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Excisional Wound Healing and the Role of Homeobox Genes

David Allen Lanning

INVESTIGATION OF A FACTOR RELEASED BY NEOPLASTIC CELLS WHICH PRODUCES A CHEMOKINETIC RESPONSE IN ACTIVATED MACROPHAGES

By

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DEDICATION

This work is dedicated to my wife, Sally, for her love and encouragement during the hard times and for her vital help in the preparation of this thesis.

This thesis by Richard Durelle Lane is accepted in its present form as satisfying the thesis requirement for the degree of Doctor of Philosophy.

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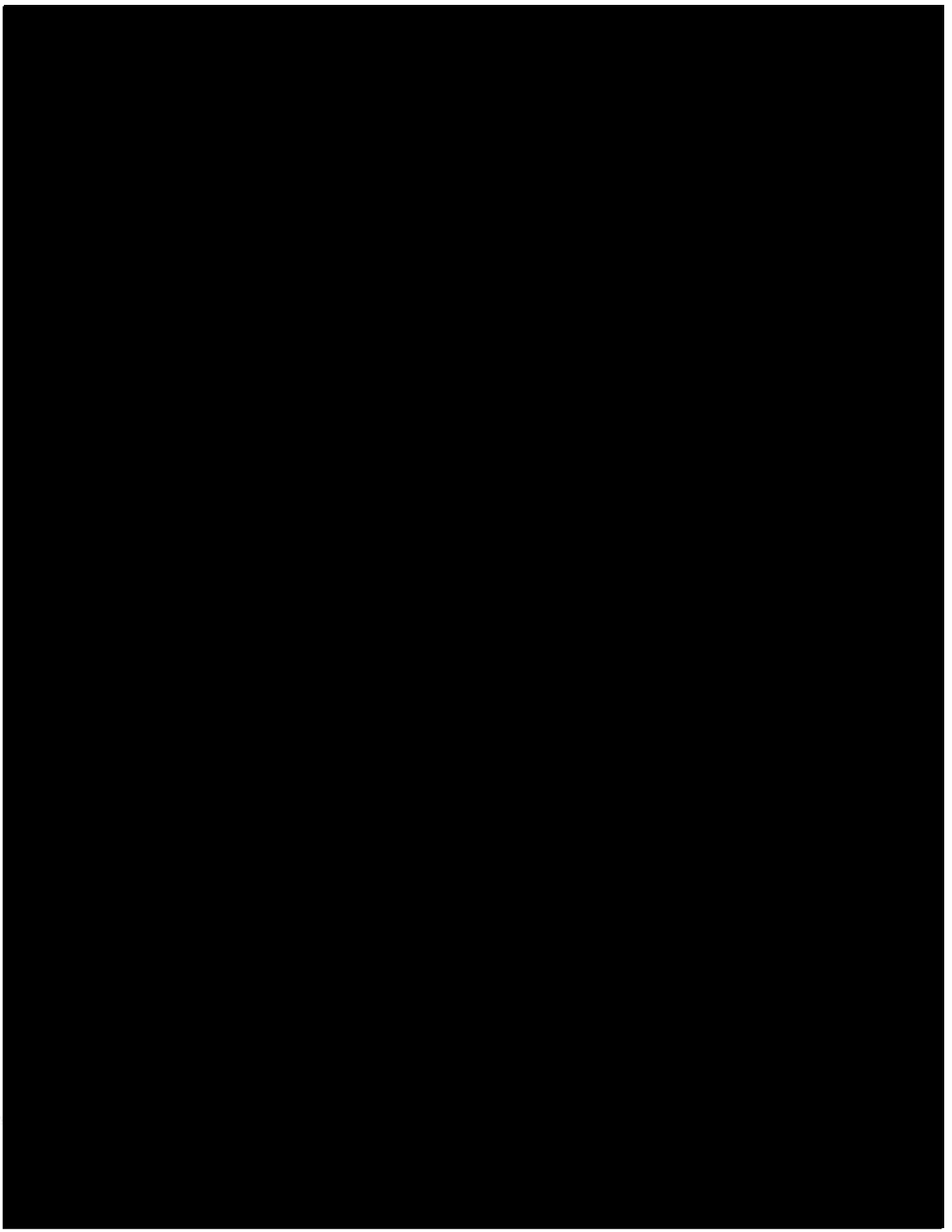

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LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cell-mediated Cytotoxicity
AMØ	Activated Macrophage
BCG	Bacillus Calmette Guerin
BS	Bovine Serum
C-AMP	Cyclic-Adenosine Monophosphate
C-GMP	Cyclic-Guanosine Monophosphate
CB	Cell Body
CEA	Carcinoembryonic Antigen
CKF	Chemokinetic Factor
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylene Dichlorotetra-Acetate
EMEM	Eagles Minimal Essential Medium
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
HBSS	Hank's Balanced Salt Solution
i.d.	intradermal
i.p.	intraperitoneal
l	lymphocyte
L	Lamellipodia
LDH	Lactic Dehydrogenase
LETS	Large External Transformation Sensitive Protein
LL	Lewis Lung Carcinoma

m	monocytic appearing macrophages
M	Mature Macrophage
MAS	Mouse Activated Serum
MCA	Methylcholanthrene
MEF	Mouse Embryonic Fibroblast
MIF	Migration Inhibition Factor
mm	millimeter
MSF	Migration Stimulation Factor
PBS	Phosphate Buffered Saline
PS	Physiologic Saline
RNA	Ribose Nucleic Acid
S.D.	Standard Deviation
SDS-PAGE	Sodium-Dodecysulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
sf	serum free
SMAF	Specific Macrophage Arming Factor
ss	serum supplemented
u	uropod
ul	microliter
µm	micron

ABSTRACT

INVESTIGATION OF A FACTOR RELEASED BY
NEOPLASTIC CELLS WHICH PRODUCES
A CHEMOKINETIC RESPONSE IN ACTIVATED MACROPHAGES

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The increase in rate of random migration (chemokinesis) of C. parvum activated macrophages in media conditioned by Lewis Lung (LL) carcinoma cells was attributed to a trypsin sensitive, heat stable, high molecular weight factor released from the membrane of the tumor cells.

A capillary tube assay was developed to expediently monitor the chemokinetic activity of macrophages incubated in whole and fractionated media. The capillary tube assay was found to be capable of detecting both chemokinetic and chemotactic (directional movement) factors present in the test media. Statistical analysis revealed the capillary tube assay provided reproducible data both within and between experiments.

Media conditioned by 6 different syngeneic and allogeneic mouse tumor cell lines demonstrated significantly higher chemokinetic activity compared to unconditioned or normal fibroblast conditioned media. The release of the chemokinetic factor (CKF) by Lewis Lung cultures was demonstrated to be maximum during the logarithmic growth phase of these neoplastic cells. Molecular seive chromatography of the Lewis Lung conditioned media revealed the CKF to have a molecular

weight of approximately 360,000 daltons. Similarly to the C. parvum activated macrophages, the pyran activated macrophages responded chemokinetically to LL conditioned media. However oyster glycogen and thioglycolate elicited macrophages demonstrated no significant chemokinetic response in the presence of the LL-CKF.

The Lewis Lung chemokinetic factor demonstrated no chemotactic activity in the Boyden chamber assay. In fact, the CKF actually inhibited the response of these macrophages to a known chemotactic factor.

Indirect immunofluorescent staining of the CKF indicated that it was a membrane protein shed by the LL cells and bound by activated macrophages. The possibility is discussed that CKF may be a glycocalyx protein shed by tumor cells which interferes with macrophage-neoplastic cell interactions involved in the tumoricidal activity of macrophages.

I. INTRODUCTION

Macrophages are the primary cell type constituting the Mononuclear Phagocyte System. Langevoort et al. (1970) and van Furth et al. (1972) defined the Mononuclear Phagocyte System as consisting of all phagocytic cells of bone marrow origin. The bone marrow origin of the macrophage was demonstrated by Balner (1963), Goodman (1964), Howard et al. (1976) and Pinkett et al. (1966). The macrophage phagocytize bacteria (Wyssokowitch, 1887) foreign particles (Von Kupffer, 1898), effete cells (Vaughn and Boyden, 1964) and antibody coated cells (Greendyke et al., 1963). The macrophage possesses other functional capabilities including the processing of antigens for lymphocyte mediated immune responses (Rosenthal and Shevach, 1973) and killing of neoplastic cells either specifically (Evans and Alexander, 1970) after immune system stimulation by the neoplastic cell, or non-specifically (Alexander and Evans, 1971), requiring no previous exposure of the host to the neoplastic cells.

Based upon the ability of the macrophages to recognize and destroy neoplastic cells in vitro and the presence of macrophages within neoplastic lesions Evans (1972) and Hibbs et al. (1978) suggested that the macrophage serves as a primary effector cell within the tumor surveillance system. Burnett (1964) originally proposed the existence of a tumor surveillance system in which immunologically competent cells function in the recognition and destruction of neoplastic cells.

Hibbs et al. (1972a) found that macrophages are involved in non-specific tumor cell killing and can distinguish a variety of different

syngeneic and allogeneic neoplastic cells from normal cells. He proposed that the macrophages recognize a particular characteristic common to all or most neoplastic cells, which is distinct from normal cells. He suggested several possibilities for the common neoplastic cell marker such as increased fluidity of tumor cell membranes and expression of C-type virus particles (Hibbs et al., 1972b). However, these features are no longer considered to be shared by all neoplastic cells and, therefore, are probably not of primary importance in recognition of neoplastic cells by macrophages. To date the mechanism by which macrophages distinguish neoplastic cells from normal cells is not well understood.

Cell movement plays an integral part of the host surveillance of neoplasms. Mobility is a basic requirement for the macrophage to locate and enter neoplastic lesions. An in vitro study by Meltzer et al. (1977) showed that macrophages respond chemotactically with a directional movement to a gradient of factors of tumor cell origin. Both Meltzer et al. (1975b) and Snodgrass et al. (1976) found that macrophages also respond chemokinetically (increase in rate of random movement) to media conditioned by neoplastic cells. It has not been determined whether the macrophages chemotactic and chemokinetic responses are mediated by the same factor. The relationship of the chemokinetic response to the tumoricidal capacity of macrophage's cytotoxic function also needs to be investigated. A study of the putative factors released by neoplastic cells into their media to produce a chemokinetic response may help answer these questions.

That neoplastic cells can proliferate and overwhelm the host's

defenses suggests that tumor cells can avoid the surveillance system. Perhaps neoplastic cells produce factors which have an inhibitory effect on the cells of the host tumor surveillance system. This possibility is supported by the findings of Hellström (1974) that tumors shed membrane antigens which bind and neutralize anti-tumor antibodies. Similarly, several factors of neoplastic origin also appear to act on the macrophage mediated arm of the host defenses including a 6,000 - 10,000 dalton molecular weight substance which inhibits the directional movement of macrophages (Snyderman et al. 1976) and a 3,500 dalton factor which inhibits the cytotoxic response of macrophages (Cheung, 1979). It is not clear whether the chemokinetic factor produced by neoplastic cells is associated with either enhancement or inhibition of tumor cell killing. Accordingly, increased macrophage movement could either increase the number of neoplastic cells the macrophage encounters and destroys, or the increased migration might be non-directable, thereby decreasing the efficiency of the chemotactic response and interfering with macrophage neoplastic cell interactions.

Therefore, a study of tumor factor(s) influencing the macrophage rate of migration is relevent to the surveillance mechanism performed by macrophages. Through either enhancement or inhibition of the antineoplastic, chemotactic response of macrophages, the tumor cell derived chemokinetic factor(s) may modulate the efficiency of macrophage-mediated tumor cell killing or cytostasis and consequently influence the survival of the host.

The overall objective of this investigation was to characterize the chemokinetic factor present in the murine Lewis Lung tumor

conditioned media. Therefore, the following projects were undertaken: (1) development of an efficient assay that measures mouse macrophage chemokinetic activity; (2) isolate the chemokinetic factor(s) present in conditioned media; (3) to determine the molecular weight, chemical nature and activity of the isolated factor; (4) to produce an anti-chemotactic antiserum for the immunological characterization and isolation of the chemokinetic factor.

II. REVIEW OF THE LITERATURE

A. The Role of Macrophages in Host Resistance to Tumors

1. The Presence of Macrophages Within Tumors

The host response to tumors is complex and multifaceted. Several different types of host cells are capable of mediating tumoricidal effects and are found infiltrating neoplastic lesions. These cells include T lymphocytes (Holden, et al., 1976), null cells, (Haskill, 1975), granulocytes (Nakayama et al., 1978) and macrophages (Evans, 1972). The macrophage involvement in the host's response to cancer is varied. The number of macrophages present within neoplastic lesions can range from 4% to 56% of all the cells present in the tumor (Eccles and Alexander, 1974). The extent of tumor infiltration by macrophages is thought to be related to tumor immunogenicity (Eccles and Alexander, 1974; Moore and Moore, 1977). It is suggested that the percentage of infiltrating macrophages is directly proportional to the immunogenicity of tumors and inversely proportional to the metastatic rates of the tumors (Eccles and Alexander, 1974).

A correlation exists between the stimulation level of macrophages and the host antitumor response. Old et al. (1959) were the first to demonstrate that mice infected with Bacillus Calmette Guerin (BCG) (a potent macrophage stimulator) displayed increased resistance to tumor grafts. BCG (Hanna et al., 1972) as well as several other macrophage stimulating agents including Corynebacterium parvum (Woodruff et al., 1973) and pyran (Snodgrass et al., 1975) when injected into

nonregressive tumors often induce regression or inhibition of tumor growth. This tumor regression is associated with the extent of hystiocytosis (influx of macrophages) at the tumor site.

The *in vitro* tumoricidal activity of macrophages removed from neoplastic lesions provides further evidence for the macrophage's role in host resistance. The tumoricidal responses of intralesional macrophages varies among tumors, from a short term cytostatic effect (inhibition of DNA synthesis and proliferation), to cytolysis (cell killing) of the neoplastic cells (Evans, 1975). Modulation of the macrophage tumoricidal response may also reflect the etiology of the tumor. The Moloney virus induced sarcoma regresses spontaneously in syngeneic hosts. The macrophages taken from the regressing tumors demonstrated stronger cytotoxic responses than those removed from non-regressors (Russell *et al.*, 1977). Fidler (1975) found the inability of the non-regressor macrophages to kill was not an innate deficiency of the macrophage, but was due to a lack of macrophage activating lymphokines within the tumors. Therefore, both *in vitro* cytotoxic assays and *in vivo* studies of intralesional host cells give supporting evidence that macrophages are a major component in the host response to tumors and are capable of inhibiting tumor growth.

2. Specificity of the Tumoricidal Capacity of Macrophages

According to the results of *in vitro* studies (Granger, 1966; Evans, 1975) macrophages respond cytotoxically to neoplastic cells: (a) specifically and (b) nonspecifically.

In the specific response, macrophages kill tumor cells which are

coated with tumor specific antibody. This is termed antibody dependent cell-mediated cytotoxicity (ADCC) (Granger, 1966). The macrophages bind by their Fc receptors to the antibody coated tumor cells. The degree and type of ADCC cytotoxicity which occurs depends upon the concentration of antibody present on the plasma membrane of tumor cells. Cytostasis occurs at low antibody concentrations and cytolysis occurs at high antibody concentrations (Evans,1975; Haskill and Fett, 1976).

Macrophages are capable of being specifically induced to a tumoricidal state by a lymphokine produced by tumor sensitized T-lymphocytes. This lymphokine is known as the Specific Macrophage Arming Factor (SMAF) (Evans and Alexander, 1972a, 1972b; Pels and Den Otter, 1973). SMAF has a specific tumor antigen binding site which confers upon the macrophage nonspecific tumoricidal ability only when the sensitizing tumor cell antigens are encountered.

The category of the nonspecific response involves a number of agents which stimulate normal macrophages to a level of cytotoxic activity. Activated macrophages express this cytotoxic response, non-specifically against any tumor cell the macrophage encounters. The activated macrophage is selective only in that it is not cytotoxic to most normal cells (Hibbs, et al., 1972a).

3. Macrophage Activating Agents

A number of different macrophage functions can be stimulated by agents such as BCG including phagocytosis (Nathan et al., 1971), adhesion to substrates (Mooney and Waksman, 1970) metabolic rate

(Nathan et al., 1971; Remold et al., 1974; Karnovsky et al., 1978), cell movement (Blanden et al., 1969) and tumor cell killing (Hibbs et al., 1972a; Cleveland et al., 1974). When macrophages are stimulated to a state of non-specific tumoricidal activity, they are referred to as being "activated." Other stimulating agents which demonstrate the capacity to activate macrophages include the lymphokine macrophage activating factor (Piessens et al., 1975), double stranded RNA (Alexander and Evans, 1971), BCG (Cleveland et al., 1974), Listeria monocytogenes (Hibbs et al., 1972a), Corynebacterium parvum (Olivotto and Bomford, 1974) and the synthetic agent pyran copolymer (Kaplan et al., 1974).

Inflamatory agents stimulate a number of the macrophage functions as do activating agents. Macrophages elicited by inflamatory agents have above normal phagocytic ability, but they have little tumoricidal capacity compared to activated macrophages (Stiffel et al., 1971). Mineral oil (Hibbs, 1974a), starch, and Brewer's thioglycollate (Krahenbuhl and Remington, 1974) are considered macrophage eliciting agents.

4. Macrophage Non-specific Tumor Cell Recognition

In their initial studies, Hibbs et al., (1972a) noted that activated macrophages destroy both syngeneic and allogeneic neoplastic cells but do not harm syngeneic or allogeneic normal fibroblasts or kidney cells. They concluded that the tumoricidal activity of activated macrophages does not depend upon tumor specific, organ specific or major histocompatability antigens. Their experiment also

suggests that normal cells upon neoplastic transformation acquire a property that triggers recognition and subsequent destruction by activated macrophages. Hibbs tested this hypothesis by comparing the response of activated macrophage to fibroblasts before and after neoplastic transformation. He found that activated macrophages distinguished between normal fibroblasts and their spontaneously transformed progeny (Hibbs et al., 1972c) and between the contact inhibited 3T3 fibroblast cell line and the virus transformed, noncontact inhibited SV-3T3 cell line (Hibbs, 1973). Therefore, activated macrophages appeared to differentiate between the normal and transformed phenotypes based upon a change associated with the acquisition of abnormal growth properties of the target cells including loss of contact inhibition. Meltzer et al., (1975b) tested the resolution of the macrophage's tumor recognition system by incubating mixtures of ^{3}H-thymidine labeled non-neoplastic and neoplastic cell lines. The activated macrophages only produced a release of the ^{3}H-Thymidine when the neoplastic cells were labeled. Therefore, the macrophage discriminates completely between normal and neoplastic cells and will not damage "innocent bystander" cells.

The possibility exists that activated macrophages do not actually distinguish between normal and neoplastic cells, instead the activated macrophage may have a toxic effect upon both and it is the neoplastic cell's higher susceptability to this toxic effect that imparts the selective killing. This possibility is supported by Keller's (1974) finding that activated macrophages inhibit the proliferation of rapidly replicating cell lines irrespective of whether they showed

normal or neoplastic growth characteristics, and only the neoplastic cells responded to the activated macrophages by dying.

