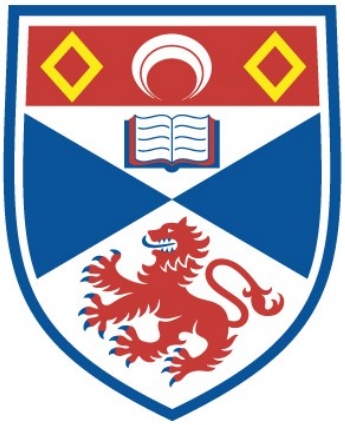
THE ROLE OF ENDOTHELIAL CELLS IN PROMOTING ADHESION, SPREADING AND MIGRATION OF B16F10 CELLS

Valerie Anne Ferro

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A thesis submitted to the University
of St. Andrews for the degree of
Doctor of Philosophy

by

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CONTENTS

ABSTRACT

For the successful establishment of secondary tumours, blood-borne metastatic tumour cells must adhere and spread on the vascular endothelium before they can migrate through it to form secondary growths in the tissue beneath. In this study an *in vitro* assay was developed to study the behavourial interactions between B16F10 cells and Bovine aortic endothelial cells.

It was hypothesized that molecules synthesized by the endothelial cells may be involved in the mediation of the adhesion, spreading and migration events and hence that such molecules may possibly be involved in the process of haematogenic metastasis. Endothelial derived extracts were obtained from the cell surface and from conditioned medium. The extracts were tested for their adhesion promoting abilities in a quick dot blot adhesion assay. To verify that these molecules promoted adhesion, antibodies were raised against the extracts. Partial characterisation of the molecules was achieved using SDS-PAGE and immunoprobing. The extracts were also tested for their spreading and migration promoting properties. An attempt was made to block the adhesion, spreading and migration events using antibodies directed against components of the extracts. Clearly, if endothelial-derived molecules are involved in metastasis, then preventing the mediation of adhesion, spreading and migration may ulitimately have relevance in the clinical situation.

DECLARATION

I, Valerie Anne Ferro, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date 1.1.89

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 in October 1985, and as a candidate for the degree of Ph.D. in October 1985.

Signed Date 1.1.89

I hereby certify that the candidate has fulfilled the conditions of the resolution and Regulations appropriate to the Degree of Ph.D.

Signature of Supervisor Date

1/1/89

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ACADEMIC RECORD

I graduated from the University of Aberdeen in July 1985, with a B.Sc.(Hons.) in Biochemistry, upper second class.

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LIST OF ABBREVIATIONS

BAE	Bovine aortic endothelial cells
BSA	Bovine serum albumin
CE	Crude BAE cell extract
CEM	Conditioned BAE cell medium
CMF-PBS	Dulbecco's calcium and magnesium free phosphate buffered saline
EC	Eagle's complete medium
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FCA	Freund's complete adjuvant
FCS	Fetal calf serum
Fg	Fibrinogen
FIA	Freund's incomplete adjuvant
Fn	Fibronectin
HAT	RPMI 1640 medium containing hypoxanthine, aminopterin and thymidine
HT	RPMI 1640 medium containing hypoxanthine and thymidine
IdU	Iodine labelled deoxy uridine
Lm	Laminin
Me	Mercaptoethanol

O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
RPMI	RPMI 1640 medium
SDS	Sodium dodecyl sulphate
SF-RPMI	Serum free RPMI 1640 medium
TEMED	N, N, N',N'-tetramethyl ethylene diamine
Tn	Tenascin
TSP	Thrombospondin
Tween 20	Polyoxyethylene sorbitan monolaurate
Vn	Vitronectin
vWF	Von Willebrand Factor

FIGURES

TABLES

PHOTOGRAPHS

SCANNING ELECTRON MICROGRAPHS

I GENERAL INTRODUCTION

1.1 MALIGNANCY

Tumour cells may tentatively be classified as either benign or malignant. The classification may be based on several factors, namely comparison with normal cells, composition, morphology, growth and cell behaviour (Sherbert, 1982). Growth and cell behaviour properties are common indices used to allow a reliable distinction to be made between the two types of tumour cells. For a more detailed account of the differences between benign and malignant tumours see the following reviews: Cole, 1973; Weiss, 1976; Sherbert, 1982; Vincent, 1985 and Weiss, 1985.

Briefly, benign tumours usually grow by expansion through cell division, remain localised and displace surrounding tissue (Woodruff, 1980). Pressure atrophy to cells surrounding the tumour, may result in the formation of a fibrous coating, around the tumour. However, this is not a universal feature of benign tumours (Sherbert, 1982). Notably, infiltration and destruction (invasion) of the tissue surrounding the tumour does not occur (Weiss, 1985).

Malignant tumours also grow by expansion through cell division and can displace surrounding tissue, but extensions from the tumours enable invasion to occur (Weiss, 1976). In addition malignant cells can also spread or metastasize to other areas of the body (Cole, 1973; Willis, 1973). Therefore the best criteria for determining malignancy are invasion and

metastasis (Vincent, 1985). A frequently cited exception to this general rule, is basal cell carcinoma. This common form of skin cancer, rarely, if ever, metastasizes, although it is capable of invading widely and may be highly destructive (Levenne *et al*, 1982). The reason that this tumour displays such a marked distinction between invasive and metastatic characteristics is not known.

1.2 METASTASIS

Metastasis may be defined as the transfer and spread of tumour cells from a primary site to one or more remote secondary sites (Roth *et al*, 1976). A series of sequential steps, leading to the formation of distant metastases was proposed by Nicolson and Winklehake (1975):

i. Invasion of the primary tumour into surrounding tissues.
ii. Spread of the tumour into body cavities and vessels and release of tumour cells for metastasis.
iii. Arrest of tumour cells at particular sites and invasion into surrounding tissue.
iv. Manipulation of the new environment to promote tumour cell survival, vascularisation and tumour growth.

1.3 INVASION

The first steps leading to metastasis are extension and local invasion (reviewed by Fidler and Nicolson, 1981). Local invasion is usually a fundamental step leading to spread of tumour. By breaching basement membranes (Trinkaus, 1976; Nicolson, 1981) (cellular sheet-like structures) (Siegal *et al*, 1981) and interstitial connective tissues (consisting of cells located in a matrix of collagen fibres, glycoproteins and proteoglycans) (Weiss, 1985), invasion of surrounding tissues can occur (Willis, 1973; Poste and Fidler, 1980).

The following observations, made *in vitro*, may help account for the release mechanisms involved in invasion and are reviewed by Hart (1981):

(1) an increase in amoeboid activity

(2) a decrease in adhesiveness

(3) loss of contact inhibition

(4) a possible release of lytic enzymes.

The relative importance of these mechanisms may vary from tumour system to tumour system (Cole, 1973). Susceptibility of host tissue to tumour cell invasion varies with the tissue type: cartilage, tendons, ligaments and arteries are relatively resistant, whereas veins, lymphatics, soft tissue and muscles are easily invaded (Willis, 1973). It would appear that tissues rich in ground substance or those containing dense elastic or collagen fibres, provide a more

successful barrier to malignant cells (Weiss, 1985). Normal host interactions at the site of invasion may also be important (Woodruff, 1980).

Once primary malignant cell invasion has occurred, the next step appears to be cell detachment when single tumour cells or tumour emboli, separate from the primary mass (Sherbert, 1982). Release mechanisms (reviewed by Weiss, 1985), include adhesive weakness, local cell rupture and enzymatic destruction, among others.

1.4 SPREAD OF TUMOURS

There are three major pathways involved in tumour cell dissemination following local invasion, as outlined by Cole (1973) and Willis (1973):

1.4.1. Spread via the lymphatic system

This route is taken particularly by carcinoma (tumours arising from epithelial cells) (Lindberg, 1972; Carter, 1978) and to a lesser extent by malignant melanoma (tumours arising from the melanin producing cells in the skin) (Carter, 1978; 1984). Primary tumours do not contain lymphatic vessels and so major points of entry are through small lymph vessels near the edge of the growing lesion (Carr *et al*, 1981). Access is generally achieved through the structural features of these small vessels which lack a basement membrane and have a high proportion of gaps between the endothelial cells (Yoffey and

Courtice, 1970). As in the case of macrophages and lymphocytes, tumour cells may enter the vessels through these gaps (Carr *et al*, 1976) and pass into the lymph nodes (Hewitt and Blake, 1975) which are able to filter clumps of cells, but presumably not single cells (Cobb and Steer, 1987).

Tumour cells may also readily enter thin walled venules (Papadimitriou and Woods, 1975) and these cells may subsequently gain access to the lymphatic system via anastamoses between venules and small lymphatic vessels (Wood, 1958; Haagenson, 1972; del Regato, 1978). There is now substantial evidence that malignant cells can pass freely between the lymphatic and circulatory system (Fidler *et al*, 1978) and the belief that tumour spread occurs exclusively by one or other, is an oversimplification.

1.4.2 Spread via the circulatory system

The most common route for metastasis is via the circulatory system. An example being melanoma (Carter, 1978). There are three routes into the circulatory system as shown in Fig. 1, p8 (taken from Weiss, 1985): lymphatic entry, direct venous invasion and invasion of tumour vessels.

(a) Lymphatic entry

This occurs if cell detachment from the primary tumour results prior to contact between tumour and blood vessel (Weiss, 1985). See Fig. 1(a).

(b) Direct venous invasion

Malignant cells may enter the bloodstream by invading blood vessels lying near the advancing edge of the tumour (Haagensen, 1972). See Fig. 1(b). It is thought that mechanical (Easty and Easty, 1978) and/or enzymatic factors, occurring at the edge of growing tumours (Kleinerman and Liotta, 1977), may contribute to penetration of the vascular system.

(c) Invasion of tumour vessels

Vascularisation of the primary tumour, is thought to be triggered off by tumour angiogenic factors (Folkman *et al*, 1971; Folkman, 1974). Invasion of these tumour vessels may occur through defects in vessel walls (Willis, 1973; Warren, 1974). These vessels are often imperfectly formed with irregular lamina, discontinuous or sometimes abnormalendothelium and defective membranes which make them more penetrable (Carter, 1984). See Fig. 1(c).

1.4.3 Spread by surface implantation

Tumour cells implant readily on the endothelial surface of pleura and peritoneum (Cole, 1973). When this occurs on an extensive scale, it is invariably associated with an effusion of fluid containing tumour cells (Woodruff, 1980). Dissemination of cancer of the lung in the pleural cavity and of cancer of the ovary, stomach and colon in the peritoneal cavity, are examples (Willis, 1973).

Tumour cells may also implant on epithelial surfaces (Cole, 1973); this occurs in the urinary tract from papillary carcinoma of the renal pelvis and in the bladder from primary bladder tumours (Willis, 1973).

Spread also occurs in the gastointestinal tract (Cole, 1973), but appears to depend on a pre-existing breach in the continuity of the epithelium (Woodruff, 1980). Implantation of tumour cells in the subcutaneous tissue and in the wall of the colon may also occur during the course of surgical operations (Cole, 1973).

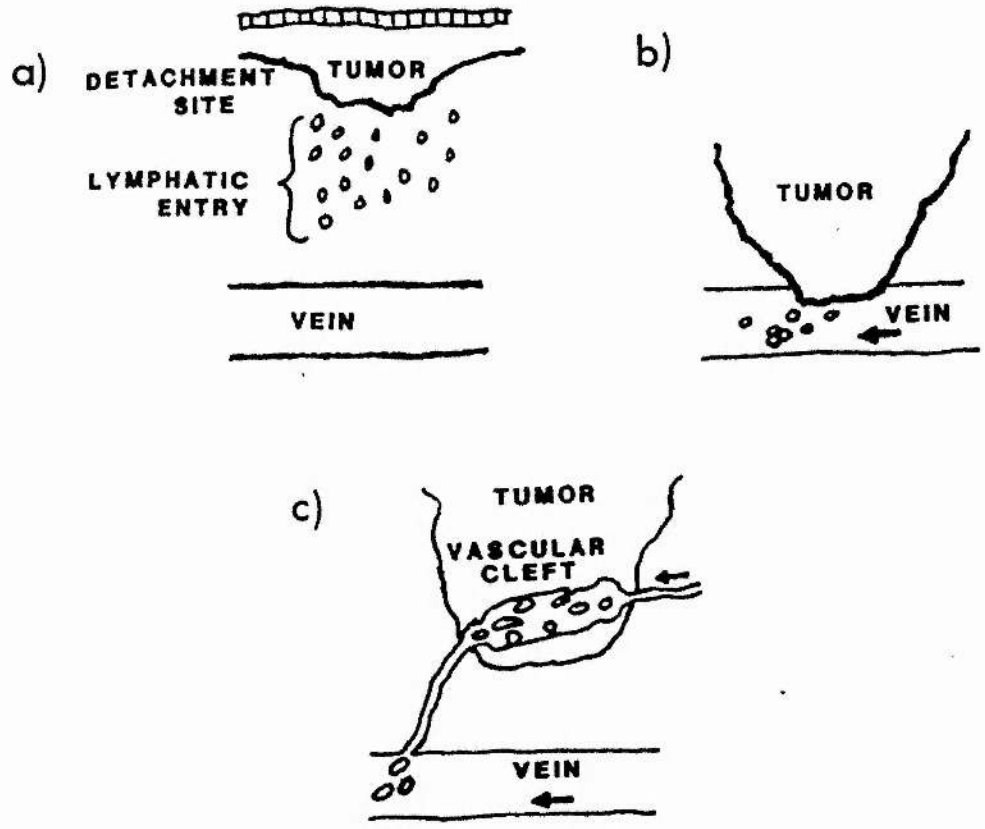


Fig. 1 shows tumour cell spread by entry into the circulatory system on the initial dissemination route.

(a) Detachment distant from the vein enables lymphatic entry to occur before direct venous entry.

(b) Detachment directly into a vein favours venous over lymphatic entry but does not exclude lymphatic entry from tumour margins.

(c) Cell shedding into tumour vessels via vascular clefts also favour venous over lymphatic entry but does not exclude the latter.

1.5 ARREST AND DISTRIBUTION OF TUMOUR CELLS IN BLOOD-BORNE METASTASIS

In this study, we are primarily concerned with events which occur in the circulatory system. First let us consider the events which occur in blood-borne metastasis, following invasion and spread.

Some tumour cells are released from the primary mass, possibly by becoming transiently less adhesive (Vollmers and Birchmeier, 1983), or by hydrolase activity of necrotic tissue which is prevalent in large tumours (Weiss, 1977), or by shear forces in the blood stream (Glucksmann and Cherry, 1964). In order to form metastses, these released circulating tumour cells must become arrested at particular sites, such as in capillary beds (Wood et al, 1966). As shown in clinical and experimental studies, the mere presence of circulating tumour cells does not constitute metastsis (Salsbury, 1975; Fidler et al, 1978).

The site of arrest on the endothelial lining of the blood vessels may be non-specific and determined by the size of the clumps of tumour cells released from the primary mass (Liotta et al, 1976). Large clumps of cells can become mechanically impacted in vessels and the size and deformability of the tumour cells, the diameter and distensibility of the capillaries and the interaction of the tumour cells with each other and with circlating components to

create multicellular emboli are relevant in this context (Poste and Fidler, 1980).

However, the arrest of smaller aggregates and single cells cannot be accounted for by mechanical means alone. The experiments of Wood (1958; 1966), described the association of tumour cells with platelets, lymphocytes and thromboplastic components during the arrest process. Various workers have since shown that tumour cells do indeed undergo interactions with these host cells and components to form stable aggregates (Pearlstien et al, 1980; Fidler, 1978; Kohga and Tanaka, 1979). In addition, Stringfellow and Fitzpatrick (1979) and Pearlstein et al (1980) suggest that prostaglandins may play a role in the arrest of many tumours. Roos and Dingemans (1979) suugest that the inflammatory events involving fibrin deposition and platelet aggregation around the tumour cells may be activated to protect the cancer cells from blood shear forces rather than in playing an active role in the arrest of tumour cells. Some reviews on the role of inflammatory reactions in metastsis include those by Gatspar (1977); Hilgard et al (1977); Sherbert (1982). In brief, arrest and emboli trigger events which couple an inflammatory response, blood coagulation and fibrinolysis, complement activation, and tissue damage, partially through products of the arachidonic acid cascade. The effects of vessel wall damage enhances further cancer arrest since exposure of the subendothelium results in platelets becoming adherent and activated.

Roos and Dingemans (1979), also suggest that more subtle

processes are required for arrest, especially in view of the fact that clinical and experimental observations show that certain tumours consistently metastasize to particular organs (organ-specificity) (Willis, 1973; Cole, 1973; Fidler and Nicolson, 1976). More recently, emphasis has been placed on cell surface properties and an excellent demonstration of this was provided by Hart and Fidler (1980). They showed that B16F10 cells inoculated intravenously, form deposits on lung and ovary tissue even when these organs are transplanted to new locations within the body. This suggests that perhaps tumour cell surface determinants (Poste, 1980) or properties of the endothelial cells within different organs (Nicolson, 1982; Auerbach and Joseph, 1984) contribute to arrest. Auerbach and Joseph (1984), postulated that capillary endothelial cells differ in their surface antigens, the differences reflecting their developmental history. These distinct organ-associated antigens on the cell surfaces could explain organ-specificity. A report by Dietrich et al (1983), suggested that heparan sulphates play a role in cell-cell recognition and adhesiveness since heparan sulphates are able to alter their configuration. To investigate organ-specificity, Alby and Auerbach (1984) used endothelial cells from mouse brain or ovaries and tested them in an in vitro assay system for adhesion. They found that ovary-derived teratoma cells and a testicular teratoma with ovary seeding properties adhered preferentially to ovary endothelial cells. Whereas glioma cells adhered preferentially to brain

endothelial cells. At present, too few endothelial cell lines are available to validate the studies. However, lymphocyte homing studies demonstrate clearly the importance of endothelial cell specificity in regulatin extravasation of circulating cells (Stamper and Woodruff, 1977; Chen and Singer, 1983; Streeter et al. 1988). Lymphocyte adherence to, and penetration through specialised endothelial cells, depends on the presence of specific adherence molecules on the endothelial cells (Butcher et al. 1979). Moreover, there is organ specificity in this lymphocyte-endothelial cell interactive system (Gallatin et al. 1983).

Historically, two simple hypotheses have been used to account for organ specificity (reviewed by Weiss, 1985). The first, the "haemodynamic" or "mechanical" hypothesis, focuses on the delivery of tumour emboli to target organs. The second, the "seed-soil" hypothesis, focuses on the differential growth of arrested emboli in different target organs subsequent to delivery. A contribution to the theory of organ specificity was made by Fidler and Nicolson (1976), using B16F1 cells (tumour cells which show low arrest in the lungs) and B16F10 cells (tumour cells which show high arrest in the lungs). Following either tail vein or left ventricular injections of ^{125}IdU-labelled melanoma cells, the initial arrest of cells depended on the injection route; after intravenous injection, more cells were arrested in the other organs than after intracardiac injections. However, 24 hours after injection, in both cell lines, more viable cancer cells

were present in the lungs after intracardiac injections. Fourteen days after the injection, similar numbers of pulmonary tumours were seen following tail vein or intracardiac administration. The results suggested that the organ pattern of tumour colonization was not determined by non-specific arrest in the first organ encountered by the injected cells.

At present, it appears that the "haemodynamic" hypothesis accounts for the majority of metastatic patterns of development up to the stage of general arterial dissemination. At the stage of general dissemination leading to end metastasis, some features of pattern are clearly not explicable solely in terms of the "haemodynamic" hypothesis. This implicates the "seed-soil" hypothesis. It seems likely that the "seed-soil" hypothesis accounts for the differential growth rate of metastases in different target organs, as distinct from the incidence of metastases in these organs.

1.6 ESTABLISHMENT OF METASTASES

Ultrastructural studies indicate, that tumour cells gain a secure foothold in the circulatory system by adhering to endothelial cells and to the underlying basement membrane (Warren, 1974: Fidler *et al*. 1978: Auerbach and Joseph, 1984). It is the initial arrest reactions which culminate in the adhesion of tumour cells to the vascular endothelium: this leads to exposure of the sub-endothelium which also acts as an adhesion site. The tumour cells then exit (extravasate) from

the vessel into surrounding tissue(s) in order to escape from the hostile environment of the circulatory system. Extravasation of malignant cells is believed to involve mechanisms similar to those responsible for the initial invasion of blood vessels (Poste and Fidler, 1980). Following successful extravasation, small tumour cell lesions are formed.

Throughout the metastatic process, malignant cells are subject to attack by host defence systems which may be immune or non-immune (Weiss, 1985). The chances of metastases developing from circulating tumour cells therefore are small (reviewed by Weiss, 1980) and may depend on:

(1) anatomical factors which influence the distribution of tumour emboli;
(2) properties of the sites at which emboli are arrested and the extent to which they can satisfy the metabolic and other requirements of the particular tumour;
(3) properties of the tumour including its capacity to produce plasmin-like enzymes and
(4) various factors which influence local and general resistance to the tumour and modify the complex process of metastasis in ways which, for the most part, remain to be elucidated.

Even following extravasation, only a small proportion of tumour cells survive and are able to become established. Little is known about the factors involved in establishment and growth of metastases in the target tissue. One factor may involve adaptation of the host tissue. For example, tumour

cells are able to induce the formation of blood vessels from the host circulatory system (Folkman and Haudenschild, 1980) and are thought to be able to do this by releasing a tumour angiogenic factor(s) (TAF). This factor(s), which was isolated by Folkman et al (1971), appears to be produced by tumours both in vitro and in vivo (Klagsburn et al, 1976; Phillips et al, 1976; Brem et al, 1977).

The failure of the rest of the extravasated cancer cells to grow may be due to a number of reasons, including the inability of the tumour cells to overcome host defence mechanisms (reviewed by Poste and Fidler, 1980; Weiss, 1985) and a non-favourable host environment (Folkman, 1975). Horak et al, (1986) showed that organs such as murine liver and thyroid gland are able to rapidly and effectively diminish the number of live tumour cells in vitro, whereas murine lung and ovary promote tumour cell attachment and survival. This effect can also be exerted by cell-free organ conditioned medium and does not require previous contact with the tumour cells (Nicolson and Dulski, 1986). This observation implicates a soluble substance in determining which organs allows the survival of different tumour types.

It would appear that the ultimately successful cells- as few as 0.1% of the circulating tumour cells (Fidler et al, 1978) - would have to undergo rigorous selection in order to survive (Roos and Dingemans, 1979; Poste and Fidler, 1980;; Poste et al, 1981). It is possible that only a small subpopulation of cells in the heterogenous primary tumour possess the characteristics to establish a successful

metastasis (Alexander, 1984: Fidler, 1978). Fidler (1973), showed that it is possible to obtain tumour lines with increasing metastatic potential by subjecting the cell population to selection procedure involving successive transplantation with intermittent in vitro culturing. Fidler injected viable B16 melanoma cells into the tail veins of C57BL/6 mice, isolated the tumour nodules which formed in the lungs of these animals and grew these isolated cells in tissue culture. The cells of the first in vivo selection for lung colonization were designated B16F1. The B16F1 cells were injected back into syngeneic mice, and the pulmonary colonies that formed were isolated and adapted for culture (B16F2). The procedure was carried out ten times to obtain the B16F10 cell line. At each stage of the selection procedure, the ability of the cells to survive and form colonies in the lungs increased (Fidler and Nicolson, 1976: Fidler and Kripke, 1977). This outcome is compatible with the view that the original unselected primary tumour contained a number of variant lines differing widely in their potential for lung colonization.

The presence of heterogeneity within a primary tumour has profound implications for the way in which research should be carried out to identify the cellular properties responsible for metastasis. Experiments to identify features unique to metastatic subpopulations require comparisons to be made with non-metastatic (but tumourigenic) subpopulations of cells from the same parent tumour. For this reason, the B16 melanoma and

its variant cell lines are a good model to work with.

From clinical studies, it is clear that the formation of metastses, is an important step in the pathogenesis of cancer, since metastatic growths are the cause of most deaths in cancer patients (Nicolson et al, 1976). A crucial step in the survival of tumour cells which have been disseminated through the circulatory system is extravasation. A greater understanding of this process may help in preventing the formation of metastases.

Bearing this in mind, the aim of the work described in this thesis is to try and investigate in greater detail the interactions between tumour and endothelial cells, with a view to increasing knowledge on the process of extravasation.

II EXTRAVASATION

2.1 INTRODUCTION

Extravasation, as defined by Wood (1958), is the process by which malignant cells adherent to endothelial cells penetrate the endothelial cell layer, bind to and finally invade the underlying basement membrane. In doing so the tumour cells escape host defence mechanisms (Weiss, 1985) and blood shear forces (Glucksmann and Cherry, 1964). This definition was arrived at from micro-cinematography observations made by Wood (1958) of cancer cells crawling out from blood vessels in rabbit ear chamber experiments. In these experiments, Wood injected V2 carcinoma cells into the central artery of a rabbit ear, proximal to a transparent window implanted in the ear. The events following this injection were photographed at high magnification. "Successful" tumour emboli were noted to form aggregates readily and to adhere strongly to the endothelium in comparison with "unsuccessful" tumour emboli. Adhesion to the endothelium was followed by formation of thrombi around the cells. Within a variable period from a few minutes to a few hours, the endothelial cells underlying the tumour thrombus appeared to become damaged. Leucocyte migration occurred beneath the tumour thrombus and the cancer cells followed, passing through breaches in the endothelial lining caused by a variety of factors, including platelets, histamines and leucocyte-mediated injury (Weiss, 1985).

Several mechanisms have been put forward to explain how tumour cells may leave the blood vessels:

1. Escape of tumour cells through defects in the endothelial wall (Sherbert 1982; Carter, 1984).
2. Endothelial penetration by breakage of endothelial intercellular junctions (Locker *et al*, 1970; Nicolson, 1981).
3. Proteolytic enzyme destruction of endothelial cells by tumour cells (Liotta *et al*, 1980; Crissman, 1985). The enzymes are released from the pseudopodia of the tumour cells.
4. Penetration of endothelial cell cytoplasm by tumour cell pseudopodia and by pinocytosis (Dingemans, 1974).
5. Endothelial wall rupture due to physical disruption as a result of proliferation of tumour cells at the site of arrest by pressure of the growing mass (Basserga and Saffioti, 1955; Chew *et al*, 1976).
6. Changes in permeability of the endothelium e.g. during inflammation, histamine release makes the endothelium leaky (Majno *et al*, 1969; Ozaki *et al*, 1971).

Whatever the method of escape, the basement membrane is the major barrier to extravasation and it is likely that more than one mechanism is utilised in this process (Crissman, 1985).

2.1.1 Investigations into tumour-endothelial cell interactions

A survey of the published literature of *in vivo* studies, reveals the use of many different biological systems in the

investigation of the interaction between tumour and endothelial cells. Workers using *in vitro* systems have added a further complication to the subject by confronting tumour cells of one species with endothelial cells of another species. The reasons for doing so are usually technical; but surprisingly, there is little evidence to show that the results obtained from studies of interactions between cells of different species and those obtained from systems utilising syngeneic tissues are markedly different (Easty and Easty, 1984). There are obvious differences between *in vivo* and *in vitro* studies. *In vivo* there is a complex interplay of hormonal, inflammatory and possibly immunological responses (reviewed by Eccles and Alexander, 1974; Sherbert, 1982; Weiss, 1985; Hanna, 1985; Robins, 1986) which may contribute to the magnitude and rates of interactions; *in vitro* these are usually absent. Thus, *in vivo* systems would appear to be the more desirable. In practice, the complexity and interdependence of *in vivo* reactions, and the difficulty of examining the effects of a single component, renders analysis of *in vivo* systems very difficult, while the relative simplicity of the *in vitro* systems facilitates analysis of mechanisms, but may yield data which are not substantiated by subsequent experiments in animals. Nevertheless, *in vitro* experiments have proven invaluable in elucidating endothelial and tumour cell interactions.

An elegant demonstration of interactions between two cell types (Kramer and Nicolson, 1979) involved a variety of tumour cells, fibroblasts, macrophages, lymphocytes and

leucocytes seeded onto monolayers of bovine aortic endothelial cells (BAE). BAE cells are commonly used *in vitro* experiments, because these cells form intercellular junctions and an extracellular matrix in culture of similar composition to the basement membrane *in vivo* (Birdwell *et al*, 1978). Using time-lapse photography, Kramer and Nicolson (1979) observed that, in general, the malignant cells, monocytes and leucocytes, which can all invade the endothelium *in vivo* (Ward *et al*, 1979), firstly adhered to the endothelial cells. This was followed by the retraction of the endothelial cells at the site of adhesion. However, only cells adjacent to endothelial cell junctions were able to migrate between the undersurface of the endiothelial and their extracellular matrices. The endothelial juctions then re-sealed, walling off the invading cells. Similar results were obtained by Zamora *et al* (1980), who inoculated monolayers of endothelial cells grown on collagen gels with multicellular spheroids of mouse mammary carcinoma. Again, retraction of the endothelial cells in the vicinity of tumour cells was observed following adhesion, and the mammary tumour cells spread on the collagen, migrating under and over the endothelial cells and into the collagen. The steps seen in these experiments correspond well with the observations made in the *in vivo* situation (Wood, 1958; 1966; Crissman, 1985).

The aim of this part of the thesis was to visualise behavioural interactions between tumour and endothelial cells.

Due to some of the difficulties arising from studying this type of interaction *in vivo*, an *in vitro* model system was developed. B16F10 cells were used for a variety of reasons in addition to their availability:

1. The cells can be easily distinguished by their morphology and colour (Kramer and Nicolson, 1979).
2. Comparison can be made with variants of the parent cell. These include cells with differing metastatic potential and metastases arising in a variety of organs, for example brain and ovary (Brunson and Nicolson, 1978; 1979) and liver (Tao *et al*, 1979).
3. The cells are easily grown in culture as well as *in vivo* (Fidler and Nicolson, 1981).

Initially, B16F10 cells were seeded onto BAE monolayers as described by Kramer and Nicolson (1979) and observations were made using light microscopy. This differed from the Kramer and Nicolson experiments, in that time-lapse photography was not used. The B16F10 cells adhered to the monolayer within 30 mins and tumour cells near endothelial cell junctions destroyed the monolayer in the regions proximal to the tumour cells after 24h. The retraction and re-sealing of the endothelium, described by other workers, could not be identified, possibly since time-lapse photography and/or magnification higher than x600 were not used.

One major disadvantage with the *in vitro* assays

previously described (Kramer and Nicolson,. 1979; Zamora *et al*, 1980) is that the tumour cells are seeded on top of endothelial monolayers. This limits the number of observations which can be made, without making transverse sections of the experimental material and thus introducing artefacts and misinterpretations into the system. Therefore the model system developed in this investigation allowed the study of interactions between the two cell types in a small volume, 10mm apart, onto different areas of a petri dish until the cells had attached (typically 30 min). Unattached cells were aspirated off and the petri dish covered with medium. Observations of interactions between the two cell types as they grew towards each other were made under light and scanning electron microscopy. One problem associated with light microscopy is that identification of specific cells can sometimes prove to be difficult. In order to overcome this difficulty and to distinguish between the two cell types, cell-specific antibodies were used to stain the cells.

This model has some modifications from those previously described. It offers the potential of studying a wide range of tumour-endothelial cell interactions, including invasion, extravasation and angiogenesis. In addition, more detailed observations could be made regarding the interactions between tumour and endothelial cells than have been possible in the past.

