treatment</td>
<td>5.565 ± 0.212</td>
</tr>
</tbody>
</table>

The mean values (shown in Table 20 above) obtained for each of the groups of macrophages treated with MAF and/or LPS in the varying combinations tested, were compared with the mean value obtained for untreated macrophages. Macrophages treated first with MAF then with LPS (row 2 above) were found to phagocytose significantly more...
(p < 0.01) yeast cells than did untreated macrophages. This is also true for macrophages treated with MAF and LPS together, but not for any of the other treatment sequences tested.

These data suggest that an obligatory treatment sequence exists in which lymphokine-treated macrophages undergo relatively rapid changes that alter the cells, making them more responsive to LPS. Activation of elicited macrophages by MAF and LPS for yeast phagocytosis occurs within a 4 hour treatment period.

Table 21: Effect Of Sequence Of Treatment With MAF And LPS For A Total Of 26 Hours On Activation Of Macrophages For Yeast Phagocytosis

<table>
<thead>
<tr>
<th>Treatment of elicited macrophages (time of exposure)</th>
<th>Mean ± SEM number of yeast cells phagocytosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAF (26 hrs.)</td>
<td>6.377 ± 0.229</td>
</tr>
<tr>
<td>MAF(2 hrs.) → LPS(24 hrs.)</td>
<td>4.124 ± 0.185</td>
</tr>
<tr>
<td>LPS (26 hrs.)</td>
<td>6.342 ± 0.230</td>
</tr>
<tr>
<td>LPS(2 hrs.) → MAF(24 hrs.)</td>
<td>5.131 ± 0.205</td>
</tr>
<tr>
<td>LPS + MAF (26 hrs.)</td>
<td>6.583 ± 0.228</td>
</tr>
<tr>
<td>No treatment (26hrs.)</td>
<td>6.644 ± 0.164</td>
</tr>
</tbody>
</table>

Using Student's t-test to analyse the results presented in Table 21 there was found to be no statistically significant increase in the
mean number of yeast cells phagocytosed by treated macrophages as compared to untreated macrophages.

The results presented in Tables 20 and 21 show that LPS and MAF synergistically activate elicited macrophages to enhanced levels of yeast phagocytosis in vitro, and that the length of exposure to LPS and MAF is very important in this response. Elicited macrophages are only activated to enhanced levels of yeast phagocytosis if their exposure to LPS and MAF is relatively brief; prolonged exposure seems actually to depress the phagocytic activity of the macrophages.

4.viii Synergistic Activation Of Macrophages By MIF/MAF And LPS For Cytotoxicity And Effect Of Length Of Exposure To MIF/MAF And LPS

In order to evaluate the synergistic effect of MIF/MAF and LPS on another parameter of macrophage activity, cytolysis, and the treatment sequence necessary, LPS at 5µg/ml (subthreshold, as defined by Schultz, 1982) and 10µg/ml and MIF/MAF were added in varying combinations and sequences for varying times to elicited macrophages before they were assayed for cytolysis against SV40-transformed 3T3 cells. 5µg/ml LPS was used unless stated otherwise. A ratio of 10:1 effector:target cells was used. Elicited macrophages were activated for either a total of 4 hours or 26 hours before co-culture with prelabelled SV40-transformed 3T3 cells for 48 hours. The results are presented below in Tables 22 and 23 for duplicate cultures.
Table 22: Effect Of Sequence Of Treatment With MIF/MAF And LPS For A Total Of 4 Hours On Activation Of Macrophages For Cytotoxicity

<table>
<thead>
<tr>
<th>Treatment of elicited macrophages (time of exposure)</th>
<th>Mean cpm ± SEM released by prelabelled SV40-transformed 3T3 cells cultured with elicited macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF + LPS (4 hrs.)</td>
<td>22,241 ± 1,190</td>
</tr>
<tr>
<td>MIF/MAF (2 hrs.) → LPS (2 hrs.)</td>
<td>23,130 ± 276</td>
</tr>
<tr>
<td>LPS (2 hrs.) → MIF/MAF (2 hrs.)</td>
<td>24,036 ± 900</td>
</tr>
<tr>
<td>MIF/MAF (4 hrs.)</td>
<td>20,615 ± 4,641</td>
</tr>
<tr>
<td>LPS (4 hrs.)</td>
<td>25,038 ± 47</td>
</tr>
<tr>
<td>No treatment</td>
<td>23,091 ± 2,940</td>
</tr>
<tr>
<td>No macrophages</td>
<td>20,666 ± 7,835</td>
</tr>
</tbody>
</table>

Using Student's t-test the amount of radioisotope released under each of the conditions given in Table 22 was compared with that released under each of the other conditions. There was found to be no significant difference between any of the treatments. The results indicate that MIF/MAF and LPS have no effect on macrophage cytotoxicity if macrophages are exposed to them for a total of only 4 hours before co-culture, in whatever combination.
### Table 23: Effect Of Sequence Of Treatment With MIF/MAF And LPS For a Total Of 26 Hours On Activation Of Macrophages For Cytotoxicity