The mechanism of tumor recognition by activated macrophages is unknown. It may depend upon changes in the membranes of both the neoplastic cell and the macrophage. The cell membranes of neoplastic cells can often be distinguished from normal cell membranes by their decreased adhesiveness to other cells and substrates (Abercrombie and Ambrose, 1962), increased negative charge (Louis, 1978), change in glycosyltransferase activity (Roth and White, 1963), marked decrease in a cell surface glycoprotein of a molecular weight of approximately 250,000 (Hynes, 1973; Critchley, et al., 1974), increased leakiness causing an increased release of intracellular enzymes into the surrounding tissue fluids, (Bissell et al., 1971), increased lectin agglutinability which is masked by glycoproteins in normal cells (Nicolson, 1974) and expression of fetal antigens (Medawar and Hunt, 1978). Keller (1979) reported evidence for involvement of fetal antigens in tumor recognition. He found activated macrophage killing of syngeneic and allogeneic tumor targets is blocked by the presence of irradiated fetal liver cells in a dose-dependent manner. This blocking feature of fetal liver cells is rapidly lost after birth. Differences between the membranes of activated macrophages and non-activated macrophages include: increased stickiness (Basic et al., 1974), increased membrane mobility (Nathan et al., 1971) and membrane glycosamine incorporation (Hammond and Dvorak, 1972). Two recent reports indicate BCG activated macrophages selectively bind tumor cells (EL-4 and P815) but not normal cells (lymphocytes and macrophages). This selectivity depends upon

trypsin sensitive structures present on the activated macrophage membrane (Marino and Adams, 1980a, 1980b). The alterations in the membranes of both neoplastic cells and activated macrophages are thought to be involved in tumor cell recognition and in the triggering of the tumoricidal mechanism of activated macrophages.

5. Mechanism of Macrophage Non-specific Tumor Cell Killing

Similar to the recognition system, the cytotoxic mechanism is poorly understood. Originally Hibbs (1974b) suggested that macrophages transported lysosomes into the tumor cells during periods of macrophage-tumor cell fusion. An ultrastructural study by Bucana et al., (1976) of macrophage-tumor cell interactions demonstrating possible lysosomal exchange supports this hypothesis.

Macrophages produce a number of hydrolytic lysosomal enzymes which play a role in the digestion of phagocytized material. Nathan et al. (1979a; 1979b) have indicated that these enzymes are involved in tumor cell killing. He found that the ability of activated macrophages to produce hydrogen peroxide through the peroxidase-hydrogen peroxide-halide system (Klebanoff, 1975) directly corresponds to their ability to kill tumor cells. Free hydroxyl radicals formed from hydrogen peroxide are considered to be the agents producing cell death (Becker et al., 1972). Inhibition of this enzyme system with catalase or ferrocytochrome C also inhibits the tumoricidal capacity of the macrophage. Non-tumoricidal thioglycolate elicited macrophages have been found to produce just as much superoxide anion (the precursor of hydrogen peroxide) as the tumoricidal BCG activated macrophages

(Johnston et al., 1978). Therefore, the lack of distinction in this enzyme system between these two groups of macrophages casts doubts upon its role in tumor cell killing.

Investigations of the cytotoxic activity of soluble products released by activated macrophages into their media have produced varied results. Media conditioned by activated macrophages have been found to be cytolytic (Currie and Basham, 1975; Sethi and Brandis, 1975), cytostatic (Keller, 1975; Calderon et al., 1974), or have no tumoricidal effect (Keller, 1973). A number of studies have reported the necessity of macrophage-tumor cell contact for the tumor cell killing to occur (Hibbs, 1974b; Kaplan et al., 1974; and Basic et al., 1975).

Several investigators attempted to determine the reason for the necessity of the proposed cell to cell contact. Alexander (1976) reasoned that the cytotoxic enzyme released by activated macrophages could be quickly antagonized by components in the serum either by competition or direct inhibition and the enzyme would only be effective in the close vicinity of the macrophage. Hibbs et al., (1977) demonstrated variation in tumor killing by activated macrophages in media containing sera from various species. More recently Adams et al. (1980) have found that the loss of tumoricidal activity from activated macrophage conditioned media was in fact due to inhibitors present in the culture serum.

Currie (1978) proposed a mechanism of macrophage non-specific cytotoxicity based upon evidence that macrophages produce the enzyme arginase. Macrophage release of arginase would deplete arginine in the microenvironment around the tumor. Arginine is not an essential

amino acid for protein synthesis by normal cells but it is essential for many neoplastic cells. Therefore, arginine depletion would have a lethal effect on these neoplastic cells. However, Nathan et al. (1979b) in his experiments found that addition of large amounts of arginine to the macrophage-neoplastic cell environment had no inhibitory effect on tumor cytotoxicity. Therefore, it is unlikely that arginase is a major component of the tumoricidal mechanism of macrophages.

Adams (1980) recently proposed that neutral serine protease(s) secreted by activated macrophages participate in the cytolytic destruction of neoplastic cells. He found that activated macrophages secrete more neutral proteases than do elicited or normal macrophages and two serine proteases inhibitors (bovine pancreatic trypsin inhibitor and diisopropylflurophosphate) reduce the cytolytic destruction of neoplastic targets by activated macrophages. In a companion article Adams et al. (1980) reported that serum free media conditioned by activated macrophages was cytotoxic to neoplastic targets but not for non-neoplastic cells. The lytic activity of this media was inhibited by both of the serine protease inhibitors and by incubation with 10% fetal calf serum.

Kaplan et al. (1978) noted a lack of DNA synthesis by neoplastic targets after culturing with activated macrophages for 16 hours. The rate of neoplastic cell division, however, remained constant for one cell division. This produced a 50% reduction in the DNA content of the neoplastic cells incubated with activated macrophages for 16 hours. Kaplan et al. (1978) suggested that the activated macrophage-induced reductive division of neoplastic cells may be related to activated

macrophage-mediated tumor cell cytotoxicity. This hypothesis accounts for the relatively long periods (18 hours) often observed (Alexander and Evans, 1971; Olivotto and Bomford, 1974; Piessens et al., 1975; Meltzer et al., 1975b) between initial macrophage tumor cell contact and tumor cell death. The reductive division phenomenae also correlates with the fact that macrophage induced tumor cytostasis has been measured as a function of the lack of incorporation of DNA base precursors.

B. Macrophage Locomotion

The ability of macrophages to migrate to sites of inflammation and developing tumors is an integral part of their response to pathogens. Macrophage locomotion is intimately associated with a system of microfilaments and microtubules. Tatsumi et al., (1973) and Hartwig and Stossel (1975a) have found that the microfilaments isolated from macrophages contain contractile proteins, specifically actin and myosin. The ratio of actin to myosin is much higher in motile leukocytes than in the non-motile skeletal muscles (Pollard and Weihing, 1974).

Hartwig and Stossel (1975b) discovered an actin binding protein present in the cytoplasm of alveolar macrophages. This protein reversibly crosslinks the abundant actin, causing the liquid cytoplasm to gel. They proposed that the excess actin is utilized to synthesize gels needed for structural support in the formation of lamellipodia and pseudopodia of motile macrophages. They correlate the hyalin appearance of these cell processes (Zigmond and Hirsh, 1973) to the

presence of actin gels. Stossel (1978) proposed that the changes in cell shape associated with cell movement are brought about by the action of a network of contractile microfilaments attached to the plasma membrane and to the actin gels. This is supported by the ability of Cytochalasin B (an inhibitor of microfilament contraction) to suppress macrophage movement (Becker 1972).

The microtubules are believed to provide a structural cytoskeleton (Bloom and Fawcett, 1975). This cytoskeleton acts as a framework to which organelles are attached and their orientation maintained. Disruption of the microtubules by colchicine does not inhibit macrophage movement but it produces the loss of directional movement such as chemotaxis (Bhissey and Freed, 1971; Allison et al., 1971; Wilkinson, 1976).

A network of intermediary reactions and messengers are needed within macrophages to transport information obtained at the plasma membrane to the microfilament-microtubule system. Two components which may act as intermediaries are cyclic-AMP and cyclic-GMP. Increased intracellular levels of cyclic-AMP has been found to inhibit macrophage migration while higher levels of cyclic-GMP enhances macrophage movements (Estensen et al., 1973).

Two mechanisms have been shown to be functional in triggering contraction of the microfilament system. One is the contractile mechanism and the other is the metabolic effector mechanism. The contractile mechanism, present in skeletal and smooth muscle cells, has been studied extensively. In this system, triggering involves depolarization of the plasma membrane. This allows for an influx of

Ca^{++} which brings about microfilament contraction as well as a break down of microtubules (Weisenberg, 1972). Evidence supporting the presence of this system in the macrophage comes from the work of Wilkinson, (1975) who found monocyte migration to be significantly depressed when the cells were incubated in Ca^{++} and Mg^{++} free media. The original rate of migration could be restored by adding the calcium inophore A23187 to the media.

The metabolic effector system is utilized by leukocytes in migration. In this case, attachment of an agonist to the plasma membrane triggers the activation of a nucleotide-cyclase. Depending upon the specificity of the enzyme, cyclic-AMP, cyclic-GMP or other cyclic nucleotides are produced. Increased levels of C-AMP and C-GMP inhibit and enhance macrophage migration respectively (Estensen et al., 1973).

Based upon the phenomenon of leukocyte capping, Stossel (1978) proposed a hypothetical mechanism for macrophage locomotion. According to this hypothesis the membrane surface receptors of the macrophage adhere to the adjacent substrate. These adherent receptors are linked to the contractile network within the cell. The contractile network which is anchored to the membrane at the sides of the cell (perpendicular to the direction of travel) pulls the substrate adherent receptors in the opposite direction of cell movement. Once the attached receptors reach the uropod (trailing region) of the cell they are either detached from the substrate and ingested or they are left attached to the substrate and are blebed off in small membrane vesicles. It remains to be seen whether this hypothesis represents

the actual mechanism by which the contractile and adherent molecules function in macrophage locomotion.

1. Chemotaxis

McCutcheon in 1946 defined chemotaxis as "a reaction by which the direction of locomotion of cells or organisms is determined by substances in their environment." Specifically, when macrophages are exposed to a chemotactic factor gradient, they respond by migrating toward the higher factor concentrations. Chemotaxis is considered to play an important role in bringing macrophages to sites of inflammation, infection, wound healing and neoplasia. The inhibition or reduction of macrophage chemotaxis that occurs in cancer patients has been correlated with a poor prognosis and decreased resistance to infection (Snyderman and Stahl, 1975; Snyderman et al., 1977).

McCutcheon (1946) proposed that to respond chemotactically the macrophage has to be able to detect the microgradient of a chemotactic factor present across its surface. Two mechanisms were suggested for the detection of the gradient by macrophages. According to one hypothesis the macrophages possess specific surface receptors for certain chemotactic agents. When the receptors at a surface site on the cell membrane become predominately occupied by the chemotactic factor, this triggers the microfilament-microtubule system to move the macrophage in that direction. Snyderman and Fudman (1980) recently reported the presence of specific high affinity receptors for the chemotactic N-formylated peptide fMet-Leu-Phe on the plasmalemma

of peritoneal macrophages. These investigators estimated approximately 10,000 binding sites per cell. This number is constant in both resident and inflammatory macrophages. Therefore, the increased chemotactic responsiveness of inflammatory macrophages probably is due to metabolic events which occur subsequent to the binding of the chemotactic factor to the cell.

Another hypothesis proposed by Snyderman and Mergenhagen (1976), based on studies with complement C5a, suggested that chemotactic factors may interact directly with the plasma membrane of the macrophages. They found that the chemotactic factor C5a is hydrophobic, which facilitated its interaction with the cell membrane of the macrophage. This factor is thought to act by first entering the phospholipid bilayer then altering the ion permeability of the membrane to allow an influx of Ca^{++} ions which activate the microfilament system. A study by Naccache et al. (1979) showed that C5a produced a displacement of membrane Ca^{++} when it binds to the surface of neutrophils. The activation would be a local effect within the macrophage and would occur in the region of the cell closest to the chemotactic source, thereby initiating cell movement in that direction. This regional effect is supported by the observations of Gallin et al. (1978) that demonstrated intracellular cations are localized at the plasma membrane lamellipodia which is extended toward a chemotaxic source.

Macrophages respond to a variety of agents chemotactically. Chemotactic factors are present on Corynebacterium parvum (an activating agent) (Wilkinson et al., 1973) in the complement system (C5a)

(Snyderman et al. 1971) and in milk protein (casein) (Keller and Sorkin, 1967). In addition, immune complexed (Ward, 1968), lymphokines produced by sensitized lymphocytes (Ward et al., 1969), and small synthetic oligopeptides such as fMet-Leu-Phe (Schiffman et al., 1975) that are present in bacterial protein degradation products (Snyderman and Gudman, 1980) are also chemotactic for macrophages. Chemotactic factors form a diverse group ranging in size from 500 daltons for fMet-Leu-Phe (Snyderman et al., 1980) to greater than 400,000 daltons for heat aggregated serum proteins (Norman and Sorkin, 1977).

2. Chemokinesis

Chemokinesis is defined as an increase in the rate of random non-directional cell movement. This response has been studied far less than the chemotactic response and, therefore, is correspondingly less well understood.

Macrophages activated in vivo by BCG (Poplack et al., 1976) or pyran (Snodgrass et al., 1977) possess an increased rate of random migration in comparison to normal macrophages. This chemokinetic response is thought to be an integral part of the enhancing effect this agent has on macrophage metabolism and function. Lymphokines produced by antigen or mitogen stimulated lymphocytes also enhance the movement of normal macrophages. These lymphokines range in size from 50,000 (Fox and Rajaraman, 1979) to 250,000 daltons (Aaskov and Anthony, 1976; Weisbart 1974). Not all the factors with chemokinetic activity are produced by stimulated lymphocytes. Fox et al. (1974)

observed that when he removed factors which inhibit macrophage movement (MIF) from fetal calf serum he could detect a macrophage stimulating factor (MSF). This MSF has a molecular weight around 67,000 daltons.

Snyderman and Pike (1978) found that blood monocytes develop enhanced random migration when incubated with lymphocyte-derived chemotactic factor under non-gradient conditions. Therefore, it appears that chemokinetic factors affect the rate but not the directional polarization of macrophage movement, while chemotactic factors may affect both.

3. Methods for Evaluating Chemokinesis

To characterize the chemokinetic factor found in tumor cell conditioned media, it is important to measure parameters of macrophage movement that distinguishes between chemokinetic and chemotactic movement. Since random macrophage movement characterizes chemokinesis, it was necessary to develop techniques suitable for assaying random lateral migration of macrophages. In order to detect random migration without constant monitoring of individual cell migration, comparative measurements of the distances the cells have migrated during a unit of time have been used. Among such techniques are the capillary tube assay of George and Vaughan (1962), the agar well (Eccles, 1977) and the agar microdrop assays (Harrington and Stastny, 1973). All of these assays involve the initial localization of a concentration of macrophages followed by the fanning out of the cell population as the result of random

migration. After a suitable period, the area covered by the cells is quantitated with the aid of a projector, or the distance the cells have migrated from the source is determined by linear measurements. These assays have the disadvantage of being insensitive to small changes in the rate of random movement. Theoretically, a cell that has increased its distance migrated from the point of origin by only 42% has actually doubled its rate of movement. Because the movement is random rather than linear, the rate of migration is a function of the area covered by the cell rather than the distance (r) from the point of origin. This follows from the formula in which area (rate) = $\pi(r)^2$. The development of an assay which physically restricts the macrophage random migration to a linear direction might provide a more sensitive assay of chemokinesis.

The modified Boyden blind well chamber is the standard assay for determining the chemotactic response of macrophages (Snyderman and Mergenhagen, 1976). The Boyden chamber consists of a plexiglass block containing an upper and a lower well separated by a filter membrane. Snyderman and Mergenhagen (1976) detected chemotaxis by placing the chemotactic factor in the lower well and the macrophages in the upper well. Diffusion of the chemotactic factor through the membrane produces a gradient to which the macrophages are attracted and they migrate through the pores of the filter into the (lower well). This assay can also be used to detect chemokinesis (Zigmond and Hirsch, 1973).

To detect chemokinesis Zigmond and Hirsch (1973) placed the same concentration of factors in both the upper and lower well. With

an increase in the rate of macrophage random movement in the upper well due to the presence of a chemokinetic factor in the medium, a greater number of macrophages randomly migrated through the filter separating the two wells and more cells were counted in the lower well.

C. Tumor Cell Products Affecting Macrophages

1. Factors Depressing Macrophage Functions

Dizon (1963) was the first to report that tumors' products can depress macrophage functions. He observed a depression of the cellular infiltration response to a sterile inflammatory agent in cancer patients. Using a skin window he compared the response of normal individuals, cancer patients and non-cancer patients. After a 6 hour test period, macrophages predominated at the test site of the healthy controls, patients with early cancer and those with other diseases. In patients with advanced cancer, however, there was a preponderance of neutrophils even at 24 and 48 hours. Based on these observations, Dizon suggested cancer patients have an impaired capacity to mobilize tissue macrophages. Soloway's (1965) study corroborated Dizon's conclusion. He tested the inflammatory responsiveness of patients with and without neoplasia. In his studies chronic bacterial inflammatory agents produced predominantly macrophage mediated inflammation in patients free of neoplasia, but in cancer patients the inflammatory response was significantly depressed.

More recently, several investigators (Boetcher, 1974; Bice, 1976; Snyderman and Pike, 1977) studied the *in vitro* chemotactic response of

blood monocytes from patients with a variety of different cancers. In each study, a substantial number of patients possessed monocytes with abnormal chemotactic capacity. Snyderman's et al. (1977) study correlated the best prognosis with those patients having normal chemotactic response prior to chemotherapy. Snyderman et al. (1978c) also noted abnormal chemotactic responses among patients with malignant breast tumors while patients with benign breast tumors had normal monocyte chemotactic responsiveness.

Direct evidence demonstrating tumor release of macrophage inhibitory factors came from the work of Fauve (1974). He found that culture media in which malignant teratocarcinoma cells were grown contained a substance with a molecular weight of 10^3 to 10^4 daltons which suppressed inflammatory responses in the peritoneal cavity. Due to the relatively short time (6 hours) involved, most of the cells inhibited by this factor were neutrophils.

In a series of detailed studies, a soluble tumor (6,000 - 10,000 daltons) factor present in the serum and urine of tumor bearers was found to produce both the reduced inflammatory response and the reduced macrophage chemotactic ability (Snyderman and Pike, 1976 and Snyderman and Cianciolo, 1979). In vitro studies by Nelson and Nelson (1977) described a similar size chemotactic inhibitor present in conditioned media of neoplastic cells. The importance of the neoplastic factors that limit macrophage chemotactic ability was correlated by several findings of Snyderman et al., (1978b). Relatively small numbers of tumor cells (1×10^4) were capable of producing enough factor to inhibit systemic macrophage chemotaxis. Mice treated

with the neoplastic factor required implantation of fewer neoplastic cells in order to get tumor growth and the tumors grew faster in the treated mice than in controls.

Recently a possible association between the neoplastic chemotactic inhibitor and viruses has been suggested. Both Stevenson, et al. (1980) and Cianciolo et al. (1980) discovered that mice infected with oncogenic or lactic dehydrogenase (LDH) virus possessed chemotactic inhibitors in their serum. The inhibitor isolated from the LDH virus envelope proteins was approximately the same size (15,000 daltons) as the neoplastic inhibitor. Chemotactic inhibition also occured in mice bearing tumors free of detectable virus. However, the possibility does exist that these tumors were infected with an undetected virus or synthesizing viral proteins. Hence, an associated viral infection and not the malignant disease may be responsible for cancer-associated macrophage chemotaxis suppression.

Several other neoplastic factors have been found to inhibit other macrophage functions. Yamazaki et al. (1977) found a lipoprotein present in the cell-free ascites of ascites tumor bearing mice that inhibits macrophage ADCC of ascites tumor cells. Cheung et al (1979) characterized a 3,500 dalton molecular weight lipid-like factor in homogenates of several murine tumors (LL, EL4, B16, mKSA and Meth 1A) which inhibited tumor cell killing by activated macrophages. North et.al. (1976) described a dialyzable factor in the serum of tumor bearing mice which inhibited macrophage mediated host resistance to bacterial parasites. A 25,000 dalton molecular weight, heat stable factor was isolated by Rhodes et.al. (1979) from supernatant of cultured human

tumors. This factor inhibited the increased expression of Fc receptors that normally occured when blood monocytes were cultures for 24 hours in 10% fetal calf serum (FCS).