2.2 MATERIALS AND METHODS

2.2.1 Cell preparation and maintenance

a) B16F10 Melanoma Cells

B16F10 cells, supplied by Dr IJ Fidler (Frederick Cancer Research Centre, Frederick, MD, USA), were maintained in Eagle's minimum essential medium with Earle's salts and supplemented with benzyl penicillin (50IU/100ml), streptomycin sulphate (50ug/100ml), glutamine (2mM), sodium pyruvate (1mM), essential vitamins, amino acids, and 10% heat inactivated fetal calf serum (FCS) (all from Gibco). This medium will be referred to as Eagle's complete (EC). Cells were subcultured every third day by the transfer of $2x10^5$ cells per 9cm petri dish (Nunclon) after harvesting with 2mM EDTA in Dulbecco's calcium and magnesium-free phosphate buffered saline (CMF-PBS). (See Appendix 1). The cells were counted using a Coulter Counter Model ZB in conjunction with a Channelyzer C1000.

b) Bovine Aortic Endothelial Cells (BAE)

BAE cells, a gift from Dr JR Starkey, Montana State University, were also maintained in EC, but with the addition of insulin (0.013IU/ml) and endothelial growth factor (5ng/ml) (both from Sigma). Cells harvested after incubation for 3min at 37°C with 2mM EDTA and 0.1% trypsin in CMF-PBS, were subcultured every third day by the transfer of $6x10^5$ cells per 9cm petri dish.

2.2.2 Rapid Lowry protein assay

The amount of protein in solution was measured using a modification of the Lowry method (Larson *et al*, 1986): 2ml stock reagent (1% $CuSO_4$, 10% 2M Na_2CO_3, 1% 10N NaOH, 16.25% tartaric acid (D+) in distilled water) were mixed with 0.1ml protein sample and 0.2ml of a 50% dilution of Folin Ciocalteau reagent in plastic micro cuvettes and left to incubate for 3min. To complete the reaction in 10min, 0.2ml 20mM ascorbic acid was added at room temperature and mixed well. After 10min, reading at 660nm were taken in a Unicam SP1800 spectrophotometer and compared against a standard curve established using BSA ranging from 0.2mg/ml-2mg/ml.

2.2.3 Antibody specificity tests

a) Dot blotting

Discs, 1cm in diameter, cut from surfactant-free nitrocellulose sheets (Millipore HATF 13750, pore size 0.45um), were fitted into the wells of a Nunc 24-well tissue culture grade plate. A confluent 9cm plate of BAE or B16F10 cells was harvested as described previously and resuspended in 1ml PBS (see Appendix 1). Samples of cell suspension (2ul) were dotted around the circumference of each disc. To each culture well was added 1ml of 10% Marvel (dried milk protein) (w/v) in PBS/0.05% Tween 20 (v/v) for 30min at 37°C and the plates incubated to block other available protein binding sites on the nitrocellulose. The discs were then incubated with 1ml of primary antibody diluted in PBS (2ug/ml-200ug/ml)

for 30min at 37°C. The discs were washed twice with PBS/Tween and then once with PBS, prior to a 30min incubation with 1ml of the appropriate phosphatase conjugated second antibody (Sigma) diluted 1:1000 with PBS at 37°C.

The discs were washed 3x with PBS and developed for 20min with Fast violet B salt (1mg/ml) and beta-naphthyl phosphate (1mg/ml) diluted in sodium borate buffer pH8.7. (See Appendix 1). The discs were then rinsed several times in distilled water and left overnight in 1ml water to reduce background staining. The discs were dried at room temperature on tissue paper. In order to score the intensity of the colour of the discs, it was necessary to include positive controls and negative controls. The positive controls consisted of dotting the phosphatase conjugated second antibody onto a nitrocellulose disc, followed by blocking and developing. The negative control consisted of dotting the test protein solution onto a nitrocellulose disc, followed by blocking, incubation with phosphatase conjugated antibody and developing.

b) Enzyme linked immunosorbant assay (ELISA)

A confluent 9cm plate of BAE or B16F10 cells was harvested as usual and resuspended in EC medium. The cells were seeded at their usual density (see Part 2.2.1) in 96-well plates and grown to confluency. The first row was used as a blank and no cells were grown in these wells.

The cells were fixed for 20mins at room temperature with

50ul of 3% glutaraldehyde in PBS/well. Excess fixative was discarded and the plate washed twice with PBS/Tween (200ul/well) before incubating with 100ul/well of diluted primary antibody for 60mins at 37°C. The plate was washed twice with PBS/Tween before incubating with 50ul of phosphatase conjugated second antibody as outlined above (Part 2.2.3a) for 2h at 37°C. The plate was washed twice with PBS/Tween and 50ul of p-nitrophenyl phosphate substrate (Sigma) (1mg/ml in 0.1M glycine buffer pH10.4, 0.001M $MgCl_2$ and 0.001M $ZnCl_2$) was added. The colour was allowed to develop for 30min. The developed solution was transferred to a new 96-well plate before reading on an ELISA plate reader. An O.D. three times higher than background was taken as positive.

2.2.4 Polyclonal antibody production

A 1ml suspension of BAE or B16F10 cells (scraped from five 9cm plates) in PBS or 1ml protein (100ug/ml) in PBS was vortexed with 1ml FCA added dropwise. The mixture was injected into a New Zealand white rabbit, at 10 subcutaneous injection sites on its back (0.2ml/site). Six weeks later a small sample of the serum obtained from an ear bleed was assayed in an ELISA system to check antibody specificity. The rabbit was reboosted with the same protein in FIA and left for 3 weeks before bleeding from the ear (30ml). Serum was obtained by allowing the blood to stand for 1h at 37°C, followed by incubation at 4°C overnight. The mixture was then

centrifuged for 10min at 680g. The supernatant obtained (serum) was then purified as described below. If the activity of the antibody appeared to be decreasing the reboosting step was repeated.

2.2.5 Polyclonal antibody purification

Saturated ammonium sulphate solution (pH7.0) was added dropwise to antibody containing serum at 4°C and the mixture stirred continuously until 40% saturation was achieved. The mixture was stirred for a further 30min before being centrifuged at 680g for 10min. The pellet was dissolved in 10ml of PBS and dialysed overnight against PBS. In the meantime, 1g of Protein A-sepharose CL-4B (Pharmacia) resuspended in 4ml PBS was poured into a plugged 10ml syringe. The column was equilibrated with 10ml 0.1M glycine-HCl buffer pH3.0 (eluting buffer) followed by 10ml PBS pH7.4. The antibody solution was then mixed with the immobilized proteinA by shaking and the protein A allowed to settle by gravitation for 15min. Unbound material was washed off with 10ml PBS, and bound material was eluted with 10ml of the eluting buffer. This solution of IgG was neutralised with 1.0M Tris-HCl pH8.0. The protein content was assayed (see Part 2.2.2) and the O.D. at 280nm read (the expected O.D. is 1.4 for IgG of concentration 1mg/ml). The solution was filtered and stored frozen in 1ml aliquots.

2.2.6 Immunostaining cells

Confluent cells were fixed with 3% glutaraldehyde for 20min at 37°C. The fixative was washed off with PBS, before adding the first antibody, diluted 1:100 in PBS, for 30min at 37°C.

The cells were washed gently with PBS before incubating with phosphatase-conjugated second antibody for 2h at 37°C. The cells were then washed once and the colour developed as described in Part 2.2.3a.

2.2.7 Tumour-endothelial cell interactions

B16F10 and BAE cells were harvested as usual and resuspended in EC medium with BAE supplements (see Part 2.2.1). The B16F10 cells were seeded in 2 areas (in 10-20ul of medium) on one side of 5cm petri dishes at $1x10^4$ cells and BAE cells at $2.8x10^4$ cells in 2 areas on the other side of the petri dishes keeping the four seeding areas about 10mm apart. The petri dishes were left at room temperature for 30min until the cells had attached to the plastic. The dots were aspirated and fresh medium gently placed into the dish. The medium was changed at three day intervals. Observations were made using a light microscope. In order to obtain some indication as to the viability of the cells, a trypan blue dye exclusion test was carried out. This involved placing 5ml of 0.1% trypan blue solution into the tested petri dish. After 1min the dye was aspirated and 5ml of PBS added to the dish. If the cells stained dark blue they were not viable. In some

cases the plates were stained with antibody as outlined in Part 2.2.6. Scanning electron microscope observations were made on some plates as outlined in Part 2.2.8.

2.2.8 Scanning electron microscopy

The cells were grown as described in Part 2.2.7. The cells were fixed with 3% glutaraldehyde in PBS, for 30min at room temperature. Discs were cut from the petri dish using a heated cork borer. The samples were sequentially dehydrated in 50% ethanol (15min), 70% ethanol (15min), 96% ethanol (15min) and 100% ethanol (15min, 2 changes). The discs were then critical point dried and gold sprayed by Mr. I. Davidson, Department of Biology and Preclinical Medicine, St. Andrews. The scanner used was a JSM-35CF, and the photographic film 120 FPA. The photographs taken were examples of interactions occurring between the two cell types.

2.3 RESULTS

In this study an *in vitro* model was developed in order to visualise interactions between B16F10 and BAE cells. Both cell types were seeded in the same petri dish and interactions were observed using light and scanning electron microscopy. Under the scanning electron microscope, specific cells were distinguished by their morphology and size, but under the light microscope this was not as easy to do. This problem was overcome by raising cell-specific polyclonal antibodies to be used to immunostain the two cell types.

2.3.1 Immunostaining of cells

One problem encountered was a result of the endogenous enzyme activity of the cells which resulted in false positives in the antibody specificity tests (Goding, 1986). For example, the BAE cells reacted with the alkaline phosphatase substrate (B-naphthyl phosphate and Fast violet B salt) used in developing the colour reaction of the antibody staining. (See Part 2.2.3a). This was attributed to alkaline phosphatase activity of the BAE cells. In order to try and remedy this problem, peroxidase conjugated second antibodies and the appropriate substrate (o-phenylenediamine - see Appendix 1) were tried. In this case the B16F10 always stained positive. This was attributed to the endogenous peroxidase activity of the B16F10 cells. This activity could be blocked using hydrogen peroxide and methanol (Farr and

NAkane, 1981). However, due to the denaturation of the antigens on the cells by these chemicals, the primary antibodies then did not interact with the cells. It was eventually decided to use antibodies raised against B16F10 cells (a-F10 - 9.2mg/ml), with alkaline phosphatase conjugated second antibodies. This combination gave a slightly stronger staining for the B16F10 cells than the background staining for BAE cells and so it was possible to distinguish between the tumour and endothelial cells (See Photographs 1 and 2).

2.3.2 Tumour-endothelial cell system

Observations were made daily. The first changes were noted 3 days after seeding and then at intervals as indicated below. The following results are representative of ten dishes for each day and a total of four repeat experiments. The observations made were highly reproducible and typically 80-90% of the dishes showed the interactions described.

a) Day 3

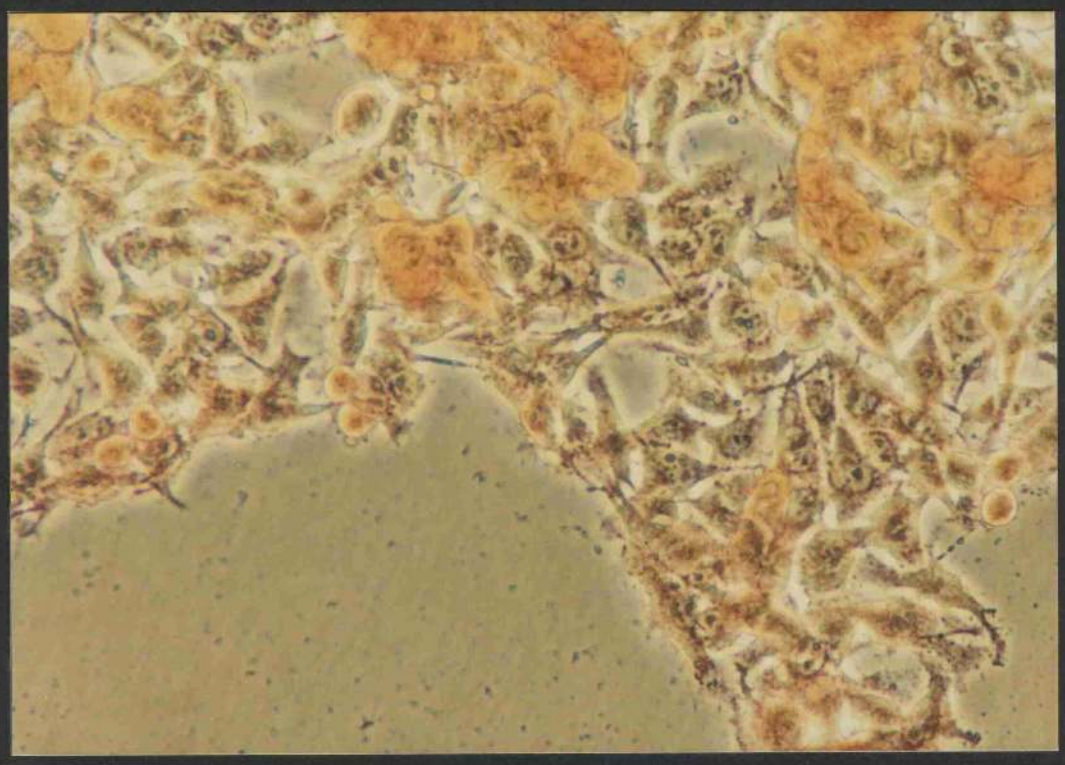
On the third day of growth the B16F10 cells appeared as two discrete compact clusters. (See Fig. 4). In the centre of the clusters (which had doubled in size from the initial seeding) the cell density was very high and the cells were rounded and overgrown into thick aggregates (See Photograph 1). On the outer edge of each cluster the cells grew outwards. The cells on the outer edge varied in morphology - a few were spread with flattened pseudopodia, but the majority

were elongated and lined sideways on (See Photograph 1 and Scanning electron micrograph 1). A few of the aggregated cells from the centre had detached and become attached elsewhere in the petri dishes and a few single cells appeared to have "escaped" from the discrete dots; so colonied developed in the initially bare area of the petri dishes.

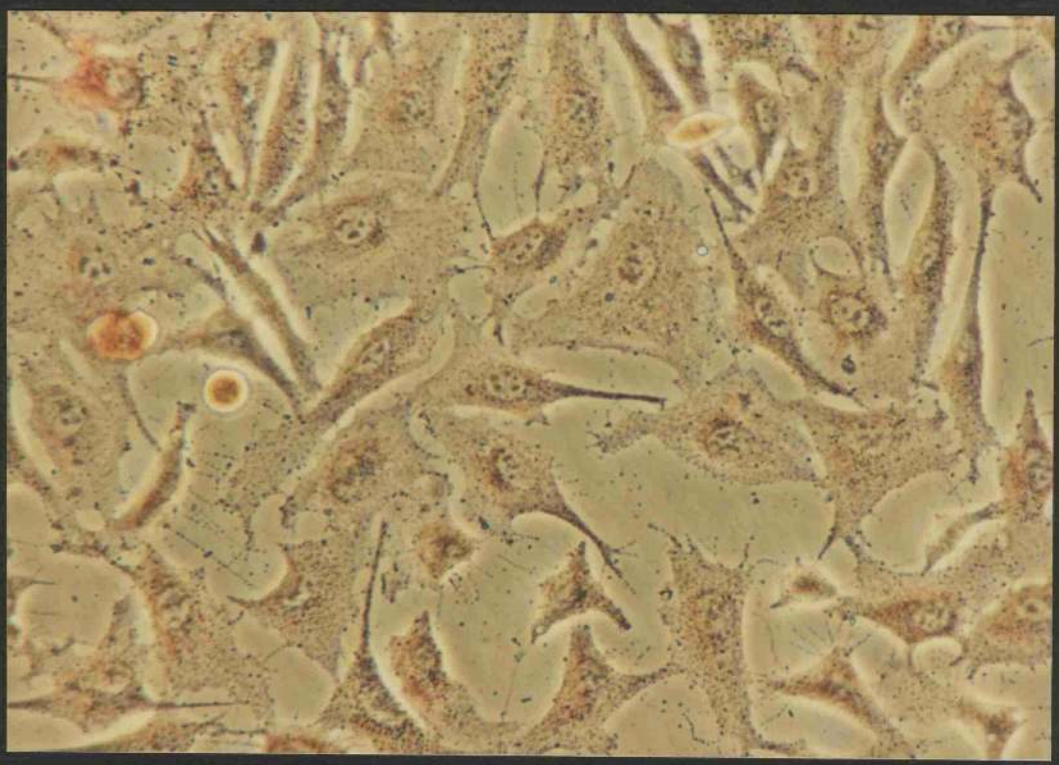
On the other hand, the BAE cells were not as compact as the B16F10 cells. The centre of each cluster of BAE cells had a typical cobblestone appearance, but at the outer edge, the cells were loosely arranged, with the cells having large widespread lamellipodia (See Photograph 2 and Scanning electron micrographs 2 and 3). In the area of the petri dish between the tumour and endothelial cells there were large numbers of single endothelial cells. However there were no areas of interaction between the tumour and endothelial cells.

Photograph 1 shows the leading edge of the B16F10 cells in the confrontation assays after three days growth. Towards the top of the photograph dense aggregated cells can be seen. Magnification x240.

Scanning electron micrograph 1 shows the leading edge of the B16F10 cells in the confrontation assays after three days growth. Magnification x1560.



Photograph 2 shows the edge of the BAE cells after three days growth. The top of the picture shows that the cells are more tightly spaced than at the edge. Magnification x270.



Scanning electron micrograph 2 shows the BAE monolayer. Towards the top right hand corner the cells are confluent. But at the leading edge the cells are loosely arranged. Magnification x172.

Scanning electron micrograph 3 shows the BAE cells at the leading edge in greater detail.
Magnification x1320.

b) Day 10

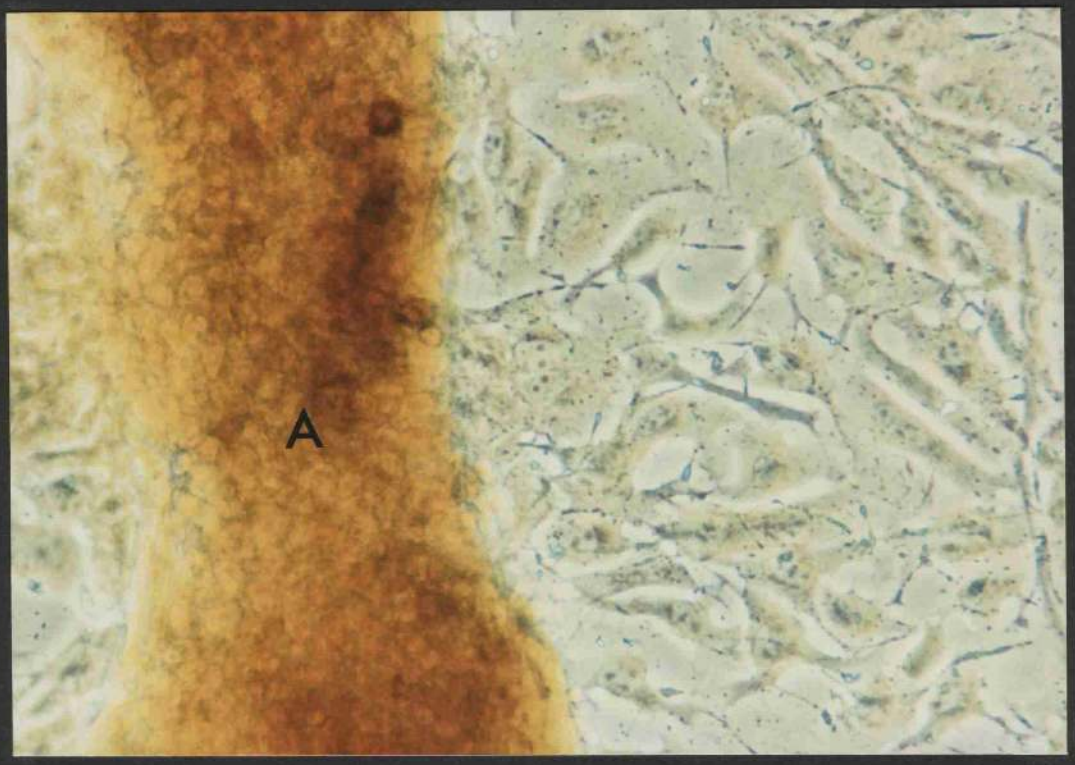
On the tenth day of growth, the B16F10 cells still appeared as discrete clusters (doubled in diameter, compared with day 3). (See Fig 4). However, the centres contained very dark aggregated cells (see Photograph 3 and Scanning electron micrograph 4). The outside edge was still compact. Only one half of the dishes was covered with tumour colonies and this area appeared to be in the area nearest to the original tumour cell seeding areas. (See Fig 4).

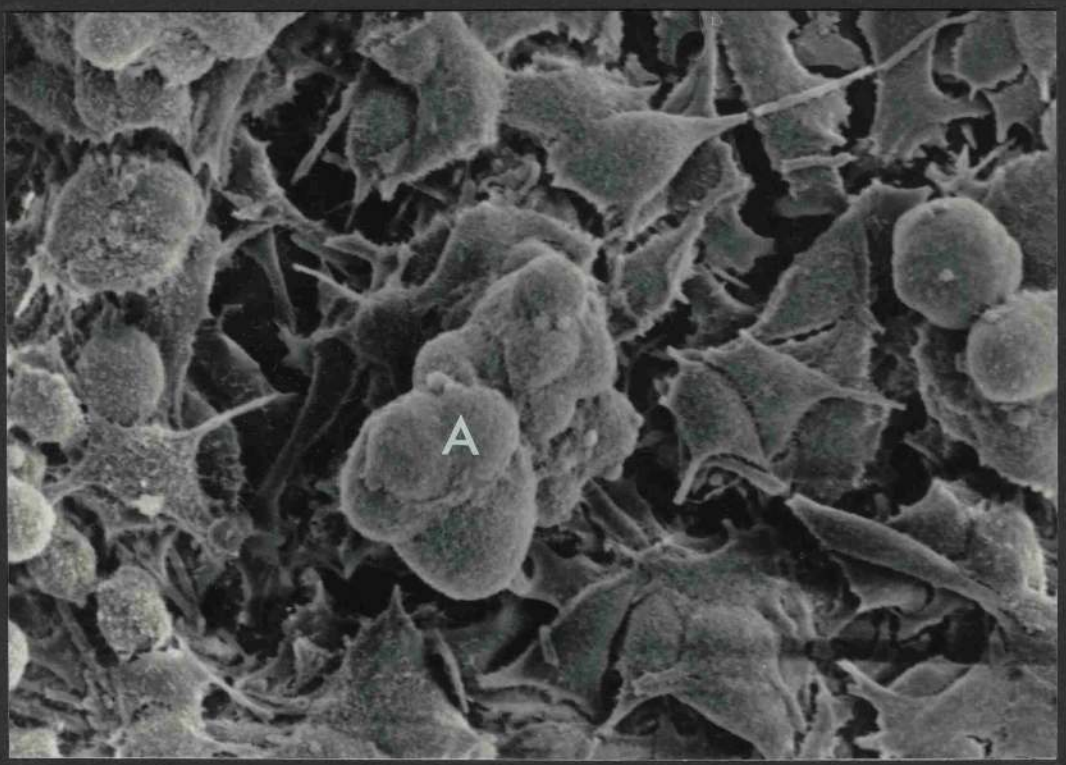
By this time single BAE cells started to appear at the leading edge of the tumour cells. The morphology of the BAE cells appeared to change and it seemed as though the BAE cells were lying across the path of the B16F10 cells. (See Scanning electron micrograph 5).

Within the BAE monolayer, there were areas where the aggregated tumour cells from the centre of the tumour cell dots had landed after detachment. This could be equeated to the *in vivo* situation where tumour cells detach from the primary tumour mass in the circulatory system and become arrested on the endothelium. The BAE monolayer, proximal to the tumour aggregate, appeared to become compressed and to be evenly pushed away from the aggregates. Results from scanning electron microscopy showed that the BAE cells were lifted off the base of the dish and rolled up as the tumour cells invaded underneath. The tumour cells began to spread and proliferate on the revealed plastic. At higher magnification it could be seen that the B16F10 cells had started to migrate under this compressed area. (See Photograph 4).

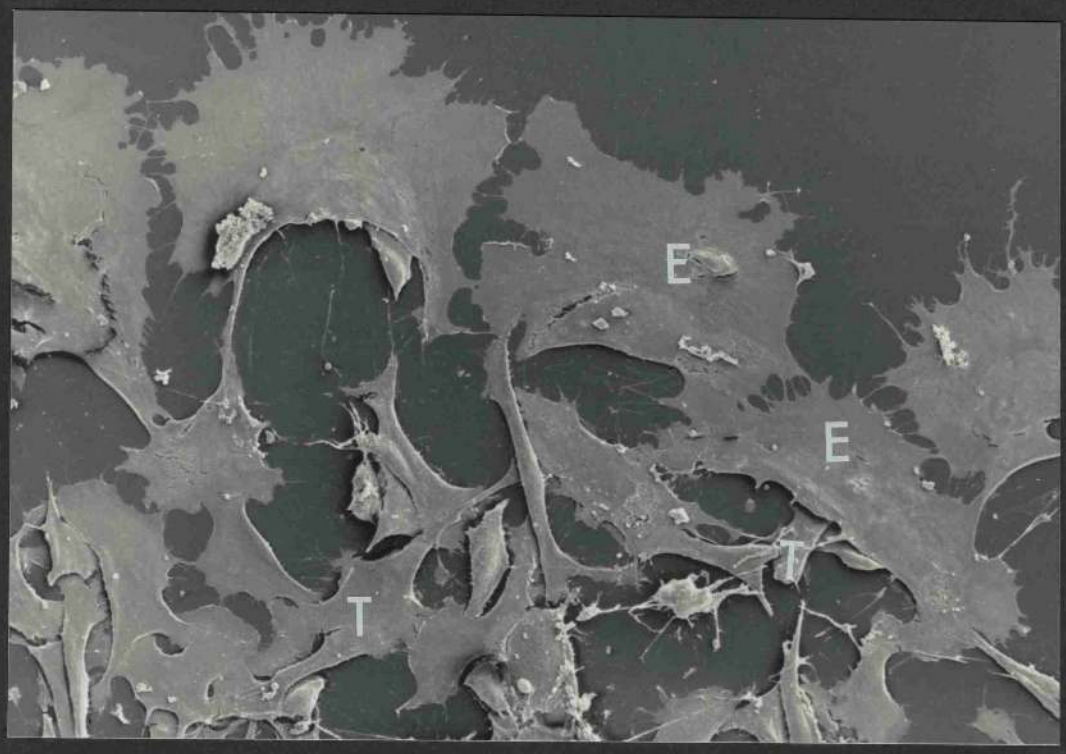
Photograph 3 shows a dense aggregate (A) of B16F10 cells which is able to detach and float to other areas of the petri dish. (After ten days growth). Magnification x240.

Scanning electron micrograph 4 shows in greater detail B16F10 aggregates (A) after ten days growth. Magnification x1592.


A

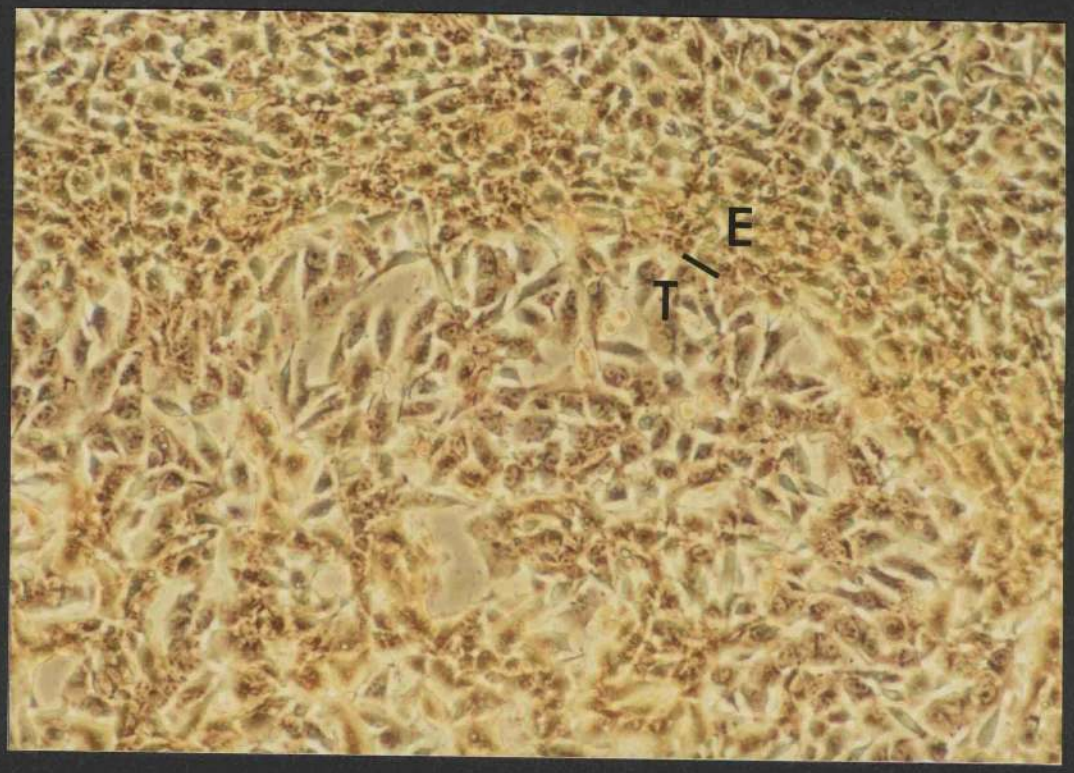

A

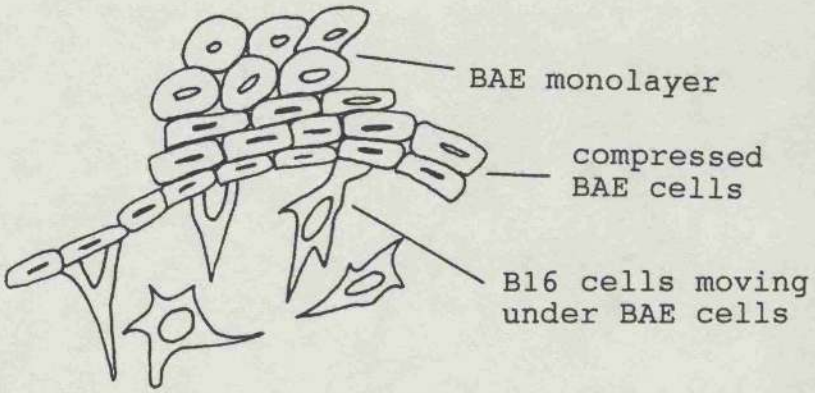
Scanning electron micrograph 5 shows the BAE cells (E) lying in the path of the B16F10 cells (T) after ten days growth. Magnification x640.


E
E
T
T

Photograph 4 shows the compression of BAE cells (E) after ten days growth. Magnification x240.

Fig 2 shows diagramatically the interaction between the endothelial cells (E) and the B16F10 cells (T) after ten days growth.