<table>
<thead>
<tr>
<th>Treatment of elicited macrophages (time of exposure)</th>
<th>Mean cpm± SEM released by prelabelled SV40-transformed 3T3 cells cultured with elicited macrophages (cytotoxicity expressed as a percent of the mean total releasable counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF + LPS (26 hrs.)</td>
<td>32,133 ± 204 (64.5%)</td>
</tr>
<tr>
<td>MIF/MAF (2 hrs.) + LPS (24 hrs.)</td>
<td>36,052 ± 870 (72.4%)</td>
</tr>
<tr>
<td>LPS (2 hrs.) + MIF/MAF (24 hrs.)</td>
<td>30,550 ± 3,002</td>
</tr>
<tr>
<td>MIF/MAF (26 hrs.)</td>
<td>32,588 ± 2,694</td>
</tr>
<tr>
<td>LPS (5ug/ml; 26 hrs.)</td>
<td>28,159 ± 7,179</td>
</tr>
<tr>
<td>LPS (10ug/ml; 26 hrs.)</td>
<td>39,201 ± 1,583 (78.7%)</td>
</tr>
<tr>
<td>No treatment</td>
<td>29,208 ± 1,460</td>
</tr>
<tr>
<td>No macrophages</td>
<td>16,046 ± 780</td>
</tr>
</tbody>
</table>

Total releasable counts 49,801 ± 2,149

By analysing the results presented in Table 23 above using Student's *t*-test it is found that significantly more SV40-transformed 3T3 cells are lysed when they are co-cultured with macrophages pretreated with MIF/MAF + LPS for 26 hours, than if the transformed cells are cultured alone (*p* < 0.01). This is also true for macrophages primed for 2 hours with MIF/MAF before activation with LPS at 5ug/ml, or if the macrophages are cultured for 26 hours with 10ug/ml LPS before the co-culture period. These
results are in agreement with those of Shultz (1982), who found that treatment with MIF/MAF increases macrophage sensitivity to the effects of a second activating stimulus rather than induce full activation for cytolysis.

Using this two-stage method of inducing macrophage cytotoxicity, these results show that at an effector:target cell ratio of 10:1 MIF/MAF can indeed play a part in activating macrophages to cytotoxicity. Applying MIF/MAF alone does not induce macrophages to cytotoxicity at this effector:target cell ratio as also shown in Sections 4.v and 4.vi.

4.ix Macrophage Cytotoxicity On 3-D Collagen
Elicited macrophages were incubated in the presence or absence of MIF/MAF on 3-D collagen gels and incubated at 37°C. After 8 hours this medium was washed off and replaced with complete medium containing prelabelled SV40-transformed 3T3 cells to give a final effector:target cell ratio of 5:1. At the same initial concentration cultures of SV40-transformed 3T3 cells alone were established simultaneously. Cultures were established in duplicate and incubation lasted 64 hours before the experiment was ended and the amount of radioisotope released by SV40-transformed 3T3 cells lysed during the incubation period was measured. The results are presented in Table 24 below. Identical cultures were established with unlabelled SV40-transformed 3T3 cells then prepared for observation by SEM by the method outlined in Section 2.8. Figure 64 is a scanning electron micrograph of SV40-transformed 3T3 cells cultured together with elicited macrophages pretreated with MIF/MAF
Elicited macrophages pretreated with MIF/MAF for 8 hours prior to co-culture with SV40-transformed 3T3 cells for 72 hours on collagen. The arrow indicates an SV40-transformed 3T3 cell which is surrounded by macrophages.

Scale bar = 10μm
for 8 hours before the start of the co-culture period. The Figure shows an SV40-transformed 3T3 cell surrounded by elicited macrophages on the collagen substrate.

Table 24: Effect Of Treatment With MIF/MAF On Macrophage Cytotoxicity On 3-D Collagen At An Effector:Target Cell Ratio Of 5:1

<table>
<thead>
<tr>
<th>Pretreatment of macrophages</th>
<th>Mean cpm ± SEM released by prelabelled SV40 transformed 3T3 cells (cytotoxicity expressed as a percent of the mean total releasable counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF</td>
<td>99,045 ± 2,786 (83.3%)</td>
</tr>
<tr>
<td>No treatment</td>
<td>68,073 ± 6,149 (57.3%)</td>
</tr>
<tr>
<td>No macrophages</td>
<td>39,898 ± 975 (33.6%)</td>
</tr>
</tbody>
</table>
| Total releasable counts    | 118,854 ± 1,047                                                                  