Of the several macrophage functions inhibited by neoplastic factors, the inhibition of chemotaxis is observed most often in tumor bearing animals. This suggests that inhibition by neoplastic cells of macrophage mobility or directional movement could play a role in insuring the escape of the tumor from the host defense.

2. Factors Enhancing Macrophage Functions

In contrast to the neoplasia mediated depression of macrophage mobilization a series of reports have demonstrated that factors produced by tumor cells can increase the proliferation of cells in the monocyte/macrophage series. This proliferative effect was observed in the bone marrow (Baum and Fisher, 1972, Khaitov et al., 1976), in draining lymph nodes (Carr, Price and Westby, 1976), in pheripheral blood (Eccles, Bandlow and Alexander, 1976) and in peritoneal macrophages (Nelson and Kearney, 1976) of the tumor bearing animals. However, no macrophage mitogenic factor has yet been isolated from neoplastic cells. It is possible that this proliferative macrophage response is mediated indirectly by macrophages and is not a reflection of the direct effect of a factor produced by tumor cells.

Blakeslee (1978) examined the effects of MCA fibrosarcoma-conditioned media upon normal resident and proteose peptone elicited macrophages. After 3 days of incubation, these macrophages developed a morphology similar to BCG activated macrophages (increased

spreading and highly vacuolated cytoplasm), an enhanced rate of migration, and cytostatic activity against fibrosacoma cells. Blakeslee concluded that a substance of approximately 12,000 daltons molecular weight in sarcoma conditioned media was responsible for the activation of the macrophages *in vitro*.

Other factors found in tumor cell conditioned media were observed to enhance the mobility of activated macrophages. Meltzer and Leonard (1977) described a 15,000 dalton molecular weight factor which behaved as a chemoattractant for BCG activated macrophages but not for normal macrophages.

A chemokinetic effect upon activated macrophages was observed by Meltzer *et al*. (1975) during the cinemicrographic analysis of macrophages incubated *in vitro* with various target cells. BCG activated macrophages exhibited a rate of lateral movement that was 5 times greater in the presence of neoplastic embryonic cells than in the presence of normal embryonic cells. When normal macrophages were tested under these conditions, their rate of lateral movement was the same in both cases.

Snodgrass *et al*. (1976, 1977) performed a more detailed cinemicrographic analysis of activated and normal macrophages in the presence of Lewis lung carcinoma (LL) cells and normal mouse embryo fibroblasts (MEF). Pyran activated macrophages incubated with MEF showed a lateral rate of movement 3 times greater than did normal macrophages. In the presence of the LL carcinoma cells, the activated macrophage moved 8.9 times faster than normal macrophages and 2.9 times faster than activated macrophages alone or with MEF.

Subsequently Snodgrass et al. (1978a; 1978b) reported similar results when activated and normal macrophages were incubated in cell free media conditioned by growing LL carcinoma, 2181 fibrosarcoma or MEF cells. They concluded that neoplastic cells produced a chemokinetic factor. As with the neoplastic chemoattractant (Melzter and Leonard, 1977), this chemokinetic factor is selective in acting upon stimulated macrophages. A macrophage migration enhancing factor of tumor origin that may be identical to Snodgrass's chemokinetic factor was characterized by Nelson and Nelson (1977). These investigators found that proteose peptone elicited macrophage migration was enhanced 29% in the presence of tumor conditioned media.

As with the inhibitory factors, the enhancing neoplastic factors act primarily on macrophage migration. The requirement of macrophage stimulation for the response to the enhancing factors indicates that these factors could be involved in triggering the stimulated macrophages to destroy neoplastic cells. In this sense, the enhancing factors could represent an integral part of the mechanism of macrophage localization and tumor cell killing at a neoplastic lesion.

III. MATERIALS AND METHODS

A. Animals

Six to eight weeks old C57BL/6J mice, male and female, were obtained from Jackson Laboratories, Bar Harbor, Maine. All mice were maintained on RMH-3000 Rodent Laboratory Chow (Charles River, Agway, Syracuse, New York) and chlorinated water ad libitum.

B. Buffers and Media

1. Physiologic Saline (PS) 149 mM: 8.7 gm NaCl was dissolved in deionized water (d.H_2O) to make 1 liter.

2. Phosphate Buffered Saline (PBS) pH 7.2, 149 mM, (for general use): dissolve 1.15 gm of Na_2HPO_4 0.2 gm of KH_2PO_4, 8.0 gm NaCl, and 0.21 gm KCl in 1 liter d.H_2O.

3. Phosphate Buffered Saline, pH 7.2, 10 mM (for Column chromatography): 56 ml. of 0.2M Monobasic Sodium Phosphate and 144 ml. of 0.2M Diabasic, Sodium Phosphate were combined with PS added to obtain a final volume of 4 liters.

4. Trypan Blue (for viability counts): dissolve 0.15 gm trypan blue in 100 ml PS.

5. Diluent (for tissue culture passage): dissolve 8.0 gm NaCl, 0.4 gm KCl, 0.11 gm $Na_2HPO_4 \cdot 7H_2O$, 0.06 gm KH_2PO_4, 1.0 gm Glucose in 1 liter d.H_2O. The solution was autoclaved and stored at 4^oC.

6. EDTA Solution (For Tissue Culture Passage): 10 ml of 2% EDTA (Sigma Chemical Company, St. Louis, Missouri) were added to 90 ml of diluent.

7. Barbital Buffer, pH 8.2, 77 mM (for Ouchterlony double diffusion): Sodium barbital 15.85 gm; and 1.9 ml conct. HCl were dissolved in 800-900 ml d.H_2O, adjusted to pH 8.2 with dilute HCl and adjusted to a volume of 1 liter.

8. Hank's Balanced Salt Solution (HBSS) pH 7.2, 149 mM, (for washing macrophages and tumor cells): 10 ml of 10X HBSS [Grand Island Biological Company (GIBCO), Grand Island, New York] , added to 90 ml sterile d.H_2O, and adjusted to pH 7.2 with sterile saturated NaH_2CO_3 in d.H_2O.

9. Culture medium used in all experiments: Eagles Minimal Essential Medium (EMEM) with Earle's balanced salt solution (GIBCO), essential amino acids (GIBCO), vitamins (GIBCO), 100 u penicillin/ml (GIBCO), 100 mcg streptomycin/ml (GIBCO), and 20% fetal calf serum (FCS) (GIBCO).

10. Tumor conditioned media was prepared by growing neoplastic cells in the same medium with or without FCS for 24-48 hours. The media are then centrifuged at 700 x g for 15 minutes to remove cell debris.

11. Mouse Embryo Fibroblast (MEF) conditioned media was prepared in the same manner.

12. Mouse Activated Serum (MAS), the chemotactic media containing

C5a was made by incubating fresh mouse serum with Zymosan (10mg/ml) at 37°C for 1 hour and at 56°C for 1 hour. The serum was then diluted in EMEM without FCS and stored at -18°C.

C. Determination of Cell Viability

For both, passage of neoplastic cell lines and preparation of macrophages, the Trypan Blue exclusion technique was utilized to determine the viability of cell preparations.

1. Add 1 part of the cell suspension to 1 part of the trypan blue PS solution.

2. Load cells into hemacytometer and count the number of unstained (viable) cells in 4 large corner squares within 3 minutes.

3. Determine the mean number of viable cells/large square

viable cells/ml = (mean number per large sq) x 10^4/ml x $\frac{1}{\text{dilution}}$

D. Neoplastic Cell Lines

The Lewis Lung carcinoma (LL) is a spontaneous pulmonary adenocarcinoma of a C57BL/6 mouse characterized by Lewis in 1951. In our facilities the tumor line was propogated in the same strain.

The B-16 melanoma arose spontaneously in a B6 mouse in 1954 (Green, 1966). In our facilities the tumor line was maintained by transplantation in the C57BL/6 strain.

The Ehrlich mammary carcinoma was first adapted to the ascites form by Loewenthal and John in 1932. In our laboratory, the Ehrlich ascites carcinoma line was maintained by transplantation in C57BL/6

mice.

The MTDl tumor line was supplied by Dr. B. Diwan of Meloy Laboratories Inc. (Springfield, Virginia). This dimethylbenz[a] anthrene induced mammary adenocarcinoma was maintained in BALB/c mice by subcutaneous transplantation.

The MCA(k)K and MCA(k)E tumor lines are methylcholanthrene induced fibrosarcomas. They were induced in the C57BL/6 mouse in 1979 by Dr. A. M. Kaplan at the Medical College of Virginia.

Cell cultures were passaged at confluence every 4 to 5 days. This was accomplished by rinsing 25 cm^2 culture flasks (Falcon, Oxnard, CA) twice with diluent, then incubating the cultures in a solution of 0.2% EDTA at 37°C for 15 minutes. After incubation, 2.0 ml of EMEM supplemented with FCS was added to the flasks and subsequently the cells were harvested by pipetting. New cultures were set up by plating 1 x 10^5 cells in 25 cm^2 culture flasks.

E. Acquisition of Normal Cells

The normal cells utilized as controls in this study were fibroblasts. These cells were obtained from C57BL mouse embryos at 15 to 17 days of gestation. The embryos were cut into small pieces and to obtain a cell suspension the tissue fragments were forced through a 10 ml syringe. The suspension was placed in a trypsinizing flask with 50 ml of EMEM containing 1.25 ml of 10% trypsin. After aggitation for 1 hour the cells were decanted, filtered through gauze, and plated at a density of 5 x 10^5 cells per 25 cm^2 culture flask containing 5 ml of EMEM. Embryo fibroblast cultures were maintained

in the same manner as the cultures of tumor cells.

F. Acquisition of Macrophage

1. Resident Macrophages

Following cervical dislocation the abdominal skin of a mouse was reflected to expose the translucent abdominal musculature overlying the peritoneal cavity. Following an intraperitoneal (i.p.) injection of 5 ml of cold HBSS the abdomen was briefly massaged and the peritoneal exudate drained. This procedure was repeated three times. The peritoneal cells obtained were washed 3 times in HBSS, and cell viability and the number of cells determined. In addition, a sample of the cells was stained for non-specific esterase to determine the number of macrophages present.

2. Elicited Macrophages

Two agents were used to elicit macrophages. Mice were injected i.p. with either sterile 2.5% type II Oyster Glycogen (SIGMA Chemical Company, St. Louis, Missouri) solution (0.25 ml/10 gm body weight) or with 1 ml of 10% thioglycollate broth (Difco Laboratories, Detroit, Michigan). The elicited peritoneal macrophages were harvested on the fifth day post injection.

3. Activated Macrophages (AMØ)

A suspension of C. parvum was obtained from Burroughs Wellcome Co. (Research Triangle, N.C.). Pyran copolymer, (lot XA124-177) was obtained from Hercules Chemical Corporation (Wilmington, Delaware).

Peritoneal macrophages were activated by injecting mice i.p. with C. parvum (35 mg/Kg) or pyran (25 mg/Kg) 7 days before harvesting of the peritoneal cells.

4. Identification by Routine Histology

Due to the heterogeneity of the peritoneal exudate population it was necessary to determine the percentage of peritoneal macrophages migrating in the capillary tubes. The differential Wright stain, the Giemsa nuclear stain and the non-specific esterase stain techniques were employed to distinguish histologically macrophages from lymphocytes and neutrophiles present in peritoneal exudates.

The differential stain consisted of drying the cells on glass slides, followed by a 15 second staining with Instant Wright Stain (Canalco, Rockville, Maryland) and a rinse in d.H_2O. Cells having a large diameter (10 to 14 µm), oval to kidney shaped nucleus, cytoplasmic granules and vacuoles were considered macrophages.

The nuclear stain involved fixing the cells with 100% methanol, staining for 15 minutes with Wolbach's modification (Wolbach et al., 1922) of the Giemsa stain and rinse in d.H_2O. Cells having a large diameter (10 to 14 µm), and a oval to kidney shaped nucleus were considered macrophages.

The identification of macrophages by non-specific esterase staining was performed according to the method of Tucker and Jordan (1977). Briefly, cells were fixed with saponin formalin for 40 seconds, then incubated in a fresh mixture of hexazonium pararosanilin and α naphthyl butyrate in cacodylate buffer, at 37°C for 12 minutes.

Cells which stained brownish-red were considered esterase positive. In the peritoneal population only the macrophage stains esterase positive. The non-specific esterase stain was routinely used to determine the percentage of macrophages in the peritoneal exudate.

5. Determination of Phagocytic Capacity

Latex beads (Difco-Bacto latex 0.81) were resuspended in EMEM at 5 x 10^8 beads/ml. This suspension was then layered over adherent cells and the cells were incubated in a humidified 95% air and 5% CO_2 atmosphere at 37°C. After two hours the cultures were rinsed several times, the cells were dried and stained with Instant Wright's stain. Cells containing three or more latex beads were considered positive for phagocytosis.

6. Scanning Electron Microscopy (SEM)

SEM was employed to determine the surface morphology of cells migrating in the capillary tubes. Capillary tubes containing activated macrophages were prepared as described in Section IV. A. (page 44). After a 6 hour incubation in experimental and control media, the tubes were removed and fixed in phosphate buffered 2% gluteraldehyde, pH 7.2. After 15 minutes the cells were washed in buffer and postfixed in phosphate buffered 1.5% OsO_4 (pH 7.2) for 15 minutes. Following osmication, the cell preparations were rinsed in buffer, and the upper surface of the capillary tube broken away to expose the migrated cells. The samples were dehydrated through acsending concentrations of acetone and finally in Freon TF. Samples

were critical-point dried with a Polaron apparatus using Freon 116 as a transitional fluid. The samples were coated with a 20 um thickness of gold-palladium in an Eiko ion coater. The surface morphology of the cells were viewed with a Hitachi S-500 scanning electron microscope (SEM).

7. Time Lapse Cinematography

Vital microscopy was used to study the migration of activated macrophages in the presence and absence of the neoplastic cell derived chemokinetic factor according to previously described procedures (Snodgrass, 1977). Capillary tubes containing activated macrophages were placed in Sykes-Moore chambers at pH 7.2. A Wild M40 inverted phase contrast microscope enclosed in a custom incubator was used for cinematography. During filming the specimen was maintained at 37^{o}C in a moist atmosphere of 5% CO_2 and 95% air. A Bellex Pollard 16 mm camera was regulated with a Sage Series 500 cinematographic apparatus (Sage Instruments, White Plains, New York). Exposures were made at 0.4 sec/exposure and 1.0 exposure/min with Kodak Plus-X reversal 16 mm film. Cells were filmed with a 10X phase-contrast objective lens and a 5X projection lens. The rate of lateral movement of macrophages was measured by mapping the position of individual cells at 10-minute intervals (as shown in Figure 2) and measuring the distance traveled with a Numonics Model 250 planimeter (Numonics Corp., Lansdale, Pennsylvania).

G. Macrophage Chemotaxis Assay

This assay was used in conjunction with the chemokinetic studies to determine whether the isolated chemokinetic factor also had any chemotactic characteristics. Dilutions of the LL conditioned media, the LL derived chemokinetic factor, unconditioned media and MAS were preheated to 37°C and placed in the lower wells of the modified Boyden chemotactic chambers. Polycarbonate membrane filters with 5 µm pores (Nucleopore Corp., Pleasanton, California) were placed over the lower wells (dull side up). The upper wells were filled with 0.2 ml of peritoneal cells (2×10^6/ml) in EMEM. The chambers were incubated at 37°C in a 95% air and 5% CO_2 humidified atmosphere for 4 hours. After incubation, the upper wells and membranes were removed and 0.02 ml of 0.2% EDTA in saline added to the lower well. Following a 15 minute incubation, the cells in the lower well were vigorously pipetted to loosen any adherent cells and then counted. All samples were assayed in triplicate. One tail student's t-test was used to assess the significance of differences. Significant differences were considered to be $P < 0.05$.

H. Concentration of Tumor Conditioned Media

1. Dialyzation and Lyophilization

The LL conditioned media, without FCS, had a very low protein concentration (less than 0.05 mg/ml). Therefore, it was necessary to concentrate these media by lyophilization before proceeding with the experiments.

Dialysis tubing, half inch in diameter was filled with tumor

conditioned media and placed in a 4-liter flask of d.H_2O on a stirring plate at 4°C. Dialysis proceeded for 48 hours with one change of d.H_2O. The material was frozen in vacuum flasks, attached to a Denton Freeze-drying apparatus and lyophilized overnight. This freeze-dried material was kept at 0°C until needed.

2. Ultrafilitration

Initially Lewis lung and Ehrlich ascites conditioned media (with FCS) were filtered through a Diaflow XM 100 membrane (Amicon Corp., Lesington, MA) with a molecular weight cutoff of 100,000 daltons to determine the molecular weight range (above or below 100,000 daltons) of the chemokinetic factor. Later it was used to concentrate the high molecular weight material in the media for molecular sieve chromatography. Media were filtered in Amicon stirring cells at a pressure of 15 psi nitrogen.

I. Molecular-Sieve Chromatography

Five milliliter samples of 10 fold concentrated LL conditioned media (molecular weight greater than 100,000 daltons) were fractionated on a hydrated Sepharose 4B (Pharmacia, Fine Chemicals AB. Uppsala, Sweden) column (bed length 70 cm, bed dia 2.5 cm) to partially purify the chemokinetic factor and to determine its approximate molecular weight.

Phosphate buffer saline, pH 7.2, 10 mM was used as an eluent to run the sample through the column at 4°C. A flow rate of 15 ml/hour was maintained during fractionation. 6 ml fractions were collected

on an ISCO fraction collector (Instrument Specialities Co., Lincoln, Nebraska). Immediately after the samples were fractionated, the relative protein concentration in each tube was determined by measuring absorbance at 280 nm with a model DU spectrophotometer (Beckman Insruments, Inc., Pasadena, California). The column was calibrated (to determine molecular size) using the following standards: thyroglobulin (670,000 daltons), catalase (230,000 daltons), aldolase (160,000 daltons) and blue dextran (5,000,000 daltons).

J. Sodium-dodecysulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

PAGE was used to determine the purity of the chemokinetic fraction obtained from the Sepharose 4B chromatography of the LL conditioned media and to compare the protein species present in the CKF with those in LL conditioned media and in FCS. A disc electrophoresis apparatus (Canalco, Rockville, Maryland) was employed. Following the procedure of Laemmli (1970), 50 µl samples of these materials were electrophoresed in 6% polyacrylamide gels for 4 hours at 2.5 mA/Gel. The gels were fixed for 1 hour in 12% acetic acid, stained in a solution of .025% Coomassie blue, 7% isopropyl alcohol and 10% acetic acid for 1 hour and then placed in a gel destainer (Canalco, Rockville, Maryland) for 48 hours. Densitometer scans of the gels were performed using an absorbtometer adjusted to 570 nm. These scans provided a method of comparing the location and relative quantity of proteins in the gels.

K. Enzymatic Digestion of the Chemokinetic Factor

The susceptability of the chemokinetic factor to digestion by the enzymes trypsin (cleaves arginine and lysine peptide linkages) and chymotrypsin (cleaves phenylalanine, tyrosine and tryptophan peptide linkages) was evaluated in an effort to elucidate the proteinaceous makeup of the factor. One ml of LL conditioned medium without FCS was incubated for 1 hour at 25°C with various concentrations of trypsin (Activity: 19 units/ml at 25°C) and chymotrypsin (Activity: 20 units/ml at 25°C) bound to agarose beads (Miles Laboratories, Elkhart, Indiana). The substrate bound enzymes were then removed by centrifugation and the chemokinetic activities of the media were determined in the capillary tube assay. The relative chemokinetic activities of each of the media were correlated with the concentration of enzyme to which the media were exposed.