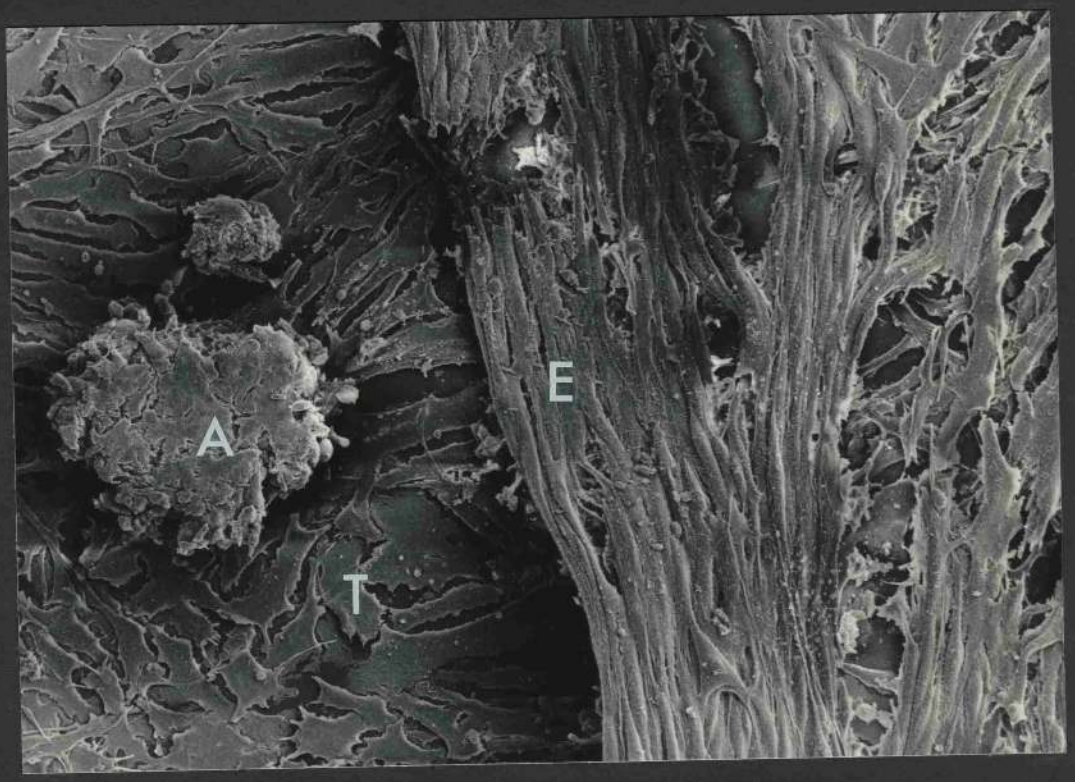

E
T


BAE monolayer
compressed
BAE cells
B16 cells moving
under BAE cells

Photograph 5 shows the BAE monolayer (E) being pushed back after seventeen days growth. Note that the B16F10 cells (T) have begun to aggregate (A) in the centre of the retraction area. Magnification x160.

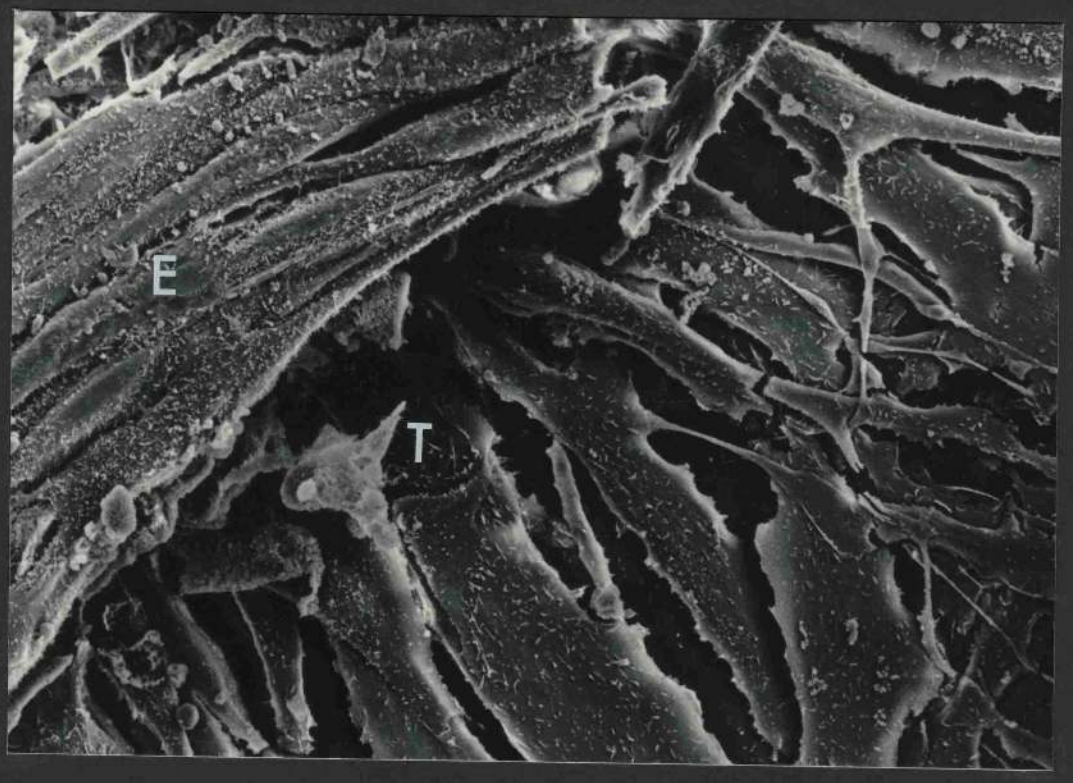
Scanning electron micrograph 6 shows the BAE monolayer (E) rolled up after seventeen days growth. it also shows the B16F10 cells (T) moving underneath the monolayer. Magnification x440.


A
T
E


A
T
E

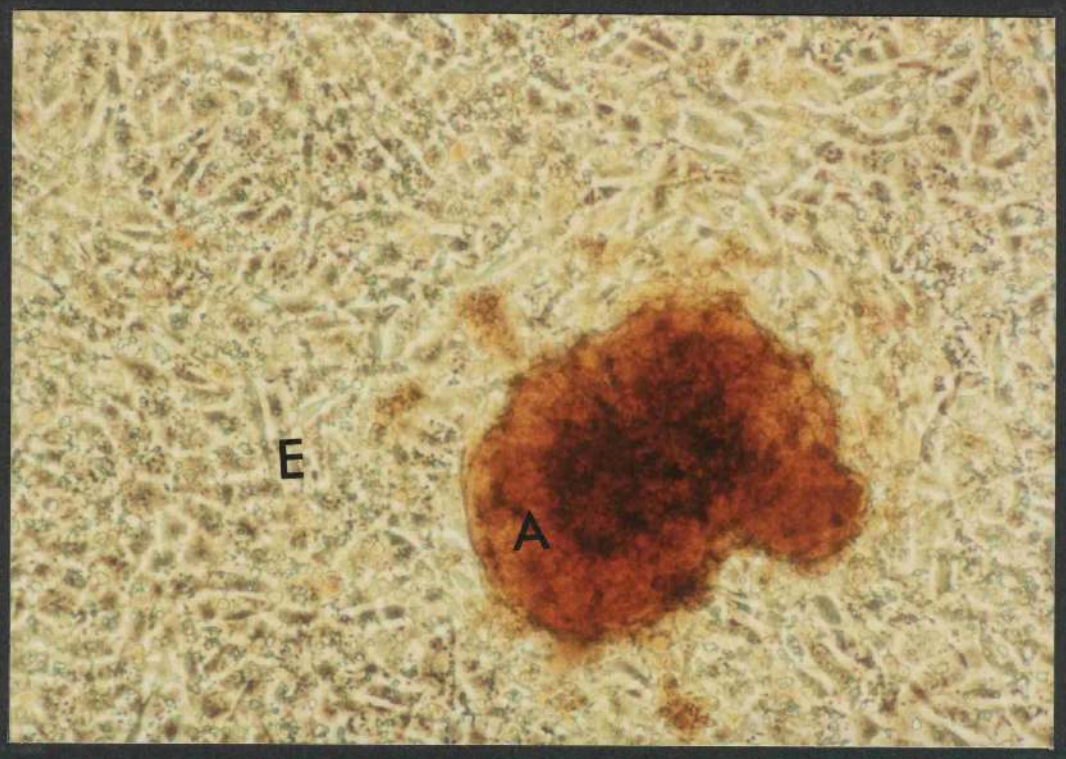
Photograph 6 and Scanning electron micrograph 7 show the rolled up monolayer of BAE cells (E) at a higher magnification and the B16F10 cells (T) moving underneath the monolayer. Magnification x270 and x1720 respectively.

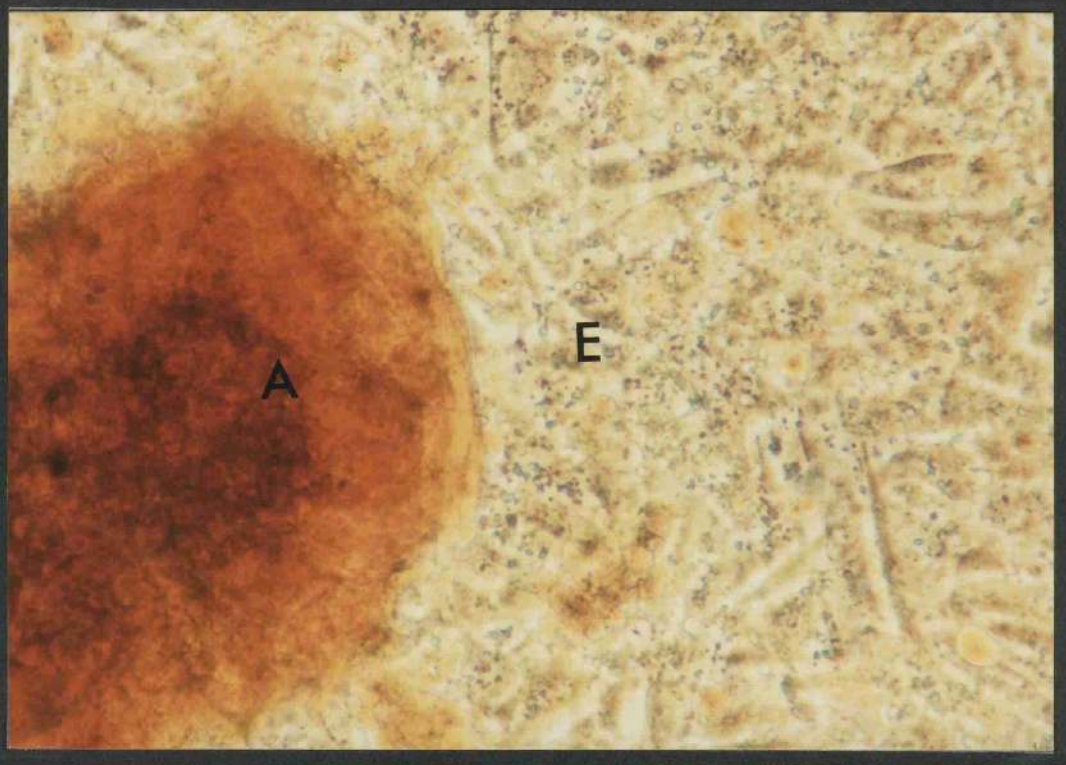

E
T


E
T

Photograph 7 shows an aggregate of B16F10 cells (A) on the BAE monolayer (E). The monolayer has started to develop vacuoles, but the cells are still viable as revealed by a trypan blue dye exclusion test. Magnification x160.

Photograph 8 shows the B16F10 aggregate in higher magnification. However, the nature of the interaction between the aggregate and the monolayer is not revealed. Magnification x280.


E
A


A
E

d) Day 30

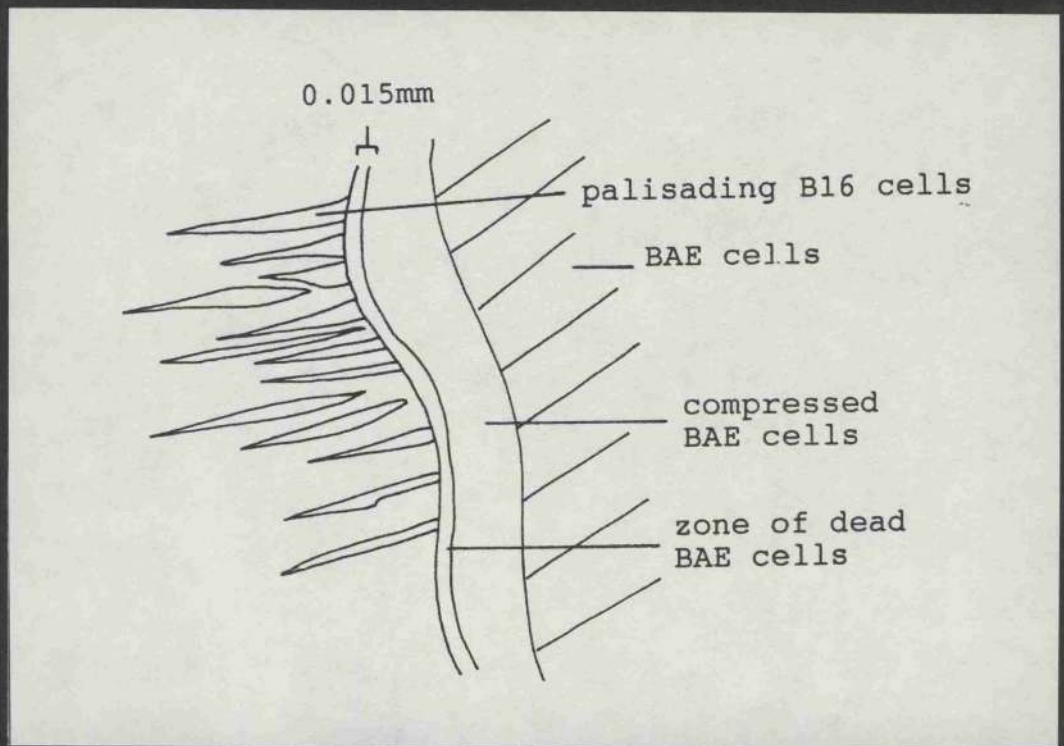
By day 30 of growth, the whole dish was covered with cells (see Fig. 4) and the whole endothelial cell layer appeared compressed. As the B16F10 cells began to move under the BAE monolayer they became elongated and began to palisade. (See Photograph 9). Once they had moved under the monolayer they regained a spread morphology which could be observed through gaps in the monolayer. (See Photographs 10 and 11). Some of the BAE cells became so compressed, they appeared to be fused into aggregates. However, by trypan blue dye exclusion test, some of these cells were still viable but at the edge and 0.015mm into the compression, the cells were non-viable.

After this time the experiment was stopped as no other changes were seen to take place after this time.

Photograph 9 shows the interaction between the two cell types after thirty days growth. Note that the B16F10 cells are exhibiting a palisading effect. Magnification x170.

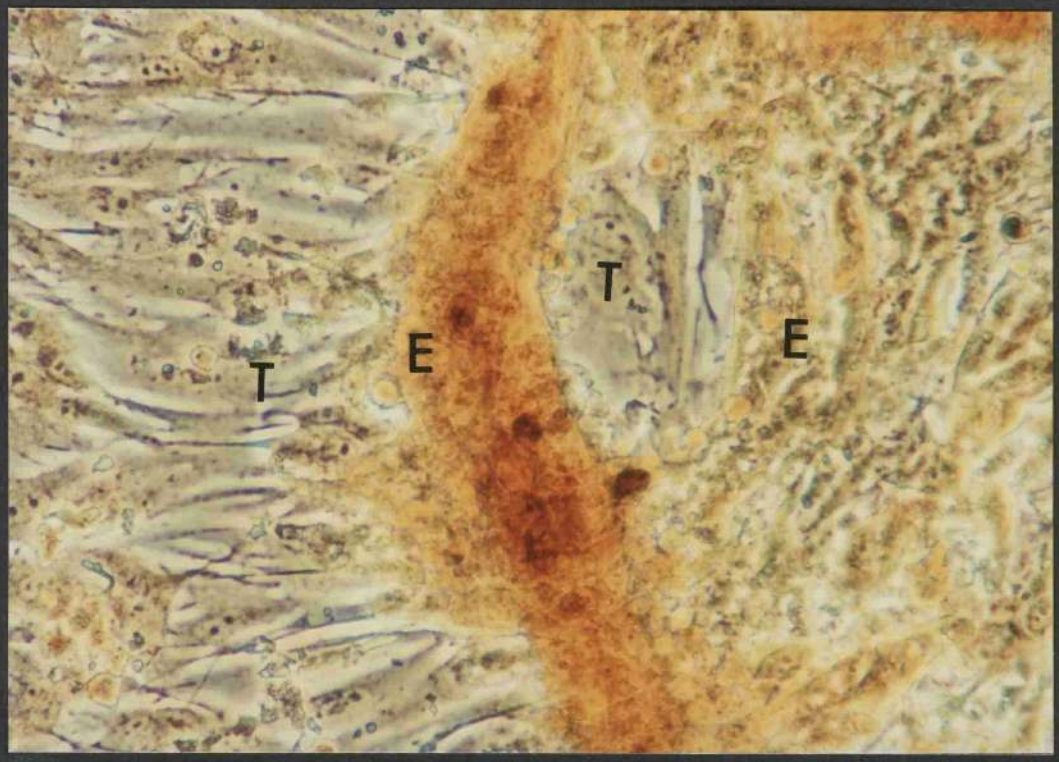
Fig 3 shows diagramatically the zone of non-viable cells.


E
T


0.015mm
palisading B16 cells
BAE cells
compressed
BAE cells
zone of dead
BAE cells

Photograph 10 shows the interaction between the two cell types after thirty days of growth. It can be seen that the B16F10 cells under the endothelial cells are taking on a spread morphology. Magnification x280.

Photograph 11 shows at a higher magnification the spread B16F10 cells underneath the BAE cells. Magnification x600.


T
E
T
E

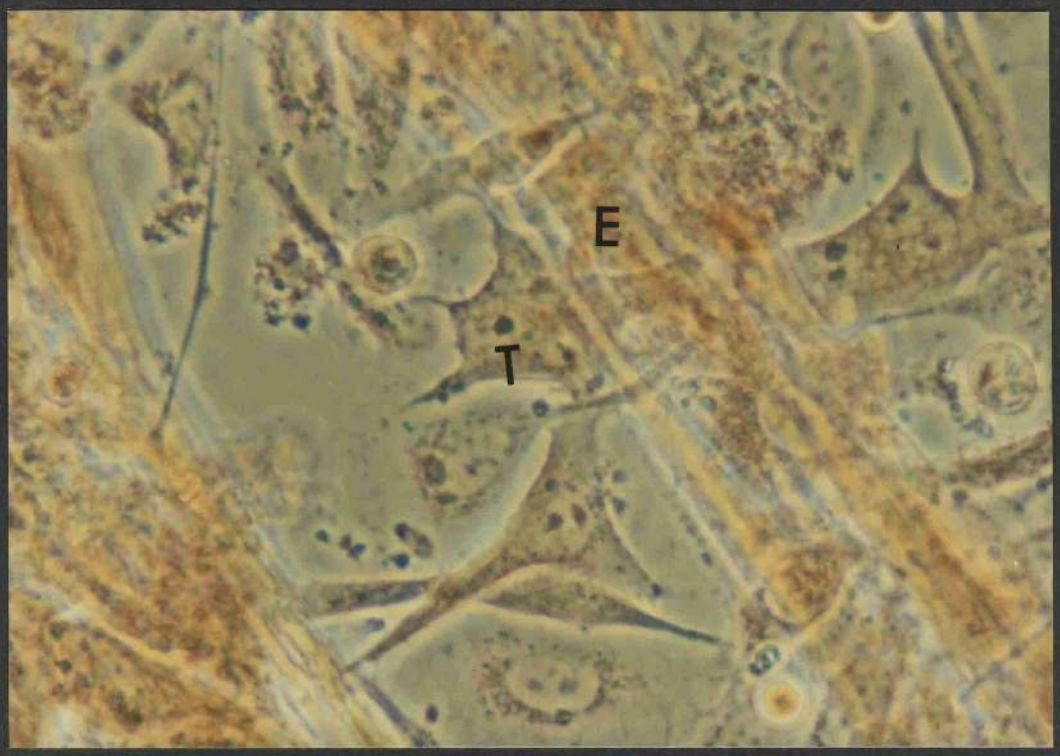
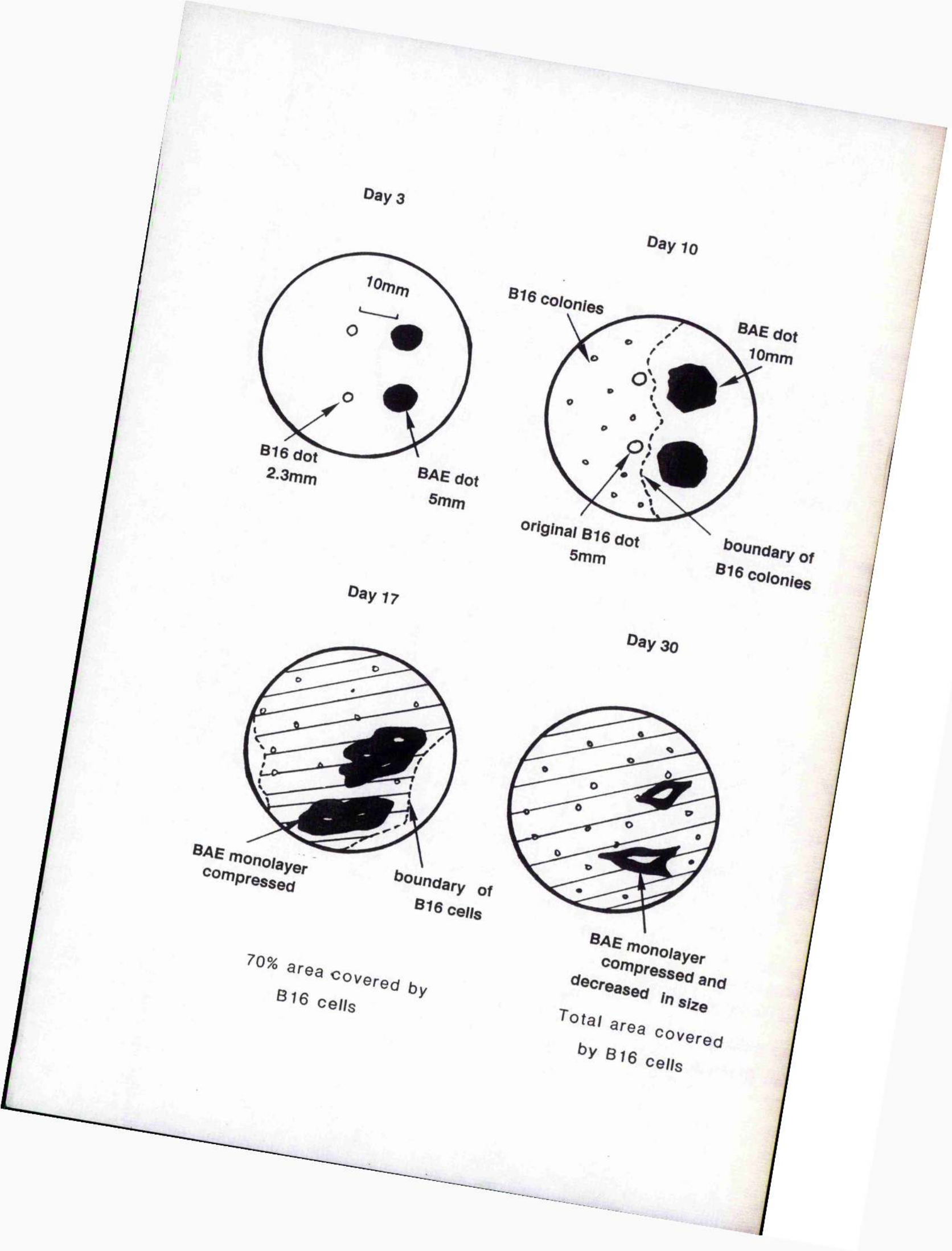

E
T

Fig 4 shows a diagram of the areas covered by B16F10 and BAE cells on a typical stained petri dish at the stated time periods. Not drawn to scale.


Day 3
10mm
B16 dot
2.3mm
BAE dot
5mm
Day 10
B16 colonies
BAE dot
10mm
original B16 dot
5mm
boundary of
B16 colonies
Day 17
BAE monolayer
compressed
boundary of
B16 cells
70% area covered by
B16 cells
Day 30
BAE monolayer
compressed and
decreased in size
Total area covered
by B16 cells

2.4 DISCUSSION

The model system developed in this study showed two types of confrontation between tumour and endothelial cells. The first type which resembled invasion, occurred between substratum-attached tumour cells and the endothelial monolayer. The second type, which resembled extravasation, occurred between aggregated tumour cells and the endothelial monolayer.

2.4.1 Substratum-attached tumour cell interaction

This type of interaction occurred as the two cell types grew towards each other in a process similar to invasion of the endothelium. It appeared as though initially, the two cell types adhered to each other, followed by the B16F10 cells inserting pseudopodia under the BAE cells.

2.4.2 Aggregated cell interaction

This interaction involved B16F10 cell aggregates and the endothelial cells. It would appear that at high densities the B16F10 cells become detached from the substrate and attached to each other (as aggregates). Initially these aggregates still adhered by one or two cells to the plastic and a trypan blue dye exclusion test revealed that these aggregated cells were viable. Following aggregation, the cells were able to detach from the substrate (although they were still aggregated) and to adhere either to other cell-free sites on

the plastic or onto the BAE monolayer. This is similar to the *in vivo* situation when clumps of cells become detached from the primary tumour and circulate in the vascular system (Sherbert, 1982). The aggregated cells adhered strongly, adjacent to endothelial intercellular junctions, and in these areas the BAE cells were seen to peel back. This may be compared with the events which occur *in vivo*, prior to and during extravasation (Wood, 1968, Crissman, 1985). At this time changes observed in behaviour of substratum-attached and aggregated tumour cells were identical.

In both types of interaction it was observed that the tumour cells adhered to the edges of the BAE cells. These observations indicate the importance of the endothelium in the initial step of causing arrest of the tumour cells. It would seem from these *in vitro* experiments, that the presence of leucocytes and platelets are not necessary for interactions to occur between the tumour and endothelial cells. This is also the view held by Warren (1976). Following adhesion, the tumour cells penetrated the endothelium, not by piercing the cells (as described by Chew *et al*, 1976), not through defects in the surface of the endothelium (Carter, 1984), but by breaking intercellular junctions (as described by Nicolson, 1981). This was followed by the BAE cells being rolled away from the tumour cells. The tumour cells then appeared to spread and move on the extracellular matrix which had been laid by the endothelial cells (Kramer *et al*, 1982).

2.4.3 Comparison with other studies

The results obtained in this study corresponded well with those of other workers (Kramer and Nicolson, 1979; Zamora *et al*, 1980). The difference between the observations made in this study and those of the other workers was that the retraction and re-sealing of the endothelial cells over the tumour cells, could not be identified. It may be possible that the re-sealing of the endothelium is a very transient process, followed closely by damage of the endothelium. If the processes follow one another closely the advantage of using time-lapse photography becomes apparent. In this study I observed that within 24 hours of tumour cell attachment, the BAE monolayer, proximal to tumour cell adhesion, was non-viable as determined by trypan blue staining. In the Kramer and Nicolson studies the experiments were stopped before this time, therefore damage was not mentioned. It is possible for B16F10 cells to damage the integrity of the endothelium by production of degradative enzymes. These include plasminogen activators and collagenase IV (Liotta *et al*, 1980; Kramer and Nicolson, 1982; Reich *et al*, 1988). The conclusion I draw from my observations is that once the endothelial cells become detached from the substrate by the action of tumour cells, re-attachment by the endothelial cells to form a contiguous structure is not observed *in vitro* (Young and Herman, 1985). If it does, it cannot do so for long. On the other hand, it is possible for re-sealing to occur *in vivo*, since repair mechanisms rapidly follow tissue injury (Sporn and Roberts, 1986).

It is also interesting to compare some of the observations made in this study with the description of *in vivo* extravasation of B16a (amelanotic) cells in C57 mice (Crissman, 1985), in which endothelial cells were gradually displaced by contact of tumour cells with the vascular basement membrane. The mechanism of endothelial cell displacment by tumour cells could not be identified, but appeared to occur at inter-endothelial cell junctions with gradual retraction of the endothelial cells as the B16a cells increased their area of basal lamina contact. Ther was no evidence of active migration by B16a cells. Extravasation occurred through a combination of inravascular tumour cell proliferation and destruction of the vascular basement membrane by the B16a cells. Hence observations made in this thesis may well illustrate some of the mechanisms by which B16a cells extravasate *in vivo*.

There are some limitations to the model which must be considered. The tumour cells used were murine and the endothelial cells bovine, but as stated earlier, experiments using different species show little difference from those using syngeneic tissues (Easty and Easty, 1984). Also, it is likely that the interactions between B16F10 and BAE cells will follow general principles and will not be affected by species source of the cells. For future experiments, it will be important to see the response of other variant cells of the B16 melanoma and other tumour cell types, with these and other endothelial cell types to determine whether the observations

made follow a general trend or are specific to B16F10 and BAE cells only. I have already carried out some preliminary experiments using B16BL6 and B16F1 cells in confrontation with BAE cells. These experiments have yielded similar results as those obtained for B16F10. This suggests that the interactions between B16 tumour cell lines and BAE cells are representative of a general trend.

2.4.4 Conclusion

The model used in this study has allowed a unique observation of the interaction which occur between B16F10 and BAE cells and shows potential for further studies. Behavioural interactions between B16F10 and BAE cells have been illustrated which are not disimilar from those of initial vascular invasion and extravasation. It must be realised that a simplified model system such as this do not include the complex haemodynamical, mechanical and electrostatic interactions which occur *in vivo* between tumour and endothelial cells. However, models such as these may provide some insight to the possible processes which occur in the *in vivo* metastatic cascade and perhaps eventually even to the way these processes are controlled. This system also enables addition of various factors such as platelets, histamine and prostaglandins to look at the effect that these components have on the interactions between tumour and endothelial cells.

Having obtained an overall view of extravasation, I thought it would be pertinent to study the processes

occurring, in greater detail. The first question to be addressed was whether the composition of the cancer cell or endothelial cell surface affects tumour arrest. From the results of this chapter, it would appear that the endothelium might prove to be a fruitful source of molecules which promote tumour cell adhesion and spreading.

III ADHESION

3.1 INTRODUCTION

The first barrier to cells which are disseminated through the vascular system, is the endothelial lining of capillaries (Nicolson, 1981). Tumour cells are seen to adhere to the endothelium before passing through it (Kramer, 1980; Kramer *et al*, 1982).

Several theories have been put forward to account for cell adhesion phenomena. Initial considerations that cellular adhesion resulted from non-specific interactions via neutralisation of negative charge on cell surfaces or van der Waal's forces acting over broad areas of surface membrane (Curtis, 1973), have now been replaced with models featuring the interaction of several classes of cell surface macromolecules such as lectins (Raz and Lotan, 1981) and glycoproteins (Terranova *et al*, 1983) with complementary binding sites. It is known that the cell surface is crucial in all cell adhesion processes and studies with animal tumour models have indicated its important role in metastasis as well (reviewed by Nicolson, 1984). Endothelial cells are thought to play a major role in extravasation because they synthesize several proteins that have been implicated in cell adhesion (Jaffe *et al*, 1976; Jaffe and Mosher, 1978; Sage *et al*, 1981). From the results of the previous chapter it would appear that tumour cells adhere to the endothelial cells. Therefore molecule(s) from the endothelial cells seemed the best candidate(s) for promoting adhesion. Thus it was decided to study the adhesion-promoting properties of molecules extracted

from endothelial cell monolayers. The most studied cell adhesion promoting factors relevant to this study which are thought to be involved in metastasis include fibronectin (Fn), laminin (Lm) and vitronectin (Vn).