The results presented in Table 24 were analysed using Student's t-test. Significantly more (p < 0.01) SV40-transformed 3T3 cells are lysed when these cells are co-cultured with MIF/MAF treated macrophages than if they are cultured in the absence of macrophages, on a collagen substrate. When untreated macrophages are tested, only slightly more (p < 0.05) SV40-transformed cells are lysed than if transformed cells are cultured in the absence of macrophages. These results indicate that MIF/MAF treated macrophages are effective cytotoxic cells against SV40-transformed 3T3 cells at an effector:target cell ratio of 5:1 when they are...
cultured on a 3-D collagen gel in vitro. This ratio was found not to induce cytotoxicity in MIF/MAF-treated or untreated macrophages when the cells were cultured on a glass substrate in vitro (Section 4.y)

4.x Effect Of Effector/Target Cell Contact On Cytotoxicity

Elicited macrophages treated with MIF/MAF were seeded onto Millipore filters. They were incubated for 72 hours with prelabelled target cells at an effector:target cell ratio of 20:1, the effector and target cells separated either by 0, 1 or 3 Millipore filters as described in Section 4.10. The amount of label released by target cells during the co-culture period was measured and is recorded in Table 25 below for 2 experiments. The amount of radioisotope released is an indicator of the amount of target cell lysis during the incubation period.

Table 25: Effect Of Cell Contact On Cytotoxicity

<table>
<thead>
<tr>
<th>No. of filters separating target and effector cells</th>
<th>Mean cpm ± SEM released by prelabelled SV40-transformed 3T3 cells (cytotoxicity expressed as percent of the mean total releasable counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>58,319 ± 1,682 (83.3%)</td>
</tr>
<tr>
<td>1</td>
<td>41,323 ± 1,678 (59.1%)</td>
</tr>
<tr>
<td>3</td>
<td>28,359 ± 3,359 (40.5%)</td>
</tr>
<tr>
<td>Total releasable counts</td>
<td>69,983 ± 1,010</td>
</tr>
</tbody>
</table>

The results presented in Table 25 were analysed using Student's
t-test. Significantly more (0.05 < p < 0.01) radioisotope was released from SV40-transformed 3T3 cells when there were no filters separating effector and target cells than when the two cell types were separated by either 1 or 3 filters. There is, however, no significant difference (p > 0.05) between the presence of 1 or 3 filters separating the two cell types. These results indicate that close proximity between effector and target cells is required for effective cytotoxicity.

4.xi Effect Of MIF/MAF On Macrophage Cytotoxicity Through 3-D Collagen

Macrophages incubated in the presence or absence of MIF/MAF were separated by 3-D collagen gels from prelabelled SV40-transformed 3T3 cells by the method described in Section 4.11. Cultures of prelabelled SV40-transformed 3T3 cells were established simultaneously. All cultures were set up in duplicate. The amount of radioisotope released from the prelabelled SV40-transformed 3T3 cells was measured after the cultures had been incubated for 72 hours at 37°C. The results are presented in Table 26 below.
Table 26: Effect Of MIF/MAF On Macrophage Cytotoxicity Through 3-D Collagen

<table>
<thead>
<tr>
<th>Treatment of macrophages</th>
<th>Mean cpm ± SEM released by prelabelled target cells (cytotoxicity expressed as a percent of the mean total releasable counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF/MAF</td>
<td>62,856 ± 565 (37.8%)</td>
</tr>
<tr>
<td>No treatment</td>
<td>50,838 ± 1,611 (30.6%)</td>
</tr>
<tr>
<td>No macrophages</td>
<td>34,857 ± 638 (21.0%)</td>
</tr>
<tr>
<td>Total releasable counts</td>
<td>166,177 ± 4,107</td>
</tr>
</tbody>
</table>

The results given in Table 26 show that macrophages can influence the amount of target cell lysis, even if separated from the target cells by a layer of collagen. There is significantly more radioisotope released from target cells when they and MIF/MAF-treated macrophages are separated by a collagen layer, than if the target cells are cultured alone \( p < 0.01 \). Untreated macrophages show a slightly less significant effect if similarly tested \( 0.05 < p < 0.01 \).

If, as the results in Section 4.x suggest, close contact between effector and target is a requirement for cytotoxicity, the macrophages here must migrate through the collagen gel to achieve close contact with the target cells. Alternatively macrophages may lyse target cells by secreting a cytolytic factor which is absorbed onto the Millipore filters.
That the effect of MIF disappears when the molecule is no longer present in the culture was discussed in Chapter 1. To test whether elicited macrophages are attracted towards transformed cells through collagen was tested and the results are presented in Section 4.xii.