L. Preparation of an Anti-Chemokinetic Factor Antiserum

Production of a specific anti-CKF antiserum provided a useful tool for the immunological identification of the chemokinetic factor by the Ouchterlony technique and could also be used for both immunohistochemical localization and affinity column isolation of the factor.

To produce the anti-serum, a mixture of 0.5 mg of column purified chemokinetic factor in 1.0 ml saline with 1.0 ml Freund's complete adjuvant (GIBCO) was emulsified by passing the mixture between two 5 ml syringes attached by Tygon tubing. After a 1 hour incubation at 4°C the suspension was mixed again, warmed to 37°C and prepared for injection. A 5 month old rabbit was primed with intradermal (i.d.)

injections at 25 different sites along the dorsal surface with a total of 1.5 ml injected. Booster injections (i.d. of .5 mg protein) were given at 6 and 8 weeks. At 10 weeks the serum was tested for anti-CKF activity by the capillary tube micro-precipitation method and the animal was bled. Serum was frozen in .5 ml aliquots at $-70^{\circ}C$ until used.

M. Absorbtion of Antisera With Aggregated Calf Serum

Due to the presence of FCS proteins in the CKF it was necessary to remove the FCS specific antibodies from the antiserum by absorbtion with FCS. Fetal Calf Serum was rendered insoluble by chemical aggregation. This insoluble immunoabsorbtion technique has the advantage of the efficient removal of the aggregated FCS and the bound FCS-specific antibodies from the rabbit antiserum after absorbtion.

Insoluble polymers of FCS were prepared by the Ethyl chloroformate coupling method of Avrameas and Ternynck (1967). Five grams of FCS was added to 100 ml of pH 4.8, 0.2M acetate buffer. Ethyl chloroformate (J.T. Baker Chemical Co., Phillipsburg, New Jersey) was added to the mixture dropwise while stirring. The mixture was stirred for 45 minutes and the pH maintained at 4.5 to 4.8 by the dropwise addition of 1 N NaOH. The mixture was allowed to stand for an additional 1 to 2 hours. The precipitate was then homogenized with a Ten Brook tissue homogenizer and washed five times with pH 7.2 PBS, twice with 0.1% Na_2CO_3, twice with pH 7.2 PBS, twice with pH 2.2, 0.2 M glycine-HCl buffer and once with pH 7.2 PBS until the pH of the final eluate equaled pH 7.2 and the OD at 280 nm of the

final eluate was less than 0.02.

Aggregated FCS immunoabsorbent was added to an equal volume of whole anti-chemokinetic factor. The mixture was incubated at 37°C for 1 hour with occasional stirring and subsequently centrifuged at 700 x g. After centrifugation the supernatant (absorbed antiserum) was collected and frozen.

N. Ouchterlony Double Diffusion Assay

The purpose of the double diffusion assay was to test the specificity of the anti-CKF antiserum to bind and form precipitation lines with proteins present in CKF, LL conditioned media and FCS. Comparison of these precipitation lines provided information concerning the specificity and cross reactions of the anti-CKF with the proteins present in LL conditioned media and FCS.

Glass slides (50 x 76 mm) were coated with a film of 1.25% Agar (Oxoid Limited, England) in barbital buffer pH 8.2 and dried at 60°C for 1 hour. Liquid 1.25% agar in barbital buffer was then poured onto the plates to a thickness of 1.5 mm and allowed to solidify. Wells were punched in the agar and the various antigens and antisera added to the appropriate wells. After a 24 hour incubation at 4°C the slides were washed for 24 hours in PBS then fixed in 12% acetic acid for 1 hour. To increase the resolution of precipitin lines, the slides were stained with Coomassie Blue for 1 hour, washed twice in 5% acetic acid, dried at 60°C and finally washed in 5% acetic acid.

O. Indirect Immunofluorescence Localization of CKF

To determine whether CKF is present on the surface of LL tumor cells and whether the attachment of CKF to putative macrophage plasma-membrane receptors is a prerequisite for chemokinetic stimulation, both LL cells and activated macrophages were subjected to indirect immunofluorescence staining utilizing anti-CKF antiserum.

The following indirect immunofluorescence staining technique is based upon the method described by Kawamura (1969). Glass slides (7.6 x 2.5 cm) were cleaned, placed in 150 mm diameter Petri dishes and sterilized. Two tenths milliliter of 1.0×10^7 ml of C. parvum activated peritoneal cells or 5×10^6/ml LL cells were placed on each slide to form a 1 - 1.5 mm diameter pool. After 2 hours of incubation at $37^{\circ}C$, in a humidified 5% CO_2 95% air atmosphere the nonadherent cells were washed from the macrophage slides and all slides were completely immersed in media. After an additional 24 hours of incubation, the slides were cooled on ice for 15 minutes and washed 3 times with PBS. Subsequently some of the slides with adherent macrophages were incubated in LL conditioned media for 1 hour then washed 3 times with PBS. All slides were incubated for 30 minutes with decomplemented anti-CKF diluted 1:5 in PBS or with control solutions (i.e. horse serum, normal rabbit serum or PBS). After washing 3 times with PBS the slides were incubated for 30 minutes with a 1:5 dilution of Fluorescein Isothiocyanate (FITC) conjugated Goat Anti-Rabbit globulin (Cappel Laboratories, Inc., Cochranville, Pennsylvania), rinsed

3 times with PBS and coverslipped with 5% glycerol in PBS. In other preparations, cell suspension of C. parvum activated peritoneal cells or LL cells were processed for immunofluorescence staining. Three million cells were placed in each culture tube (8 x 70 mm). The peritoneal cells were incubated in 1 ml of ice cold unconditioned or LL conditioned media for 1 hour then washed 3 times with 1 ml of PBS. All anti-Bovine Serum (BS) and anti-CKF incubations were performed using 0.1 ml of 1:5 diluted antiserum per tube. The cells in each tube were washed 3 times in PBS, incubated in 0.1 ml of FITC conjugated goat anti-rabbit globulin for 30 minutes, washed 3 times in PBS and resuspended in 0.1 ml of 5% glycerol in PBS. A single drop of suspended cells from each preparation was placed on an acetone cleaned slide and coverslipped.

All slides were examined for fluorescent staining with a Zeiss Photomicroscope III using a No. 51 barrier filter and UV illumination. The degree of membrane staining of each preparation was judged on a scale from 0 to 4+, with 0 representing the absence of fluorescence and 1+ to 4+ representing increasing intensities of membrane fluorescence.

IV. RESULTS

A. The Capillary Tube Migration Assay as a Means of Detecting Chemokinetic Activity in Tumor Conditioned Media

Incubation of BCG (Meltzer, et al., 1975a) and pyran (Snodgrass, et al., 1977) activated macrophages in neoplastic cell conditioned media has previously been shown by time lapse microcinematography to increase the random migration rate. For a more precise and reproducable assay of this activity we investigated the use of a capillary tube migration method that measures the migration of C. parvum activated macrophages in neoplastic cell conditioned media.

The initial procedure used to perform this assay involved placing varying amounts of a peritoneal cell suspension (1.5×10^7 cells/ml) in sterilized glass capillary tubes (I.D.1.1 to 1.2 mm , length 75 mm), plugging the bottom of the tubes with Seal-Ease (Clay Adams, Parsippany, New Jersey), centrifuging the tubes at 200 x g for 10 minutes, scoring and fracturing the tubes 2 mm in front of the cell pellet, placing two tubes in each migration chamber (Universal XAB, Enskede, Sweden) containing the media to be tested and incubating the chambers at $37^{o}C$ in a humidified 95% air, 5% CO_2 atmosphere. The position of the free edge of the cell pellet within the length of the tube was measured with a linear micrometer (SGA Scientific Inc., Bloomfield, New Jersey) attached to a steroscopic microscope (American Optical Corporation, Buffalo, New York). The migration of the cells in the free edge of the pellet was determined by subtracting the value of the position of the free edge at the beginning of the incubation period from its position at

the end of incubation. Routinely, samples were assayed in triplicate. One tail student's t-test was used to assess the significance of differences. Significant differences were considered to be $P < 0.05$. Using this procedure we found that when 8×10^5 C. parvum-stimulated peritoneal cells were placed in capillary tubes, the distance they migrated in 18 hours was greater in LL conditioned than in MEF conditioned media (Figure 1).

1. Identification of Migrating Cells in the Capillary Tube Assay

The peritoneal cell population 7 days post i.p. injection of C. parvum was determined by differential staining to be 19% lymphocytes, 17% granulocytes and 64% monocytes/macrophages. Due to the cellular heterogeneity of the peritoneal exudate, it was necessary to confirm the identity of the migrating cells in the capillary tubes. The use of shortened capillary tubes allowed the cells responding to the LL conditioned media to migrate out of the tubes and onto glass coverslips. By the three criteria of morphology, non-specific esterase staining and latex bead phagocytosis the cells migrating onto the coverslips were found to be enriched with macrophages when compared to the peritoneal population examined before being placed in the tubes (Table 1). Morphology also revealed that many responding macrophages possessed monocytic features (size 8-10 μm and indented nuclear shape) (Figure 2).

FIGURE 1

Capillary tube migration of C. parvum stimulated peritoneal cells. Peritoneal cells (8×10^5/tube)were centrifuged at 200g for 10 minutes in plugged capillary tubes to produce a cell pellet at the plugged end. Each capillary tube was fractured 2 mm in front of the cell pellet. Two capillary tubes were placed in each migration chamber containing the test media. The media tested were FCS supplemented unconditioned EMEM (A) and FCS supplemented EMEM conditioned by LL cells for 48 hours (B). Chambers were incubated for 18 hours in a humidified 95% air, 5% CO_2 atmosphere. The distances migrated during the 18 hours were expressed in millimeters. Magnification, X50.

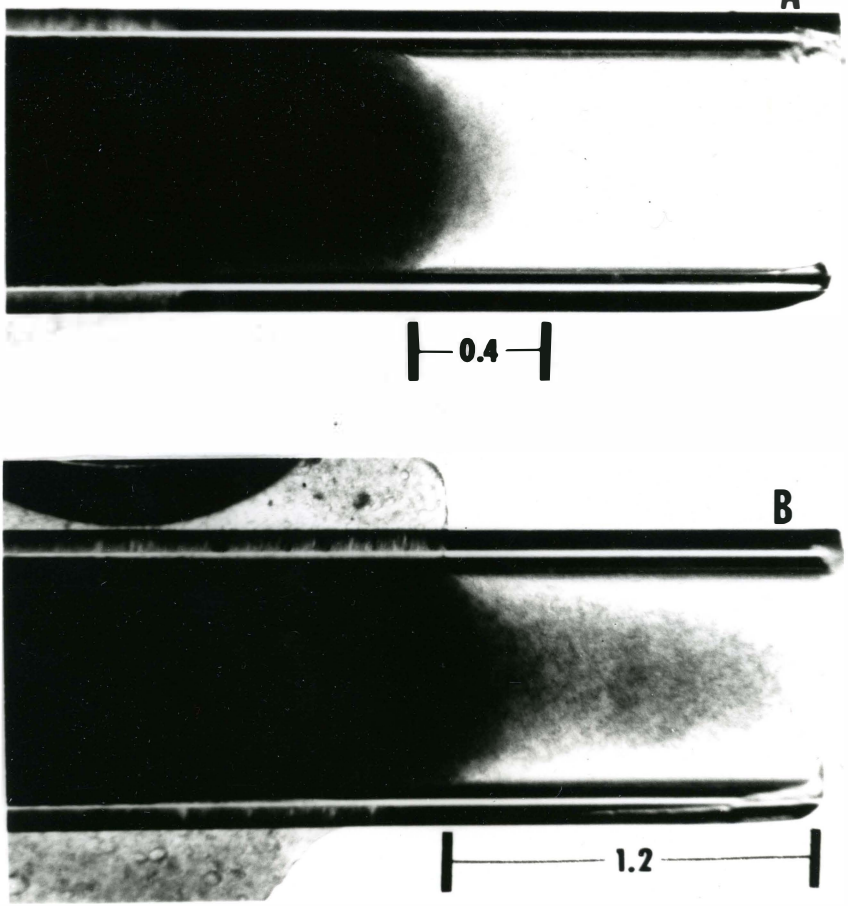

1
A
0.4
B
1.2

TABLE 1

Comparison of the Macrophage Population Among Cells Migrating[2] in the Capillary Tube With Those in the Peritoneal Exudate

Source	% Macrophage By Morphology	% Non-Specific Esterase	% Phagocytize Latex Beads
Direct from Peritoneal Cavity[1]	65 ± 3.2	67 ± 3.9	61 ± 5.1
Leading Edge of Capillary Tube[3]	88 ± 1.6	92 ± 1.7	95 ± 3.6

[1] Mice stimulated i.p. with C. parvum 7 days prior to removal of peritoneal cells

[2] Cells migrating in response to FCS supplemented media conditioned by LL cells for 48 hours.

[3] The cells were allowed to migrate out of shortened capillary tubes (fractured 1.0 mm in front of cell pellet) onto coverslips.

FIGURE 2

Corynebacterium parvum stimulated peritoneal cells which have migrated out of shortened capillary tube (fractured 1.0 mm in front of cell pellet) incubated in LL conditioned media during a 6 hour incubation at 37°C in a humid 5% CO_2, 95% air atmosphere. Present are mature macrophages (M), immature monocytic appearing macrophages (m), a lymphocyte (L) and a neutrophil (N). Giemsa stain, Magnification, X 1250.


2
N
M
m
m
M
L

2. Determination of the Optimal Peritoneal Cell Concentration for the Capillary Tube Assay

Starting with 1.6×10^6 peritoneal cells, decreasing cell concentrations were tested. At all concentrations used in these experiments the distance migrated by the cells in the LL conditioned media was greater than the distance migrated in unconditioned media (Table 2). The largest number of cells (1.6×10^6) proved to be the lowest migration for detecting the stimulation of migration. When 8×10^5 and 4×10^5 cells were placed in the capillary tubes the distances migrated were significant in comparison to controls ($p < 0.005$). The lowest number of cells tested (2×10^5 and 1×10^5) showed a greater than 90% increase in distance migrated in comparison to controls. Due to the large variance, however, the difference was not significant. Therefore, we elected to use 4×10^5 cells/capillary tube, since this number routinely gave a low variance with significant increase in migration when stimulated by tumor-cell conditioned media.

3. Determination of Optimal Migration Time

In order to determine the optimal incubation time for migration of peritoneal cells in the assay, the distance the cells traveled in the capillary tubes was measured at five successive time points during an 18 hour incubation period. The results showed that the distance traveled during the first hour was the greatest and then decreased rapidly with time (Figure 3). However, the relative difference between the distance migrated in the LL and unconditioned media was larger at the later time points. Therefore, a full 18

TABLE 2

Comparison of Various Numbers of C. parvum Treated Peritoneal Cells Employed in the Capillary Tube Migration Assay

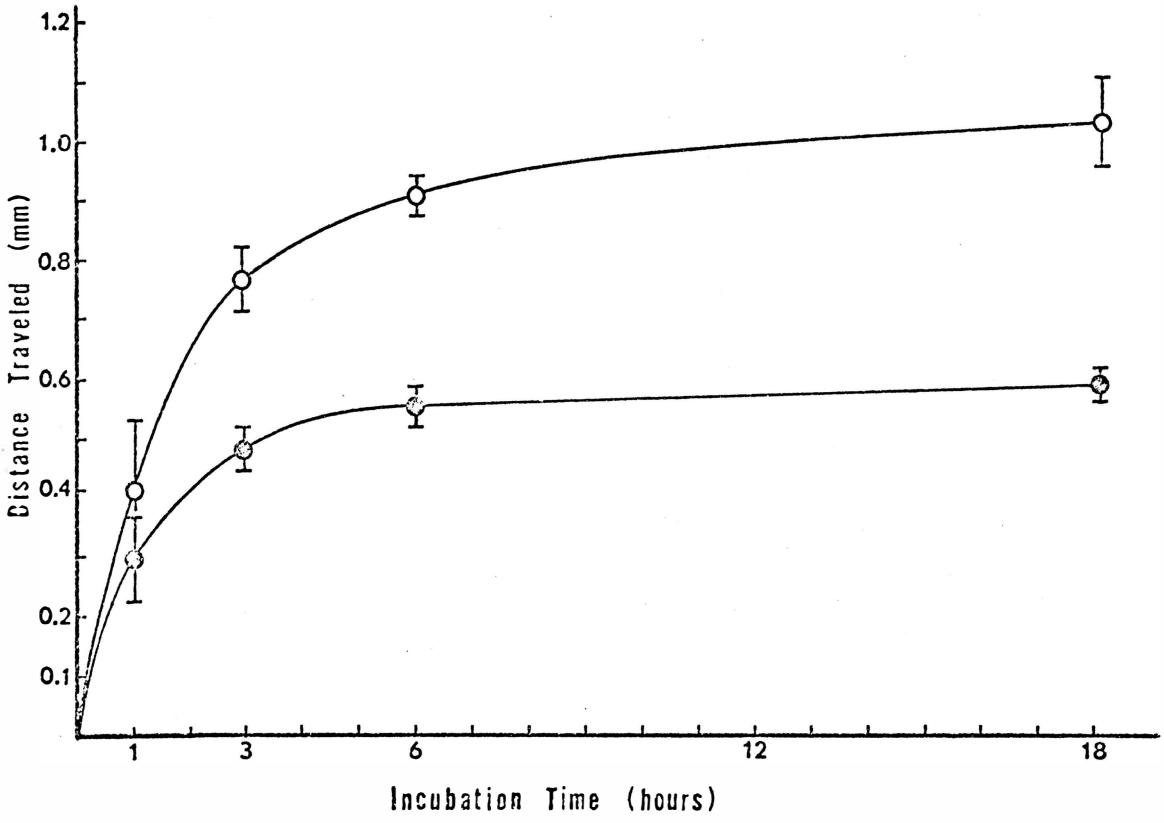
Number of Peritoneal Cells/Tube	Distance Traveled in Unconditioned Media[1] ± S.D. (mm)	Distance Traveled in LL Conditioned Media[1] ± S.D. (mm)	Percent Difference	Significant Difference	
$16x10^5$	1.23 ± .11	1.43 ± .04	16	N.S.[2]	P >.05
$8x10^5$	0.58 + .07	1.25 ± .14	116		P <.005
$4x10^5$	0.5 ± .14	1.14 ± .13	128		P <.005
$2x10^5$	0.25 ± .35	0.53 ± .25	112	N.S.	P >.10
$1x10^5$	0.20 ± .38	0.38 ± .10	90	N.S.	P >.20

[1]Distances of migration were measured after an 18 hour incubation period.

[2]Not Significant, P value greater than .05.

FIGURE 3

Capillary tube migration versus incubation time of C. parvum stimulated peritoneal cells. Migration distances of C. parvum stimulated peritoneal cells were determined at 1, 3,6 and 18 hours of incubation. Cells were incubated in LL conditioned (o) and in unconditioned media (●). The points represent the distance traveled ± S.D. in mm.


1.2
1.0
0.8
0.6
0.4
0.2
0.1
Distance Traveled (mm)
1
3
6
12
18
Incubation Time (hours)

hour incubation period was selected as the time interval, to attain the largest difference in macrophage migration between LL conditioned and unconditioned media.

4. Individual Cell Migration

Data concerning the rate of individual cell migration in the capillary tubes was obtained for comparison with the data of Meltzer et al. (1975a) and Snodgrass et al. (1977). With the aid of time lapse microcinematography the migration response of individual cells was studied in capillary tubes. The analysis of the film sequences revealed a 2.8 fold increase in the rate of migration for cells in the LL conditioned media over those in unconditioned or MEF conditioned media (Figure 4). The highly directional cell movement toward the open end of the capillary tube suggested the possibility that the cells were responding to a chemotactic factor. This possibility was further investigated after a crude sample of the chemokinetic factor was isolated from the LL media (see Page 84).