3.1.1 Fibronectin

A vast literature has developed on the subject of fibronectin over the past fourteen years. Fibronectins, comprise a class of closely related glycoproteins of). They are found in two forms: a soluble plasma, amniotic and cerebrospinal fluid protein and an insoluble protein in most basement membranes, on the surface of some cells and in soft connective tissue matrices (Yamada and Olden, 1978). Major producers of fibronectin are, fibroblasts, myoblasts and endothelial cells, but other cells produce lesser amounts. Some cells secrete significant amounts into their culture medium, possibly due to proteolytic enzyme degradation, but Fn is more likely to be found as fibrillar networks between cells and between cells and substratum (Hynes and Yamada, 1982).

a) Structure of Fibronectin

Fibronectin is a multi-functional protein (Paul and Hynes, 1984; Yamada *et al*, 1985) and in addition to promoting cell adhesion (Piershbacher *et al*, 1982; Yamada, 1983), has several binding sites for various components including DNA (McMaster and Zardi, 1982), collagen (Yamada, 1983),

proteoglycans (Yamada, 1983) and bacteria (Van de Water, 1983). The various domains of fibronectin are shown in Fig 6 (taken from Yamada *et al*, 1984).

Extensive amino acid sequencing of cellular fibronectin has not been possible since it is less abundant than plasma fibronectin. Information on the primary sequence of fibronectin and elucidation of the origin of the differences between the Fn subunits have therefore come from the sequencing of cDNA and genomic clones of plasma Fn (Petersen *et al*, 1983; Skorstengaard *et al*, 1984; Kornblihtt, 1983). Ninety percent of the primary structure of plasma fibronectin consists of a series of homologous repeats. See Fig 5 (taken from Hynes, 1985). There are three types of repeat: Type I, Type II (disulphide-bonded loops, each 45-50 amino acids long) and Type III (90 amino acids long with no disulphide bonds). There are 12 Type I homologies, 2 Type II homologies and 15-16 Type III homologies. Differences in the subunits are shown in Figs 5 and 6. In evolutionary terms, the repeating homologies strongly suggest that the Fn gene arose by endoduplication of several primordial minidomains, corresponding with the present day homology of Types I, II and III (Odermatt *et al*, 1985).

The recombinant DNA analyses reveal that although the subunits do differ in parts of their primary sequence, they all arise from a single gene, and are identical over much of their sequence. The structure of the Fn gene is extremely complex, comprising of approximately 50kb of DNA and 50 exons (Hynes, 1985). The precise correspondence between exons and

homologous repeats has yet to be defined. The variations in fibronectins must arise from alternative splicing of the primary transcript. One gene can give rise to 6-10 different variations (Tamkun *et al*, 1984). Different cell types can apparently regulate this alternative splicing (Hynes, 1985) but it remains unclear how or why they do so.

b) Cell Adhesion Function of Fibronectin

The reason for interest in the cell adhesion promoting properties of Fn arose from its apparent involvement in a variety of biologically important functions. For example, there is a good, but not perfect correlation between transformation and loss of Fn (Vaheri and Ruoslahti, 1975). Loss of Fn also correlates with tumorigenicity *in vivo* (Chen *et al*, 1976). Investigations designed to elucidate the significance of the loss of Fn for the transformed phenotype (Yamada *et al*, 1976) revealed a role for Fn in cell adhesion. For example, addition of purified Fn to transformed cells which lack it, produces increased cell attachment. A role of fibronectin in cell adhesion was also shown by studies of the role of serum in cell adhesion (Klebe *et al*, 1978, Hayman *et al*, 1982). Many cells require serum in order to attach in culture, either to plastic or collagen and the serum component responsible for this appears to be Fn (Klebe, 1974; Kleinman *et al*, 1978; Grinnell and Hays, 1978).

The cell attachment region of Fn was determined by Ruoslahti (1982), who proteolytically digested fibronectin, until only a small peptide remained which still retained cell

adhesion activity. By making synthetic oligopeptides to the digests, Piersbacher and Ruoslahti (1984) found that the peptide consisted of Arg-Gly-Asp (RGD). Knowledge of the active site of the cell-attachment domain has also facilitated investigations of the cellular side of this interaction. In its free form the RGD peptide inhibits cell attachment, but when coupled to a substrate via a spacer arm, the RGD peptide promotes adhesion of cells to the substrate (Ginsburg *et al*, 1985). (See Fig 7).

Other cell adhesion regions are also thought to exist. In rat Fn, expression of a second RGD sequence was found to be controlled by alternative mRNA splicing (Schwarzbauer *et al*, 1983), raising the possibility that some Fn molecules have two, if not several cell attachment sites. In melanoma cells, attachment to Fn occurs through an REDV site (Humphries *et al*, 1986) which is found in domain V of human Fn. In rat Fn the sequence is RGDV, which is also found in domain V.

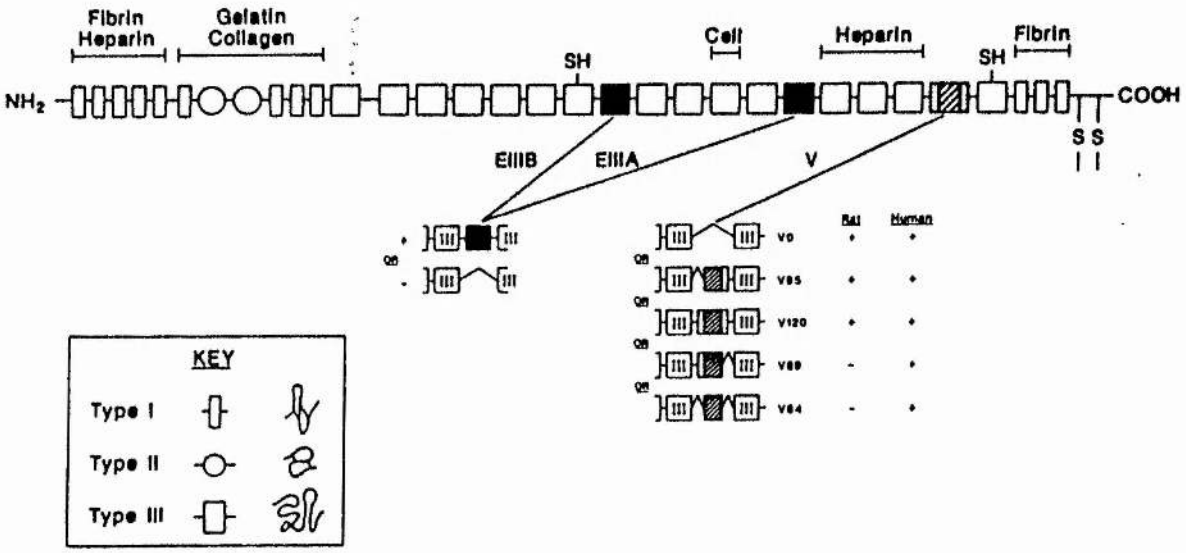


Fig 5 shows the primary structure of Fn

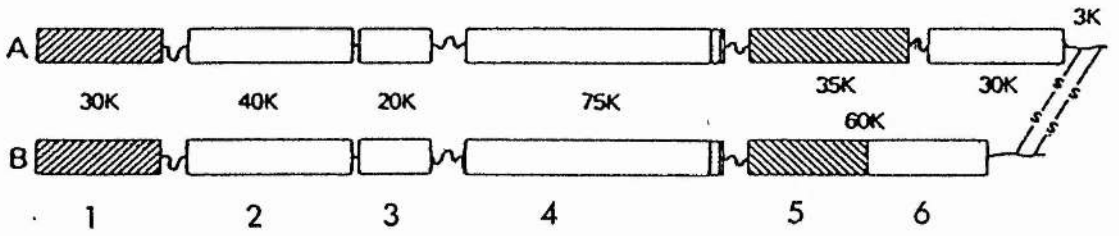


Fig 6 The functional domain structure of fibronectin.

1 Binds to a variety of ligands including heparin, fibrin, actin, transglutaminase and *S. aureus*

2 Collagen binding domain

3 Weak fibrin binding site - easily destroyed by proteases

4 Cell binding - the middle fragment has a high degree of homology between species. The actual binding site is thought to consist of three amino acids. ARG-GLY-ASP (RGD)

5,6 There is a difference in the dimers at this point which is a possible requirement as a recognition mechanism between complementary sites on the subunits, which would function during the assembly of a dimeric fibronectin molecule from monomers. This domain also contains heparin and fibrin binding sites.

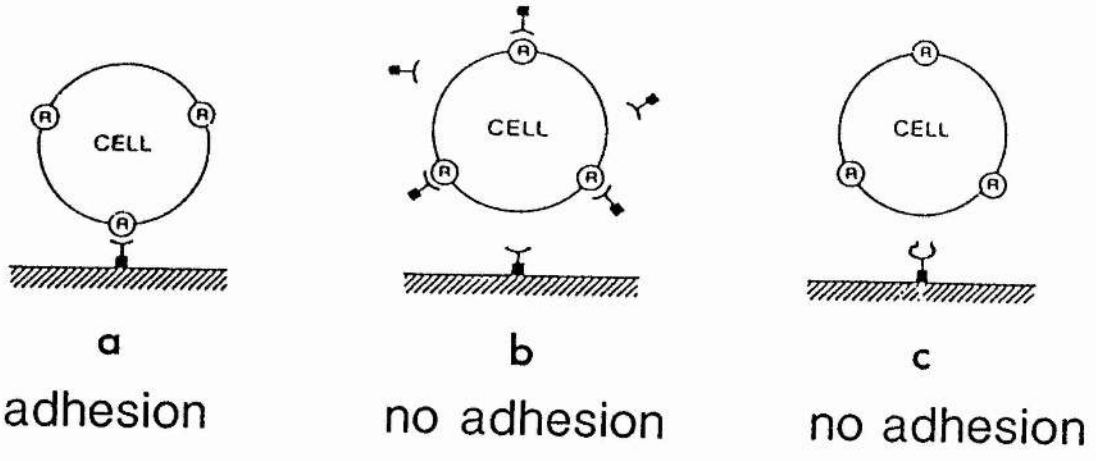


Fig. 7 shows the effect of the RGD peptide on adhesion of cells.

3.1.2 Laminin

In addition to promoting cell adhesion (Martin and Timpl, 1987), Lm has binding sites for collagen Type IV (Rao *et al*, 1982), heparin sulphate (Woodley *et al*, 1983), nidogen (Paulsson *et al*, 1987), bone osteonectin (Mason *et al*, 1986), complement components C1q and C3 (Bohnsack *et al*, 1985; Leivo and Engvall, 1986) and plasminogen and plasminogen activator (Salonen *et al*, 1984).

a) Structure of Laminin

Laminin is a glycoprotein (Mr 1000kD) found predominantly in basement membranes and is synthesized by epithelial cells. It consists of three alpha chains of 200kD

and one beta chain of 400kD held together by disulphide bonds (Kleinman *et al*, 1984). It has a cruciform shape as shown in Fig 8. Digestions of Lm by various proteases and characterisation of the large fragments by electron microscopy and immunochemical methods have proved useful approaches for dissecting the multi-domain structure of Lm (Timpl *et al*, 1983; Martin and Timpl, 1987).

cDNA clones have been isolated and some primary sequences have been obtained (Barlow *et al*, 1984; Pikkarainen *et al*, 1988). The primary structure of Lm is shown in Fig 9 (taken from Martin and Timpl, 1987). Information from the B1 arm shows that Domains I and II are alpha-helical structures of about 600 amino acids (interrupted by 6 cysteine residues whose function is unknown, but may involve neurite outgrowth or cell attachment). Domains III and V are cysteine rich and consist of 50 amino acids. This repeat structure occurs 5 times in V and 8 times in III. This type of cysteine rich repeat structure is also found in precursor EGF, coagulation factors and thrombospondin (Sasaki *et al*, 1987). Domains IV and VI consist of 250 amino acids, are low in cysteine residues and are thought to be the globular regions.

The size difference between the B2 and B1 arms is about 190 amino acids, but shows the same arrangement of domains (Martin and Timpl, 1987: Pikkarainen *et al*, 1988). Other differences include a lower number of EGF-like repeats and the absence of the 6 cysteine residues. B2 and B1 are therefore the products of related but distinct genes that are probably derived from a common ancestor gene. The question is raised

that since B2 and B1 have different biological activities it is also possible that different molecular species exist as is found for Fn. The characterisation of the A chain is still incomplete but it is known that the N-terminal segment shares homology to comparable domains in the B1 and B2 chains (Pikkarainen *et al.* 1988).

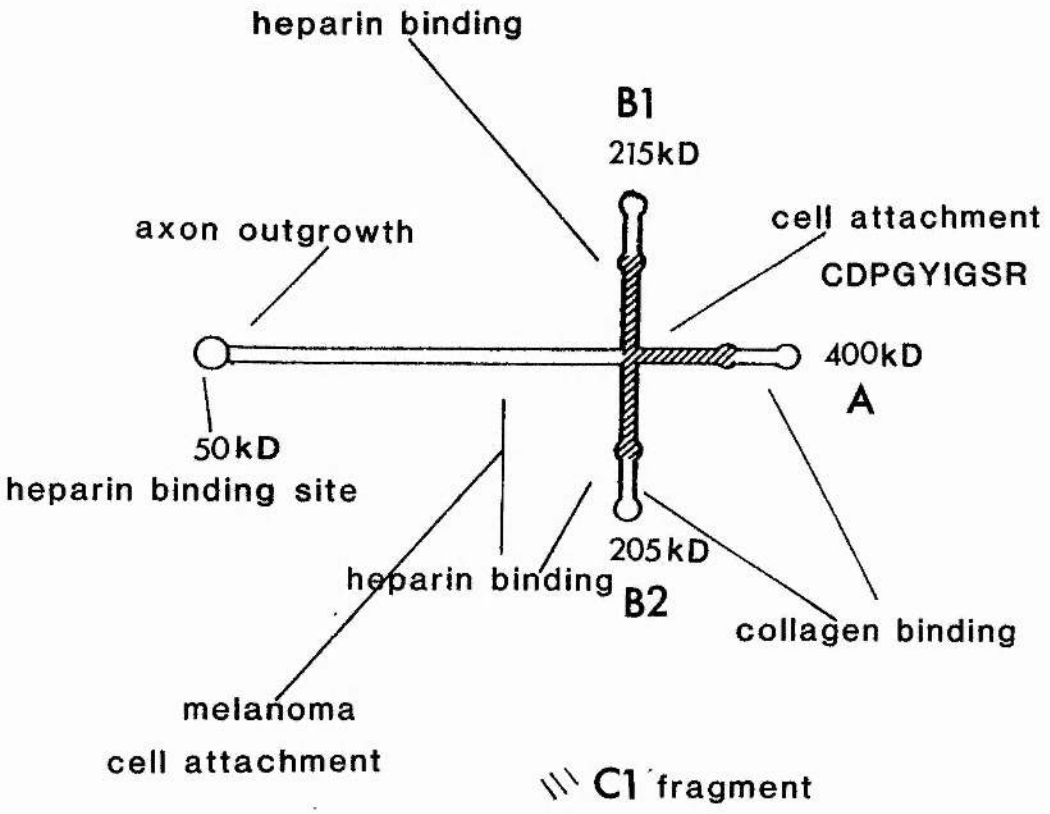


Fig 8 The functional domain structure of laminin.

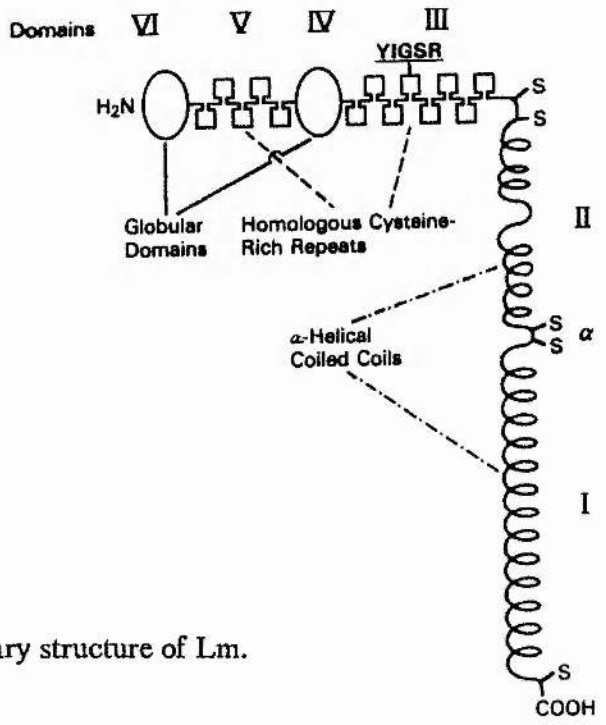


Fig 9 shows the primary structure of Lm.

b) Cell Adhesion Function of Laminin

In common with Fn, Lm promotes adhesion of some cells (Terranova *et al*, 1982). Information about the amino acid sequence of Lm has revealed a specific nonapeptide binding site known as the CDPGYIGSR site after the amino acid sequence of the active peptide region (Rao *et al*, 1982; Graf *et al*, 1987) which promotes epithelial cells to adhere to Lm. This binding site is found in domain III of Lm (Graf *et al*, 1987) and was able to inhibit attachment of the cells to Lm by up to 80%, indicating that it is one of the major sites of cell attachment in Lm.

There has been some controversy as to whether the cell attachment site in Lm involves an RGD peptide as it does in Fn. However Ruoslahti (1988b) has presented evidence for its existence. The evidence is that the RGD peptide in its free form can slightly inhibit cell adhesion to Lm (Ruosalahti, 1988a). Since the RGD sequence has not been found in either the B2 or the B1 arms, it is probably located in the A chain.

Skubitz *et al*, (1987) described another cell adhesion domain in Lm. Using antibodies to chymotryptic fragments of Lm, they found an antibody which reacts to a region close to the cross region of the long arm of Lm. This antibody inhibited adhesion of murine metastatic melanoma cells (K1735M4). This adhesion site is also a heparin binding site, as determined by the same antibody (Skubitz *et al*, 1988). In addition to this heparin binding site, three others are shown in Fig 8. The complex process of cell adhesion to extracellular matrices may depend on heparin binding since

other extracellular matrix molecules also have heparin binding sites: such as vitronectin (Suzuki *et al*, 1985), thrombospondin (Dixit *et al*, 1984), von Willebrand factor (Fujimura *et al*, 1987) and fibronectin (Yamada, 1983). The extracellular matrix molecule interaction with heparin might be used in conjunction with or as an alternative way for cells to adhere rather than just by the known adhesion sites.

3.1.3 Vitronectin

Vitronectin is found in the form of two non-covalently bound polypeptides (65kD and 75kD) on the surfaces of many cells (Owens and Miller, 1980; Tullis *et al*, 1981; Barnes and Silnutzer, 1983), in the extracellular matrices of various tissues (Hayman *et al*, 1983; Simonton *et al*, 1985) and in serum (Hayman *et al*, 1985). In addition to promoting cell adhesion (Yamada *et al*, 1985), Vn is also thought to have a role in the latter stages of the complement and coagulation pathways (Jenne *et al*, 1985). It should be noted at this point that S protein found in serum and vitronectin are immunologically and functionally the same (Tomasini and Mosher, 1986; Hayman *et al*, 1985). Vn binds to a thrombin-anti thrombin III (82kD) complex (Ill and Ruoslahti, 1985; Jenne *et al*, 1985). The anti thrombin (ATIII) (58kD) glycoprotein is a major protease inhibitor for the coagulation system and functions by binding to thrombin (Marcum and Rosenberg, 1985). Binding of Vn to this complex probably results in protection of thrombin from inactivation by ATIII. The binding of thrombin-ATIII to vitronectin also exposes an

extra heparin binding site on vitronectin (Preissner *et al*, 1985). It may be that the heparin binding site serves to increase the affinity of the interaction of the extracellular molecules with the cell surface by binding to heparin sulphate proteoglycan in the plasma membrane (Yamada *et al*, 1985). Vitronectin also binds to various complement components - C5b7, C8 and C9 (Bhakdi and Roth, 1981; Jenne and Stanley, 1985).

a) Structure of Vitronectin

The structure of vitronectin is shown in Fig 10 (taken from Juliano, 1987). The domain structure for Vn has been elucidated by Suzuki *et al* (1984; 1985) and Jenne and Stanley (1985). This analysis reveals no sequence homology between Vn and Fn except with an RGD peptide. Another homology is observed with somatomedin B over the first 30 amino acids at the NH2-terminal.

b) Cell Adhesion Function of Vitronectin

The cell adhesion function of Vn is thought to be mediated by the tri-peptide RGD (Suzuki *et al*, 1985). Further evidence comes from the inhibition of cell adhesion by the RGD peptide (Ruoslahati and Pierschbacher, 1986). In addition since plasma vitronectin binds to an anti-thrombin III/thrombin/heparin complex (Choay *et al*, 1983: I11 and Ruosalahti, 1985; Lane *et al*, 1987; Carlsson *et al*, 1988), and since antithrombin III (ATIII) has been shown to be produced by endothelial cells (Owens and Miller, 1980) and malignant

cells (Tullis *et al*, 1981) it is thought that in some way this interaction may facilitate cell adhesion.

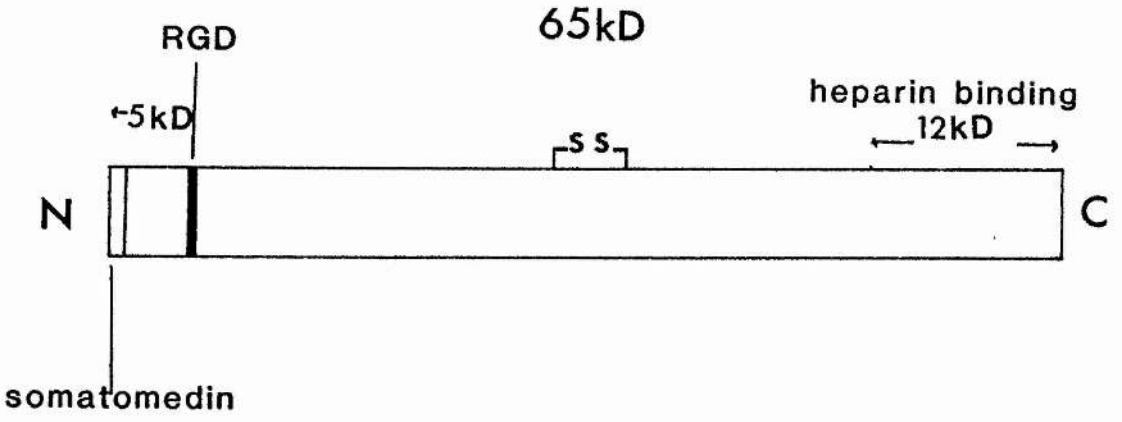


Fig 10 The domain structure of vitronectin.

3.1.4 Other glycoproteins

There are several other extracellular matrix glycoproteins which have been implicated in cell adhesion, and which interact with and have properties in common with Fn, Lm and Vn. (See TABLE 1). These glycoproteins include fibrinogen (Doolittle, 1984), heparin sulphate (Birdwell *et al*, 1978) and entactin (or nidogen) (Dziadek *et al*, 1985). More recently, other molecules have been increasingly reported

in the literature, and include thrombospondin, tenascin and von Willebrand factor (see below).

a) Thrombospondin

Thrombospondin is secreted by a variety of cells (Dixit *et al*, 1984;), including endothelial cells (Mosher *et al*, 1982; Raugi *et al*, 1982) and is also a platelet alpha granule protein, secreted on platelet activation (Lawler *et al*, 1978). It consists of three polypeptides of Mr 180kD (Dixit *et al*, 1985) and some domain organisation (Coligan and Slayter, 1984) and stretches of protein sequence have been determined (Dixit *et al*, 1984). It is known to bind heparin (Lawler *et al*, 1978), collagen (Lahav *et al*, 1982), fibrinogen (Leung and Nachman, 1982) and fibronectin (Lahav *et al*, 1982). In addition it causes attachment in some cells including melanoma cells (Varani *et al*, 1986; Roberts *et al*, 1987; Riser *et al*, 1988).

b) Tenascin

Tenascin (240kD) is an extracellular matrix molecule, found in various tissues (Thesleff *et al*, 1987; Maier and Mayne, 1987; Mackie and Thesleff, 1987). It has a "six arm" structure which would enable it to have multiple interactions with ECM components and to be involved in cell attachment. However, it has the least activity of the extracellular matrix (ECM) molecules (Chiquet-Ehrismann, 1988). It is also involved with cell aggregation (Chiquet and Fambrough, 1984; Chiquet-Ehrismann *et al*, 1986).

c) Von Willebrand factor

Von Willebrand factor (220kD) is a disulphide linked glycoprotein, synthesized by endothelial cells and megakaryocytes and is also found in plasma, as a component of the sub-endothelium and in the alpha granules of platelets (Santoro, 1987). There is no sequence homology with any other proteins except as regards an RGD sequence (Ginsburg *et al*, 1985; Gartner and Bennett, 1985) which may promote the adhesion of platelets to endothelium (Santoro, 1987). There is, however, a lot of internal repetition (Bonthron *et al*, 1986; Shelton-Inloes *et al*, 1986).

In summary, these adhesion promoting proteins are large complexes with a variety of functions. They share common structural/functional features and are comprised of a series of proteinase stable functional domains which have binding activities for other macromolecules, linked by proteinase sensitive interdomain regions. The question of the possible homology of these glycoproteins with each other is of some interest and the information on this is beginning to accumulate at an increasing rate. Most, if not all these molecules, contain an RGD sequence(s) (reviewed by Ruoslahti and Pierschbacher, 1986) which is thought to be involved in cell attachment. This RGD sequence is also found in epidermal growth factor (EGF) and beta-transforming growth factor (TGF) precursor (Gartner and Bennett, 1985).

TABLE 1

Interaction of extracellular matrix proteins with other components

Glycoprotein	Mr	Interaction
Type IV collagen	550k-600kD	heparin sulphate and Fn
Laminin	1000kD	proteoglycans, entactin, actin, collagen type IV, heparin sulphate
Heparin sulphate	550k, 300k and 230kD	collagen type 1V, Lm and Fn
Chondroitin sulphate	200k-300kD	Vn
Entactin	150kD	Lm, Fn and Coll
Fibronectin	440kD	Coll types I-V, fibrin, actin and heparin sulphate
Vitronectin	75k, 65kD	chondroitin SO_4^{2-} heparin SO_4^{2-} and anti TIII
Tenascin	240kD	fibronectin, proteoglycans
von Willebrand factor	270kD	collagen
Thrombospondin	580kD	collagen, Fn fibrinogen thrombin and vWF

3.1.5 Extracellular matrix receptors

Since the RGD sequence appears to be the common link between the extracellular matrix proteins previously discussed, it would seem appropriate to consider the receptors for this sequence first. The RGD receptors appear to belong to a super family, all in the 100-160kD range which consist of transmembrane glycoprotein receptors for extracellular matrix proteins. They share common structural an functional features and are thought to link the cytoskeleton to the extracellular environment; thus they have been called "integrins". This name was coined for them by Hynes (1987) and his classification of the RGD receptors and related proteins is based on DNA sequence information and immunological cross reactivity.

The RGD receptors (or integrins) generally consist of two beta chains and two alpha chains (Juliano, 1987). The RGD receptor is found on the surfaces of a wide variety of cell types (Ruoslahti, 1988) including platelets (glycoprotein IIa/IIIb or gpIIa/IIIb) (Bennett *et al*, 1983), M21 human melanoma cells (Cheresh and Spiro, 1987), avian fibroblast cells (cell substrate attachment antigen - CSAT) (Horwitz *et al*, 1986) macrophages (MAC-1) (Juliano, 1987), *drosophila* epidermal cells (PS antigen) (Leptin, 1986) lymphocytes (LFA-1) (Ruoslahti and Pierschbacher, 1986) and T-cells (VLA) (Takada *et al*, 1987). It also arises in the lambda receptor of *E. Coli* (Charbit *et al*, 1984) and in some viruses - yellow fever and foot and mouth (Ruoslahti, 1988b).

The cellular RGD receptor for Fn has been difficult to

elucidate, largely because the binding of cells to Fn is of relatively low affinity (e.g. K_d $8x10^{-7}$ in fibroblasts) (Akiyama and Yamada, 1985). Pytela *et al* (1985a), proposed protein(s) of Mr 140kD (non-reduced) and 160 and 120kD (reduced) as the receptor for Fn. The direct binding of Fn to these proteins and the inhibition of Fn-binding to the proteins by the RGD peptide support this model.

In the case of Lm, there may also be a cell adhesion receptor in the integrin family (Gehlsen *et al*, 1988). However, much less is known about the RGD receptor in Lm and until recently it was not though to exist. Two polypeptides with affinity to Lm have been described with Mr of 120kD and 180kD (Kleinman *et al*, 1988; Smalheiser and Schwartz, 1987) and it is yet to be seen whether these bind to the RGD sequence in Lm.

The cellular RGD receptor for Vn consists of two chains of 115kD and 125kD (reduced conditions) and were isolated using a hexapeptide containing RGD peptide (Pytela *et al*, 1985b). The degree of homology between the amino acid sequences of the larger subunits of the RGD receptors for Vn and Fn is between 34-48% (Martin and Timpl, 1987).

The integrin sequences which have so far been characterised, are the alpha and beta subunits of human Fn receptor (Argraves *et al*, 1986), the alpha and beta subunits of Vn (Suzuki *et al*, 1987), the alpha and beta subunits of gpIIb/IIIa (Poncz *et al*, 1987) and the beta subunit of CSAT (Tankum *et al*, 1986).

From this and immunological information it can be seen that the beta subunit is conserved (Fitzgerald *et al*. 1987; Ruoslahti, 1988b). For example, the Fn receptor, VLA and avian integrin are thought to be related and their beta subunit is termed beta 1. The MAC-1 and LFA-1 receptors are related and their beta subunit is termed beta 2. The Vn receptor and gpIIb/IIIa are related and their beta subunit is termed beta 3. There are also 10 alpha bands which are distinct and which are denoted by the nature of the ligand i.e. alpha- is the alpha subunit for fibronectin. The alpha subunits of each integrin contains several sites that are homologous to calcium binding site in other proteins (calmodulin). Fig 11 (taken from Ruoslahti. 1988b) shows a proposal for the structure of an RGD-receptor. Calcium is known to bind to gpIIb, Fn and Vn, and is important in the role of receptors (Oppenheimer-Marks and Grinnell. 1984; Edwards *et al*, 1987; Ruoslahti. 1988b). Other divalent cations might also be important - for example, manganese. It had been thought that the Fn RGD receptor was only specific to Fn; however a recent article by Gailit and Ruoslahti (1988) suggests that manganese enhances binding of the Fn receptor to other RGD-containing proteins.