4.xii Chemoattraction Of Elicited Macrophages Through Collagen Towards SV40-Transformed 3T3 Cells

Using the method described in Section 4.12 transformed and non-transformed 3T3 cells were cultured on top of collagen gels and their effect on macrophage migration through the collagen was determined. The following three situations were set up:

A. 3T3 cells on one side of the gel, elicited macrophages on the other.

B. SV40-transformed 3T3 cells on one side of the collagen, elicited macrophages on the other.

C. SV40-transformed 3T3 cells on one side of the collagen gel, no cells on the other.

The gels were fixed after 64 hours' incubation, pieces of the gels embedded in Paraplast and transverse sections of the gels cut, stained and observed using a Zeiss Photomicroscope. The number of invading cells of both cell types on each gel was counted and the distance migrated into the gel by each of these determined. The proportion of invasive cells was calculated as follows:

\[
\frac{a + \sqrt{a(1-a)}}{n} \left( \frac{1}{n} \right)
\]

where 
- \(a\) = number of invasive cells
- \(n\) = total number of cells counted

192
The results are presented below in Table 27

<table>
<thead>
<tr>
<th>Cells</th>
<th>% of cells invadedSEM</th>
<th>Mean distance migrated by invasive cells(μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elicited macrophages on gel A</td>
<td>27.42 ± 3.37</td>
<td>19.92 ± 3.49</td>
</tr>
<tr>
<td>Elicited macrophages on gel B</td>
<td>42.52 ± 4.77</td>
<td>147.36 ± 14.56</td>
</tr>
<tr>
<td>3T3 cells on gel A</td>
<td>5.56 ± 1.21</td>
<td>33.75 ± 6.48</td>
</tr>
<tr>
<td>SV40-transformed 3T3 cells on gel B</td>
<td>10.98 ± 1.92</td>
<td>41.69 ± 11.69</td>
</tr>
<tr>
<td>SV40-transformed 3T3 cells on gel C</td>
<td>10.08 ± 0.52</td>
<td>35.54 ± 6.31</td>
</tr>
</tbody>
</table>

The results presented in Table 27 indicate that elicited macrophages migrate more readily through a layer of collagen towards SV40-transformed 3T3 cells than towards normal 3T3 cells. This is illustrated both in the proportion of cells which migrate through the collagen and in the distance migrated by the elicited macrophages.

The migratory behaviour of SV40-transformed 3T3 cells was not influenced by the presence or absence of elicited macrophages.
This was shown by comparing the proportion of SV40-transformed cells which migrated into gel B with that which migrated into gel C, where no other cell type was present.

It can be concluded from these results that elicited macrophages are attracted towards SV40-transformed 3T3 cells, presumably by some chemoattractant released by these cells.

It may be useful at this point to briefly summarise the results presented in this Chapter. Resident macrophages were found not to respond to substances which activated elicited macrophages to enhanced levels of cytotoxicity. Also, elicited macrophages incubated in the presence or absence of these substances are not cytotoxic towards non-transformed 3T3 cells.

A 1 hour incubation with activating substances prior to co-culture is insufficient to render elicited macrophages cytotoxic towards SV40-transformed 3T3 cells. A treatment period of at least 8 hours is required.

Elicited macrophages have a cytostatic effect on SV40-transformed 3T3 cells at a much lower effector:target cell ratio than is required for a cytolytic effect. At a very high effector:target cell ratio both MAF treated and untreated elicited macrophages are cytotoxic towards SV40-transformed 3T3 cells; however, MAF treated macrophages show enhanced levels of cytotoxicity for longer than do untreated macrophages. At lower effector:target cell ratios, pretreatment with MAF is only effective over a given period since
the start of the co-culture period, but this effect is crucial to overall cytotoxicity.

The requirement for a high effector:target cell ratio for effective cytolysis was found to be diminished by the pretreatment of macrophages with LPS then MAF before the start of the co-culture period. At an effector:target cell ratio of 10:1, MAF treated or untreated elicited macrophages exhibit little cytotoxic effect. If elicited macrophages are pretreated with LPS plus MAF prior to co-culture at this effector:target cell ratio, however, there is a dramatic and statistically significant increase in cytolysis of target cells.

There was found to be a synergistic and obligatory treatment sequence for macrophage activation for both yeast phagocytosis and target cell lysis by LPS and MAF. The same treatment sequence was found to be effective for both yeast phagocytosis and target cell lysis by macrophages, but total macrophage treatment time was found to be important in determining which functions macrophages perform. A longer pretreatment time is required for macrophage activation for cytolysis than for yeast phagocytosis.

Incubation on a collagen substrate was also found to decrease the effector:target cell ratio required for cytolysis of target cells.

Evidence is also given here suggesting that macrophages are attracted through collagen towards transformed cells, but not towards their non-transformed counterparts, and that macrophages do
not require physical contact with SV40-transformed cells for effective cytolysis. The theory that macrophages kill SV40-transformed 3T3 cells by secreting cytotoxic substances is supported here.