5. Surface Morphology of Migrating Cells

Scanning electron microscopy of the cells migrating within the capillary tubes revealed a morphology which is typical of motile cells (Zigmond and Hirsch 1972). Large, flat, vellum-like processes or lamellipodia extended from the cells in the direction of the tube opening (Figure 5), and small knob-like processes or uropods were located at the end of the cells furthest from the tube opening (Figure 6).

FIGURE 4

Maps illustrating the migration of C. parvum stimulated peritoneal cells within capillary tubes during a one hour period. The peritoneal cells were incubated in a, EMEM; b, MEF condition media; c, LL conditioned; d, LL-CKF. Average rates of migration were determined from the movements of the cells illustrated in each frame. In each map the opening of the capillary tube is to the right.

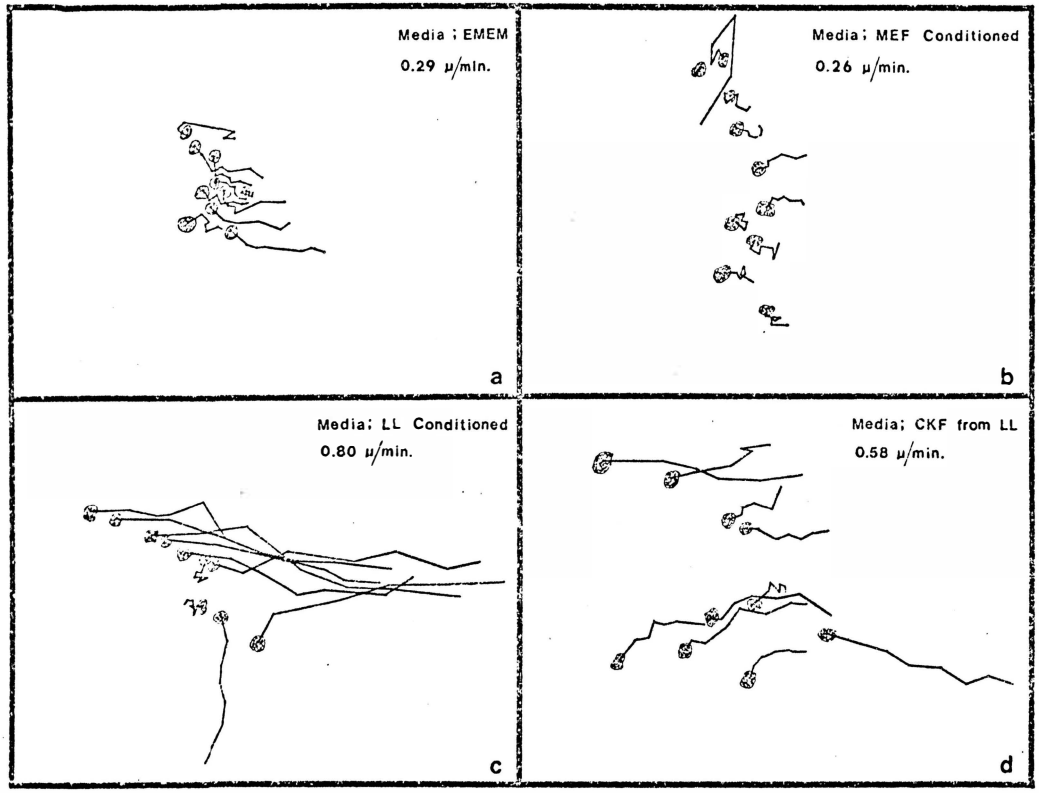
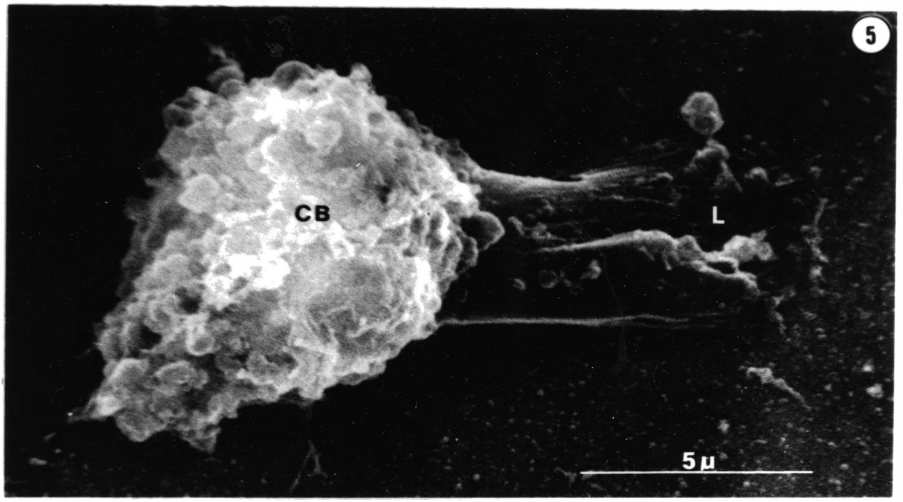

Media ; EMEM
0.29 μ/min.
a
Media; MEF Conditioned
0.26 μ/min.
b
Media; LL Conditioned
0.80 μ/min.
c
Media; CKF from LL
0.58 μ/min.
d

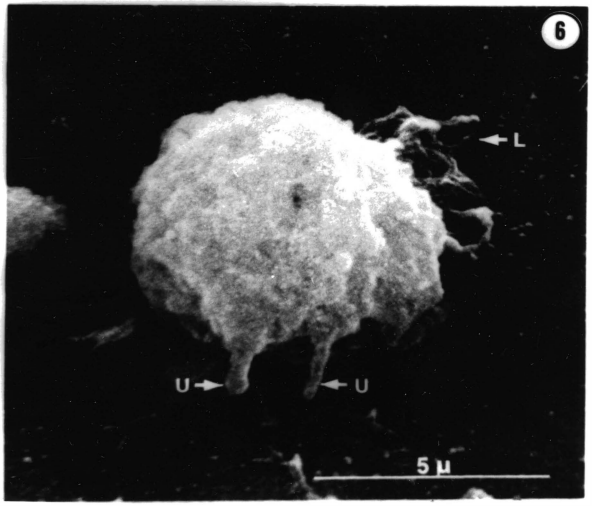
FIGURE 5

Macrophage adhering to the interior of the capillary tube. The cell body (CB) is positioned with a lamellipodia (L) extending toward the capillary tube opening. Magnification, X 7,400.

FIGURE 6

Macrophage adhering to the interior of a capillary tube. The surface of the macrophage oriented toward the cell pellet is exposed. Knob-like processes or uropods (U) are extended toward the surface of the capillary tube. A lamellipodia (L) is visible extended from the opposite side of the cell toward the capillary tube opening. Magnification, X 74,000.


5
CB
L
5 µ


6
L
U
U
5 µ

To examine whether the above described response was unique for C. parvum activated macrophages or for activated macrophages, pyran activated macrophages were also tested in the assay. The results indicated that pyran activated macrophages responded identically to C. parvum activated macrophages in LL conditioned and in MEF media (Table 3). The only difference between C. parvum and pyran activated macrophages was a higher variance observed in the migrational response of the pyran activated macrophages. Therefore, the observed macrophage response to tumor conditioned media is a response common to activated macrophages from two different sources.

B. The Occurance of Chemokinetic Activity in Tumor Conditioned Media

The release of the chemokinetic factor into the culture media by proliferating LL cells was determined at 24 hour intervals for a period of 7 days (Figure 7). Tumor cells were initially plated at 1×10^5 cells/25 cm^2 flask. These cultures reached confluence by the 4th or the 5th day. Continued growth resulted in crowding of cells and an increase in cell numbers to approximately 3×10^6 cells/flask by the 7th day. The chemokinetic effectiveness of the LL conditioned media, as assessed by its stimulation of macrophage migration in the capillary tube assay, increased through day 4 after which there was a steady decline through the 7th day.

Media from five additional syngeneic and allogeneic neoplastic cell lines were tested and compared to unconditioned media and normal mouse embryonic fibroblases (MEF) conditioned media for macrophage migration enhancing activity in the capillary tube assay

TABLE 3

Comparison of C. parvum and Pyran Activated Macrophage Migration in the Capillary Tube Assay

Media[2]	Distance Traveled ± S.D. (mm)	
	C. parvum Activated Macrophages	Pyran Activated Macrophages
Unconditioned	0.48 ± .10	0.48 ± .19
MEF[3]	0.40 ± .07	0.40 ± 0.10
LL[3]	0.93 ± .08[1]	0.90 ± .35[1]

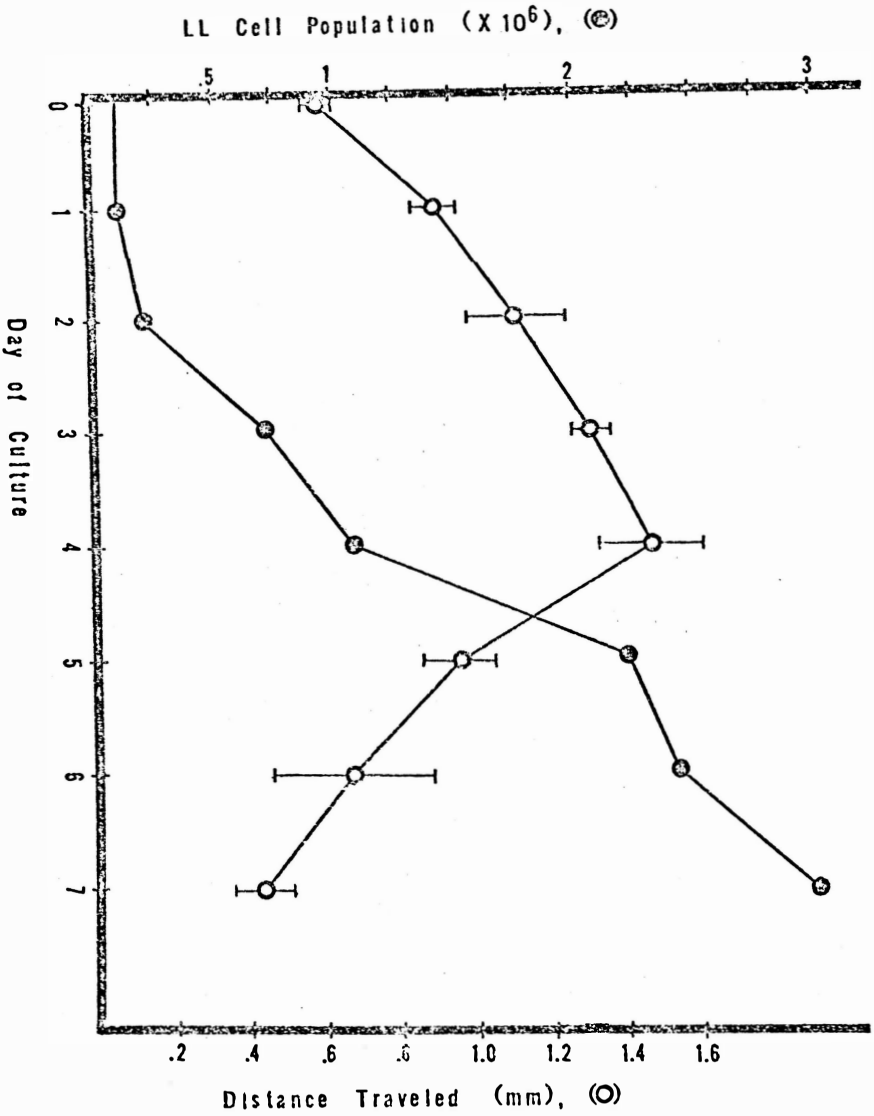
[1] Significantly different from distance migrated by cells in unconditioned or MEF conditioned media

[2] All media were supplemented with 20% FCS

[3] Media were conditioned for 48 hours by an initial concentration of 2.5×10^5 tumor cells/25 cm^2 culture flasks.

FIGURE 7

Comparison of tumor cell proliferation (●) and chemokinetic (○) activity. Lewis lung tumor cells were cultured at 1 x 10^5 viable cells per 5 ml in medium with 20% FCS in 25 cm^2 plastic flasks. Culture fluids were harvested at 24 hour intervals and assayed for chemokinetic activity in the capillary tube assay. Each circle represents the mean distance migrated per 4 tubes $\pm$ S.D. (mm). The number of tumor cells/flask were determined daily for 7 days.


LL Cell Population (X 10⁶), (●)
.5
1
2
3
0
1
2
3
4
5
6
7
Day of Culture
.2
.4
.6
.8
1.0
1.2
1.4
1.6
Distance Traveled (mm), (○)

(Table 4). In each case, the tumor cell conditioned media stimulated the migration of activated macrophages was significantly ($p < .005$) greater than MEF or unconditioned media.

C. Physicochemical Characterization of the Lewis Lung Tumor-derived Chemokinetic Factor

In order to determine the approximate molecular weight of the chemokinetic factor, media from both LL and Ehrlich ascites cultures were filtered through an Amicon XM100A membrane. In both cases, the majority of the chemokinetic activity remained in the non-filterable fraction with a molecular weight above 100,000 daltons (Table 5). However, the material below 100,000 daltons also possessed some activity. In the case of the Ehrlich ascites conditioned media, the activity of the low molecular weight material was significantly above that of the unconditioned media.

A Sepharose 4B column (which fractionates proteins according to their molecular weight from 100,000 to 10,000,000 daltons) was used to fractionate the Amicon concentrate of high molecular weight material from the LL conditioned medium (Figure 8). Three protein peaks were detected in the fractions by their ability to absorb light at 280 nm. A single peak of chemokinetic activity was detected by the capillary tube migration assay. Time lapse cinematography confirmed that the activity of this peak enhanced the rate of migration of individual macrophages (Figure 4). By calibrating the column with the molecular weight standards of thyroglobulin (670,000), catalase (230,000) and aldolase (160,000), the chemokinetic fraction was determined to have a molecular weight of approximately 360,000

TABLE 4

Chemokinetic Activity of Culture Media
Conditioned by Various Cell Types

Cells Used to Condition Media[1]	Distance Traveled ± S.D. (mm)	Significance of Difference in Comparison to Unconditioned Media
Lewis Lung Carcinoma	0.93 ± .08	P < .005
B-16 Melanoma	1.05 ± .3	P < .005
MCA (K) F Fibrosarcoma	1.0 ± .18	P < .005
MCA (K) D Fibrosarcoma	1.05 ± .35	P < .005
Ehrlich Ascites	1.08 ± .03	P < .005
MID-1 Mammary Adenocarcinoma	.97 ± .33	
Embryonic Fibroblast	.40 ± .07	
Unconditioned	.48 ± .10	

[1] All media were conditioned by 48 hours of incubation with dividing tumor or normal cells. Approximately 5 x 10^5 cells/25 cm^2 culture flask were present at the beginning of media conditioning.

TABLE 5

Chemokinetic Assay of Culture Media Filtered through an Amicon XM100A Membrane

Media	Distance[1] Traveled ± S.D. (mm)	Significance of Difference in Comparison to Unconditioned Media
Lewis Lung[2]	.93 ± .08	P < .005
High Molecular[3] Wt Residue of Lewis Lung	1.08 ± .12	P < .005
Filtrate of[4] Lewis Lung	.69 ± .27	N.S.
Ehrlich Ascites[2]	1.08 ± .03	P < .005
High Molecular[3] Wt Residue of Ehrlich Ascites	1.12 ± .13	P < .005
Filtrate of[4] Ehrlich Ascites	.79 ± .19	P < .005
Unconditioned[5]	.49 ± .12	

1 Chemokinetic activity as measured by the capillary tube assay

2 FCS supplemented media was conditioned for 48 hours

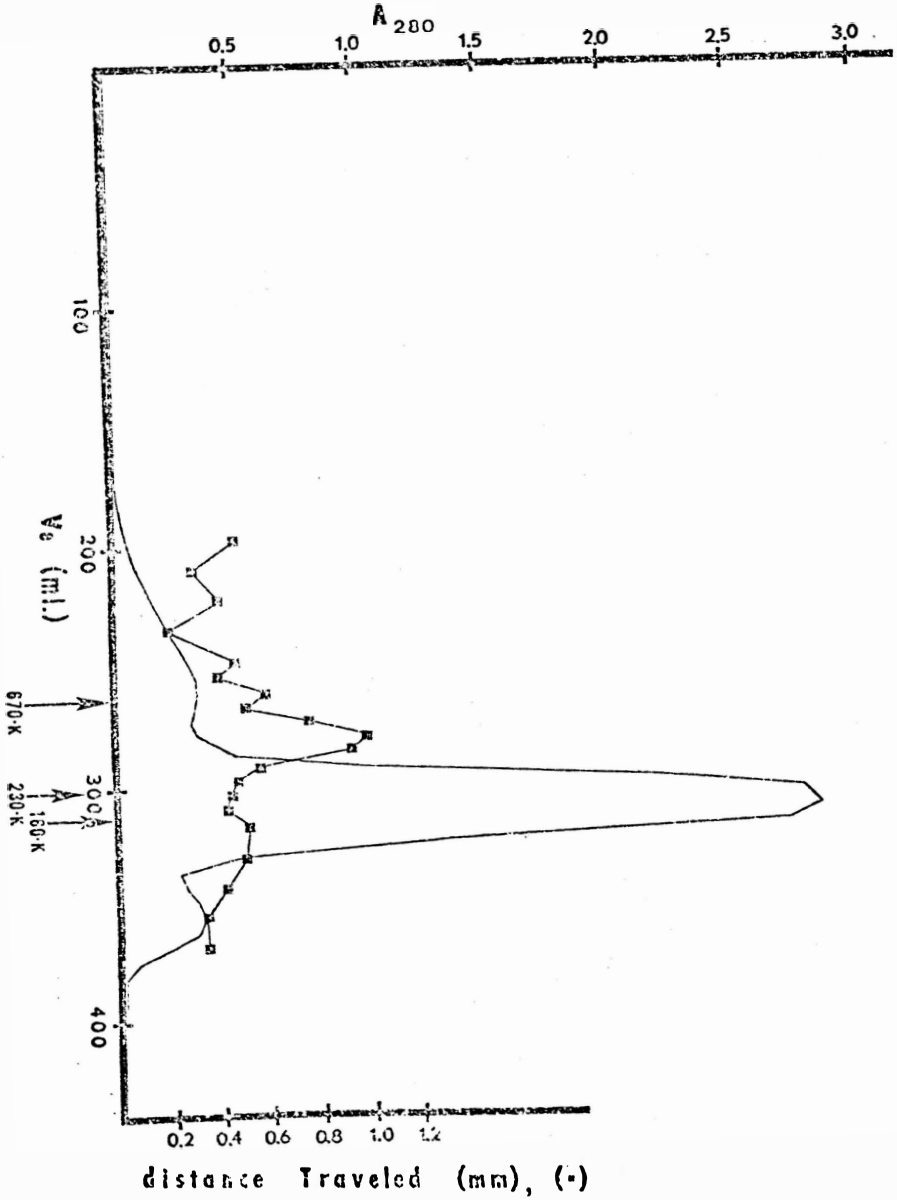
3 Fifteen fold concentrate of conditioned media (molecular weight above 100,000 daltons) diluted 1:5 in FCS supplemented EMEM before assaying chemokinetic activity.