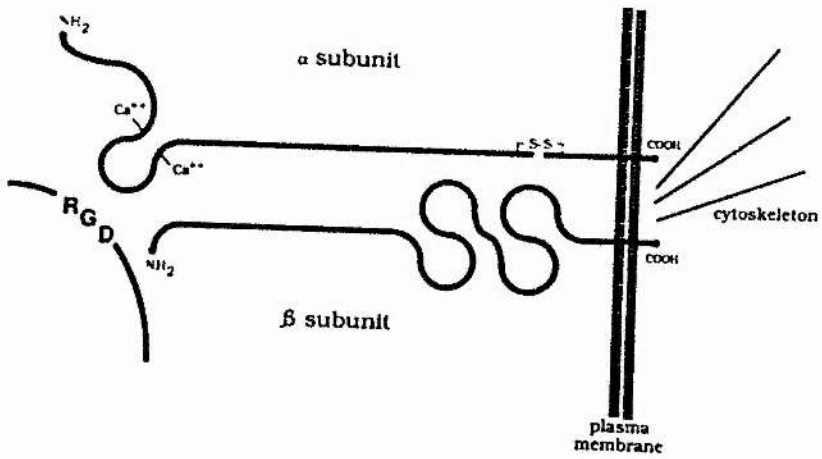


Fig 11 shows the structure of an RGD-receptor.

It is thought by Ruoslahti and Pierschbacher (1986) that divergent evolution gives rise to multiple mammalian receptors that have subtly different specificities. In contrast, the adhesion proteins may have arisen through convergent evolution by means of simple mutations, since these proteins do not seem to share other homologies.

Other types of cell receptors to the extracellular matrix also exit. These are of lower molecular mass than the RGD receptors. For example in melanoma cells, attachment to Fn is achieved through an REDV site (Humphries *et al*, 1986) and since the RGD receptors are highly specific, there must be another receptor for this site which has not yet been

elucidated. Another candidate for a low Mr receptor for Fn is a 47kD glycoprotein found in BHK cells (Yamada *et al*, 1985).

Much more is known about the low molecular weight receptors to Lm. A high affinity receptor to Lm has been described, which is thought to bind the CDPGYIGSR peptide (K_d 2 x 10^{-9}M) (Brown *et al*, 1983; Von der Mark and Kuhl, 1985) and has been found in various cells including melanoma cells. This receptor has a Mr of about 68kD under reduced conditions (Rao *et al*, 1983) and has been partially characterised (Kitten *et al*, 1986).

A cellular receptor for TSP has recently been discovered (88kD) in platelets (Asch *et al*, 1987) which appears to be RGD-independent. Similarly a platelet receptor for vWF (150kD) has been described (Santoro, 1987).

There are also numerous polypeptides of Mr 31kD, 47kD, 65kD and 75kD, which show an affinity for collagen (Yamada *et al*, 1985; Juliano *et al*, 1987).

Little is known of the regulation of any of these adhesion receptors in terms of expression or function. However, there is increasing evidence of their possible regulation by protein kinases. For example, there is a tyrosine kinase site on the CSAT antigen in RSV-transformed cells which becomes phosphorylated by pp60src. Other observations have led to the suggestion that both cAMP-dependent protein kinases (Cheung and Juliano, 1985; 1987) and C-kinase (Juliano, 1987) may also regulate Fn-dependent adhesion. It is not certain if the effects of cAMP-dependent

kinase or C-kinase are mediated at receptor phosphorylation level or if it is post receptor elements of the adhesion pathway which are regulated by these kinases (Juliano, 1987). It is possible that amongst the membrane receptors for extracellular components that there is growing evidence for a similar type of "cross talk", as occurs in the case of hormone and growth factor receptors, where there are numerous examples of the stimulation of one receptor affecting the behaviour of another.

3.1.6 Extracellular matrix molecules in malignancy

Cell adhesion molecules may be important in a variety of interactions occurring in the metastatic process. For example once tumour cells detach from the primary mass and are released into the circulatory system (Sherbert, 1982), they can interact with normal cells such as lymphocytes (Fidler, 1975), platelets (Pearlstein *et al*, 1980) and thromboplastic components (Kohga and Tanaka, 1979). Tumour cell aggregation with platelets may involve Fn, thrombospondin and von Willebrand factor which are secreted from platelets following activation by thrombin or collagen (Zucker, 1979; Dixit *et al*, 1985; Ginsburg, 1985b). It is possible that these molecules on the surfaces of platelets cause tumour cells to become attracted to them. An example of this might be found on M21 melanoma cells which have an RGD receptor which can recognize Vn, fibrinogen (Fg) and vWF (Cheresh and Spiro, 1987). On the other hand, the RGD binding receptors gpIIa/IIIb on platelets may be used for recognising the RGD sequences on Vn, Fn, Fg or

vWF on tumour cells or in serum, in order to form stable tumour cell aggregates.

Receptors on the tumour cell surface may then be involved in implantation of blood-borne tumour cells to Vn, Fn and vWF on the endothelium (Nicolson and Winklehake, 1975; Phondke *et al*, 1981; Netland and Zetter, 1986), and may induce the endothelial cells to withdraw from each other, thus exposing patches of the underlying basement membrane (Kawaguchi *et al*, 1985).

Following adhesion, tumour cells may utilise Fn, Lm and Vn receptors to migrate through the BM (Liotta, 1986; McCarthy *et al*, 1985) since these adhesion proteins have been shown to affect the motility and migration of the tumour cells. Further evidence for the role of adhesion proteins in metastasis has come from inhibition of formation of experimental metastases by use of the RGD peptide. The mechanism of RGD anti-metastatic effects, however, remains unclear.

In tumour cell adhesion, emphasis has been placed by a number of workers, on Lm and Fn receptors, who have reported differences in the abilities of high and low metastatic lines to adhere to extracellular matrix molecule. They have also shown differences in the abundance and/or composition of the cell-surface glycoproteins of these cell lines (Terranova *et al*, 1984). Interactions of adhesion promoting proteins with receptors on the cell surface can modulate the adhesive, motile, invasive and metastatic behaviour of tumour cells.

Thus Terranova *et al* (1982) found that exposure of B16 cells to laminin *in vitro* enhanced binding of the cells to the endothelium *in vivo* and metastatic ability increased. On the other hand, Nicolson *et al*, (1981) showed that exposure to fibronectin reduced laminin binding and reduced metastasis.

Another question still to be answered is how the adhesiveness of cells can be modulated. Since the various adhesion promoting glycoproteins discussed are ubiquitous, it is difficult to understand how tumour cells can adhere at tissue-specific sites in order to form secondary deposits. One obvious way would be to vary the number of adhesion receptors expressed at the cell surface. For example, an experiment by Reiber *et al*, (1986) demonstrated that melanoma cells were unable to attach to collagen type I and formed aggregates even in the presence of Fn. In these aggregated cells, the Fn receptor (140kD) was much decreased in expression. There may also be a difference in expression of Lm receptors. For example, there are more Lm receptors in breast carcinoma than normal breast tissue (Hand *et al*, 1985). Other possibilities might involve modulation of surface display of adhesion receptors (degree of clustering or interaction with other membrane components) (Aplin and Hughes, 1981). There is also a difference in distribution of Lm receptors between benign and malignant cells, e.g. in breast cancer the benign tumours express their receptors at the base of the cells adjacent to the basement membrane whereas the malignant cells express their receptors over the entire surface of the cell (Hand *et al*, 1985).

However, Brown and Juliano (1986), described the situation where ample 140kD protein is expressed but adhesion still does not take place. This maybe due to tyrosine phosphorylation (as occurs in transformed cells) or control by cAMP-dependent kinases and protein kinase-C (Saga *et al*, 1988).

3.1.8 *In vitro* assays for testing adhesion promoting molecules

The aim of this part of the study was to find a suitable means of extracting molecules from the surface of endothelial cell monolayers and from the conditioned medium without destroying any adhesion-promoting activity of the molecules. In a study by Hatcher *et al* (1986), endothelial cells were grown in serum-free medium and 20 hours later the conditioned medium was removed and tested for adhesion promoting molecules. Since this seemed to be a rather detrimental procedure to the cells, which could result in the release of toxins as the cells started to die, it was decided that the cell surface proteins should be extracted using EDTA and to concentrate the conditioned medium. In order to determine which molecules were derived from the cells and which were derived from the serum, cell-free controls were run concurrently.

The next step was to develop a suitable assay for testing adhesion. Adhesion assays described in the literature are based on the adsorption of putative adhesive molecule(s) onto an artificial substrate such as plastic or

nitrocellulose, followed by incubation of the molecule with a suspension of cells. The first use of this kind of adhesion assay was performed by Klebe *et al* (1978), whereby serum was electrophoresed on cellulose acetate and a diffusion replica made by overlapping onto a gel of 0.25% type I collagen. Molecules not bound to collagen were washed away and the bound molecules to the collagen were incubated with Chinese hamster ovary cells for a fixed time of 90mins at 37°C. The replica was then rinsed, fixed and stained (0.1% toludine blue).. Two adhesive bands were identified in this way: one by its isoelectric point (pI 4.8) and the other by its extremely large size (failure to run into the gel). The major drawback of this study was that only adhesive molecules with collagen-binding properties would be detected.

Hayman *et al* (1982) developed this technique further by separating plasma proteins (depleted of albumin and IgG) using SDS-PAGE, followed by diffusion blotting of the separated proteins onto nitrocellulose (as described by Bowen *et al*, 1980). The blot was then incubated with rabbit kidney (NRK) cells, fixed and stained (0.1% amido black). Two adhesive proteins were identified with Mr of 220kD and 70kD (corresponding to fibronectin and vitronectin respectively). One of the limitations pointed out by Hayman *et al* (1982) was that the properties of the adhesion promoting proteins could be destroyed by treatment with SDS and reducing agent such as mercaptoethanol (2-Me).

In this study, adhesion promoting molecules extracted from the endothelium were initially probed with B16F10 cells, in the presence of divalent cations, in a dot blot assay which was developed for quick determination of the extent of the adhesion promoting activity of molecules. An advantage of this assay was that problems associated with blotting techniques could also be investigated (Appendix 3). One such problem was associated with the use of SDS and 2-Me as pointed out by Hayman *et al* (1982).

Having shown that these molecules could promote adhesion, verification could be obtained by using an immunological approach. This involves the production and characterisation of anti-cell surface antibodies which inhibit adhesion (Damsky *et al*, 1979; 1982 Knudsen *et al*, 1981). These workers developed polyclonal antibodies to fibroblasts which caused cell rounding and detachment. They then extracted fibroblast membrane glycoproteins which could block the antibody activity. During the last 15 years, laboratories have produced a variety of polyclonal antibodies which can inhibit cell-cell adhesion or prevent the interaction of cells with extracellular components (Goodman *et al*, 1983; Edelman, 1983). The dot blot assay system developed in this study could also be adapted for use in testing antibodies, raised to adhesive molecules or to cell surface molecules, for their ability to inhibit adhesion.

Having established that the extracted molecules promote adhesion they could then be characterised by SDS-PAGE and

immunoprobing in order to determine whether they are known adhesion-promoting proteins such as Fn, Lm, Vn, or a new protein.

3.2 MATERIALS AND METHODS

All chemicals used were obtained from BDH and were Analar grade, unless otherwise stated.

3.2.1 Protein extraction

a) Fibronectin (Fn)

Obtained from Dr N. Hunter (Scottish Blood Transfusion Centre, Edinburgh).

b) Laminin (Lm)

Laminin was purchased directly from Sigma.

c) Crude Cell Extract (CE)

Cell extract was prepared from BAE cells seeded at 6.5×10^4 cells and grown to confluence (3 days) in roller bottles (growth area 750cm^2). The roller bottles were gassed for 1min with 95% air, 5% CO_2 and rolled at 0.5rpm. The spent medium was retained (cell extract medium - CEM). Each bottle was washed once with 30ml calcium- and magnesium-free phosphate buffered saline (CMF-PBS) before extracting the confluent monolayer with 25ml 2mM EDTA in CMF-PBS for 10min at 37°C (cell extract - CE). (See Fig 12).

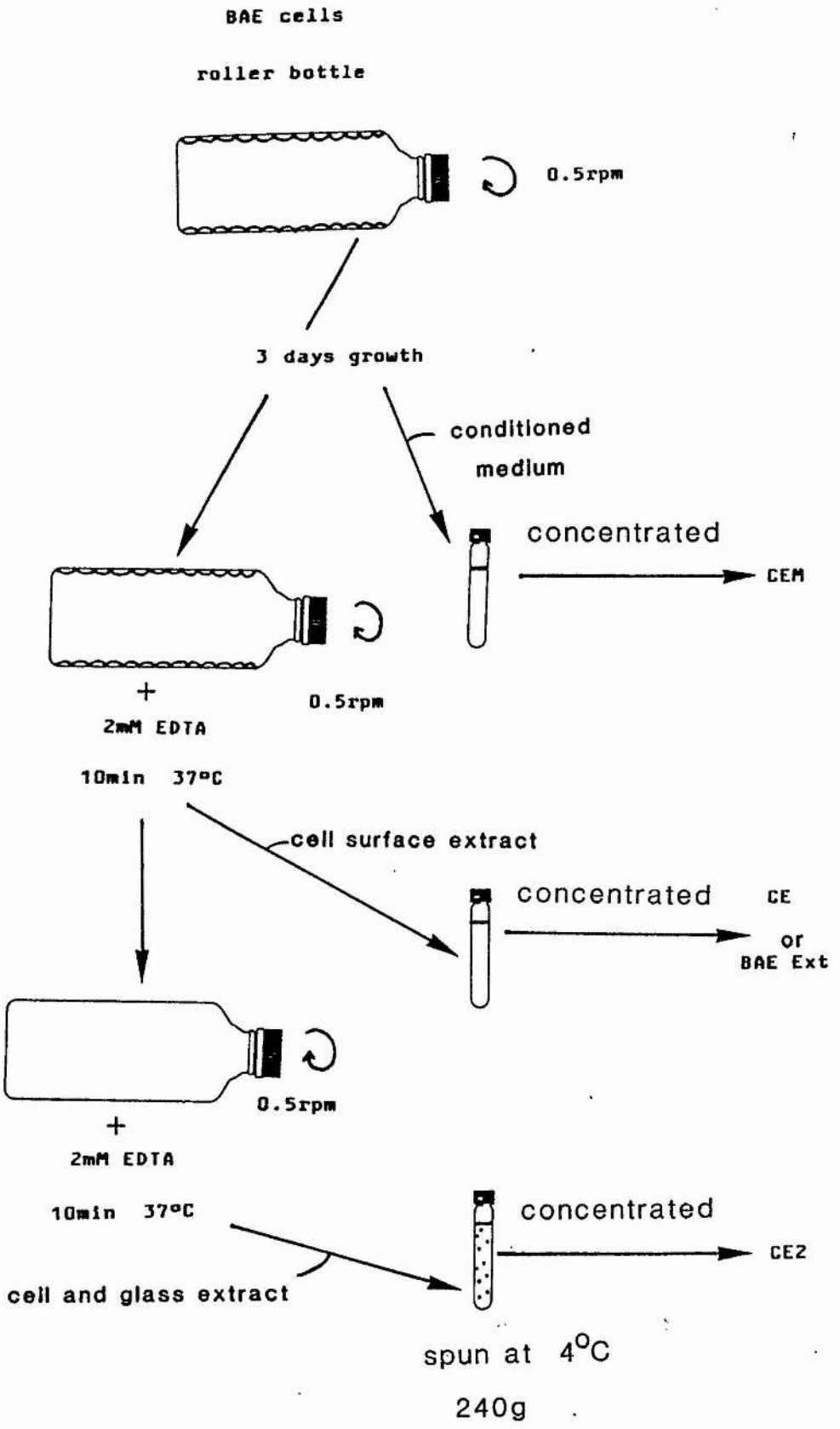
Initially the extracted proteins were concentrated against aquacide III (flake polyethylene glycol, Mr 8kD, Behring Diagnostics), until reduced 10x and then dialysed

against PBS overnight at 4°C. However, it was found that CE precipitated irreversibly upon concentrating. A new protocol was adopted whereby the proteins were concentrated to half the initial volume, dialysed against distilled water overnight before concentrating as already described. This reduced the amount of precipitation and so enabled the proteins to be filter sterilised without loss of protein. For some experiments CE which had been concentrated by the former method, were used. In this case it was denoted BAE Ext. (See Fig 12).

d) Cell-free controls

Roller bottles containing medium but no cells, incubated under the same conditions as above, were extracted in the same way to give an indication that the effects observed were due to cell-derived or serum-derived components.

Fig 12 shows the procedures used in the extraction of CE, BAE Ext and CEM.



CONCENTRATION PROCEDURE

TO OBTAIN CEM, CE1 and CE2

concentrated against
aquacide III
at room temperature
until reduced to
half the initial volume

↓

dialysed overnight
against water 4°C

↓

concentrated against
aquacide III at
room temperature
until reduced 10x

↓

dialysed overnight
against PBS 4°C

↓

filter sterilised

BAE Ext

concentrated against
aquacide III at
room temperature
until reduced 10x

↓

dialysed overnight
against PBS 4°C

↓

spun at
240g 4°C
for 20min

↙ ↘

supernatant — precipitate

↓

filter sterilised

3.2.2 Gel electrophoresis

a) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples containing SDS at a final concentration of 0.625% were boiled for 2min (non-reduced conditions), and in some cases 2-mercaptoethanol (2-Me) was added (final concentration 10%) and the solution boiled for a further 2min (reduced conditions). The non-reduced and reduced samples were separated on slab gels (without stacking gel) using an adaptation of the Laemmli method (Laemmli, 1970).

Each gel cartridge consisted of two sheets of glass separated by plastic spacers on two sides and held together with sticky tape. The gel solution was made up according to the range of molecular masses present in the sample (i.e. higher Mr proteins were run on lower percentage acrylamide gels). See Appendix 1.

The electrophoresis running buffer contained 0.025M tris-HCl, 0.192M glycine and 0.01% SDS, pH8.3.

Protein samples (40ul/well) containing 10% glycerol were run at 50mA/gel for about 4h on gels (7.5 x 15 x 1.5mm). Molecular weight standards (Sigma SDS-7) were run simultaneously if the molecular masses were being determined.

b) Gel staining

In order to visualise protein bands, the gels were fixed in 7% acetic acid in distilled water for 30min immediately after electrophoresis and stained overnight in 0.02% Coomassie blue in 7% acetic acid. The gels were destained in 25%

methanol and 10% acetic acid in distilled water. At this stage the gels were dried down and photographed.

c) Gel drying

Polyacrylamide gels were placed into a plastic sandwich box containing a 500ml slurry of aquacide III (flake polyethylene glycol, Behring Diagnostics) in distilled water (1g/ml) and left at room temperature until reduced about 16X. The amount of time for dehydration was a function of the percentage of acrylamide in the gel: it took about 12 min for each percent of acrylamide (Ferro *et al*, 1989 - see Appendix 4). The dehydrated gels were rinsed in distilled water and placed on filter paper (Whatman No 2) in a gel drier (Pharmacia) with a hot air blower placed about 15cm above the gel. Four gels could be processed at one time. The gels were left to dry completely for three hours and then left under a 1kg weight overnight before being stored permanently.

3.2.3 Western blotting

Following electrophoresis, gels were placed in tris-HCl/glycine buffer pH8.3 (with 20% methanol in the case of SDS-containing gels). The blotting sandwich consisting of a Scotchbrite pad, Whatman filter paper No. 2, nitrocellulose paper (Millipore cat. no. HATF 13750, pore size 0.45um), the gel, and another Whatman filter paper and Scotchbrite pad was lowered into the blotting tank (Biorad). The nitrocellulose was placed to the anodal side and blotting carried out for

30min at 100V. A strip of nitrocellulose was cut and stained in 1% amido black for 3min to check that protein had been transferred. Destaining was carried out in 70% ethanol to visualise transferred proteins.

3.2.4 Cell attachment

a) Dot blots

Proteins were dotted onto nitrocellulose discs as described in Part 2.2.3a and the discs placed into a 24-well plate. The upper chamber (consisting of a bottomless 5ml Teklab tube) was placed over the discs and 1ml 10% BSA in distilled water was used to block the filters for 30min at 37°C. (See Fig 13).

The cells were harvested as usual and resuspended in serum-free EC medium to $1x10^6$ cells/ml. 1ml of cell suspension was placed into each chamber and left at 37°C for 90min, after which the discs were placed in CMF-PBS, followed by 2mM EDTA for 2min and finally washed extensively using 10ml PBS dispensed from a 10ml syringe through a 19 gauge needle. The cells attached to the filters were then stained as described in Appendix 1 and the dried filters mounted in emulsion oil so that the cells could be observed using light microscopy.

b) Western blots

Western blots of gels containing adhesion promoting proteins were blocked with 10% BSA in distilled water for

30min at 37°C. The blots were then cut in order that one track fitted into a plastic holder with a fitted upper chamber which was 1ml deep with a total volume capacity of 20ml (See Fig 14). The B16F10 cells were harvested and 10ml of cell suspension ($1x10^6$ cells/ml) was placed into the upper chamber and left at 37°C for 90min. After this time the filter was placed into CMF-PBS, followed by 2mM EDTA for 2min to remove cells which had bound non-specifically to the substrate and then washed extensively using 50-100ml PBS dispensed from a 20ml syringe through a 19 gauge needle. The filter was stained and mounted as for the dot blots (Part 3.2.4a). (See Fig 15).

Fig 13 shows a schematic representation of the apparatus used in probing dot blots with cells.

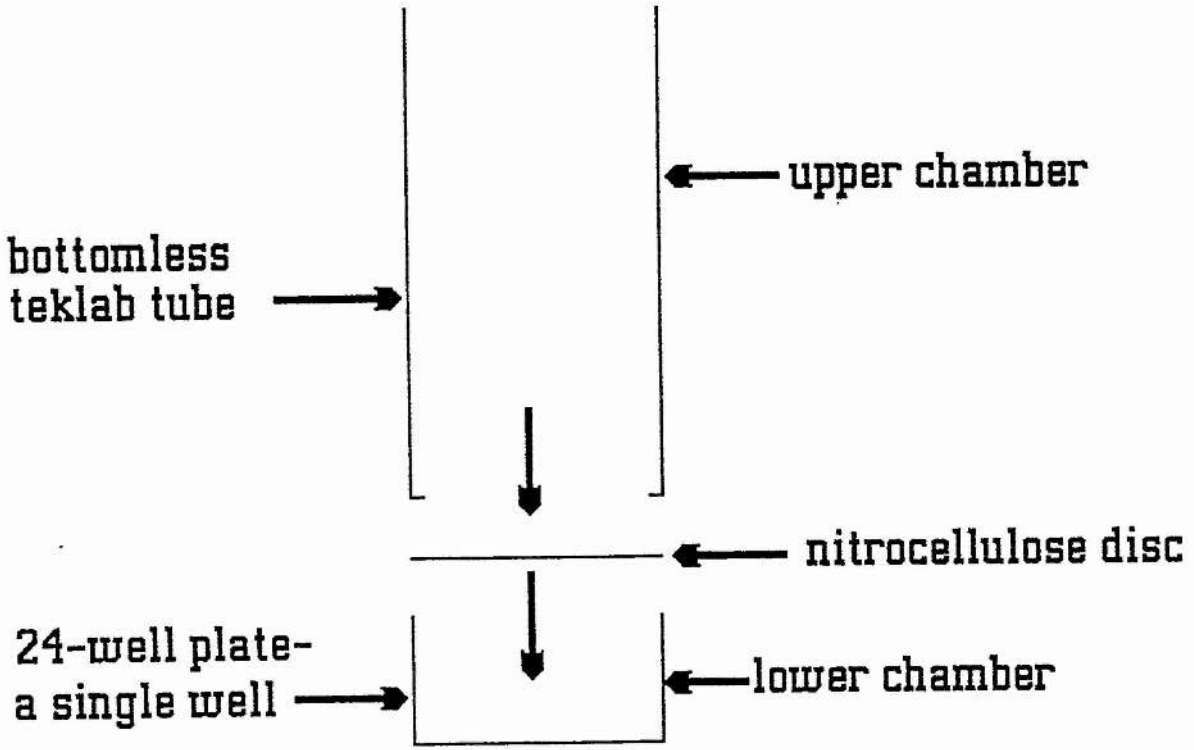


Fig 14 shows a schematic representation of the apparatus used in probing Western blots with cells.

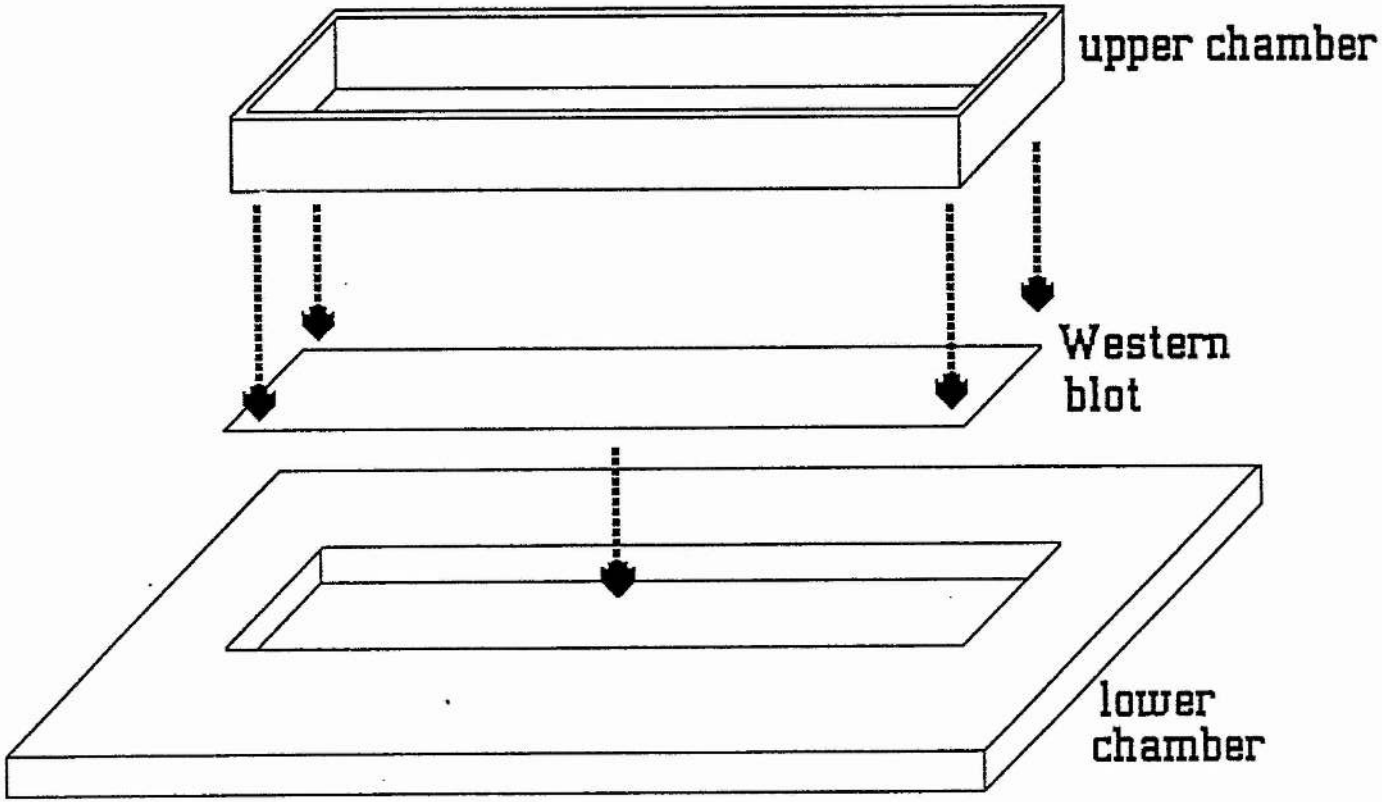
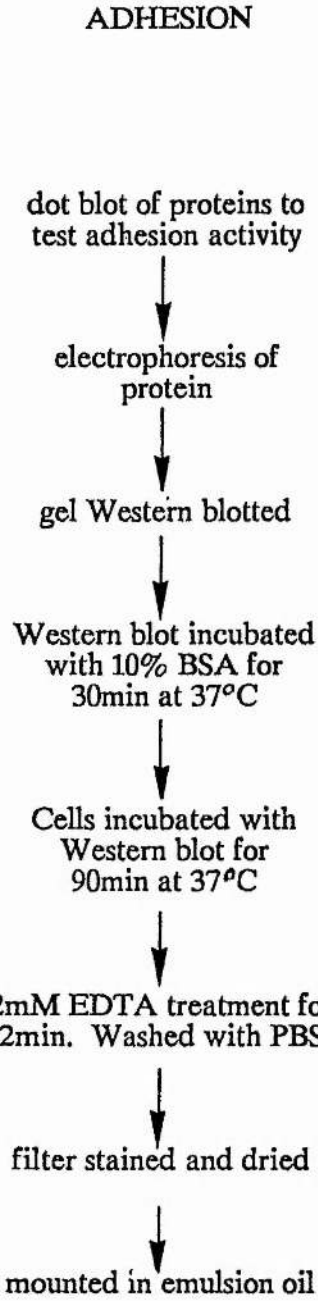


Fig 15 shows the protocol used in cell attachment.



3.2.5 Antibody Specificity Tests

a) Dot blotting - using soluble protein as antigens

The method was followed as described in Part 2.2.3a except that 2ul of protein solution (various dilutions made up in PBS) was dotted around the circumference of each of the discs.

b) ELISA - using soluble protein as antigens

The method as described in Part 2.2.3b was followed with the following modifications. Instead of using cells, 100ul of protein solution, made up to a final concentration of 20ug/ml in carbonate-binding buffer pH11.0 (see Appendix 1), was incubated in each well of a 96-well plate for 2h at 37°C. The first row was used as a blank and no protein was placed in these wells. Excess liquid was discarded and the plate washed twice with PBS/0.05% Tween (200ul/well) before blocking with 10% Marvel (dried milk protein) in PBS/Tween (150ul/well) for 1h at 37°C. Diluted primary antibody was incubated, followed by phosphatase-conjugated second antibody and phosphatase substrate, as described in Part 2.2.3b. The colour was allowed to develop for 30min before reading the plate directly on an ELISA reader. An O.D. three times higher than background was taken as positive.

3.2.6 Raising polyclonal antibodies to soluble antigens

The method as described in Part 2.2.4 was followed but instead of using cells, 1ml of protein solution (100ug/ml) was vortexed with 1ml FCA. Purification of the antibodies was as described in Part 2.2.5.