**Discussion**

In the system tested here inflammatory-elicited macrophages were found to be cytotoxic towards SV40 virally-transformed 3T3 cells, but not their non-transformed counterparts. That the macrophages involved were inflammatory-elicited was found to be important since normal or resident macrophages were found not to be cytotoxic towards SV40-transformed 3T3 cells. Nor were these normal macrophages capable of responding to treatment with MIF/MAF by becoming cytotoxic towards SV40-transformed 3T3 cells.

The main aim of this Chapter was to investigate the effect of the lymphokine(s) MIF/MAF on macrophage tumoricidal activity. Elicited macrophages were routinely incubated in complete medium containing MIF/MAF for 8 hours before co-culture with the target cells was begun. This length of time was chosen after finding that incubation of elicited macrophages for only 1 hour with MIF/MAF failed to have any effect on tumoricidal activity. This is in agreement with the findings of Meltzer (1981b) who found that optimal levels of cytotoxic activity occur after 8-12 hours of
lymphokine treatment.

The effector:target ratio was found to have a crucial effect on whether or not elicited macrophages are cytotoxic towards SV40-transformed 3T3 cells in vitro. The influence on cytotoxicity of pretreating elicited macrophages with MIF/MAF was also found to depend on this ratio.

Irrespective of whether or not elicited macrophages are pretreated with MIF/MAF, at an effector:target cell ratio of 5:1 SV40-transformed cells are not lysed by macrophages except when cultured on a collagen substrate (discussed later). Elicited macrophages (+/- MIF/MAF) have only a slight effect on target cell lysis at an effector:target ratio of 10:1. At a ratio of 25:1, however, pretreatment of elicited macrophages with MIF/MAF activates these cells to enhanced levels of cytotoxicity towards the target cells. MIF/MAF-activated macrophages were found to be cytotoxic towards the target cells between 17 and 41 hours into the co-culture period, and increased cytotoxicity during this period was found to influence overall cytotoxicity at the end of 112 hours' co-culture. When tested at effector:target cell ratios of 40:1 and 50:1, both MIF/MAF activated and non-activated macrophages were found to be cytolytic towards SV40-transformed 3T3 cells. When investigated, however, it was found that at these high ratios MIF/MAF activated macrophages are cytotoxic for a longer period and at a higher level than are non-activated macrophages.

These results indicate that, at relatively low ratios, lymphokine
activation is necessary for macrophage cytotoxicity, but at high ratios activation serves only to increase the cytotoxicity already shown by elicited macrophages towards transformed cells.

Both MIF/MAF-activated and non-activated macrophages were found to be cytostatic towards SV40-transformed 3T3 cells at 5:1 effector:target cells, although neither activated nor non-activated macrophages were found to be cytolytic at this low ratio. Keller (1975) found that target cell (in this case lymphocyte) proliferation is markedly enhanced when activated macrophages are few in number (effector:target cell ratio 1:10) but lymphocyte proliferation, irrespective of how it is induced, is completely blocked when activated macrophages are present in a majority (effector:target cell ratio 10:1). Keller also found that this inhibition of cell proliferation could be duplicated by a soluble factor produced by macrophages.

Cytostatic factors produced by human monocytes activated with lymphokines and lipopolysaccharide (LPS) have been purified and found to have molecular weights in the range of 60,000-30,000 daltons (Nissen-Meyer and Hammerström, 1982) and are physically similar to the 55,000-dalton tumour necrosis factor (TNF) found in sera of endotoxin-treated mice and rabbits infected with Mycobacterium bovis (Ruff and Gifford, 1980). The mechanism whereby cytostatic factors induce cytostasis is unknown (Nissen-Meyer and Seim, 1983).

It can be concluded from these results that the effector:target
cell ratio is very important in determining effector (macrophage) function.

The presence of high numbers of macrophages is not always beneficial, however. In some instances macrophages can enhance tumour growth and the presence of too many macrophages may suppress the immune response of lymphocytes (Rhodes, 1977). It would seem, therefore, that a given number of macrophages is required for an optimal host defence mechanism; too many may be harmful.

The finding by Schultz (1982) that treatment with MAF increases macrophage sensitivity to the effects of a second activating stimulus, such as LPS, was confirmed here. Schultz used peritoneal macrophages from CF1 mice as effector cells and P815 mastocytoma cells as targets. Both here and in the report by Schultz a 2 hour incubation with MAF followed by a 24 hour incubation with a subthreshold level of LPS was found to induce enhanced levels of cytotoxicity. It was found here that besides the treatment sequence, the length of treatment time is also important in determining effector function. If elicited macrophages are treated with MAF for 2 hours then with LPS for only 2 hours instead of 24, macrophages do not show enhanced levels of cytotoxicity towards SV40-transformed 3T3 cells. In contrast, elicited macrophages pretreated for 4 hours with MAF plus LPS were found to be activated for yeast phagocytosis, but not if pretreated for a total of 26 hours. Again the sequence of MAF then LPS was found to be required for optimal activation for yeast phagocytosis.
The substrate on which the cells were cultured was found to affect macrophage cytotoxicity against SV40-transformed 3T3 cells. MAF-activated macrophages were found to be cytotoxic towards the target cells at an effector:target cell ratio of 5:1, although no cytotoxicity was reported for macrophages cultured on glass at this ratio.