4 Conditioned media (molecular weight below 100,000 daltons) was diluted 1:2 in FCS supplemented EMEM before assaying chemokinetic activity

5 FCS supplemented EMEM

FIGURE 8

Chromatography of tumor-cell conditioned media. Sepharose 4-B gel filtration of 10X concentration of high molecular weight (above 100,000 daltons) LL conditioned media. Absorbance of 280 nm light by fractions (-); chemokinetic activity in capillary tube assay of fractions diluted 1:5 with EMEM (■). Each square represents the mean value of 4 tubes. Arrows represent the elution volumes of molecular weight standards thyroglobulin (670,000 daltons), catalase (230,000 daltons) and aldolase 160,000 daltons).


A 280
0.5
1.0
1.5
2.0
2.5
3.0
100
200
300
400
A s (mi.)
670 K
300 K
230 K
0.2
0.4
0.6
0.8
1.0
1.2
distance Traveled (mm), (•)

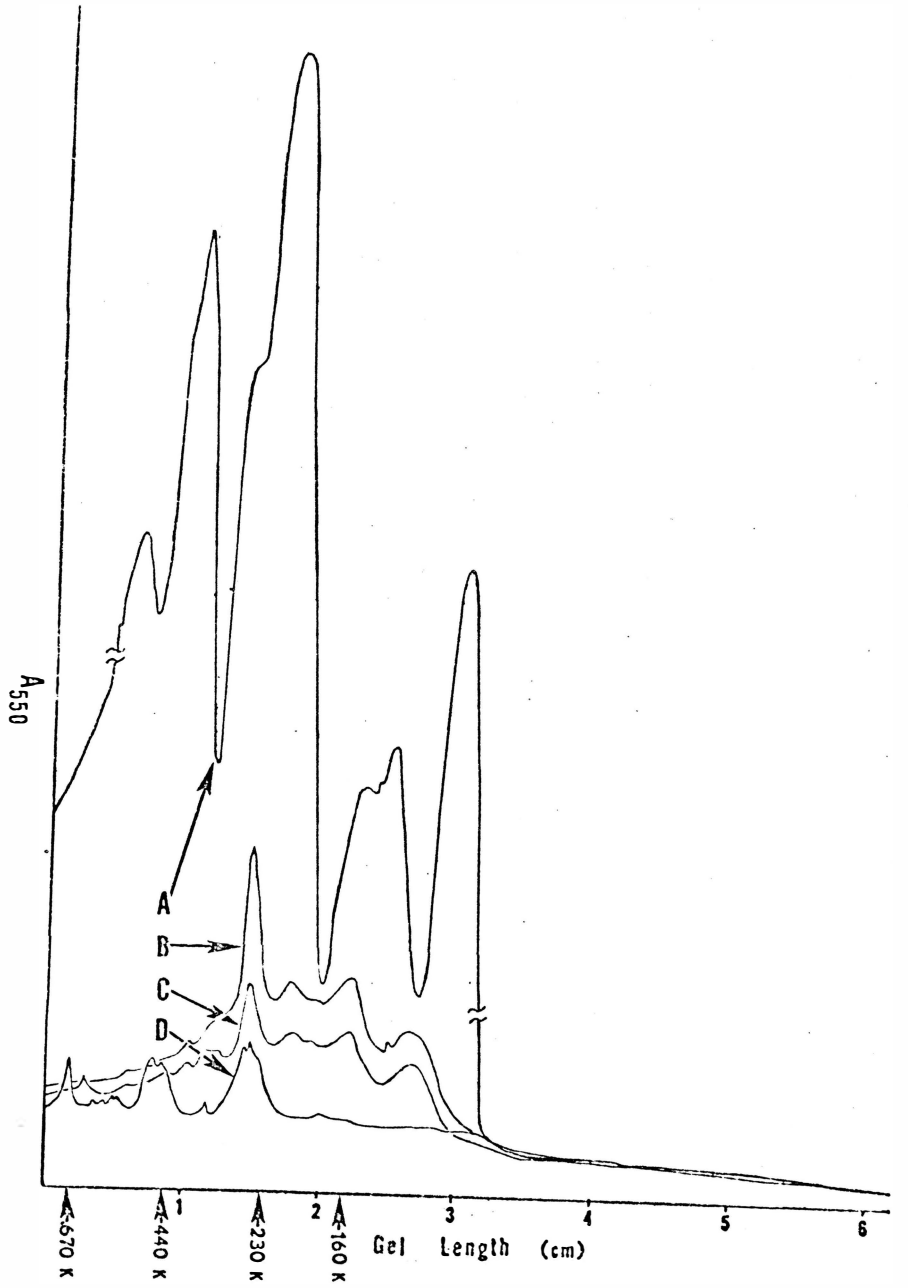
daltons.

Sodium-dodecysulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of this chemokinetically active fraction revealed the presence of 3 major and 6 minor separate protein peaks (Coomassie blue stained) (Line D, Figure 9). It is likely that most of these proteins originated from the FCS used to supplement the culture media. Additional LL conditioned media was prepared from serum free media exposed to LL cultures that had previously grown to 50% confluence in serum supplemented media. The electrophoretic patterns of day 1 and day 2 LL conditioned media without FCS were very similar to one another (Lines B and C, Figure 9). The location and relative amplitudes of the protein peaks in these two patterns correlated poorly with the pattern produced by electrophoresing whole FCS (Line A, Figure 9). Comparison of the FCS free LL conditioned media without FCS with the chemokinetically active fraction demonstrated a region of similarity positioned at 1.1 cm to 1.7 cm of gel length. Calibration of the gels with molecular weight standards revealed this region to contain proteins in a molecular weight range of 200,000 to 380,000 daltons.

In the absence of serum, Lewis Lung cells do not survive in culture for long periods of time (greater than 5 days). The condition of the LL cells as defined by their ability to divide during a 48 hour incubation in serum-free media was examined along with the chemokinetic activity of the serum free LL media. The LL cells continued to divide during the initial 24 hours following removal of the FCS supplemented media. They divided at a reduced

FIGURE 9

Electrophoresis of tumor-cell conditioned media. Components of LL conditioned media were electrophoresed through SDS-polyacrilamide cylindrical gels. Location and relative intensity of proteins was determined by staining gels with Coomassie blue and subsequently scanning the length of the gel for light absorbance at 570 nm (—). Fetal calf serum (A); day 1 LL conditioned media without FCS (B); day 2 LL conditioned media without FCS (C); and the chemokinetic fraction isolated from LL conditioned media with FCS (D). Arrows indicate the position of molecular weight standards thyroglobulin (670,000 daltons), Ferritin (440,000 daltons), catalase (230,000 daltons) and aldolase (160,000 daltons).


A_{550}
A
B
C
D
670 K
440 K
230 K
160 K
1
2
3
4
5
6
Gel Length (cm)

rate during the next 24th and 48th hour. Both of these media and the 360,000 dalton fraction prepared from these media were found to possess chemokinetic activity (Table 6). However, this activity was only detectable when FCS was added to the media at the time of the assay.

In order to investigate the role of FCS in producing the chemokinetic response, FCS was separated by filtration into three molecular weight fractions. Each fraction was tested for its ability to restore activity to the serum free LL conditioned media. The results illustrated in Table 7 indicate that: (a) the low molecular weight FCS (10^4 daltons) has no restorative ability; (b) the mid range FCS (10^4 to 10^5 daltons) restores the full activity; and (c) the high molecular weight FCS (10^5 daltons) has no restorative activity but it did raise the background activity of both the unconditioned and LL conditioned media to the level of the FCS supplemented unconditioned media.

D. Characteristics of the Anti-Chemokinetic Factor Antiserum

A rabbit anti-CKF antiserum was prepared using the 360,000 dalton chemokinetically active fraction prepared from the Sepharose 4B fractionation of LL conditioned media containing 20% FCS as the immunizing agent. This antiserum, when initially tested formed definitive precipitation bands against the CKF. Subsequently, the Ouchterlony double diffusion technique was utilized to compare the specificity of the antiserum for CKF (which was believed to contain components of FCS) versus neat FCS used for supplementing the LL

TABLE 6

Chemokinetic Activity of Media Conditioned by
LL Neoplastic Cells in the Absence of Fetal Calf Serum

Media	Distance Traveled (mm) ± S.D. in the Absence of FCS	Significance of Difference in Comparison to Unconditioned Media Without FCS	Distance Traveled (mm) ± S.D. with FCS[1]	Significance of Difference in Comparison to Unconditioned Media Supplemented with FCS
Day 1 LL Conditioned without FCS[2]	0.45 ± .07	N.S.	0.95 ± .04	$P < .005$
Day 2 LL Conditioned without FCS[3]	0.46 ± .09	N.S.	0.89 ± .10	$P < .005$
360,000 dalton fraction[4] prepared from 24 hr. LL Media without FCS	0.4 ± .06	N.S.	1.5 ± .04	$P < .0005$
Unconditioned Media	0.4 ± .09		0.65 ± .08	

1 FCS added to serum free media at time of assay to obtain a 20% FCS solution

2 75% increase in LL cells/flask during this interval

3 29% increase in LL cells/flask during this interval

4 Fractionated on a Sepharose 4B column, diluted 1:5 in EMEM without FCS

TABLE 7

The Chemokinetic Activity of Activated Macrophages in the Presence of Fetal Calf Serum Fractions[2]

Media Assayed	Distance Traveled in Capillary Tubes ± S.D. (mm)				
	No FCS	Whole FCS	FCS (10^4 Daltons)	FCS (10^4 to 10^5)	FCS (10^5)
Unconditioned	0.40 ± .04	0.80 ± .04	0.35 ± .07	0.40 ± .04	0.82 ± .11
Lewis Lung Conditioned	0.45 ± .08	1.27 ± .23	0.38 ± .03	1.44 ± .23	0.83 ± .00
Significant Difference	N.S.	P < .025	N.S.[1]	P < 0.005	N.S.[1]

[1] Not significant, P value is greater than 0.05

[2] FCS fractions prepared by membrane filtration

media. Double diffusion tests demonstrated no definitive antigen-antibody precipitation lines unique for CKF between the anti-serum and the active CKF prepared from FCS free, LL conditioned media (Figure 10A). This suggests that either the antiserum was not specific for CKF, or the CKF was present in too low a concentration to form a visible precipitation band with the antiserum. However, these bands did form between anti-CKF and CKF prepared in FCS supplemented media.

It is possible the observed bands were produced by high molecular weight FCS proteins present in the CKF fraction, since there was FCS in the original LL conditioned media used in the isolation of CKF. The lyophilized material prepared from non-serum supplemented LL conditioned media (L-LL), however, was found to produce two precipitation bands with the Anti-CKF. These bands shared identity with two of the bands from the CKF prepared from LL media containing FCS (Figure 11). Neither of these bands demonstrated non-identity (spurs) with the bands formed between FCS and anti-CKF. Absorption of the anti-CKF with aggregated FCS greatly reduced all the reactivity between the anti-serum and either CKF or L-LL (Figure 10 B). Therefore, the antisera was not recognizing any antigenic determinants in the CKF or the L-LL material which were not also present in the FCS.

Anti-bovine serum (Anti-BS) was used to confirm the cross reactivity or identity of the precipitating proteins with FCS present in the CKF (Figure 12). When anti-BS was reacted with CKF, two precipitation bands were formed which were continuous with the two bands formed between anti-CKF and CKF. Furthermore, these two bands showed

FIGURE 10

Ouchterlony double diffusion showing specificity of anti-CKF antiserum for components of LL conditioned media

Center Well A - Anti-CKF: Anti-CKF antiserum, unabsorbed
Center Well B - Anti-CKF: Anti-CKF antiserum absorbed with aggregated FCS

Outer Wells

CKF sf	Chemokinetic Factor prepared from serum-free[1] Lewis Lung conditioned media
CKF ss	Chemokinetic Factor prepared from serum-supplemented Lewis Lung conditioned media
EMEM sf	Serum-free unconditioned media
EMEM ss	Serum-supplemented unconditioned media
L-LL sf	Serum-free[1] lyopholized LL conditioned media

FIGURE 11

Ouchterlony double diffusion comparing specificity of anti-CKF antiserum for CKF prepared from LL conditioned media containing FCS and for concentrated LL conditioned media lacking FCS.

Wells

CKF ss	Chemokinetic factor prepared from serum-supplemented Lewis Lung conditioned media
L-LL sf	Serum-free lyopholized Lewis Lung conditioned media
Anti-CKF	Anti-Chemokinetic Factor antiserum, unabsorbed

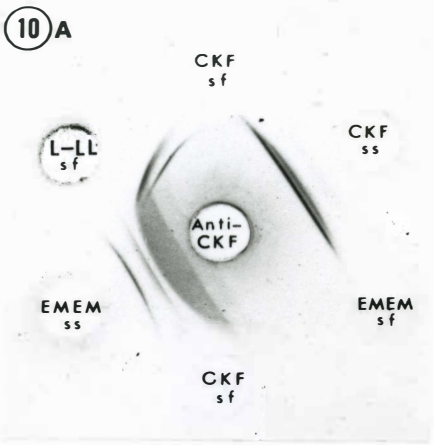
FIGURE 12

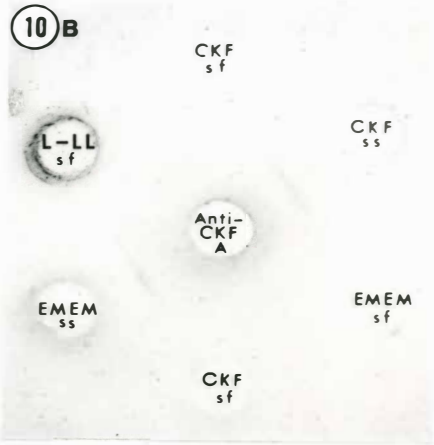
Comparison of Anti-CKF and Anti-BS reactivity toward CKF and toward FCS.

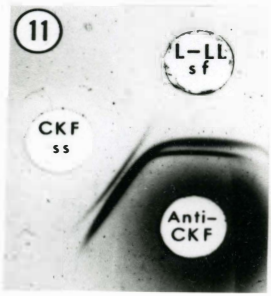
Wells

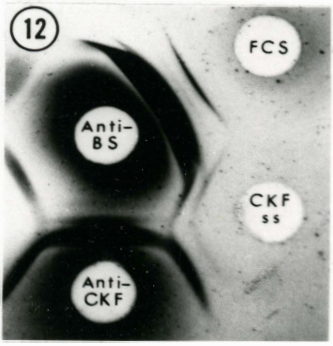
FCS	Fetal Calf Serum
Anti-BS	Anti-Bovine Serum antiserum, unabsorbed
CKF ss	Chemokinetic Factor prepared from serum-supplemented Lewis Lung conditioned media
Anti-CKF	Anti-Chemokinetic Factor antiserum, unabsorbed

[1] Serum-free indicates that no FCS was added to media before conditioning with LL cells. However, contamination of this media by FCS adhering to the LL cells and their culture flasks is a possibility.


10 A
CKF
sf
L-LL
sf
CKF
ss
Anti-
CKF
EMEM
ss
EMEM
sf
CKF
sf


10 B
CKF
sf
L-LL
sf
CKF
ss
Anti-
CKF
A
EMEM
ss
EMEM
sf
CKF
sf


11
L-LL
sf
CKF
ss
Anti-
CKF


12
FCS
Anti-
BS
CKF
ss
Anti-
CKF

continuity with bands formed between anti-BS and FCS. Therefore, all detectable reactivity in double diffusion tests indicated that the CKF antigens were identical in their antigenic determinents to some FCS components.

The CKF specificity of the anti-CKF antiserum was detectable when LL conditioned media was tested in the capillary tube assay in the presence of the antiserum (Table 8). Addition of anti-CKF to either CKF or LL conditioned media significantly reduced the migrational responses of the activated macrophages. The specificity of the anti-CKF antiserum was indicated by the inability of either normal rabbit serum or anti-bovine serum to reduce the CKF induced migration of macrophages.

If the LL tumor cell derived CKF is shed from the plasma membrane of LL cells to act on macrophages via attachment to the macrophage membrane, then it should be possible to localize the CKF on the plasma membrane of both the tumor cells and macrophages. The results of preliminary experiments utilizing anti-CKF antiserum in conjunction with immunofluorescence supported this hypothesis (Tables 9 and 10). The anti-CKF antiserum produced varying degrees of localized membrane fluorescence among LL cells cultured in the presence of FCS and less intense staining of LL cells cultured for 48 hours in serum free media (Table 9). A lesser degree of membrane fluorescence was apparent when the anti-CKF was absorbed by FCS. LL membranes treated with either absorbed or unabsorbed anti-CKF demonstrated a patchy fluorescence, often including 1 to 6 small blebs per cells (Figure 13). Lewis Lung cells exposed to either unabsorbed

TABLE 8

Effect of Anti-CKF Upon the Chemokinetic Activity of Lewis Lung Conditioned Media in the Capillary Tube Assay[1]

Media	Antisera	Distance Traveled ± S.D. (mm)	Significance of Difference
LL Conditioned[2]	None	0.93 ± .03	P < .005 relative to EMEM
LL Conditioned	Anti-CKF[3]	0.46 ± .09	N.S. relative to EMEM
LL Conditioned	Rabbit serum[3]	0.88 ± .10	P < .005 relative to EMEM
LL Conditioned	Anti-Bovine Serum[3]	0.89 ± .04	P < .005 relative to EMEM
CKF[4]	None	0.71 ± .06	P < .005 relative to EMEM
CKF	Anti-CKF[3]	0.52 ± .04	N.S. relative to EMEM
CKF	Rabbit serum[3]	0.81 ± .09	P < .005 relative to EMEM
EMEM	None	0.52 ± .05	

1 All assays were done with media supplemented with FCS

2 LL conditioned media was prepared without FCS. The media was conditioned for 24 hours by LL cells.

3 0.2 ml of antiserum or serum were incubated in 0.8 ml of media for 1 hour at 25°C prior to assaying

4 CKF was prepared from LL conditioned media with FCS and diluted 1:5 in EMEM containing 20% FCS

TABLE 9

Indirect Immunofluorescent Staining of Lewis Lung Cells with Anti-CKF and Anti-BS Antiserum

Cells	Media Used for Cell Culture	Primary[1] Antiserum	Degree of Membrane Fluorescence
LL	FCS supplemented EMEM	Anti-CKF	0 to 4+, + Avg
LL	FCS supplemented EMEM	FCS Absorbed Anti-CKF	0 to 2+, + Avg
LL	FCS supplemented EMEM	Anti-BS	0[2]
LL	FCS supplemented EMEM	FCS Absorbed Anti-BS	0
LL	FCS supplemented EMEM	Horse serum	0
LL	FCS supplemented EMEM	PBS	0
LL	Serum free EMEM[3]	Anti-CKF	0 to 2+, + Avg
LL	Serum free EMEM	FCS Absorbed Anti-CKF	0 to 1+, ½+ Avg
LL	Serum free EMEM	Anti-BS	0[2]
LL	Serum free EMEM	FCS Absorbed Anti-BS	0
LL	Serum free EMEM	Normal rabbit serum	0
LL	Serum free EMEM	PBS	0

1 Anti-serum used for incubation prior to incubation with FITC conjugated goat anti-rabbit globulin.

2 A low percentage (5%) with 1 to 3 large fluorescent blebs on surface

3 Cells had been cultured 48 hours in serum free media previously to staining.

TABLE 10

Indirect Immunofluorescent Staining of C. parvum Activated Macrophages and Peritoneal Exudate Cells With Anti-CKF and Anti-BS Antisera

Cells	Media[1]	Primary[2] Antiserum	Degree of Membrane Fluorescence
AMØ[3]	FCS supplemented EMEM	Anti-CKF	+
AMØ	FCS supplemented EMEM	FCS Absorbed Anti-CKF	0
AMØ	Serum free EMEM	Anti-CKF	+
AMØ	Serum free EMEM	FCS Absorbed Anti-CKF	0
AMØ	FCS supplemented LL conditioned	Anti-CKF	2+ to 4+, 3+ Avg
AMØ	FCS supplemented LL conditioned	FCS Absorbed Anti-CKF	2+ to 4+, 3+ Avg
APEC[4]	FCS supplemented LL conditioned	Anti-CKF	2+ to 4+[5,6] 3+ Avg
APEC	FCS supplemented LL conditioned	FCS Absorbed Anti-CKF	2+ to 4+, 3+ Avg
APEC	Serum free LL conditioned[7]	Anti-CKF	2+ to 4+, 3+ Avg
APEC	Serum free LL conditioned[7]	FCS Absorbed Anti-CKF	1+ to 3+, 2+ Avg
APEC	FCS supplemented EMEM	Anti-BS	0[8]
APEC	FCS supplemented EMEM	FCS Absorbed Anti-BS	0
APEC	FCS supplemented LL conditioned	Anti-BS	0[8]
APEC	FCS supplemented LL conditioned	FCS Absorbed Anti-BS	0

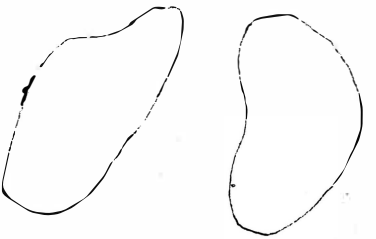
[1] Cells were incubated in this media for 1 hour on ice prior to incubation with primary antiserum.
[2] Anti-serum used for incubation prior to incubation with FITC conjugated goat anti-rabbit globulin.
[3] C. parvum activated peritoneal macrophages adhered to glass slides.
[4] C. parvum activated peritoneal exudate cell suspension.
[5] 71% of the peritoneal population had membrane fluorescence in this range, the remaining 29% had no membrane fluorescence.
[6] The membrane fluorescent subpopulation consisted of the larger (greater than 8u diameter) exudate cells.
[7] Media conditioned during the second consecutive 24 hours of LL cell incubation without FCS.
[8] A low percentage (5%) of cells with 1 to 3 large fluorescent blebs on their membrane.