3.2.7 Raising monoclonal antibodies

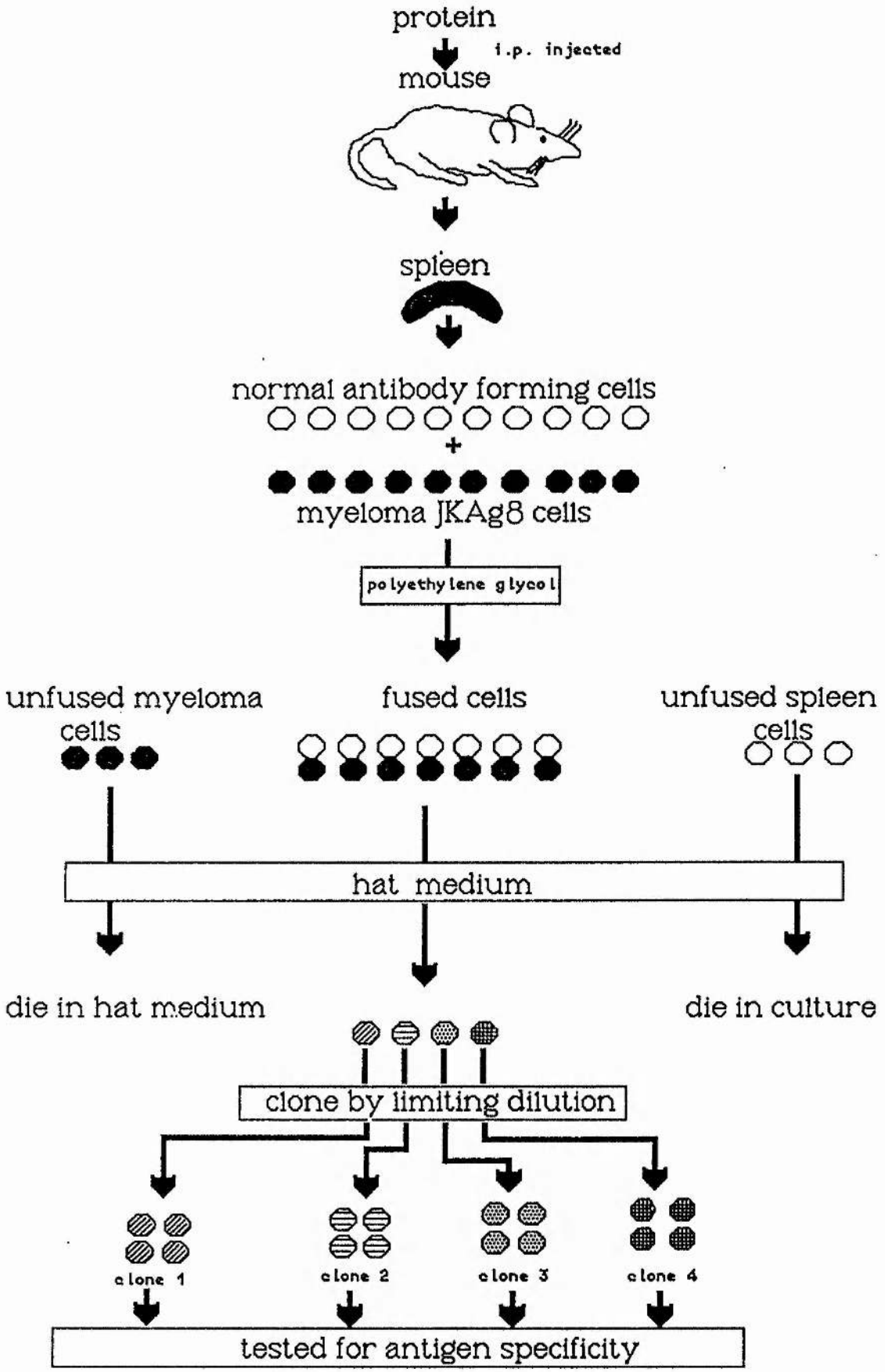
0.5ml solution of protein in PBS (100ug/ml) was vortexed with 0.5ml FCA as described by Goding (1986). A dose of 200ul of the mixture was injected into each of the peritoneum of 5 Balb/c mice. After a few months, a tail bleed was taken from each mouse and the serum tested on an ELISA system (see Part 2.2.5b) for an O.D. three times greater than background. The mice were each reboosted with 400ul of protein solution vortexed with a 1:1 ratio of Freund's incomplete adjuvant (FIA). Concurrently the myeloma cell line JKAg8 was grown up in 75cm^2 flasks (Sterilin) in RPMI 1640 medium with 10% FCS, glutamine (2mM), benzyl penicillin (50IU/100ml), streptomycin sulphate (50ug/100ml), 2-Me (50ul/100ml) and sodium pyruvate (1mM). This medium will be referred to as complete RPMI. Mouse peritoneal cell feeder layers were also prepared in ten 96-well plates (Nunc). This was done by washing out the peritoneal cavity of a dead mouse with 1ml cold serum-free RPMI medium. The harvested cells were seeded at 2x10^4 cells/0.2ml RPMI medium. When a sufficient number of JKAg8 cells (5x10^7 - 1x10^8) were obtained, the next procedure was carried out.

A spleen from an immunised mouse was removed surgically

under asceptic conditions and teased through a fine mesh in a Swinnex holder to obtain a single cell suspension. The spleen and myeloma cells were both washed with serum-free RPMI medium (SF-RPMI), counted on a haemocytometer and adjusted to give a ratio of 2:1 of spleen:myeloma cells. These cells were mixed in a universal and centrifuged for 3min at 240g. Concurrently 1ml of SF-RPMI was added to 1ml of sterile polyethylene glycol (PEG, Mr 6kD) which had been pre-warmed to 40°C. The PEG/medium solution (1ml) was then added slowly to the cell pellet over a period of 1min. The cell suspension was stirred for 90 seconds and then 1ml SF-RPMI was added to the suspension, stirring for a further minute. This latter step was repeated for 60sec and then again for 30sec. Finally 20ml SF-RPMI was added over 2min. The cell suspension was left standing for 5min at 37°C to standardize conditions and then centrifuged for 4min at 240g. The supernatant was aspirated off and the cells resuspended in 50ml HAT medium (complete RPMI containing hypoxanthine (1.36×10^{-4}mg/ml), aminopterin (1.76×10^{-4}mg/ml) and thymidine (3.86×10^{-4}mg/ml)). Aliquots of the cell suspension (50ul) were distributed into each well of the ten 96-well plates containing a peritoneal cell feeder layer. After a week the cells were fed with fresh HAT medium and about a month later HT medium containing hypoxanthine (1.36×10^{-4}mg/ml) and thymidine (3.876×10^{-4}mg/ml) was substituted for HAT. The medium was permanently changed to complete RPMI after a few more weeks. Appendix 2 shows the metabolic pathways involved in the selection of hybridoma cells in HAT medium.

Once the cells were stable in complete RPMI, an ELISA test was carried out. Positive colonies were transferred to a 24-well plate. As soon as the cells were confluent the cells were cloned by dilution in which each 100ul of cell suspension from a confluent well of a 24-well plate was diluted 10x with complete RPMI. 1ml of each suspension was further diluted with 10ml medium and then finally 100ul was diluted with 20mls with complete RPMI containing catalase (500IU/ml). This method enabled each 100ul of cell suspension/well to contain 3 cells/ml. Samples of 100ul were plated out into 96-well plates. After 2 weeks the antibody in the wells was tested on an ELISA system. Those cells showing a positive staining for the antigen were transferred into a 24-well plate and fed regularly. On reaching confluence, the supernatant was tested against a panel of antigens (see Part 3.4.3, TABLE 5). The rest of the cells were grown in mass culture in 200cm^2 flasks. The supernatant was collected from the flasks weekly and the antibody containing solutions were centrifuged at 240g for 20min. The clear solutions were then stored at 4°C until purified. Fig 16 summarises the protocol used in the production of hybridoma cells.

Fig 16 shows the production of hybridomas.



3.2.8 Purification of monoclonal antibodies on a protein A column

The antibody containing supernatant was concentrated against aquacide III (polyethylene glycol, Behring Diagnostics) until reduced 10x, followed by overnight dialysis against PBS.

The method described by Goding (1986) was used as follows: A 10ml immobilised protein A column (Biorad) was equilibrated with phosphate buffer pH8.0. (See Appendix 1). Prepared antibody (10ml) was loaded onto the column using a flow rate of 0.5ml/min. Unbound material was washed off with phosphate buffer and bound material was eluted succesively with citrate buffer pH6.0; 4.5 and 3.5 (see Appendix 1). The eluate was collected in 1ml fractions via a fraction collector.

The column was washed with citrate buffer pH3.0, followed by phosphate buffer pH8.0 with 0.02% (w/v) sodium azide and then stored in the dark at 4°C until required for further use.

The peaks of the fractions were pooled, filtered through a nitrocellulose membrane (Millipore HATF 13750, 0.22um pore size) and stored sterile at 4°C until assayed on a dot blot system. Protein concentrations were worked out for antigen specific fractions and frozen in 1ml aliquots until required.

3.2.9 Inhibition of attachment

A dose response curve was set up with test protein as described in Part 3.2.4a. The optimum protein concentration

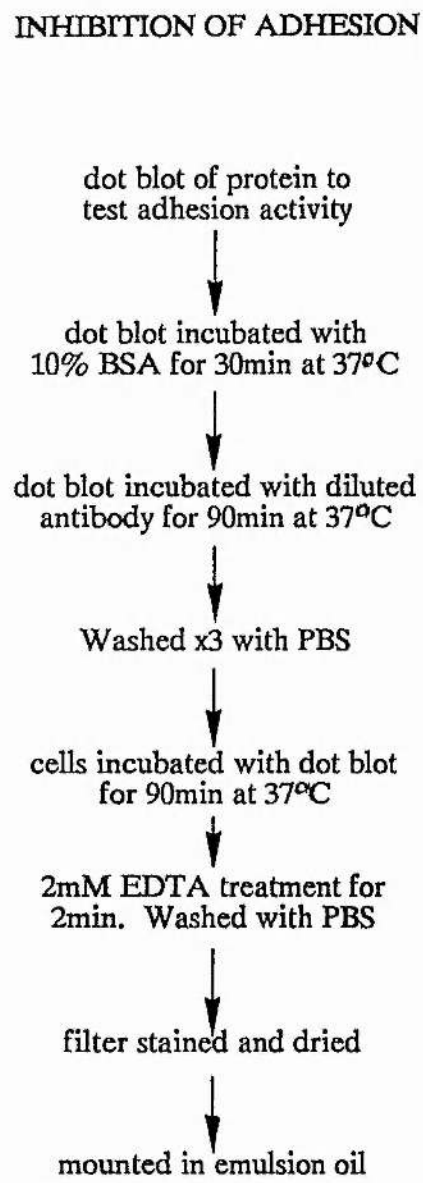
was used to test inhibition of adhesion by antibodies. Before incubating cells with the filters, additional adhesive binding sites on the filters were blocked with 10% BSA for 30min at 37°C. Diluted antibody (1ml) at varying concentrations was then incubated with the filters for 30min at 37°C. The filters were washed twice with PBS before incubating with cells as described in Part 3.2.4a. (See Fig 17).

3.2.10 Immunoprobing Western blots

A Western blot was blocked with 10% Marvel in PBS/Tween 1h at 37°C. The blot was incubated with 20ml of a 1:100 dilution of purified antibody solution in PBS for 90 min at 37°C and washed 3x with PBS/Tween before being incubated with 20ml of phosphatase conjugated second antibody (Sigma) in PBS for 2h at 37°C.

The blot was washed 3x with PBS/Tween and the colour developed as described in Part 2.2.3a.

Fig 17 shows the protocol used in inhibition of cell attachment.



3.3 RESULTS

Adhesion is thought to be the first step in extravasation as observed in the previous chapter. It was possible that the endothelial cells might prove to have molecules which would promote the adhesion of tumour cells to the endothelium (Jaffe *et al*, 1976; Jaffe and Mosher, 1978; Sage *et al*, 1981; Hatcher *et al*, 1986). Therefore proteins were extracted from the surface of endothelial cell monolayers (CE) and from the conditioned medium (CEM). The extracted molecules were tested for adhesion-promoting activity of B16F10 cells.

3.3.1 Attachment assay - probing proteins in a dot blot assay

A dot blot assay was developed whereby proteins to be tested for adhesion promoting ability were dotted onto nitrocellulose discs. Fibronectin and laminin were used as positive controls as they are known to promote adhesion (Yamada *et al*, 1985). Cells were applied to the filter and after aan incubation period of 90mins the cells which had not attached to the filter were washed off using EDTA treatment. Using this assay, adhesion to Fn by the B16F10 cells was detectable down to 0.5ng; tp Lm down to 50ng; to CE down to 300ng; and to CEM down to 400ng. In comparison FCS and cell-free controls, at a wide range of concentrations, did not promote adhesion.

3.3.2 Inhibition of adhesiion

In order to confirm that the molecules extracted from the endothelial cells promoted adhesion, polyclonal antibodies were raised against the extracted endothelial cell surface molecules (anti-BAE EXT) and used to block adhesion of the B16F10 cells to CE and CEM.

A 1:100 dilution of anti-BAE EXT (3.2mg/ml) blocked adhesion to CE (0.7ug) and to CEM (40ug). This confirms that CE and CEM promote adhesion. (See TABLE 4).

3.3.3 Antibody probing

The next step was to test anti-BAE EXT in a dot blot immunoprobing assay to see if this antibody would cross react with other proteins known to promote adhesion. The antibody was tested for cross-reactivity to Fn and Lm. The results are shown in TABLE 2. BAE cells were not used since they caused a positive reaction with the conjugated antibody (due to their alkaline phosphatase activity - see Part 2.4.1).

TABLE 2 shows the relative cross reactivity of anti-BAE EXT with Fn, Lm, CE and CEM

Antibody	Fn 0.5ug	Lm 0.5ug	CE 0.7ug	CEM 40ug
anti-BAE EXT	2+	3+	1+	1+

5+ is the highest score achieved when the conjugated second antibody is developed with its substrate.

These results seemed to indicate that fibronectin and laminin were the active molecules in either or both the cell extracts. Therefore it was decided to test antibodies raised against Fn and Lm to see if they cross-reacted with and/or blocked adhesion to CE and CEM. Other antibodies were also tested and included anti-S protein, anti-thrombospondin and anti-anti thrombin III. The results are summarised in TABLES 3 and 4.

Monoclonal antibodies

Antibodies were raised and purified against Fn as described in Parts 3.2.7 and 3.2.8. 1:100 dilutions of anti-Fn1 (250ug/ml) and anti-Fn2 (1390ug/ml) were tested against Fn (0.5ug), Lm (0.5ug), CE (0.7ug) and CEM (40ug) in either immunodot blot/ELISAs and in adhesion blocking assays. (See TABLES 3 and 4).

Polyclonal antibodies

The source of these antibodies was as follows: The anti-endothelial cells extract (a-BAE EXT) was raised in rabbits and purified as described in Parts 2.2.4. The anti-fibronectin (a-FN) and anti-anti thrombin III (a-ATIII) were raised in sheep and obtained from the Scottish antibody production unit, Carluke. The anti-S protein (a-SP) obtained from Calbiochem. anti-laminin (a-LM) obtained from Collaborative Research and anti-thrombospondin (a-TSP)

obtained from Dr N. Hunter, Scottish Blood Transfusion Centre, Edinburgh, were raised in rabbits.

Dilutions of antibody (1:100) - a-BAE EXT (3.2mg/ml), a-FN (18mg/ml), a-ATIII (18mg/ml), a-SP (1mg/ml), a-TSP (1mg/ml) and a-LM (1mg/ml) were tested against Fn (0.5ug), Lm (0.5ug), CE (0.7ug) and CEM (40ug) in immunodot blot/ELISa and in adhesion blocking assays. (See TABLES 3 and 4).

TABLE 3 Test panel of antibody specificities

Antibody	Fn 0.5ug	Lm 0.5ug	CE 0.7	CEM 40ug
a-FN1 (m)	3+	-	-	-
a-FN2 (m)	2+	-	-	-
a-BAE EXT (p)	2+	3+	1+	1+
a-FN (p)	5+	1+	-	-
a-ATIII (p)	-	-	-	3+
a-SP (p)	-	-	-	2+
a-LM (p)	-	5+	1+	-
a-TSP (p)	-	-	-	-

5+ is the highest score and is achieved when the conjugated second antibody is developed with its substrate.

p=polyclonal antibody

m=monoclonal antibody

The results from TABLE 3 indicate that CE contains laminin and CEM contains anti thrombin III and S-protein. These results are in agreement with those of Martin and Timpl, 1987; Owens and Miller, 1980; Preissner *et al*, 1988.

TABLE 4 shows the relative cross reactivity of some antibodies

Antibody	cell extract	cell extract medium
	adhesion	adhesion
a-FN1 (m)	(5+)	(3+)
a-FN2 (m)	(5+)	(2+)
a-BAE Ext (p)	(4+)	(2+)
a-FN (p)	(5+)	(4+)
a-ATIII (p)	(5+)	(2+)
a-SP (p)	(5+)	(5+)
a-LM (p)	(4+)	(5+)
a-TSP (p)	(5+)	(5+)

The results from TABLE 4 indicate that CE contains laminin and CEM contains fibronectin and anti thrombin III. These results are in agreement with those of MArtin and Timpl, 1987; Owens and Miller, 1980; Hatcher *et al*, 1986.

3.3.4 Protein analysis

In order to determine if the adhesion promoting molecules extracted from endothelial cells were known molecules such as fibronectin. laminin or vitronectin. protein characterisation using SDS-PAGE and immunoprobing of Western blots was carried out.

The band patterns are shown in Photograph 12; the percentage concentration of each protein component was estimated from scanning densitometer traces.

a) Cell extracts (CE and CEM)

CE is a complex mixture of proteins containing more than 11 resolvable bands under reduced conditions (TRACKS 4 and 8).

CEM showed 9 resolvable bands under reduced conditions and 6 resolvable bands under non-reduced conditions (TRACKS 5 and 9).

b) Fibronectin

Fibronectin (Fn) is a large glycoprotein of about 440kD and is present as a dimer (220kD and 210kD) (Yamada and Olden, 1978). Using SDS-PAGE analysis there were 9 resolvable bands under reduced conditions and 12 resolvable bands under non-reduced conditions (TRACKS 2 and 6). The 440kD, 220kD and 210kD components comprised 65.5% of the total protein analysed under non-reduced conditions. The smaller Mr bands (35.5%) seen in Photograph 12 are probably fragments of Fn or serum protein contaminants.

c) Laminin

Laminin (Lm) is a large flycoprotein of about 100kD which consists of three 200kD chains and one 400kD chain (Kleinman *et al*, 1984). In this analysis, Lm appeared to have 4 resolvable bands under reduced conditions and 3 resolvable bands plus a precipitate at the origin under non-reduced conditions (TRACKS 3 and 7). The 1000kD component is seen as a precipitate at the origin and the 200kD and 400kD components comprise 92% of the total protein content under reduced conditions. The smaller Mr bands (8%) seen in this sample may be fragments of Lm (Photograph 12).

Photograph 12 shows the protein bands for Fn, Lm, CE and CEM

Lanes 1, 6. SDS-7 Mr markers (Sigma) myosin (205kD), B galactosidase (116kD), phospholipase (97.7kD), BSA (66kD), egg albumin (45kD) and carbonic anhydrase (29kD).

Lane 2. Fn reduced (20ug) 440kD, 220kD, 210kD, 200kD 180kD, 142kD, 120kD, 89kD, 56kD and at front.

Lane 3. Lm reduced (20ug) 400kD, 200kD, 120kD and 110kD.

Lane 4. CE reduced (30ug) 330kD, 300kD, 186kD, 116kD, 103kD, 94kD, 66kD-56kD, 43kD,37kD and at front.

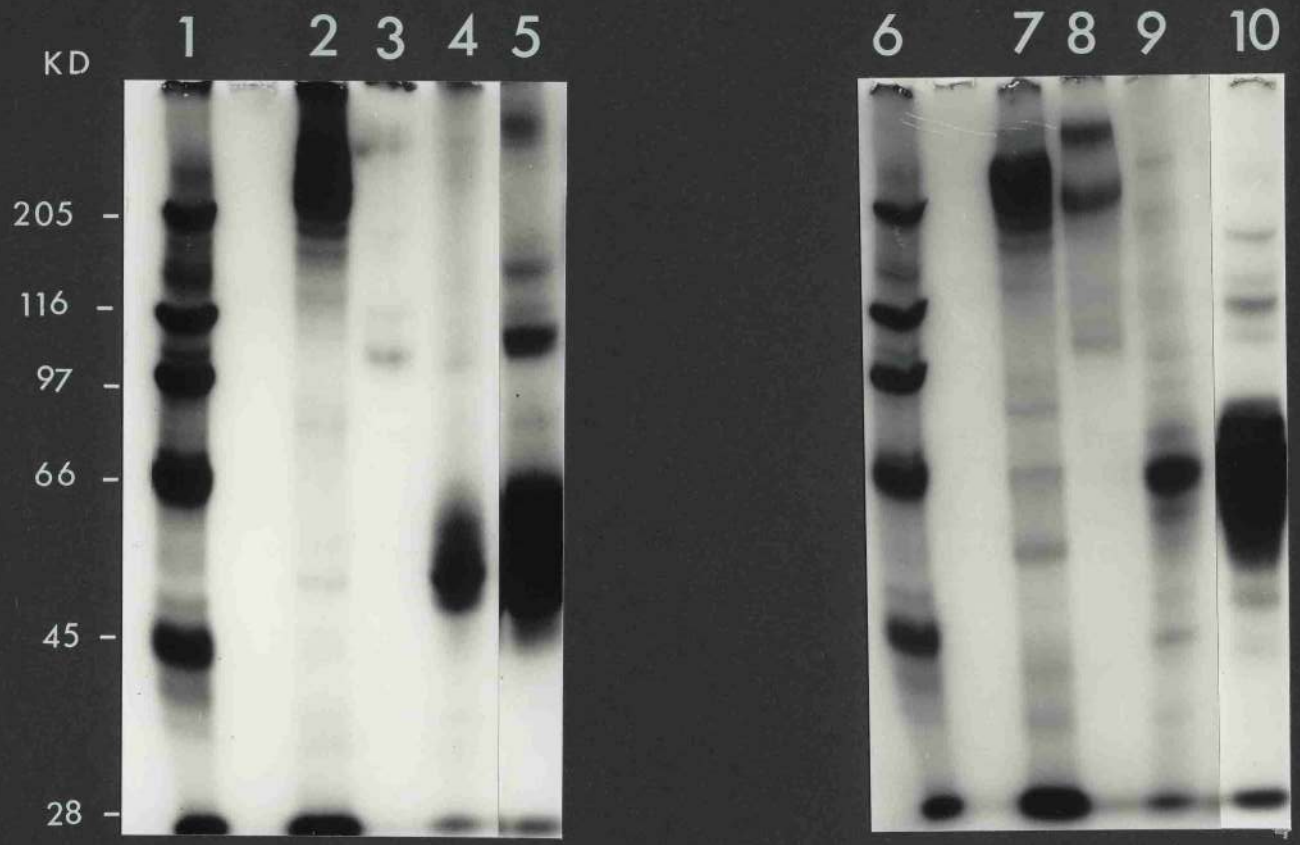
Lane 5. CEM reduced (160ug) 330kD, 300kD, 179kD, 140kD, 116kD, 105kD, 95kD, 75-63kD, 49kD, 43kD and at front.

Lane 7. Fn non-reduced (20ug) at origin, 440kD, 220kD, 210kD, 193kD, 187kD, 175kD, 135kD, 113kD, 77kD, 60kD, 54kD and at front.

Lane 8. Lm non-reduced (20ug) at origin, 400kD, 193kD and 107kD.

Lane 9. CE non-reduced (30ug) 440kD, 105kD, 61kD, 54kD, 50kD and at front.

Lane 10. CEM non-reduced (160ug) 600kD, 400kD, 155kD, 107kD, 63kD-53kD and at front.


KD
1
2
3
4
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205
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28

When comparing the cell extracts (CE and CEM) with each other it was found that CE contained an exclusive band at 37kD (reduced). CE lacked components at 179kD and 140kD (reduced) and 155kD (non-reduced) which were found in CEM. Some of the components had similar electrophoretic mobilities to those found in CE. Although comparison was made with cell free controls and fetal calf serum. it was not possible to determine whether these proteins were derived from the endothelial cells or the serum used in the medium. In the cell derived extracts and cell free controls. the major component (between 65-80%) was observed with mobilities around 70kD-60kD (reduced) and 65kD-55kD (non-reduced). This component ran with similar electrophoretic mobilities to bovine serum albumin.

When comparing CE and CEM with laminin and fibronectin, it was not possible to identify the Fn and Lm components in the extracts, which had been indicated by immunodot blot analysis (see TABLE 3).

Although CE and CEM were shown to promote cell attachment, from this SDS-PAGE analysis it was not possible to say which band(s) caused it nor whether the various bands are Fn, Lm, Vn, fragments of these proteins or a novel protein. It had been hoped that probing Western blots of SDS-polyacrylamide gels would be a useful tool in identifying the actual molecular masses of the adhesion-promoting components, but this proved to be unsuccessful. (See Appendix 3).

3.3.5 Western blot analysis

In order to try and work out the Mr of the cross-reacting components, Western blotting analysis was carried out instead. In most cases the antibody sensitivity resulted in the detection of smears rather than specific bands.

The antibodies which cross reacted with Fn (see TABLE 3), commonly immunoprecipitated an 187kD (non-reduced conditions) and 52kD (reduced conditions) band.

The antibodies which cross reacted with Lm, commonly immunoprecipitated a 440kD (non-reduced conditions) band.

The antibodies which cross reacted with CE, commonly immunoprecipitated a 37kD band (reduced conditions).

Out of the antibodies which cross reacted with CEM, anti-BAE EXT immunoprecipitated bands at 187kD (non-reduced conditions) and 52kD (reduced conditions), anti-S protein and anti-ATIII immunoprecipitated two bands at 250kD and 105kD (under non-reduced conditions). This latter result suggests that S protein and anti thrombin III in CEM are complexed together under non-reduced conditions. However, under reduced conditions they become denatured and so fail to interact with these antibodies.

3.4 DISCUSSION

In the dot blot adhesion assay developed in this study, the extracted endothelial cell proteins (CE and CEM) were found to promote adhesion to varying degrees, but not to as great an extent as Fn and Lm.

3.4.1 Immuno-analysis of components of CE and CEM

In order to confirm that CE and CEM do promote adhesion, a polyclonal antibody raised against endothelial cell surface proteins (anti-BAE EXT) was used to try and inhibit adhesion. Using this analysis, it was found that this antibody did indeed block B16F10 cells from adhering to CE and CEM (the latter being inhibited to a greater degree).

On further analysis with this antibody, it was found to cross-react with Fn and Lm. It was therefore decided to test antibodies raised against Fn and Lm, as well as those against vitronectin, thrombospondin and anti thrombin III to see if they would cross react with either CE and CEM and also to test them in inhibiting adhesion. It was found that although the Fn antibodies did not appear to cross react with either CE or CEM, they blocked adhesion to CEM. The anti-FN may not cross react with CEM for a variety of reasons, one of which might be that fibronectin in CEM may not be detectable by immunological methods but may by adhesion-promoting activity (Ferro *et al*, 1988). Anti-LM cross reacted with, and slightly blocked adhesion to CE, while anti-anti thrombin III cross reacted

with and blodked adhesion to CEM. On the other hand, anti-S protein cross reacted with, but did not block adhesion to CEM. This apparently contradictory result is not easily explained. It is likely that a complex of S-protein and anti-thrombin III is contained in CEM. It might be that the antibodies in the polyclonal anti-SP are able to react with some epitopes of S-protein, but not with the cell-adhesion promoting epitopes. Alternatively, this may be a function of the different configurations taken up by the molecule on plastic or nitrocellulose. Anti-TSP did not cross react or block adhesion to either CE or CEM. Although thrombospondin is synthesized by the endothelial cells, it is produced in very small quantities and this may explain these results (Raugi *et al*, 1982).

In order to determine which components of the extracts were cross reacting, Western blotting analysis was carried out. This analysis revealed that the cross reaction of anti-LM with CE could not be detected, possibly due to the conditions of electroblotting and electrophoresis denaturing the cross reacting moiety. However, a 37kD Mr component (reduced) of CE was detected by anti-BAE EXT.

On the other hand, CEM cross reacted with anti-SP and anti-ATIII at the same positions (205kD and 105kD) and this showed that the two proteins were probably associated with each other as described by Ill and Ruoslahti, (1985). Preissner *et al* (1988) indicated that this Vn/ATIII/thrombin complex has a molecular mass of 350kD as worked out by SDS-

PAGE analysis. In addition to Vn, there may also have been Fn present since anti-BAE EXT cross reacted with an 187kD band (thought to be an Fn fragment). Turther evidence for this was that the anti-fibronectin monoclonal antibodies prevented cell adhesion. It would therefore appear that adhesion by CEM is promoted by an anti thrombin III/ thrombin/Vn complex and by Fn.

3.4.2 SDS polyacrylamide gel analysis of components of CE and CEM

Characterisation of the molecules extracted in this study was achieved by SDS-PAGE. The results showed that CE and CEM contained a mixture of proteins. CE had an exclusive band at 37kD (under reduced conditions) and lacked components 155kD (non-reduced conditions), 140kD and 179kD (reduced conditions), found in CEM.

Comparison was also made with Fn and Lm, but this did not reveal any similarities. The pitfalls involved in making comparison using gel electrophoresis should be borne in mind. Although most soluble peptides migrate linearly according to the logarithm of their molecular weights (Weber and Osborn, 1969) it is not certain that this is true for extremely hydrophobic polypeptides or all glycoproteins. Therefore all molecular masses determined for surface or membrane proteins should be considered as nominal only. Furthermore, the values obtained depend on the exact conditions of the electrophoretic

analysis, degree and nature of cross-linking of the gels and Mr of the standards used. Similarity and/or co-migration or Mr do not necessarily indicate identity. One way of resoving this problem might be to use isoelectric focussing. Alternatively antibody analysis can be used as well, as was employed in this study.

When comparison of CE and CEM was made with FCS and cell free controls, it was difficult to discern which of the proteins in the extracts were derived from the serum or from the endothelial cells themselves. For future experiments, it would be better ro radio-label the metabolites used in culture by the cells, in order that the cell derived proteins be detected by radioactivity (Browning *et al*, 1983).

3.4.3 Conclusion

In conclusion, the results seem to indicate that adhesion was caused by proteins on the endothelial cell surface (a 37kD protein whose identity is not known and possibly Lm) and in secretions into the medium (Vn/ATIII and Fn). A 37kD protein has been described in FS9 Sarcoma cells which is thought to be involved in metastasis (Steinman *et al*, 1984). It will remain to be seen if these two proteins are the same. One way of doing this would be to use antibodies raised in this study (such as anti-BAE EXT) to see if they cross-react with the 37kD component described by Steinman *et al*.

The results of this chapter indicate tha Vn, Fn and Lm

which are synthesised by endothelial cells, are important in promoting adhesion of B16F10 cells. The way that these results could fit in with the observations made in Chapter II, would be as follows: Adhesion-promoting molecules such as Vn and Fn could have been released into the medium (CEM) (Preissner *et al*, 1988; Birdwell *et al*,1978; Hatcher *et al*, 1986). The released molecules may then have either become adsorbed to the plastic or to the surfaces of the tumour cells. This would then enable tumour cell aggregates which had detached from the initial seeding areas to attach to the monolayer or to the plastic as seen in Part 2.3.2. (See Fig. 18). On the other hand, adhesion promoting molecules on the endothelial cells themselves (Lm and possibly 37kD) (Martin and Timpl, 1987) would have promoted attachment to the monolayer only as these molecules were not released into the medium. (See Fig 18).

Now that the identity of the adhesive proteins in these extracts have been tentatively established it will be possible to purify them using gel filtration and immunoaffinity chromatography. For future experiments, I would suggest using a monoclonal antibody such as anti-FN1 to purify the 187kD component in CEM and gel filtration as a further step in the purification of the 37kD component in CE. Monoclonal antibodies should be used because they can be screened for their ability to interfere with specified cell interactions and can be produced against minor surface components of a

particular cell type. In contrast to polyclonal antibodies, monoclonal antibody preparations are highly specific and can be made in unlimited amounts. Furthermore heterogenous material (such as whole cells) can be used for initial immunization and the relevant antigens subsequently purified by immunoaffinity chromatography.

The purified extracts could then be further characterised and tested in adhesion assays to confirm that they do promote adhesion. Monoclonal antibodies could be raised against these components and used in animal models and experimental metastases as have been done by Vollmers and Birchmeier, (1983a; 1983b) - this being the ultimate aim of a study such as this. Other experiments which could be done, would be to test combinations of antibodies to try and totally inhibit adhesion. This would show whether these molecules work in concert or individually in promoting adhesion.