Substrate effects on cytotoxicity of monocytes has been reported elsewhere, although with a different outcome. If monocytes are cultured on collagen gels with no contact with glass, no cytotoxic activity is induced (Kaplan, 1983). Kaplan attributes this lack of activity to the differentiation of monocytes, when they are cultured on collagen, into cells resembling resident tissue macrophages, which also do not show cytotoxic activity. Monocytes cultured on glass, however, appear to differentiate into cells resembling activated macrophages and are cytotoxic against transformed cell lines. Here the substrate effects on cytotoxicity of elicited macrophages rather than monocytes or resident macrophages were studied and found to be important. These results support the need to take the nature and composition of the substrate into account when assessing macrophage behaviour.

Separating effector and target cells by Millipore filter(s) was found to cause a marked decline in macrophage cytotoxicity towards target cells. Macrophages were, however, found to induce cytolysis of SV40-transformed 3T3 cells through a layer of collagen. It would appear from the experiments where effector and target cells are separated by Millipore filter(s), that direct physical contact
with the target is a requirement of macrophage cytotoxicity. This theory, however, is revoked by the finding that macrophages can induce cytolysis when separated by a layer of collagen. Although elicited macrophages were found to be attracted towards the SV40-transformed 3T3 cells through the layer of collagen gel, they did not invade in numbers sufficient to cause the level of cytotoxicity observed. It is proposed, therefore, that although close contact between macrophages and target cells may be required, direct contact is not. It follows that macrophage cytotoxicity, in the system described, is mediated via factors secreted by the macrophage. This theory could be tested by measuring cytotoxicity in cultures of target cells overlaid with medium in which anti-tumour macrophages have been incubated.

One of the key unresolved problems in macrophage cell biology today is the mechanism by which macrophages acquire the capacity to recognise and subsequently lyse or inhibit DNA synthesis of tumour cells. Cells transformed by viruses, such as those used in the experiments described here, can be recognised by specific antigens on their surface. That macrophages were activated to lyse SV40-transformed cells following incubation in medium in which SV40-transformed 3T3 cells had been grown was probably due to the presence, in the medium, of specific antigens characterising the transformed cells. Unfortunately for the clinician, most human tumours arise spontaneously and the cell surface antigens are generally not different from those on the host's normal cells.

Several workers have looked at alterations in surface proteins that
accompany tumouricidal activation and their potential functional significance (Cohn, 1978; Karnovsky, 1978; North, 1978), and a recent study has been made on macrophage surface carbohydrates (Mercurio et al., 1984). Mercurio and his colleagues characterised the major glycolipid constituents of the mouse peritoneal macrophage and demonstrated that alterations in the amount and in the accessibility of specific glycolipid species to galactose oxidase/NaB₃H₄ labelling, an indicator of glycolipid surface exposure, occur in response to inflammation and as a consequence of activation to a tumouricidal state. They also found that a spatial reorganisation of surface glycolipids accompanies the in vitro acquisition of tumouricidal capacity for thioglycollate-elicited macrophages in the presence of gamma interferon and LPS. Other work by the same group (Robbins et al., 1984; Springer et al., 1984) substantiate the hypothesis that macrophage activation is accompanied by widespread alterations in surface carbohydrates. It remains to be shown that such surface alterations are involved in the recognition by activated macrophages of tumour cells.

Based on the observation that activation of macrophages leads to an increased capacity to bind tumour cells (Piessens, 1978; Marino and Adams, 1980), and on ultrastructural evidence for transfer of lysosomes from macrophages to tumour cells which subsequently lyse (Hibbs, 1974b), it has been proposed that tumour cell destruction by activated macrophages requires an intimate contact between the two cell types. Alternatively, soluble substances such as neutral proteases (Adams et al., 1980), activated factors of the complement system (Schorlemmer et al., 1977), arginase (Currie, 1978),
thymidine (Stadecker et al., 1977), tumour necrosis factor (Hoffmann et al., 1978), or oxygen metabolites (Nathan, 1980), have been implicated as possible mediators of extracellular cytolysis by different types of phagocytes. Individual activated macrophages have also been shown to inhibit DNA synthesis in tumour target cells by direct contact with the target cells (Krahlenbuhl and Lambert, 1975).