FIGURE 13

Illustrations of the Indirect Immunofluorescent Labeling of LL Tumor Cells and C. parvum Activated Peritoneal Cells

Positive Fluorescence | Control (No Staining)

Lewis Lung tumor cell incubated in FCS supplemented media for 36 hours. Indirect immunofluorescent labeling of cell membranes with either neat or FCS absorbed Anti-CKF.



Positive Fluorescence | Control (No Staining)

Lewis Lung tumor cell incubated in FCS supplemented media for 36 hours. Indirect fluorescent labeling of cell membranes with neat Anti-BS.

Positive Fluorescence | Control (No Staining)

C. parvum activated peritoneal cells incubated for 1 hour on ice with FCS supplemented media conditioned for 48 hours by LL tumor cells. Indirect immunofluorescent labeling of cell membranes with either neat or FCS absorbed Anti-CKF.

Positive Fluorescence | Control (No Staining)

C. parvum activated peritoneal cells incubated for 1 hour on ice with serum free medium conditioned for 24 hours by LL tumor cells. Indirect immunofluorescent labeling of cell membranes with either neat anti-CKF or FCS absorbed anti-CKF.

or FCS absorbed Anti-BS showed no membrane fluorescence except for a rare fluorescent bleb seen on the surface of a few LL cells exposed to the unabsorbed anti-BS (Figure 13).

The unabsorbed anti-CKF produced activated macrophage membrane fluorescence in all media tested (Table 10). However, only the activated macrophages incubated in LL conditioned media fluoresced when exposed to the FCS absorbed anti-CKF. Cell suspensions of C. parvum activated peritoneal cells incubated in both FCS supplemented and unsupplemented LL conditioned media and exposed to either neat anti-CKF or FCS absorbed anti-CKF displayed membrane fluorescense among 71% of the population. The fluorescing cells comprised the larger cells (8-14 µm) present in the suspension. A variation in the quality of the peritoneal cell membrane fluorescence was noted between the peritoneal cells exposed to FCS supplemented LL condition media and serum free LL conditioned media. The membranes exposed to the supplemented LL media produced an amorphous, blebby fluorescent staining of the membranes, while the serum free media produced a uniformly thin granular fluorescent staining of the membranes (Figure 13). Peritoneal cells exposed to FCS supplemented LL conditioned or unconditioned media did not produce detectable membrane fluorescence with either neat anti-BS or FCS absorbed anti-BS, except for a rare bleb on the surface of a few cells incubated in FCS supplemented media and exposed to neat anti-BS.

Since the presence of FCS was required to obtain a migrational response to CKF or LL conditioned media, the hypothesis was entertained that the high molecular weight CKF could be an enzyme which

acted upon substrates in the FCS to produce chemokinetically active fragments. If this hypothesis is correct, inactivation of the high molecular weight factor from FCS supplemented media immediately before assaying the media for migration stimulating activity should not significantly reduce the activity of the LL conditioned medium, since the hypothetical enzyme would make more active FCS fragments. This hypothesis was tested utilizing the anti-CKF antiserum to inhibit the high molecular weight CKF from FCS supplemented and non-supplemented LL conditioned media. The results demonstrated that the chemokinetic activities of FCS supplemented LL conditioned media and unsupplemented LL conditioned media were both reduced to control levels (Table 11). Therefore, the high molecular weight factor did not appear to be generating chemokinetically active fragments from the FCS during an 18 hour period of incubation.

E. Susceptibility of the Chemokinetic Factor to Inactivation by Proteolytic Enzymes

In order to determine the protein nature of the chemokinetic factor in LL conditioned media, the susceptibility of this media to inactivation of the agarose bound proteolytic enzymes trypsin and chymotrypsin was assessed. The results indicated a loss of activity when LL conditioned (serum free) media was incubated with trypsin (which cleaves lysine and arginine peptide linkages) but not when incubated with chymotrypsin (which cleaves phenylalanine, tryptophan and tyrosine peptide linkages (Figure 14). As indicated by the results with trypsin degestion, lysine and arginine peptide linkages are present in the chemokinetic factor and these are required for chemokinetic activity. Since digestion

TABLE 11

Comparison of the Ability of Anti-CKF to Remove Activity from LL Conditioned Media in the Presence and Absence of FCS

Media	FCS Added to Media 24 hours Before Antiserum Treatment	Anti-CKF	FCS[2] Added After Antiserum Treatment	Distance Traveled (mm) ± S.D.	Significance to Unconditioned Media with FCS
LL[1]	-	-	+	1.2 ± .13	P < .005
LL	-	+	+	0.68 ± .07	N.S.
LL	-	+	-	0.58 ± .12	N.S.
LL	-	-	-	0.46 ± .08	N.S.
LL	+	-	-	1.14 ± .23	P < .01
LL	+	+	-	0.70 ± .10	N.S.
LL	+	+	+	0.74 ± .18	N.S.
LL	+	Anti-BS[3]	-	1.14 ± .09	P < .005
EMEM	-	-	+	0.50 ± .14	

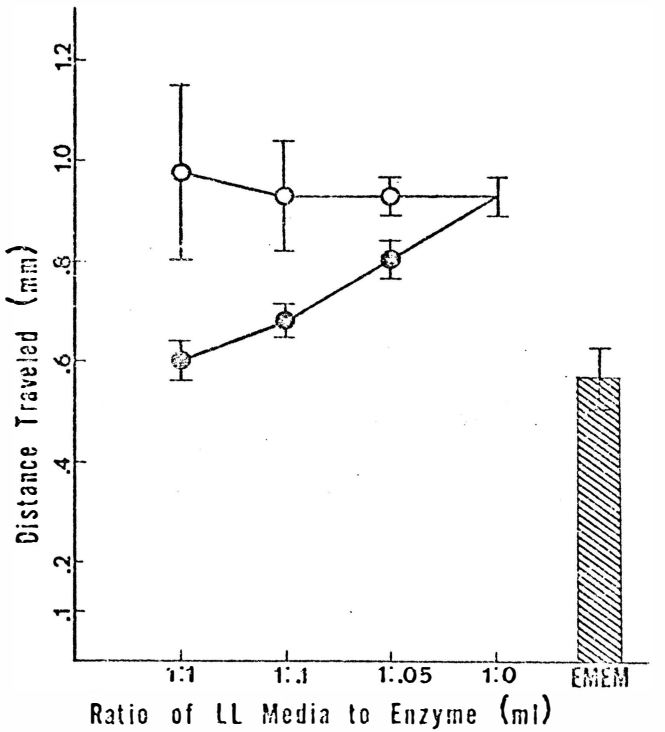
1 All media in this experiment were prepared from serum free EMEM. Media were conditioned by LL cells for 24 hours. At the start of media conditioning there were approximately 5 x 10^5 cells/25cm^2 culture flask.

2 All FCS supplementations produced a 20% FCS concentration

3 Undiluted antisera were added to the test media to achieve a 1:5 antisera media ratio. Antisera-media mixutres were incubated 15 minutes at 25°C before proceeding with the experiment.

FIGURE 14

The effect of proteolytic enzymes upon the chemokinetic activity of LL conditioned media. Agarose bound trypsin (19 units/ml at 25°C) and chymotrypsin (20 u/ml at 25°C) were each incubated with serum free LL conditioned media at the indicated volume ratios (abscissa). The enzymes were removed, FCS was added to the media and their chemokinetic activities assayed by the capillary tube migration of C. parvum activated macrophages. Chemokinetic activity of chymotrypsin treated LL media (o); and chemokinetic activity of trypsin treated LL media (●). Points represent chemokinetic activity ± S.D. The bar represents the chemokinetic activity of unconditioned FCS supplemented ± S.D.


Distance Traveled (mm)
.1
.2
.4
.6
.8
1.0
1.2
1:1
1:.1
1:.05
1:0
EMEM
Ratio of LL Media to Enzyme (ml)

with chymotrypsin did not destroy the activity of LL conditioned media, phenylalanine, tryptophan and tyrosine peptide linkages either are not present on the CKF, not crucial to the activity of the factor, or sequestered from the proteolytic activity of this enzyme.

F. Susceptibility of the Chemokinetic Factor to Inactivation by Heat

Sensitivity to heat inactivation is a standard criterion used to characterize factors. For this reason, the activity of the LL chemokinetic factor after heating was investigated. The results demonstrated a 14% decrease in activity after 30 minutes at 57°C and a 33% decrease after 30 minutes at 100°C (Table 12). This indicates the CKF is a fairly heat stable entity.

G. Chemotactic Activity of the Chemokinetic Fraction from Lewis Lung Conditioned Media

A highly directional movement of the macrophages was observed in the capillary tubes (Figure 4). This suggested a chemical gradient may exist between the media in the capillary tubes and the chamber. Experiments were then undertaken to test the chemotactic capacity of the CKF. This response of activated macrophages to CKF and to the known chemotactic substance, Mouse Activated Serum (MAS), was compared in both the capillary tube assay (Figure 15) and in the Boyden chamber assay (Figure 16).

To establish gradient conditions for the capillary tube assay, the factors were placed in the chamber containing the

TABLE 12

Heat Sensitivity of the Chemokinetically Active Fraction from Lewis Lung Conditioned Media

Media	Treatment of CKF[2]	Distance Traveled in Capillary Tube Assay (mm ± S.D.)	Percent Loss of Activity
1:5 CKF in EMEM[1]	None	1.40 ± .09	
1:5 CKF in EMEM[1]	56°C, 30 min	1.32 ± .07	14
1:5 CKF in EMEM[1]	100°C, 30 min	1.22 ± .03	33
EMEM[1]	None	0.85 ± .07	

1 EMEM supplemented with 20% FCS

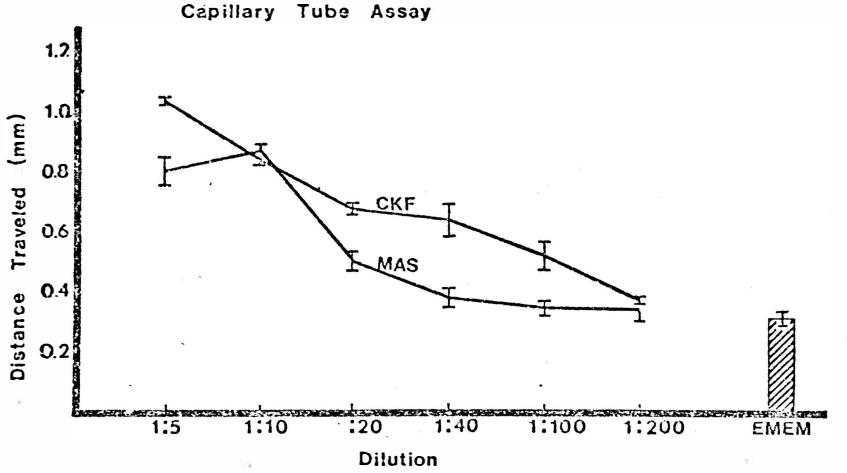
2 CKF was produced by sepharose 4B fractionation of FCS supplemented media conditioned for 48 hours by LL cells

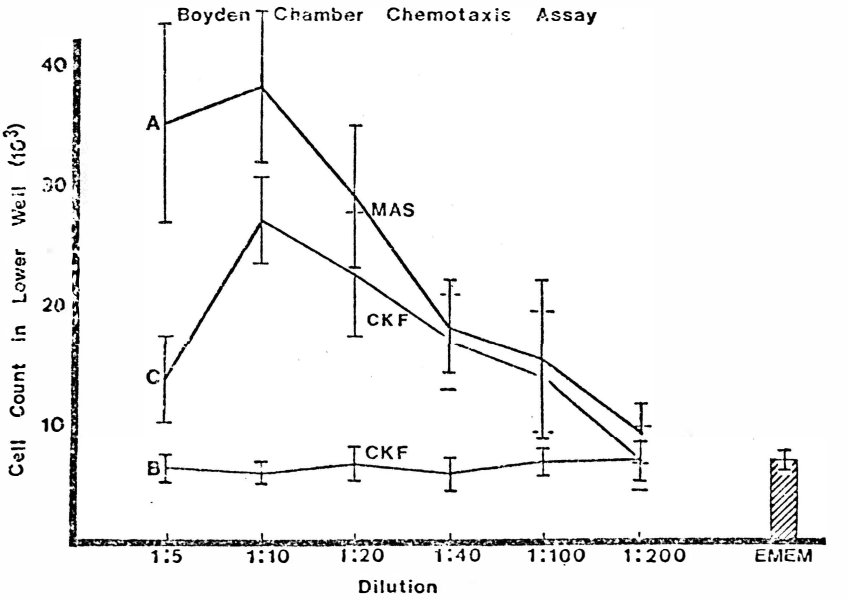
FIGURE 15

Comparison of activated macrophage capillary tube migration in LL-CKF and in MAS supplemented media. Both CKF and MAS were diluted in EMEM containing FCS. Each point represents average ± S.D. chemokinetic activity of 4 replicates. The bar represents the chemokinetic activity of unconditioned media.

FIGURE 16

Comparison of activated macrophage chemotactic migration in LL-CKF and in MAS supplemented media. Both CKF and MAS were diluted in EMEM containing FCS and placed in the lower wells of the Boyden chambers (Graphs A and B). In addition CKF was also tested under non-gradient conditions (identical dilutions of CKF in upper and lower wells, Graph C). Each point represents average ± S.D. chemotactic activity of 3 replicates. The bar represents the migrational activity of unconditioned media.


Capillary Tube Assay
Distance Traveled (mm)
1.2
1.0
0.8
0.6
0.4
0.2
CKF
MAS
1:5
1:10
1:20
1:40
1:100
1:200
EMEM
Dilution


Boyden Chamber Chemotaxis Assay
Cell Count in Lower Well (10^3)
40
30
20
10
A
MAS
CKF
C
CKF
B
1:5
1:10
1:20
1:40
1:100
1:200
EMEM
Dilution

capillary tube. For the Boyden chamber assay, the factors were placed in the lower wells. MAS produced migrational responses in both assays, the maximum response occuring at a 1:10 dilution. CKF produced a maximum migration in the capillary tube assay at the highest concentration (1:5). Under gradient conditions (chemotactic) in the Boyden chamber no migration to the lower well was observed at any of the CKF dilutions. However, under non-gradient conditions (chemokinetic) at a 1:10 dilution, CKF demonstrated a significant increase in activity over control levels.

The above results also indicated that MAS produced a chemokinetic response, either by acting upon the cells in the capillary tube, or by producing a gradient between chamber and capillary tube. To obtain information on these possibilities, the effects of CKF and MAS gradients upon migrating cells in the capillary tube and Boyden chamber assay were investigated in subsequent experiments (Tables 13 and 14).

CKF was placed in both the capillary tube and the chamber to avoid the creation of a CKF gradient, which may be present when CKF is placed only in the chamber. The migration of macrophages exposed to CKF under non-gradient conditions was enhanced in comparison to conditions in which CKF is placed only in the chamber. In this sense, a gradient was not required for the macrophages to respond optimally to CKF. In contrast, the migration of the macrophages was reduced when they were exposed to MAS under non-gradient conditions as compared to MAS-gradient conditions. Both CKF and MAS had little effect upon migration when placed only within the capillary tubes. The presence of either CKF or MAS in the capillary

TABLE 13

Migration of Activated Macrophages Under Gradient and Non-Gradient Conditions in the Capillary Tube Assay

Media in Capillary Tube	Media in Chamber	Average Distance Traveled ± S.D. (mm)	Significance of Difference in Comparison to EMEM in Upper and Lower Wells
EMEM[1]	EMEM	0.55 ± .08	
EMEM	CKF[2]	0.80 ± .08	$P < 0.005$
CKF	CKF	0.90 ± .27	$P < 0.025$
CKF	EMEM	0.53 ± .11	
EMEM	MAS[3]	0.90 ± .15	$P < 0.005$
MAS	MAS	0.59 ± .16	
MAS	EMEM	0.58 ± .05	
CKF	MAS	0.88 ± .08	$P < 0.005$
MAS	CKF	0.76 ± .05	$P < 0.005$

1 EMEM containing 20% FCS

2 CKF diluted 1:5 in EMEM. This dilution produced the maximum migration response in the capillary tube assay (Figure 14).

3 MAS diluted 1:10 in EMEM. This dilution produced the maximum migration response in the capillary tube assay (Figure 14).

TABLE 14

Migration of Activated Macrophages under Gradient and Non-Gradient Conditions in the Boyden Chamber Assay

Media in Upper Well	Media in Lower Well	Average # Cells in Lower Well (X 10^3)	Significance of Difference in Comparison to EMEM in Upper and Lower Wells
EMEM	EMEM	3.2 ± 0.77	
EMEM	CKF[2]	2.5 ± 0.58	
CKF	CKF	2.4 ± 0.38	
CKF	EMEM	4.9 ± 1.2	
EMEM	MAS	30.6 ± 4.4	P < 0.005
MAS	MAS	2.2 ± 0.74	
MAS	EMEM	3.6 ± 1.0	
CKF	MAS	21.9 ± 3.3	P < 0.01
MAS	CKF	2.2 ± 0.41	

1 EMEM containing 20% FCS

2 CKF diluted 1:5 in EMEM. This dilution produced the maximum migrational response in the capillary tube assay (Figure 14).

3 MAS diluted 1:10 in EMEM. This dilution produced the maximum migrational response in the Boyden chemotactic assay (Figure 15).

tube had little or no effect upon the positive migrational response to a gradient of the other factor in the chamber.

Gradient and non-gradient conditions for MAS in the Boyden Chamber assay produced significant increases in macrophage migration into the lower well only when a gradient of MAS was present. The presence of CKF partially inhibited the macrophage response to this gradient (Table 14). The data (Tables 13, 14 and Figures 15, 16) indicate that chemotactic activity can be detected by both assays under gradient conditions. However, chemokinetic activity was detectable in the capillary tube assay under both gradient and non-gradient conditions, while in the Boyden chamber assay, chemokinetic activity was detectable only under non-gradient conditions.