For future work it would also be of interest to test other tumour cell lines and variants of the B16 melanoma against these extracts. In addition, extracts from other endothelial cell lines could also be tested against to see if tumour cells adhesion to endothelial cells is organ-specific as has been suggested (Alby and Auerbach, 1984; Horak *et al*, 1986; Nicolson and Dulski, 1986).

Fig 18 shows possible roles for CEM and CE in promoting substrate adhesion *in vitro* during the extravastion experiment of Part 2.4.

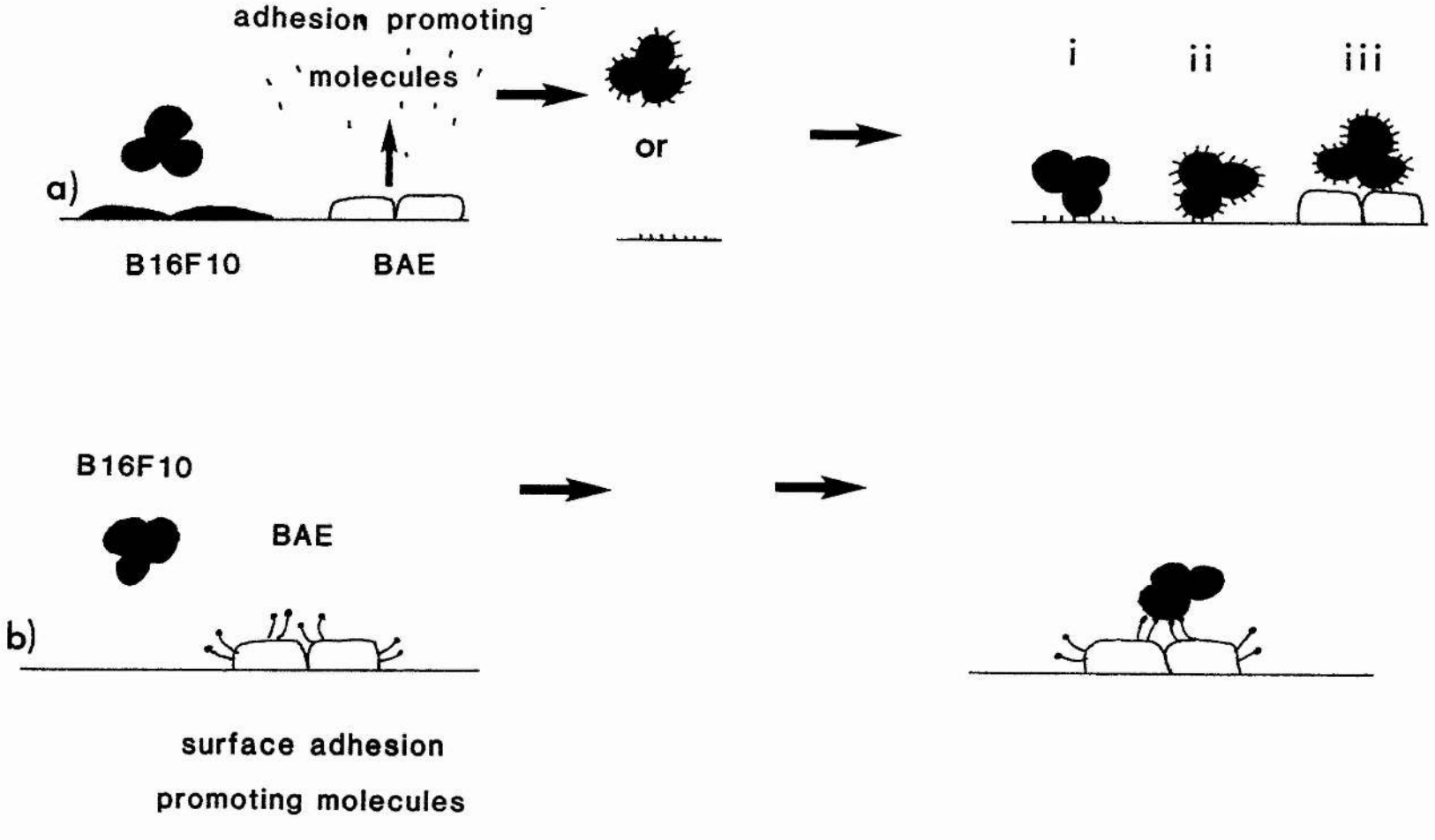
a) shows the BAE cells producing substrate adhesion promoting molecules (CEM) which may bind either to the substrate or to the B16F10 cells.

i) The molecules which attach to the plastic substrate may cause B16F10 cells to attach to the plastic.

ii) The molecules which attach to the cells may cause the B16F10 cells to attach to the plastic or

iii) to the endothelial cells.

b) shows the B16F10 cells attaching to cell surface proteins on the endothelial cells.


adhesion promoting
molecules
or
i
ii
iii
a)
B16F10
BAE
B16F10
BAE
b)
surface adhesion
promoting molecules

IV SPREADING

4.1 INTRODUCTION

During extravasation, adhesion to the endothelium is thought to be followed by spreading of tumour cells. Spreading increases the surface area of a cell which enables a greater interaction with the substrate which presumably maintains a stronger hold on the endothelium. Spreading requires the extension, attachment and contraction of pseudopodia and is thus dependent on activities of the cytoskeleton which are regulated by calcium (Lackie, 1986).

4.1.1 Molecules involved in spreading

Since spreading is, in part, dependent on adhesion it seemed pertinent to investigate whether the molecules which had promoted adhesion would also promote spreading. It is known that various glycoproteins which promote adhesion also promote spreading. Some of these have already been considered in the previous chapter.

4.1.2 The spreading Function of Fibronectin

To date, fibronectin is the most studied adhesion-promoting glycoprotein which also stimulates cell spreading in many cells (Yamada *et al*, 1985). Among the effects of Fn on transformed cells, the cells generally lack well-organized microfilament bundles but on addition of Fn, microfilament bundles are restored (Ali and Hynes, 1977). This effect could reflect increased spreading, but it seems probable that it

reflects a more direct effect of Fn on the cytoskeleton. Cellular Fn is thought to mediate attachment in close association with the cytoskeletal elements. Support for this has come from various observations. To correlate the organization of actin with Fn, it was necessary to use methods which allowed visualisation of both actin and Fn at the same time in the cell. This was accomplished by double label immunofluoresence. While these studies showed a high degree of correspondence between Fn and actin, an obvious drawback is that they are limited by the resolution of light microscopy (0.2um). Higher resolution was achieved by Singer (1979) using electron microscopy. He showed that bundles of Fn fibrils outside the cell were apparently continuous with microfilament bundles inside the cell. Further information was achieved using reflection interference microscopy (Avnur and Geiger, 1981). In this technique, the thin film of medium between the base of the cell and the substratum is exploited as an interference film and the intensity of the reflection of monochromatic light, or less often, the colour of the reflection of whole light provides information about the thickness of the film. Areas of very close contact (eg attachment plaques) produce strong negative interference in monochromatic light and appear dark. Use of this technique, in combination with electron microscopy, established that attachment plaques are points of termination of actin microfilament bundles. Other experiments (Chen *et al*, 1980) showed Fn to be found adjacent to focal adhesion sites in the

close contact sites and so it is possible for coordination of adhesion and spreading to occur by the transmembrane linkage of Fn with actin.

There is also thought to be some sort of transmembrane linkage between the focal contact sites (as opposed to the close contact sites) and the intracellular microfilaments. For some time vinculin was thought to play this part, mainly because it was enriched in this area and because it was thought to interact diretly with actin. The latter has since been shown to be untrue (Wilkins and Lin, 1986). Furthermore, vinculin was the first adhesion plaque protein found to contain elevated (8x) phosphotyrosine levels in cells transformed by Rous sarcoma virus (Selton *et al*, 1981). This result, combined with localization of the RSV oncogene product $pp60^{src}$ within adhesion plaques (Rohrschneider, 1980), led to the proposal that phosphorylation of vinculin is a major event in the disruption of adhesion plaques in transformed cells. This model has not been supported by subsequent work (Kellie *et al*, 1985). Weigant *et al* (1986) suggested that vinculin phosphorylation has little significance in the coordination of adhesion and spreading, since the level of vinculin phosphorylation is low at best and is a coincidental event reflecting its proximity to the oncogene product $pp60^{src}$. Recently, talin (another adhesion plaque protein) has also been found to contain phosphotyrosine and to have elevated levels in cells transformed by RSV (De Clie and Martin, 1987). But as in the case of vinculin, it does not correlate with loss of

stress fibres or rounded morphology. Other possible cellular substrates of $P60^{v-src}$ that are phosphorylated on tyrosine which are relevant to this study are the Fn receptor, the avian fibroblast integrin receptor and calmodulin (Hirst *et al*, 1986).

As yet there is much controversy in this subject area and it is difficult to envisage how all the effects of ECM molecules can be mediated via a simple transmembrane linkage to cytoskeletal components.

4.1.2. Laminin

Laminin induces spreading in some cells (Terranova, 1980). Since the RGD peptide in its free form inhibits cell spreading (Ruosalahti, 1988a), it is likely that spreading is mediated via an RGD peptide. This would indicate that spreading and adhesion to Lm are related. However it may be that the 68kD Lm receptor to the CDGYPSIR site, which also binds specifically (Kd $6x10^{-7}$) to actin filaments (Brown *et al*, 1983), may connect the intracellular cytoskeleton to the extracellular matrix and influence cell shape (Cody and Wicha, 1986).

4.1.3 Other glycoproteins

Other molecules which are known to cause spreading and adhesion are vitronectin (Yamada, 1983), vWF (Santoro, 1987) and thrombospondin (Varani *et al*, 1986). However, tenascin has been shown not to promote spreading (Chiquet-Ehrismann *et al*, 1988).

4.1.4 In vitro assays to investigate spreading

Since the spreading phenomenon is of potential importance in haematogenic metastasis, many *in vitro* assays have been developed to investigate the properties of spreading. Since spreading is, in part dependent on adhesion it seemed pertinent to investigate whether the molecules which had promoted adhesion, would also promote spreading.

One method of investigating spreading molecules depends on the binding characteristics of putative molecules to a variety of substrates - in particular to tissue culture-grade plastic (Yamada, 1983). In these experiments, test proteins were bound to culture-grade petri dishes and seeded with tumour cells. Light microscopy was used to give a direct measurement of the spreading-promoting activity of the test molecule. I took these experiments one step further by measuring the mean surface area of cells spread on numerous test molecules. This substrate also proves useful for testing inhibitors of spreading, such as antibodies directed against the spreading molecules or against the cell surface. Therefore the aim of this part of the thesis was to see if the adhesion molecules extracted from BAE cells could also promote spreading of B16F10 cells. This together with the use of the antibodies used in the previous chapter would give some indication of whether the processes of adhesion and spreading are related.

4.2 MATERIALS AND METHODS

4.2.1 Cell spreading assay

100ul of sterile protein solution in PBS, at a wide range of dilutions, was incubated in a tissue culture grade 96-well flat bottomed plate (Nunc) for 2h at 37°C. Excess protein solution was aspirated off and additional binding sites on the plate were blocked with sterile 1% BSA in PBS for 1h at 37°C.

B16F10 cells were harvested as usual and resuspended in serum free EC medium with 1% BSA to a final concentration of $4x10^4$ cells/ml. 100ul of cell suspension was added to each well. The percentage of spread cells to total number of cells was determined in one field of view at x20 objective lens magnification at 30 min and 60 min (See Fig 19).

Photographs were taken of cells spread after 3h on protein, dotted onto tissue culture-grade petri dishes. This time was chosen in order to achieve maximum spread for all the proteins. In order to quantify spreading, the surface area of the spread cells was determined for each protein using a computer programme written by Mr K Thom in the Department of Biology and Preclinical Medicine, St Andrews. This involved tracing 10 cells onto a digitized pad and the computer calculated the mean surface areas from this data.

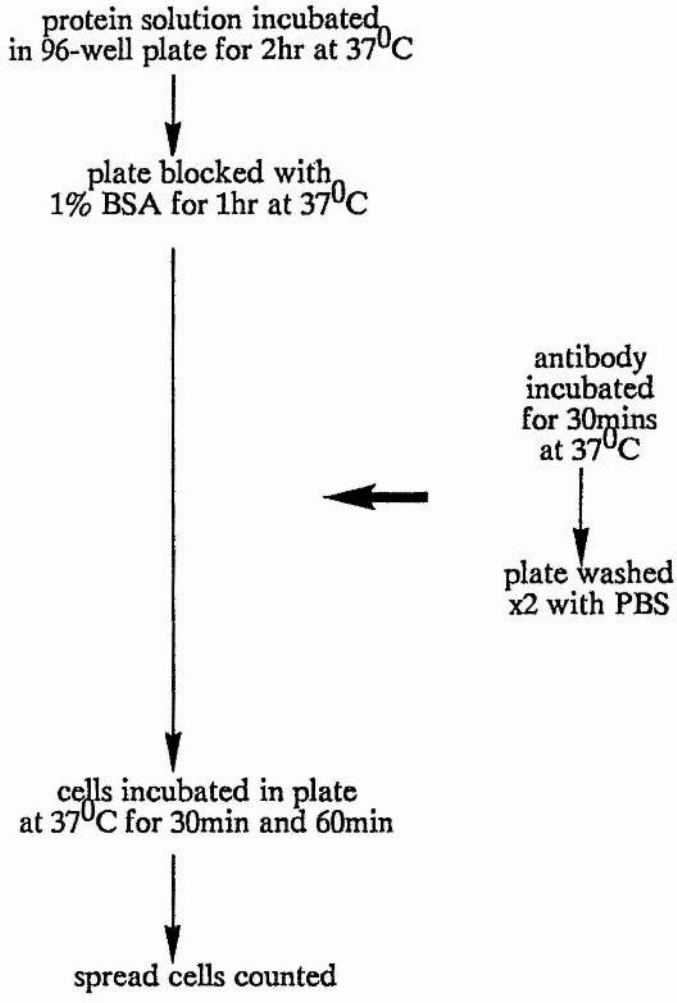
4.2.2 Inhibition of spreading

A dose-response curve was set up for each test protein. The optimum protein concentration which promoted spreading was

used when inhibition of spreading by antibodies was being tested. The method followed was as for Part 4.2.1, but prior to cell incubation, a 100ul dilution of antibody was incubated in each well at 37°C for 30 min. The antibody was washed out with PBS before adding the cells. (See Fig 19).

Fig 19 shows the protocol used in spreading and inhibition of spreading

SPREADING AND INHIBITION OF SPREADING



4.3 RESULTS

4.3.1 Spreading assay

A comparison was made of the spreading activities of the B16F10 cells on the extracts obtained from endothelial cell monolayers, which had been shown to promote adhesion in the previous chapter. A spread morphology was defined as a cell where the nucleus was clearly visible and where the cell either possessed a thin, well-spread cytoplasm or had mor than three cytoplasmic extensions. The spreading responses are shown in TABLES 5-7.

The spreading response to BAE cell surface extract (CE) was 34% and 80% at 700ug/ml after 30min and 60min respectively. On conditioned medium of the BAE cells (CEM) the spreading responses were 40% and 70% at 4mg/ml after 30min and 60min respectively.

In comparison, the controls Fn and Lm promoted greater spreading. On Fn a peak response of 80% at 40ug/ml after 30min and 86% after 60min was achieved. On Lm a peak response of 85% at 30ug/ml after 30min and 98% after 60min was achieved. On fetal calf serum (FCS) 19% of the tumour cells spread at 4mg/ml after 30min. This value increased to 78% after 60min. The cell-free extracts showed little, if any tumour cell spread-promoting ability. These differences in concentration are due to the extracts not being pure. If the active components are purified, the peak responses would probably be at concentrations similar to those for Fn and Lm.

4.3.2 Spreading morphology

Under the light microscope the morphologies appeared to vary although it was noted that all the extracts met with the criteria for a spread morphology.

Photographs 13-18 show the spread morphologies of tumour cells after 3h incubation (in order to obtain maximum spread of cells), using light microscopy.

TABLE 5 shows the % number of spread cells for CE and CEM after 30min.

n=4

CE conc	% spread ± S.D.
0.07ug/ml	0.0 ± 0.0
0.7ug/ml	0.0 ± 0.0
7.0ug/ml	0.0 ± 0.0
70ug/ml	0.0 ± 0.0
700ug/ml	34.0 ± 2.1

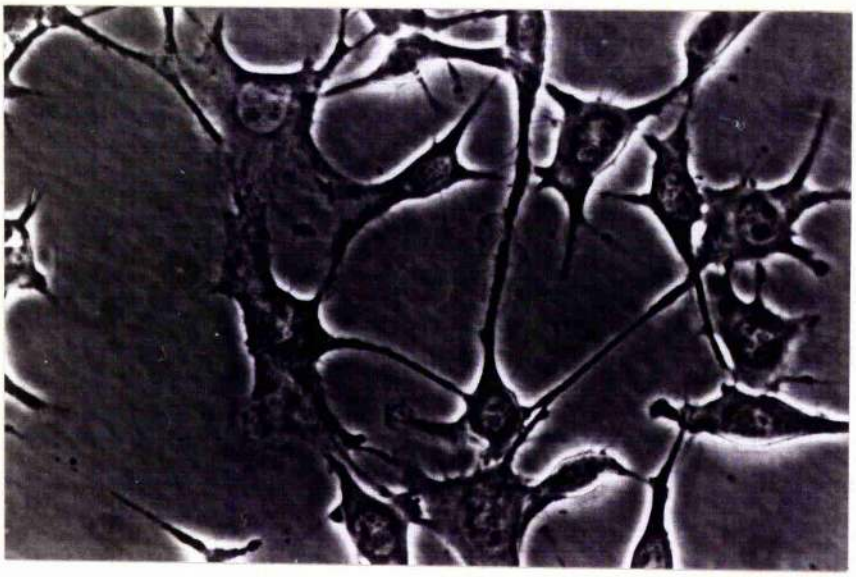
CEM conc	% spread ± S.D.
4ug/ml	0.0 ± 0.0
40ug/ml	0.0 ± 0.0
400ug/ml	15.0 ± 1.2
4mg/ml	34.2 ± 3.1
40mg/ml	40.0 ± 2.2

Photograph 13

Shows the spread morphologies of B16 cells on 700ug/ml CE after 3 hours. Magnification x270.

Photograph 14

Shows the spread morphologies of B16 cells on 4000ug/ml CEM after 3 hours. Magnification x280.



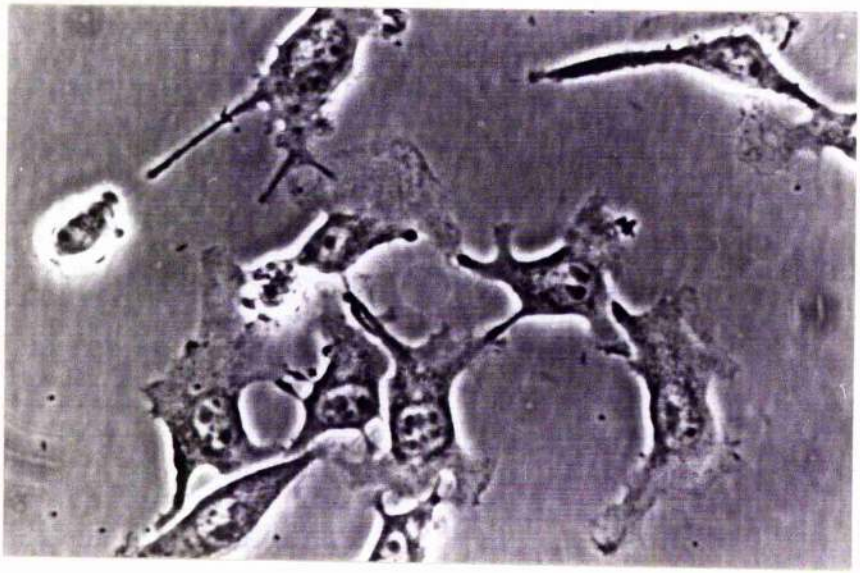


TABLE 6 shows the % number of spread cells for Fn and Lm after 30min.

n=4

Fn conc	% spread ± S.D.
10ug/ml	55.4 ± 2.1
20ug/ml	63.7 ± 6.1
30ug/ml	70.3 ± 2.6
40ug/ml	80.2 ± 3.0
50ug/ml	69.5 ± 2.4

Lm conc	% spread ± S.D.
10ug/ml	57.2 ± 1.0
20ug/ml	80.4 ± 2.4
30ug/ml	85.8 ± 1.3
40ug/ml	78.1 ± 4.8
50ug/ml	74.4 ± 3.2

TABLE 7 shows the % number of spread cells for FCS

after 30min.

n=4

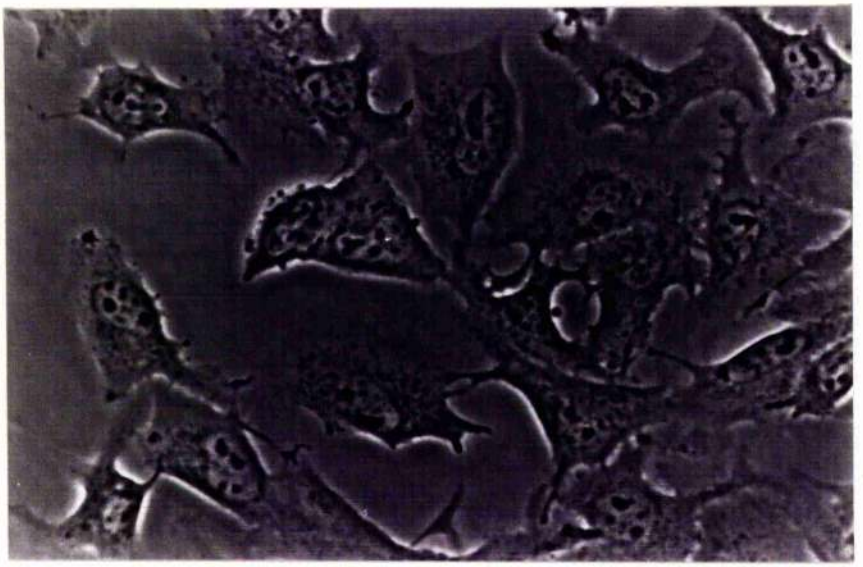
FCS conc	% spread ± S.D.
4ug/ml	12.4 ± 1.3
40ug/ml	14.2 ± 2.4
400ug/ml	12.1 ± 6.2
4mg/ml	19.5 ± 4.8
40mg/ml	10.3 ± 3.2

Photograph 15

Shows the well spread morphologies of B16 cells on 40ug/ml Fn after 3 hours. Magnification x550.

Photograph 16

Shows the spread morphologies of B16 cells on 40ug/ml Fn after 3 hours. Magnification x270.



Photograph 17

Shows the spreading morphologies of B16 cells on 30ug/ml Lm after 3 hours. Magnification x270.

Photograph 18

Shows the spread morphologies of B16 cells on 4000ug/ml FCS after 3 hours. Magnification x270.

Quantification of the surface areas of the spread cells was made from photographs of the spread cells. The results are as follows.

TABLE 8 shows the surface areas of spread cells on various proteins

n=10 number of spread cells

Protein conc.	Mean Surface Area um^2 ± S.D.	% unspread ± S.D.
Fn 40ug/ml	884.7 ± 236.5	11.5 ± 3.2
Lm 30ug/ml	1018.4 ± 362.7	5.3 ± 0.2
CEM 4000ug/ml	875.5 ± 116.1	16.1 ± 1.5
CE 700ug/ml	989.5 ± 248.7	13.0 ± 2.1
FCS 4mg/ml	510.4 ± 115.5	20.2 ± 0.5

The results indicate that there is little difference in the surface areas occupied by cells spread on Fn, Lm, CE and CEM. However, from t-tests, FCS is significantly different from Fn (t=2.93, p=0.013), Lm (t=3.15, p=0.0092), CEM (t=4.15, p=0.0013) and CE (t=4.15, p=0.0013).

4.3.3 Antibodies in spreading assays

The optimum protein concentration used for testing inhibition of spreading by antibodies, was obtained from the dose-response curves set up in Part 4.3.1a.

a) Endothelial cell extract

None of the tested antibodies showed any inhibition of spreading to CE.

b) Conditioned medium extract (CEM)

The antibodies which showed a significant inhibition to 40mg/ml CEM were anti-FN1 (2.5ug/ml), anti-FN2 (14ug/ml), anti-Fn (18mg/ml), anti-BAE EXT (3.2mg/ml) and anti-ATIII (1.8mg/ml). The F ratios (as determined by analysis of variance) are indicated in Fig 20. TABLE 9 and Fig 20 show a summary of the responses of the tested antibodies.

TABLE 9 shows the effect of active antibodies in inhibiting cell spread to CEM

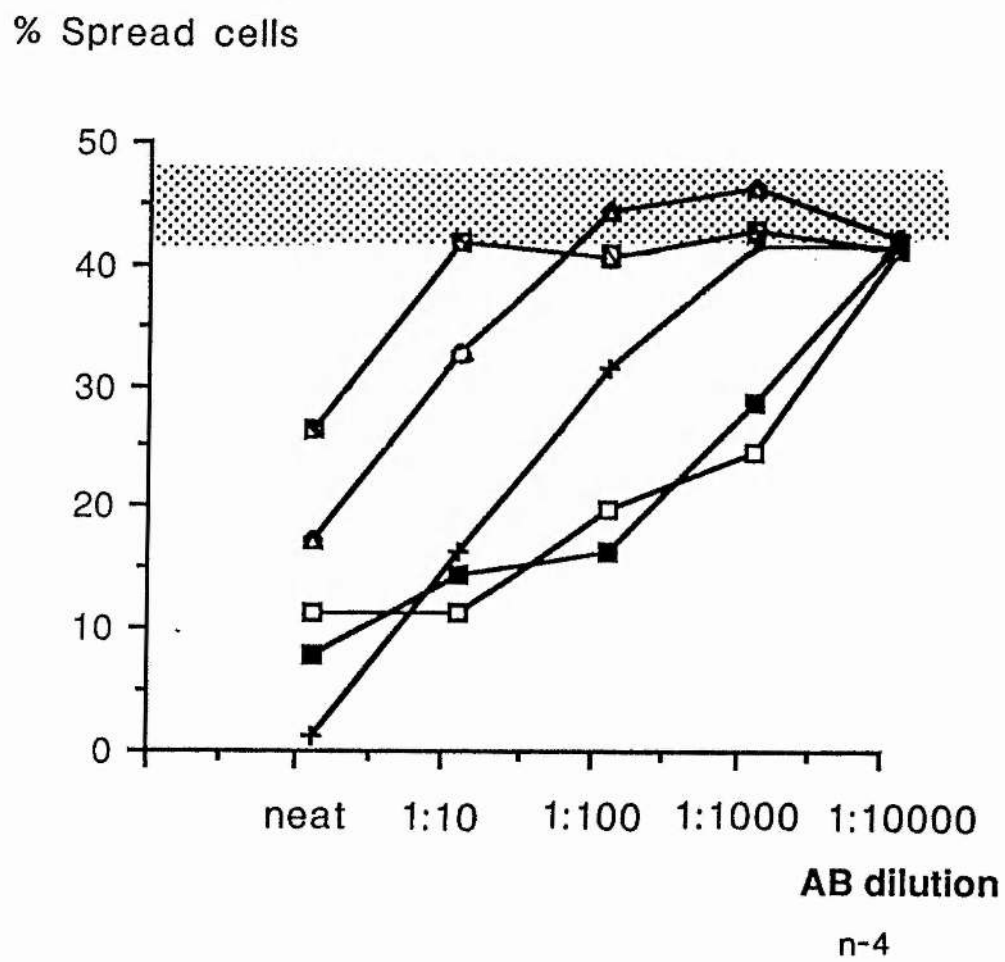
n=4

	% number of spread cells ± SD				
antibody	neat	1:10	$1:10^2$	$1:10^3$	$1:10^4$
a-FN1 (m) (250ug/ml)	10.1 ± 1.0	10.2 ± 1.3	18.5 ± 3.2	23.4 ± 4.2	40.0 ± 5.1
a-FN2 (m) (650ug/ml)	6.5 ± 2.1	13.2 ± 2.3	15.1 ± 1.6	27.4 ± 5.1	40.7 ± 5.3
a-BAE EXT (p) (3.2mg/ml)	27.6 ± 2.3	30.8 ± 4.1	38.4 ± 3.2	50.1 ± 5.3	40.1 ± 1.3
a-FN (p) (18mg/ml)	25.1 ± 3.1	44.6 ± 2.5	39.2 ± 4.1	41.6 ± 2.4	39.9 ± 4.5
a-ATIII (p) (17mg/ml)	0.0 ± 0.0	15.1 ± 2.6	30.3 ± 7.1	40.1 ± 5.3	40.1 ± 4.7
a-SP (p) (1mg/ml)	38.2 ± 4.2	39.3 ± 3.8	36.1 ± 4.1	37.5 ± 3.2	38.2 ± 3.2
a-LM (p) (1mg/ml)	39.1 ± 2.3	39.0 ± 2.6	37.3 ± 3.4	38.5 ± 4.1	40.1 ± 3.5

The neat concentration of the antibodies is shown in brackets.

Fig 20 shows the effect of a) anti-FN1 (m), b) anti-FN2 (m), c) anti-ATIII (p), d) anti-BAE EXT (p) and anti-FN (p) on the spreading response of B16F10 cells on CEM (40mg/lml).

S.D. is as shown on TABLE 9.



4.4 DISCUSSION

It was observed in Chapter II that following adhesion to the endothelium, the B16F10 cells spread on the substrate. Morphological changes also occurred in the tumour and endothelial cells during their interaction with each other, which have been previously discussed. It therefore appears that adhesion and spreading of tumour cells onto the vascular endothelium are important processes which occur during extravasation. Kramer and Nicolson (1979), suggested that molecules present in serum and on the endothelial cells may have a role to play in promoting the spreading of metastasising tumour cells onto the vascular endothelium as they did in adhesion.

From the results obtained in Chapter III, it was established that adhesion of tumour cells was promoted by molecules from the endothelium.

The spreading assay used in this study indicated that both CE and CEM promoted spreading of the B16F10 cells. In contrast, the controls (FCS and CFE) promoted little spreading. Analysis of the surface areas covered by the cells on the various extracts revealed that on the spreading-promoting substrates the surface areas were very similar and in contrast to those on the inactive substrates. This therefore appears to be a more satisfactory means for measuring spreading promoting properties of a molecule than just by looking at the morphology of the cells.

4.4.1 Immunological approach to spreading

Spreading on CE was not inhibited by any of the antibodies used in this study. The reasons for this can only be speculated at and may be verified by the fact that adhesion to CE was only slightly inhibited by anti-BAE EXT and anti-Lm. For future work it would be interesting to see if both these antibodies together would inhibit spreading.

Spreading to CEM was significantly inhibited by the fibronectin monoclonal antibodies anti-FN1 and anti-FN2 and polyclonal antibodies anti-Fn, anti BAE-EXT and anti-ATIII. These are the same antibodies which inhibited adhesion. Also from previous analysis it may be presumed that these antibodies are reacting with Fn (187kD fragment) and an ATIII/thrombin/Vn complex. This indicates that spreading is dependent on the same molecules in spreading and adhesion on CEM. From this comes the suggestion that spreading is an extension of adhesion and not an independent process. It will remain to be seen if control of these two processes is also the same.