From experiments in which target cells (SV40-transformed 3T3 cells, EMT-6 mammary tumour cells and P815-X-2 mastocytoma cells) were in direct contact with activated macrophages or separated by a Gelman filter disc, Philippeaux and Mauel (1984) concluded that cytotoxicity is mediated by a highly diffusible compound. That cytotoxicity was not observed here when effector and target cells were separated by Millipore filter(s) may be due to the cytotoxic compound which mediates cytotoxicity having been absorbed onto the Millipore filter(s) and so not reaching the target cells. That cytotoxicity occurs when macrophages are separated from target cells by a layer of collagen may be due to any such factor(s) not binding to the collagen. Philippeaux and Mauel (1984) found that supernatants of activated macrophages incubated with target cells for 1 to 4 hours, but not of activated macrophages incubated alone, were toxic for tumour cells, suggesting that tumour cells provide a signal to activated macrophages, in the absence of which toxic intermediates are not released. Addition of peroxidase or catalase considerably reduced target cell destruction by activated macrophages in Philippeaux and Mauel's experimental model indicating that oxygen metabolites might play a role as mediators.
of activated macrophage cytotoxicity.
GENERAL DISCUSSION AND CONCLUDING REMARKS

Since the first lymphokine, MIF, was described twenty years ago, up to one hundred biological activities have been found in stimulated (usually T-cell) supernatants. In the years following its discovery, a great number of macrophage-activating properties were attributed to MIF. More recently work has begun on the thorough characterisation of these factors. From the preliminary work carried out to date, it is clear that MIF activity is separate from MAF activities but associated with several molecular weight species and that guinea pig, murine and human MIF at least are oligomers of common subunits (of molecular weight 15,000 daltons and isoelectric point 5.2 for guinea pig MIF) (Sorg, 1982).

The determination of structural homologies between different lymphokines or lymphokine species requires quantities sufficient for biochemical analysis. This could be achieved by cloning the cDNA which codes for the protein component of the precursor molecules of the lymphokine, then expressing these proteins in large quantities in E. coli. The majority of lymphokines so far characterised have been found to have large carbohydrate moieties associated with them. From the DNA sequence the amino acid sequence could be determined offering predictions on possible glycosylation sites and proteolytic cleavage sites. This sequence could then be compared with other cloned lymphokine species giving information on possible precursor molecules. Alternatively, and under investigation as mentioned in Chapter 1, is the establishment
of T-T cell hybrids, preferably human, which produce only a few lymphokines (Ratliff et al., 1982; Kelso et al., 1982). From these T-T hybrid cell cultures, supernatants can be assayed for the various lymphokine activities of interest and then lines producing suitable amounts of the various lymphokines of interest grown in large amounts and the lymphokines purified from the supernatants by standard biochemical procedures. Once purified to homogeneity, lymphokines could be compared, for example, by peptide analysis and sequencing. A full understanding of the molecular nature of the various lymphokines would show how closely related these molecules are, and might also give an insight into which molecular features are important for the various lymphokine activities. This could lead to their use in clinical situations or the construction of, for example, new anti-viral, anti-tumour and anti-Leishmania drugs, and drugs against other intracellular macrophage parasites.

The extent of the controversy surrounding lymphokine heterogeneity is matched by that surrounding the functional heterogeneity of macrophages, which was discussed in Chapter 3. That mature macrophages are functionally heterogeneous has been well documented, and is not questioned. How this heterogeneity is generated, however, remains to be elucidated. One assumption is that the heterogeneity is due to true subpopulations of cells as is known for the T-cell/B-cell lineages. An alternative possibility is that macrophages pass through different maturation stages or intermediate, relatively stable, phenotypes on their way to maturity and senescence, expressing characteristic functions in each maturation stage. This latter hypothesis is favoured here in
the light of the results reported in Chapter 3. In order to confirm those results, however, methods must be developed whereby individual macrophage precursor cells in the bone marrow can be cloned to provide sufficient numbers of identical cells to test in several different assays.

There is some evidence in the literature to link macrophage heterogeneity to the cell cycle (Neumann and Sorg, 1980). Phorbol myristate acetate (PMA) is a tumour promoter which is also mitogenic to macrophages and induces them to plasminogen activator production. Between 2 and 22 hours after exposure to PMA, macrophages produce substantial amounts of plasminogen activator. After 22 hours there is a steady decrease in the amount of plasminogen activator produced. In contrast, the number of cells in S-phase peaks later, at 26 to 46 hours after exposure to PMA. These results indicate that after exposure to PMA, macrophages are pushed towards the S-phase of the cell cycle and that on their way to S, probably in late G1, macrophages express plasminogen activator as a constitutive trait. It was also found that they become sensitive to activation by lipopolysaccharides at this time.

From the recent data Sorg (1982) put forward the following theory. After a phase of intensive proliferation of precursors and differentiation into macrophages, young macrophages continue to cycle and accumulate in late G1, which is characterised, for instance, by plasminogen activator production, inducibility of interferon, and their response to MIF and certain chemoatotic factors. As proliferation fades away, the cells gradually
differentiate in $G_1$, lose a series of constitutive functions and pass on to a $G_0$-like state characterised, for instance, by the production of fibrinolysis inhibitors. The model proposes that the functional state of normal resident macrophages should be compatible with $G_0$, and that of proteose peptone-elicited macrophages with early and thioglycollate-induced macrophages with late $G_1$. The model further proposes that macrophage differentiation in $G_1$ is reversible and that macrophages of various functional states have to go through the bottleneck of late $G_1$ before entering $S$.