H. Response of Elicited Macrophages to the Lewis Lung Chemokinetic Factor

The responses of Oyster glycogen and thioglycolate elicited macrophages to CKF and MAS were examined in the capillary tube and the Boyden chamber assays. In the capillary tube assay neither of the elicited macrophages responded significantly to CKF or MAS (Table 15). Using the Boyden chamber chemotactic assay, the elicited macrophages responded significantly to MAS but not to CKF (Table 16). Thus, CKF appeared to be selective in enhancing migration of activated macrophages but not of elicited macrophages. Similarly, MAS produced a greater chemotactic response by activated macrophages than from elicited macrophages.

TABLE 15

A Comparison of the Migration Response of Macrophages Elicited With Osyter Glycogen and Thioglycolate versus C. parvum Activated Macrophages

Media[2] in Chamber	Oyster Glycogen Elicited	Significance of Difference in Comparison to EMEM	Thioglycolate Elicited	Significance of Difference in Comparison to EMEM	C. Parvum Activated	Significance of Difference in Comparison to EMEM
EMEM	0.52 ± 0.08		0.73 ± 0.03		0.43 ± .03	
CKF[3]	0.46 ± 0.09	N.S.[1]	0.71 ± 0.09	N.S.	0.85 ± 0.07	P < .0005
MAS[4]	0.75 ± 0.15	N.S.	0.94 ± 0.25	N.S.	0.95 ± 0.07	P < .0005

1
N.S., Not significant, P < .05

2
All media were supplemented with 20% FCS

3
CKF was prepared from FCS supplemented LL conditioned media and was diluted 1:5 in EMEM

4
MAS was diluted 1:10 in EMEM

TABLE 16

A Comparison of the Chemotactic Response of Macrophages Elicited With Oyster Glycogen and Thioglycolate Versus C. parvum Activated Macrophages

Media in Lower Well	Oyster Glycogen Elicited	Significance of Difference in Comparison to EMEM	Thioglycolate Elicited	Significance of Difference in Comparison to EMEM	C. Parvum Activated	Significance of Difference in Comparison to EMEM
EMEM	1.5 ± 0.5		16.0 ± 6.2		6.2 ± 1.3	
CKF	2.1 ± 1.1	N.S.[1]	22.1 ± 5.7	N.S.	6.0 ± 2.0	N.S.
MAS	3.1 ± 0.8	$P < .05$	45.8 ± 1.8	$P < .01$	21.2 ± 0.4	$P < .005$

[1] N.S., Not Significant, $P < .05$

V. DISCUSSION

The tumor induced increase in chemokinetic activity of pyran activated macrophages was described originally by Snodgrass et al. (1977) using time lapse microcinematography. The possible importance of this response to macrophage-neoplastic cell interactions in vivo led us to characterize the physicochemical properties of the chemokinetic factor in neoplastic cell conditioned media. In order to expediently monitor the chemokinetic activity of fractions isolated from tumor cell conditioned media and to determine optimal experimental conditions to measure the chemokinetic effect, a sensitive macrophage capillary tube migration assay was developed. The capacity of this assay to detect both chemokinetic and chemotactic factors present in FCS supplemented media was also evaluated.

The chemokinetic activity was detectable by the capillary tube assay in the culture media of five neoplastic cell lines. The production and/or release of chemokinetic activity into the media of LL cultures correlated with the log phase of LL cell proliferation in vitro. The factor responsible for the chemokinetic activity was found to be a high molecular weight (i.e. 360,000 daltons), heat stable protein which was susceptable to digestion by trypsin, but not by chymotrypsin. A hypothesis that the CKF is a shed glycocalyx protein with an inhibitory influence upon macrophage/tumor cell interactions and possibly on the tumoricidal capacity of activated macrophages is proposed. This hypothesis is based upon data indicating that CKF inhibits chemotactic migration and that CKF is shed from LL cell membranes and attaches to activated macrophage cell membranes.

A. The Chemokinetic Assay

The development of the chemokinetic assay was based on the theory that by limiting random macrophage migration linearly, the vectors of random movement would become linearly additive and consequently provide an increase in linear displacement which could be measured with greater sensitivity. This was accomplished by limiting macrophage migration to the confines of capillary tubes. Under experimental conditions, an increase in the rate of random movement was expressed linearly toward the open end of the tube (Table 4). This assay was more accurate in detecting movement of macrophages because it measured the combined movements over an 18 hour period as opposed to microcinematographic measurement of movement at given time intervals. The capillary method of measuring the distance of macrophage movement was more sensitive than the method used in microcinematography, because the capillary method measured the actual distance traveled by macrophages migrating along a linear pathway in the capillary tubes (Figure 4) while microcinematography measured apparent distance along straight lines between consecutive positions of randomly migrating macrophages. When measuring apparent distance at intervals with microcinematography, distance was lost since the macrophages did not normally migrate along straight lines during chemokinetic responses.

To determine whether this directional movement is expressed in response to both chemokinetic (CKF) and chemotactic factors (MAS), the assay was tested under gradient conditions (a requirement of chemotactic assays) and under non-gradient conditions. A gradient of either CKF or MAS through the open end of the capillary tube produced significant migration. However, only the CKF produced significant migration under

non-gradient conditions. Thus it is postulated that the directional movement within the capillary tubes as indicated by microcinematographic observations (see Figure 4, page 57) was primarily due to the physical resistance provided by the shape of the capillary tube forcing the macrophage movement toward the region of least resistance.

By testing FCS supplemented and unsupplemented LL conditioned media in the capillary tube assay it was observed that FCS was required for macrophage migration similarly as in the MIF assay (Hughes, 1972). For example, no macrophage migration occured in the MIF assay in serum-free media, but migration doubled when the concentration of FCS was increased in the medium from 1.9% to 60% (Pick and Manheimer, 1974). Leonard and Skeel (1976) isolated a 100,000 dalton molecular weight heat stable factor present in human serum which enhanced the random migration of mouse peritoneal cells in the Boyden chamber assay. This report is consistent with our observations that macrophage migration occured in capillary tubes only if the assay medium was supplemented with a 10,000 dalton or greater molecular weight fraction of FCS. The mechanism by which the FCS factor(s) enhance macrophage migration was suggested by evidence for a 500,000 dalton serum protein which coated the ionic surface of certain substrates (i.e. glass and Falcon polystyrene) and was required for spreading of baby hamster kidney cells (Grinnell, 1976).

Statistical comparison within single experiments of both LL conditioned media and CKF versus unconditioned media routinely indicated significant ($P < 0.05$) differences in mcarophage

capillary tube migration activity. When variations in the means of replicate capillary tube assays, between different experiments were examined, the coefficients of variations (standard error/mean) were 0.0449 for LL conditioned media, 0.1058 for CKF containing media and 0.0818 for unconditioned media. These statistics strongly suggest that the capillary tube assay provides reproducible data between experiments with an average error of about 8 percent. Variations between experiments could be related to varying degrees of activation of the macrophages in response to the C. parvum. This possibility is supported by variations in the tumoricidal activity of C. parvum activated macrophages. When calculated from the data of Fray et al. (1975), the average error between the tumoricidal activities of 4 different sets of day 7 post C. parvum i.p. injected peritoneal cells was 18 percent.

B. The Chemokinetic Factor

Utilizing the capillary tube assay we investigated the occurance and identity of the chemokinetic factor. We found that several different syngeneic and allogenic mouse tumor cell lines elaborated factors in vitro which were chemokinetic for C. parvum activated peritoneal macrophages. No chemokinetic activity was detected in media conditioned by normal mouse embryonic fibroblasts. Therefore, the production of chemokinetic factors appeared to be linked to the expression of the neoplastic state. The appearance of tumor-derived chemokinetic activity in LL conditioned media correlated with the log growth phase of the tumor cells in vitro. This suggested close association between the production and/or release of CKF and the maximum levels of synthetic activity in tumor cells.

Whether the tumor-derived chemokinetic factor was secreted by the cells in culture, shed from the cell surface membrane, or formed through interaction with FCS components *in vitro* is not known.

It is possible that the LL derived CKF could be a tumor cell modified or concentrated FCS protein. This possibility was suggested by evidence that, (a) macrophage migration stimulating factors are present in FCS (Fox *et al.*, 1974), and (b) the Ouchterlony precipitation lines formed using anti-CKF antiserum detected only FCS proteins in the LL conditioned media. Data which indicates that LL-CKF is not a FCS related protein include: (a) the 60,000 dalton molecular weight of the FCS derived macrophage stimulating factor (Fox and Gregory, 1972), which is 1/6 the molecular weight of CKF, and (b) the activity of CKF which is still detectable in the media of LL cultures washed free of FCS at two consecutive 24 hour intervals prior to harvesting of the tumor cell conditioned media. Any FCS proteins remaining after such extensive washing would be tightly adherent to the culture flasks or the surface of LL cells. The FCS adherent to the flask probably would not be released into the culture medium in high enough concentrations to produce a significant chemokinetic activity detectable in the media unless the LL cells produce a proteolytic enzyme. Evidence against the enzyme nature of the LL cell product was obtained by utilizing anti-CKF to inactivate the high molecular weight CKF in the various media (Table 11). The possibility that LL cells retain FCS attached to their cell membrane is unlikely since a turnover of the cell membrane components is expected during the 24 hour period between the first and second washing of the culture with FCS-free medium prior to collecting the LL conditioned

medium. The non-FCS nature of the CKF is further supported by indirect immunofluorescent staining of LL cells incubated for 48 hours in serum free media when FCS absorbed anti-CKF was used as the primary antiserum. Overall the LL-CKF appears to be distinct from the FCS.

The Lewis Lung chemokinetic factor was found to be a heat stable high molecular weight (360,000 daltons) protein containing lysine and/or arginine amino acids susceptable to cleavage by trypsin. The molecular weight determination of CKF was based on the assumption that CKF was a globular protein. If, in fact, the CKF has a linear configuration its actual molecular weight could be much less. A high molecular weight factor which enhanced macrophage random migration was also described by Aaskov and Anthony (1976). This factor was isolated from culture supernatants of BCG stimulated human peripheral lymphocytes. It was slightly larger than IgG (150,000 daltons M.W.) and stimulated movement of normal mouse splenic macrophages in the MIF assay. A similar factor was found in supernatants of BCG stimulated lymphocytes which enhanced the random movement of human buffy coat leukocytes from a well cut in agarose gel. This factor was heat stable at 56° for 30 minutes and had an electrophoretic mobility in the gammaglobulin region (Weisbart et al., 1974). Therefore the high molecular weight characteristic of LL-CKF is not unusual when compared to chemokinetic factors from other sources.

Reports of the molecular size of factors isolated from tumors which enhance macrophage migration, has been vague. A factor present in tumor conditioned media which enhanced migration by proteus peptone elicited macrophages in the MIF assay was only defined as being above 10,000 daltons (Nelson and Nelson, 1977). Another factor

isolated from fibrosarcoma-conditioned media, which also enhanced the migration of proteus peptone stimulated macrophages in cultures, was characterized only as being non-dialysable (molecular weight above 12,000 daltons) (Blakeslee, 1978). Since the CKF molecular weight was above 12,000 daltons, it is possible that both of the above factors are identical to the LL-CKF.

Tumor associated proteins with molecular weights in the range of the CKF (i.e. between 200,000 and 500,000 daltons) include the Carcinoembryonic Antigen (CEA) with a molecular weight of 200,000 daltons, and the cell coat or glycocalyx glycopropteins with a molecular weight range of 200,000 to 500,000 daltons (Codington et al., 1972). The CEA is found in normal fetal colon and in the serum of patients with tumors of the gastrointestinal tract, breast, bladder and lung. Indirect immunofluorescence on frozen sections with anti-CEA indicates that CEA is localized on the tumor or embryonic cell surfaces, probably as part of the extracellular material forming the glycocalyx (Alexander, 1972). The possibility exists that the CKF could be CEA shed from the glycocalyx of LL cells.

Another tumor associated fetal antigen is alpha feto-protein. This protein is a major fetal serum protein. It is also found in the serum of cancer patients with hepatocarcinomas or teratoblastomas. Since alpha feto-protein is found in fetal serum of mammalian species (Sell, 1979) it is likely to be present in FCS used in the capillary tube assay. However, it is unlikely that it is identical to LL-CKF because of its lower molecular weight (70,000 daltons).

The appearance of a specific group of fucose-containing membrane glycopeptides was demonstrated on a group of dimthylnitrosamine induced Syrian golden hamster embryo tumor cell lines (Glick et al., 1973). The expression of these large glycopeptides was pronounced in the more tumorigeneic cell lines. Kim et al. (1975) demonstrated that spontaneously metastasizing tumors shed membrane glycopeptides, which could be detected in the blood of tumor bearing rats, while no dectable tumor glycopeptides were present in the blood of rats bearing non-metastasizing tumors. The LL carcinoma is also a highly metastatic tumor, and considering the molecular size (360,000 daltons) of CKF, the CKF could well be a component of the LL-glycocalyx which is readily shed as previously suggested in relation to the CEA.

The possibility cannot be excluded that CKF is a product of an oncogene or a latent virus. This possibility could be tested by using immunological methods to compare CKF antigenic determinants with those of viral products. A radioimmunoassay would provide a sensitive method for making this comparison.

The data in Table 13 indicates that a short term (30 minutes) exposure of activated macrophages to optimal CKF concentrations die not produce enhanced macrophage migration. To obtain enhanced macrophage migration in the capillary tube, a continuous (18 hour) exposure of the activated macrophages to optimal concentrations of the CKF is required. Therefore, the mechanism of migration is apparently

not the result of a short-term triggering of a long-term intracellular process, but it is maintained as the consequence of a continuous interaction between CKF and the activated macrophage.

The chemokinetic activity of the LL-CKF was easily detectable in the capillary tube assay. Preliminary experiments examining the activity of CKF under non-gradient conditions in the Boyden chamber assay indicated no chemokinetic activity (Table 14). Since this was contrary to findings by other investigators, CKF activity was re-examined systematically using serial dilutions of CKF and a different lot of membranes (Figure 16). This experiment corroborated observations on chemokinetic migration by other laboratories and showed that under non-gradient conditions a significant number of macrophages migrated across the membrane at a 1:10 dilution of CKF. Other agents which can produce chemokinetic activity under non-gradient conditions in the Boyden chamber assay are the chemotactic factors MAS, Casein (parrott, 1980) and a lymphocyte derived chemotactic factor (Snyderman and Mergenhagen, 1976). The high molecular weight lymphokine described by Aashov and Anthony (1976) as stimulating macrophage migration in the MIF assay, similarly to CKF under gradient conditions, had no migration enhancing activity in the Boyden chamber assay. This factor was not tested under non-gradient conditions. Information is completely lacking on the Boyden chamber migration characteristics of the chemokinetic lymphokine isolated by Weisbart et al. (1974).

The results (Figure 16) showed the LL-CKF had no chemotactic activity in the Boyden chamber assay. The lack of chemotactic activity along with its large size (360,000 daltons) indicates that LL-CKF is distinct from the neoplastic chemotactic factor (molecular weight, 15,000 daltons) described by Meltzer, et al. (1977). In

fact, data available suggests that in the Boyden chamber assay the presence of CKF in the upper chamber produces a significant inhibition (i.e. 30% reduction, see Table 14) of the chemotactic migration of macrophages to the MAS containing lower chamber. This inhibitory effect of CKF on chemotaxis may have relevance to the in vivo survival of tumors and should be investigated.

C. Anti-CKF Specificity Versus CKF Localization by Anti-CKF

Anti-CKF showed specificity in Ouchterlony double diffusion tests only for FCS components. However, the ability of the antiserum to abrogate the activity of LL conditioned media suggested that the anti-CKF also contained CKF specific antibodies. It is thought that a low concentration of CKF made its presence undetectable by the Ouchterlony double diffusion assay. Similar observations were reported by Snyderman and Pike (1978a) for antiserum raised against a tumor-derived chemotaxis inhibitory factor. This antiserum prepared against the chemotaxis inhibitor also removed activity from tumor conditioned media but did not produce specific anti-chemotactic factor precipitin lines in the double diffusion assay.

The presence of CKF specific immunoglobulins in the antiserum was supported by the indirect immunofluorescent staining of both LL cells, and activated macrophage plasma membranes exposed to LL conditioned media using FCS absorbed antiserum. Further evidence that membrane adherent FCS was not involved in the membrane fluorescence produced by anti-CKF came from the observation that Anti-BS which cross reacted with the FCS specificities of the anti-CKF in the

Ouchterlony plates did not produce significant LL or activated macrophage membrane fluorescence. The rare bleb of fluorescent material observed on anti-BS treated macrophages and LL cells may have been produced by an adherent aggregate of FCS proteins. The lack of macrophage membrane staining by anti-BS also indicated that anti-CKF was binding CKF molecules attached to the macrophage membrane. It is theoretically possible that the macrophage membrane fluorescence is produced by macrophage Fc receptors binding either aggregated FITC conjugated anti-rabbit globulin or FITC conjugated anti-rabbit globulin/anti-CKF complexes. However, evidence against this possibility comes from a study by Thrasher, et al. (1975). He found that the fluorescein conjugate of the anti-rabbit globulin blocks the Fc portion of the immunoglobulin from being bound by the macrophage Fc receptor due to its large size.

The decreased anti-CKF LL membrane fluorescence, which occured after 48 hours of LL culture in serum free media, could be due to a reduction in the synthesis of CKF as a consequence of the lack of nutrients due to the absence of FCS supplement. In the absence of FCS the majority of LL cells die after 48 to 96 hours of incubation. Thus, the reduced macrophage surface fluorescence observed after incubation with FCS-free LL conditioned media may be explained by a lower concentration of CKF in the conditioned medium.

The anti-CKF selective staining of the larger peritoneal cells making up 71% of the total population suggests that the LL-CKF was selectively bound by the activated macrophages averaging 65% of the total population of C. parvum activated peritoneal cells as determined

by differential and esterase staining. Therefore, it appears that CKF which is a membrane protein shed by the LL cells, is bound by the activated macrophages of the peritoneal exudate population.

Production of an antiserum using CKF isolated from serum-free LL conditioned media as the immunogen, would have significantly less of the undersirable FCS specificity of the current antiserum. Such an antiserum could be used to resolve the question of possible cross-reactivity between CKF and FCS.

D. Overview

In summary, we have demonstrated that the chemokinetic activity of activated macrophages incubated in LL conditioned media is due to stimulation by a heat stable, high molecular weight factor present in the media. CKF appears to be a trypsin sensitive portein which may be shed from the cell membrane or glycocalyx of LL cells during cell proliferation and adheres to the cell membrane of activated macrophages.

The functional significance of the CKF to macrophage-neoplastic cell interactions is unknown. However, the significant inhibition of the activated macrophage response to the chemotactic factor (MAS) mediated by CKF suggests that CKF may block receptor sites for tumor derived chemotactic factors. The concept of a tumor produced inhibition of the macrophage chemotactic response is supported by several studies of reduced monocyte chemotaxis among cancer patients (Boetcher, 1974; Bice, 1976; Snyderman and Pike, 1977) and the isolation of a lower molecular weight (6,000 - 10,000 daltons) tumor factor

which inhibits macrophage chemotaxis (Snyderman and Pike, 1976).

If CKF is a shed surface glycoprotein, it is likely that *in vivo* activated macrophages would be exposed to high concentrations of CKF in the vicinity of growing tumors. Due to its large size and ability of CKF to bind to the macrophage cell surface, CKF may also interfere with cell-cell contacts necessary for the tumoricidal activity of macrophages. Therefore, further studies of the concentration of CKF required to produce the chemokinetic response of activated macrophages versus the concentration of CKF required to inhibit chemotactic or cytocidal responses of activated macrophages would provide relevant information for the *in vivo* significance of CKF in tumor-host interactions.

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