From Sorg's model and the results presented in Chapter 4 it is proposed that the elicited macrophages used here are probably mostly in early $G_1$, from which they can be pushed by MAF into late $G_1$, where they are susceptible to activation by LPS to cytotoxicity or yeast phagocytosis depending on how far through the cell cycle they are pushed.

The findings which show that activation for cytotoxicity by MAF alone requires a higher effector:target cell ratio than that required when elicited macrophages are MAF-primed then activated by LPS, also fits into this model. The model proposes that only those cells which are in late $G_1$ are responsive to activating signals which will render them cytotoxic towards transformed cells. Within a population of elicited macrophages freshly harvested from the peritoneum of a mouse, there is likely to be a proportion of cells already in late $G_1$ and so responsive to activation for cytotoxicity MAF alone. It is this proportion of responsive cells which is
increased when elicited macrophages are MAF-primed.

Using purified lymphokines and other cytokines, it should be possible in the future to define the precise nature of a cytokine signal and the nature of the cellular response to it, and so define differentiogenic, mitogenic, and activating signals. That differentiation, activation and homeostasis of the mononuclear system is regulated by a complex network of lymphokines and other factors seems inevitable from the literature available to date.

It should be recognised that it is not always possible to directly relate models described in vitro to the situation in vivo where many, often conflicting, factors come into play. For instance, any conclusions drawn regarding the role of macrophages in destroying tumour cells in vivo must for instance take into account the immunogenicity of the tumour in question and the possibility of immunosuppression accompanying progressive tumour growth. Although it is not possible to guard against these problems in any in vitro models described to date, work reported in Chapters 2 and 4 here indicate the importance of mimicking the in vivo situation as closely as possible. It was found, for instance, that macrophage behaviour including the cytotoxic response is affected by the culture substrate.

The work described in Chapter 2 on the effects of substrate and MIF on the morphology, adhesion and locomotion of macrophages indicate that MIF might inhibit migration by affecting macrophage adhesive forces. Using purified preparations of MIF it would be interesting
to follow up this theory. This could be achieved *in vitro*, for instance, by studying the effects of MIF on the proportion of the cell surface covered by and the distribution of areas of close contact between the substrate and MIF-treated macrophages using interference reflection microscopy. Also an investigation into the effects of MIF on the production of fibronectin and other cell adhesive molecules by macrophages would contribute to an understanding of the mechanism by which MIF prevents macrophage migration both *in vitro* and *in vivo*.

Finally it is hoped that this thesis has drawn attention to the impressive role played by macrophages in many homeostatic functions. From being primitive scavenging cells they have evolved into cells essential to mammalian immunology. They also handle fat and iron and control fibroblasts, make lysozyme, contribute to the complement system and quell unruly mutants. The manipulation of these cells and factors affecting them by the clinician in the treatment of many diseases in the future seems promising, desirable and inevitable.
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THE EFFECT OF THE LYMPHOKINE(S) MIF/MAF ON MURINE MACROPHAGE BEHAVIOUR
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ABSTRACT
Migration inhibition factor (MIF) was isolated from the mouse, rat, Xenopus laevis and the newt Triturus cristatus. These MIFs were used to investigate the specificity of the molecule and it was found that MIF is not species specific and that animals with an evolutionarily sophisticated immune response are responsive to MIFs produced by animals which show much less complex immune responses.

Macrophages are one of the few normal adult cell types which show motility as part of their functional phenotype and this process can be manipulated in vitro by the presence or absence of MIF. Using LM and EM the effects of MIF and substrate on locomotion and macrophage-substrate adhesion were investigated.

The question of macrophage heterogeneity was addressed to determine whether macrophages can be activated for a wide range or only a limited number of functions. The results obtained suggest that macrophages become committed along particular pathways depending on external stimuli from the micro-environment surrounding the cell and that performance of particular functions precludes the cell from performing certain other functions.

Macrophage activating factor (MAF) co-chromatographs with MIF and was isolated with MIF. The effect of MIF/MAF and effector:target cell ratio on macrophage anti-tumour activity was also investigated. Macrophages were found to have a cytostatic effect at a much lower effector:target cell ratio than that required for cytolysis of transformed cells. Also, macrophages lyse target cells at a lower effector:target cell ratio when cultured on a collagen rather than a glass substrate. Macrophages are also attracted through collagen towards transformed cells preferentially. Pretreatment with MAF enhances the cytolytic response of macrophages. This response is increased further by pretreating macrophages with LPS and MAF when both pretreatment time and sequence are important in determining which functions macrophages perform.