4.4.2 Conclusion

It would appear from the results obtained in this chapter that CEM promotes spreading of B16F10 cells by an 187kD fragment of Fn and a Vn/ATIII/thrombin complex. For CE it is not possible to deduce the spreading moiety since none of the antibodies inhibited it. Therefore it can be seen that it is likely that endothelial cell proteins play an important role

in inducing spreading of the tumour cells and may do so through molecules such as Fn and Vn. However, there may be other molecules invoved, since spreading to CE could not be inhibited.

The way in which these results fit in with the observations made in Chapter II are as follows: *In vitro* molecules such as Fn, Vn and ATIII réleased by the BAE cells into the culture medium (CEM) (Hatcher *et al*, 1986; Preissner *et al*, 1988) could attach to the plastic and cause the B16F10 cells to spread on the substrate.

The way in which CE may promote spreading is less clear. One possibility is that as the BAE cells retract from the tumour cells, they leave behind surface molecules onto which the B16F10 cells can spread. This could explain why prior to rolling the monolayer, the tumour cells were elongated, but on moving underneath the monolayer, the tumour cells appeared to be well spread (see Part 2.4.2). An alternative explanation would be that the tumour cells are spreading on the extracellualr matrix laid down by the BAE cells (Birdwell *et al*, 1978; Kramer and Nicolson, 1982).

V MIGRATION

5.1 INTRODUCTION

A natural conclusion to this thesis seemed to be to study migration of the tumour cells through the endothelium. Three steps leading to migration were proposed by Liotta (1986):

(1) tumour cells attach to components of the matrix via receptors to molecules such as Lm and Fn.
(2) hydrolytic enzymes are secreted - the matrix is degraded, including attachment components.
(3) locomotion occurs by pseudopodial extension and contraction, and by further attachment to components of the matrix. A repetition of these three steps may occur until the tumour cell is through the barrier. (See Fig 21 taken from Liotta, 1986).

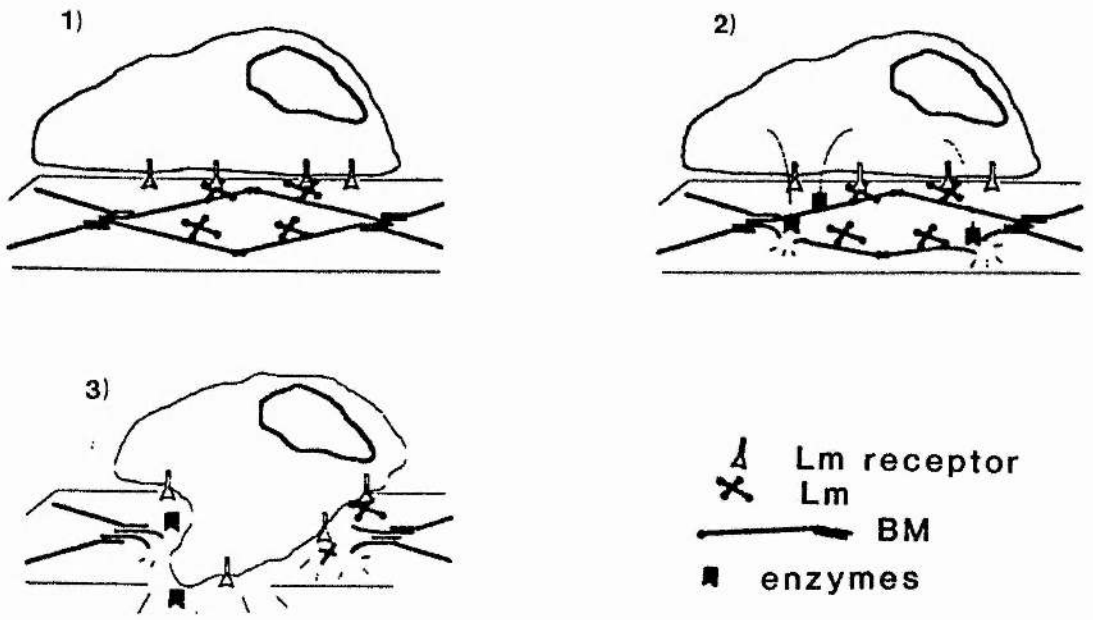


Fig 21 shows the three steps to extravasation.

After implantation in the microcirculation, blood-borne metastatic cells must penetrate the endothelial basal lamina in order to extravasate: thus it is likely that they have the capacity to degrade all the major components of the extracellular matrix (Jones and De Clerck, 1982). The way in which tumour cells might penetrate the basement membrane can be seen using re-constituted matrix gels of collagen, Lm and entactin. Initially, some of the tumour cells penetrate the barrier leaving channels in the gel (Kramer *et al*, 1986) and others follow through the channels. At the front of the invading cells, irregular pseudopodia are projected in the direction of migration which implies that focal hydrolysis of the matrix occurs at the adhesion contact between pseudopodia and matrix molecules. Presumably, at the site of invasion, proteolytic enzymes are released from the pseudopodia of tumour cells and once the matrix substance has been removed the tumour cells are able to migrate forward (Terranova, 1986). Proteinase activity released by the tumour cells therefore helps the cells to penetrate the barriers. Evidence supporting this is that a greater proteinase activity is released by malignant tissue compared with control tissues (Liotta *et al*, 1980). Furthermore, using a metabolically labelled, basal lamin-like matrix to monitor the release of radio-label on exposure to tumour cells, it is possible to identify the components which are susceptible to enzymatic degradation (Nakajima *et al*, 1983). The evidence that proteolytic degradation of BM plays a role in invasion

includes the following: 1) that tumour cells can actively degrade BM or BM-related products *in vitro* (Liotta *et al*, 1980); 2) that purified proteases have been derived from tumour cells which degrade BM collagen (Weiss, 1985); and 3) that protease inhibitors block or partially inhibit invasion in some model systems (Jones and De Clerck, 1982).

The major proteinases released by tumour cells (and sometimes by host tissue as a result of the tumour cells) include plasminogen activators, collagenases, proteoglycanases and cathepsins. Minor proteases include elastase, gelatinase and stromolysin (Tryggvason *et al*, 1987).

Plasminogen activators are serine proteolytic enzymes and play a part in the migration of tumour cells as follows: Plasminogen activators (PA) convert plasminogen in the extracellular matrix to plasmin which can degrade Lm, Fn and entactin (Liotta *et al*, 1981; Balian *et al*, 1979), and can activate collagenases to degrade collagen. B16F10 cells use PA and collagenase IV to invade the BM (Reich *et al*, 1988). Control of PA is achieved through the tumour-promoting phorbol ester (TPA) and by oncogenic transformation (Quigley, 1979). Proteoglycanases degrade BM and connective tissue components during invasion and may expose the ground substance so that collagen is exposed to further collagenase activity. Cathepsins can degrade Lm and proteoglycans (Laug *et al*, 1983).

One way in which it is thought that the tumour cells are activated to migrate through the BM is by following a chemical

gradient either chemotactically or haptotactically (Varani *et al*, 1985).

Chemotaxis may be defined as the directed migration of cells in response to a soluble gradient of attractant (Lan, 1987). Haptotaxis, on the other hand, may be defined as the directed migration of cells in response to a substrate bound insoluble gradient of attractant (Lackie, 1986). Some of the factors known to promote chemotactic and/or haptotactic migration include the adhesion-promoting molecules discussed in Chapter III.

5.1.1 Molecules involved in migration

Perhaps the best characterised of the adhesion proteins which also promotes haptotaxis is Fn (Lacovara *et al*, 1984). Lm and Vn also promote directional haptotactic migration over substratum bound gradients of the molecule (McCarthy *et al*, 1985; Basara and McCarthy, 1985; Iwamoto *et al*, 1988).

The involvement of Fn, Lm and Vn in cell adhesion and spreading and in possible interactions with the cytoskeleton suggests a possible involvement in cell migration and, as has been reported, these molecules can promote cell migration *in vitro* (McCarthy *et al*, 1986). This raises the possibility that Fn-, Lm- and Vn-containing extracellular matrices might be involved in promoting and/or directing cell migration *in vivo* (Wewer *et al*, 1987). Since it was shown in the previous chapters that the endothelial cells synthesize Fn, Lm and Vn,

the aim of this part of the study was to demonstrate the role of the endothelial cells in tumour migration.

One way in which the tumour cells might migrate through the endothelial monolayer could be due to an increasing concentration (gradient) of molecules such as Fn, Lm and Vn from the apical surface of the endothelial cell to the interstitium *in vivo* (McCarthy *et al*, 1985). For example, it is known that Lm and Fn are present at a high concentration in the sub-endothelium compared with that on the apical surface of the endothelial cells (Kramer *et al*, 1980). It is possible that the mechanism may be due either to adhesion to a natural gradient set up by proteins on the surface of the endothelial cells or in the extracellular matrix. On the other hand migration may be induced by some other mechanism which is not dependent on adhesion.

5.1.2 Boyden chamber assay

In 1962, Boyden introduced a technique for the measurement of chemotaxis of leucocytes. The chemotactic substance was separated from the cells by a micropore filter with pores of a size such that the cells were able to squeeze through the filter by actively migrating, but not to drop through it passively. The cells were allowed to settle on top of the filter and the chemotactic factor in solution was placed underneath. The chemotactic factor then diffused through to the top surface of the filter from below. Thus a concentration gradient was set up which was recognised by the cells on top of the filter. The gradient may either be

soluble and non-established or insoluble and pre-established. Investigations using Boyden chambers included work on Fn (McCarthy and Furcht, 1984), Lm (McCarthy and Furcht, 1984) and Vn (Basara and McCarthy, 1985) on the assumption that tumour cells in blood-borne metastasis encounter gradients of these or other proteins in and around the endothelial lining.

This method, is a useful technique for measuring the migration of cells towards chemoattractants. The technique is versatile since the chemoattractants may or may not be substrate bound (haptotaxis and chemotaxis respectively) and may or may not be in the form of a gradient (taxis as opposed to kinesis). Furthermore, it is useful in that inhibitors to migration can also be used.

5.2 MATERIALS AND METHODS

5.2.1 Migration assay in Boyden Chambers

A 13mm diameter polycarbonate filter (Nucleopore - 8um pore size), was spplied dull side up with UHU glue to a 2.5ml syringe which had been sawn off at the 0.2ml level. This formed the upper chamber, while the lower chamber consisted of a 5ml Teklab tube (see Fig 22).

The B16F10 cells were harvested as usual and resuspended in serum free EC medium to $1x10^6$ cells/ml. Cell suspension (160ul) was placed in the upper chamber with 40ul serum free EC medium in the cases of chemotaxis[1] and random[2] locomotion or with 40ul presumptive attractant in PBS in the case of chemokinesis[3]. In the lower chamber, 100ul chemoattractant solution was added to 400ul serum free EC medium for chemotaxis or chemokinesis. For random testing only 500ul medium and no protein was added. In the case of haptotaxis[4] the lower chamber contained 100ul protein to 400ul medium and the upper chamber 200ul medium. This apparatus was left overnight at 37°C. The next day both chambers were washed twice with PBS and re-assembled. This time the lower chamber contained 500ul medium and the upper chamber contained 160ul cells and 40ul medium. TABLES 10 and 11 show a summary of the constituents in the upper and lower chambers.

1 *Directional locomotion of cells to a concentration gradient of a soluble attractant*

2 *Locomotion of cells in the absence of an attractant*

3 *Locomotion of cells in the presence of a soluble chemical attractant, but not in the form of a gradient*

4 *Directional locomotion of cells to a concentration gradient of a substrate bound chemical attractant*

Fig 2 shows a schematic representation of a Boyden Chamber.

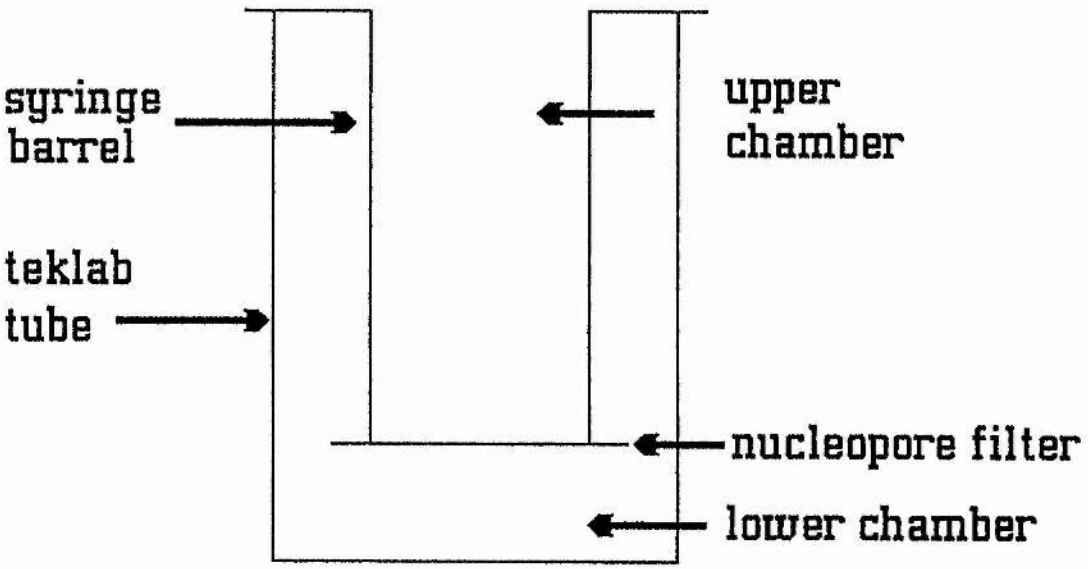


TABLE 10

Chemokinesis, chemotaxis and random locomotion:

Chemotaxis - soluble gradient of attractant in lower chamber

Chemokinesis - soluble attractant in lower and upper chambers

Random - no attractant

LOWER CHAMBER		
chemokinesis	chemotaxis	random
100ul protein	100ul protein	500ul medium
400ul medium	400ul medium	

UPPER CHAMBER		
chemokinesis	chemotaxis	random
160ul cells	160ul cells	160ul cells
40ul protein	40ul medium	40ul medium

For all types of migration the chambers were covered over with cling film and left at 37°C for 4h in an incubator. At the end of the incubation period the syringe barrels were placed into a holder and immersed in 96% alcohol for 2.5min to enable the filters to be peeled off. The filters were then re-placed into the holder dull side up and stained as described in Appendix 1.

TABLE 11

Haptotaxis:

Haptotaxis - insoluble gradient of attractant within the filter

LOWER CHAMBER	
overnight incubation	experimental conditions
100ul protein	500ul medium
400ul medium	

UPPER CHAMBER	
overnight incubation	experimental conditions
200ul medium	160ul cells
	40ul medium

The top of the filters (dull side) were then wiped with cotton wool soaked in 100% ethanol before being left in xylene for 2min. After this time the filters were mounted onto glass slides (dull side up) using styrene. Spread cells which had migrated through each filter were scored in 18 fields of view at x20 objective lens magnification. The mean number of cells/cm^2 was calculated.

5.3 RESULTS

In this part of the study, proteins extracted from the endothelium were studied in a Boyden chamber assy to see if they would promote migration. A comparison was made of the chemotactic, chemokinetic, haptotactic and random movements of B16F10 cells to the endothelial derived extracts (CE and CEM). Fn and Lm were used as positive controls and FCS and CFE were used as further controls.

5.3.1 Boyden Chamber assay

Chemotaxis, haptotaxis and chemokinesis dose-response curves were set up using CE (0.07-700ug/ml), CEM (4ug/ml-4mg/ml), Fn (5-40ug/ml), Lm (10-50ug/ml) and FCS (4ug/ml-4mg/ml). The mean number of migrated cells at x20 objective lens magnification was calculated from 18 readings per filter.

a) CE

There was no migration response to CE.

b) CEM

There was no migration response to CEM.

c) FCS and cell free controls

There was no migration response to these proteins.

d) Fn

The peak responses for chemotaxis, chemokinesis and haptotaxis caused by Fn were at 20ug/ml, 20ug/ml and 30ug/ml respectively as shown in TABLE 12. The peak responses for chemotaxis and haptotaxis were similar (although haptotaxis was higher), but for chemokinesis the peak response was about 12x lower than that for chemotaxis or haptotaxis. Using analysis of variance (ANOVA) the results indicated that chemotaxis and chemokinesis were significantly different ($F=2775.7$, $(df:1,20)$) ; values obtained for chemotaxis and haptotaxis were significantly different ($F=3791.4$, $(df:1,20)$) as were values obtained for chemokinesis and haptotaxis ($F=10988.7$, $(df:1,20)$).

c) Lm

The peak responses for chemotaxis, chemokinesis and haptotaxis for Lm were at or above 50ug/ml, 50ug/ml and at 40ug/ml respectively as shown in Fig 23 and TABLE 13. It may be that the peak responses for chemotaxis and chemokinesis are above 50ug/ml, but without using more concentrated solutions (due to the high cost of Lm) this could not be determined. However it could be seen that the peak response for chemotaxis would be higher than that for haptotaxis which is in contrast to what occurs for Fn.

It was observed that prior to wiping the surface of the filters, the way in which the cells settle on the filter differs for the various proteins. For Fn and Lm, the cells

settled thickly on the top surface. However, for FCS, CE and CEM, very few cells appeared to cover the surface of the filter. When the filters were examined using an inverted microscope, before wiping, it was observed that for FCS, CEM and CE that the cells were aggregated. One possibility worth considering is that the aggregated cells were unable to migrate through the pores. This aggregation phenomenon was observed when cells were suspended in a solution of these proteins but to a much lesser extent in Fn and Lm. (See Appendix 8).

TABLE 12 shows chemotaxis, chemokinesis and haptotaxis locomotion of B16F10 cells to Fn. n=3

Fn conc	Chemotaxis cells moved /cm^2 ± S.D.	Chemokinesis cells moved /cm^2 ± S.D.	Haptotaxis cells moved /cm^2 ± S.D.
5ug/ml	340.9 ± 13.0	13.6 ± 2.1	25.0 ± 1.0
10ug/ml	363.6 ± 13.0	13.6 ± 1.2	288.6 ± 4.2
20ug/ml	397.7 ± 29.5	47.7 ± 3.2	350.0 ± 13.6
30ug/ml	211.4 ± 13.5	20.5 ± 2.2	431.8 ± 12.1
40ug/ml	179.5 ± 9.5	11.7 ± 4.4	284.1 ± 7.2

TABLE 13 shows chemotaxis, chemokinesis and haptotaxis locomotion of B16F10 cells to Lm. n=3

Lm conc	Chemotaxis cells moved /cm^2 ± S.D.	Chemokinesis cells moved /cm^2 ± S.D.	Haptotaxis cells moved /cm^2 ± S.D.
10ug/ml	0.0 ± 0.0	13.6 ± 3.0	211.4 ± 9.5
20ug/ml	22.7 ± 3.5	47.7 ± 3.2	468.2 ± 13.0
30ug/ml	145.5 ± 8.2	50.0 ± 2.9	518.2 ± 20.3
40ug/ml	195.5 ± 10.2	113.6 ± 8.1	540.9 ± 21.2
50ug/ml	570.5 ± 29.0	293.2 ± 12.0	475.0 ± 19.6

Fig 22 shows the migration response of B16F10 cells to Fn.

SD is shown in TABLE 12.

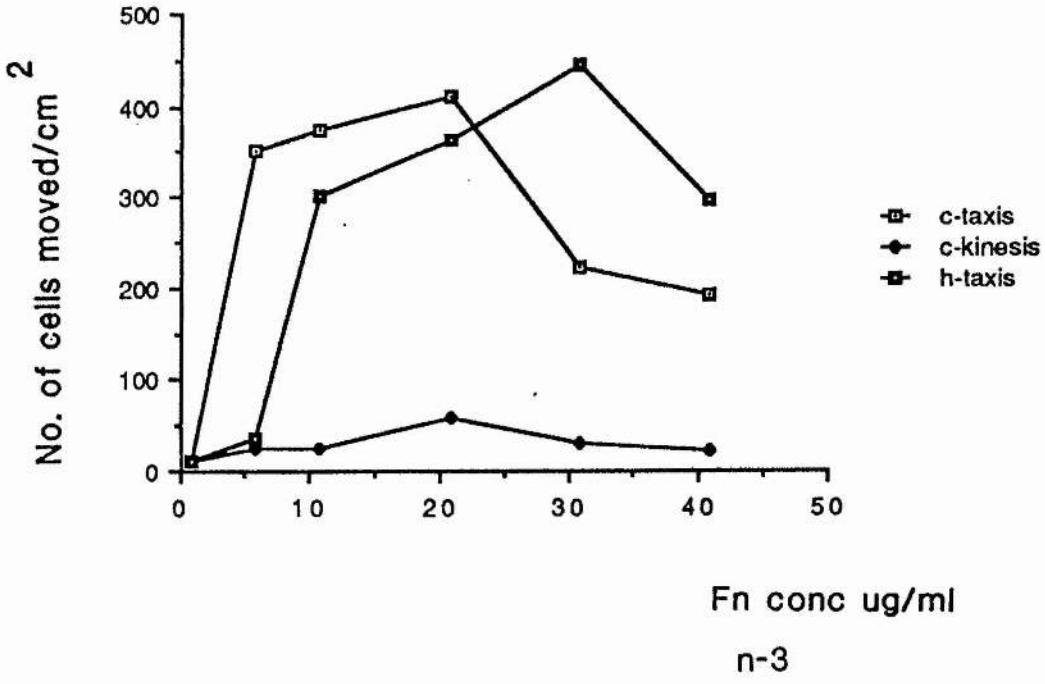
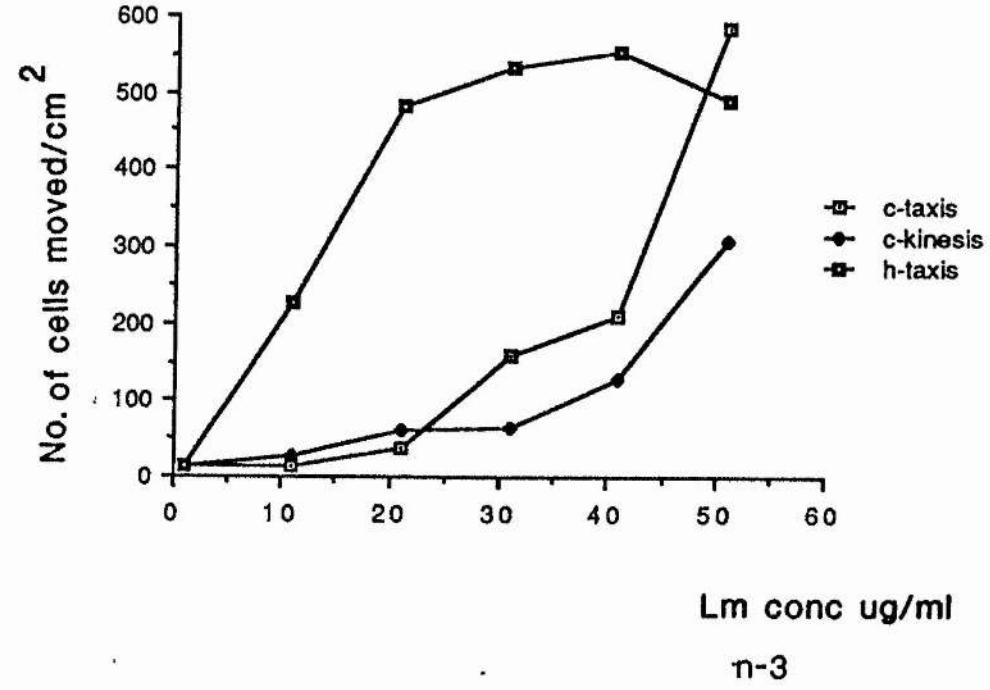


Fig 23 shows the migration response of B16F10 cells to Lm.

SD is shown in TABLE 13.



The B16 cells showed a greater response to Lm than to Fn for chemotaxis (nearly 1.5x) and chemokinesis (nearly 6x) but for haptotaxis migration was about the same, at their peak responses. For each protein, the response was also different in that for Fn the cell migrated in greater numbers to an insoluble gradient (haptotaxis), whereas for Lm they migrated in greater numbers to a soluble gradient (chemotaxis) and with an increase in random movement in the presence of Lm (chemkinesis).

All control values obtained in the absence of attractant were zero and tests of significance performed between controls and data for chemotaxis, chemokinesis and haptotaxis gave highly significant p-values except where the cells failed to migrate.

5.4 DISCUSSION

The Boyden chamber assay used in this study has enabled an investigation of the molecules which may be involved in promoting migration.

5.4.1 Migration response

Although the endothelial derived proteins have been shown to contain Lm (in the case of CE) and Fn and Vn (in the case of CEM), in the Boyden chamber assay these proteins did not promote migration. One possibility is that the aggregation promoting properties which these extracts also exhibit (see Appendix 8), result in the B16F10 cells becoming aggregated. In the Boyden chamber assay, this would mean that the tumour cells would be unable to migrate through the 8um pores of the nucleopore filter. Alternatively, there may be an inhibitory molecule present in the extracts, which while allowing spreading and adhesion to take place, inhibits migration. One way to investigate either of these suggestions would be to purify the endothelial extracts by immunoaffinity chromatography and gel filtration as has been outlined before and then to test the purified molecules in the Boyden chamber for inhibition and promotion of migration of B16F10 cells.

The results for migration to Fn indicated that it occurred mainly by chemotaxis and haptotaxis. It would appear that the peak response for chemotaxis occurred at a lower concentration (30ug/ml) than it did for haptotaxis (40ug/ml).

This suggests that functionally one of two mechanisms might be employed during migration depending on the concentration of Fn present. It would appear that the concentration gradient is important whether soluble or insoluble.

The results for Lm indicated that migration occurred through chemotaxis, haptotaxis and possible to lesser extent chemokinesis. It would appear though, in contrast to Fn, that chemotaxis occurred at a higher concentration (not determinable but at or higher than 50ug/ml). Although chemotaxis is higher than haptotaxis, haptotaxis is probably physiologically more relevant. It should also be noted that random movement in the presence of Lm was significantly increased. This was in agreement with the results obtained by Situ *et al* (1984) who used highly malignant murine fibrosarcoma cells. The potential sigfnificance of haptotaxis to Lm and Fn in the metastatic process is suggested by the studies of Varani (1982) which show a correlation between tumour cell metastatic potential and cell attachment rates to insolubilised endothelial and subendothelial components.

5.4.2 Comparison with other studies

Although Lm and Fn have been shown to promote the migration of a number of tumour cell types in the Boyden chamber assay system, there seems to be little agreement amongst investigators as to the dose-response range and peak response values for B16 cells. Lacovara *et al* (1984) have reported that concentrations of Fn below 50ug/ml do not elicit

an increase in migration over 300ug/ml. McCarthy (1984), on the other hand, has reported that the stimulation of migration occurred over a concentration range of 1-100ug/ml for Lm and Fn, with a peak value occurring between 12 and 25ug/ml for haptotaxis. These results compare better with the results of this study where a peak response for Fn was 30ug/ml and Lm was 40ug/ml. High concentrations of the Lm and Fn used in McCarthy's experiments and in the experiments of this thesis indicate that there is a fall-off from the peak values. This suggests that at high concentrations of Fn and Lm that the cells may become desensitised and no longer react to the molecules. The difference in results suggests that the Fn used by Lacovara was either impure or inactive through bad handling during its preparation.

5.4.3 Migration in malignancy

Although the finding that tumour cells migrate along insoluble, positive gradients of Lm and Fn is relevant to metastasis, it is not necessarily the case that gradients of adhesive proteins exist *in vivo*, i.e. proteins could be distributed randomly throughout the extracellular matrix. If this is the case, then it is still possible that active metastasis could occur as both Fn and Lm have been shown to accelerate the random movement of cells in the Boyden chamber assay system in this investigation and also by other workers (McCarthy *et al*, 1984; Lacovara *et al*, 1984). There is also the fact that cells use specific enzymes to degrade matrix

proteins, thereby creating gradients in their paths by loss of Lm and collagen (Barsky *et al*, 1983).

It is also plausible to propose that a natural gradient of protein(s) exists across the endothelial monolayer. Lm and Fn are present at relatively high concentrations in the subendothelial basal lamina in contrast to the low levels found on the endothelial cell apical surface (Birdwell *et al*, 1978; Kramer *et al*, 1980) and this might represent a natural gradient. This might also explain why CE does not promote migration. Similarly, Lm, which is localised in the BM has been shown in this study to be much more potent at attracting B16 cells than Fn, and therefore it is possible that the relative localisation of these proteins *in vivo* may serve to provide a natural gradient which stimulates tumour cells to extravasate.

VI CONCLUDING REMARKS

6.1 CONCLUDING REMARKS

The successful colonization of distant host sites by metastatic tumour cells proceeds by a series of complex sequential steps (Nicolson and Winklehake, 1975), that depend upon the abilities of the tumour cells to invade surrounding tissues (Weiss, 1976), enter and circulate in the blood stream (Kramer *et al*, 1982), implant in the circulatory system (Fidler *et al*, 1975), extravasate or invade the capillary endothelium (Poste and Fidler, 1980), migrate to extravascular sites (Terranova *et al*, 1986) and establish a microenvironment for subsequent vascularization and growth (Folkman and Haudenschild, 1980).

Blood-borne metastatic tumour cells adherent to the capillaries must invade both the endothelial cell layer and the sub-endothelial BM (Jones and De Clerck, 1982). This is thought to occur by a combination of enzymatic and mechanical destruction (Liotta *et al*, 1980; Chew *et al*, 1976). Since the basement membrane consists of collagen, fibronectin, laminin and a variety of other proteoglycans and glycoproteins, destruction of the matrix by invading cells may require a variety of hydrolases which act sequentially to solubilize the matrix and permit its penetration by metastatic cells (Liotta, 1986).

6.2 TUMOUR-ENDOTHELIAL CELL INTERACTIONS

Using an *in vitro* model, certain morphological and biochemical aspects of tumour cell (B16F10) interactions with endothelial cells (BAE) can be studied. The results show that B16F10 cells (either as single cells or as aggregates) are capable of adhering to the apical surfaces of endothelial cells, induce rupture of endothelial cell junctions, followed by peeling back of the endothelial cells from their underlying matrix and migration to the exposed subendothelial matrix. The exact mechanisms employed by the tumour cells could not be identified. However, it is likely that adhesion was caused by macromolecules on the surface of the endothelial cells, that rupture of the intercellular junctions occurred through enzymic degradation and the underlying matrix was important in the processes of spreading and migration.

6.3 THE ROLE OF BAE CELLS IN TUMOUR CELL ADHESION

Since it seemed likely that the endothelium might prove to be a source of adhesion promoting molecules, proteins were extracted from the surface of the BAE cells using 2mM EDTA (CE) and from the conditioned medium (CEM). These molecules were shown to promote adhesion in a dot blot assay and partial characterisation of them was achieved using SDS-PAGE and antibody immunoprobing. From these analyses it was concluded that the active components in CE were Lm and possible a 37kD protein which was not identified, but which might be similar

to a molecule reportedly involved in metastasis (Steinman *et al*, 1984). The active molecules in CEM were an 187kD fragment of Fn and Vn complexed with ATIII. These results are in agreement with reports by Hatcher *et al* (1986) and Preissner *et al* (1988). However, Preissner *et al* (1988) put forward the suggestion that these molecules are involved in inflammation and repair mechanisms. It may be that the processes of inflammation, tissue repair and tumour cell extravasation are closely inter-related, as reviewed by Sporn and Roberts (1986). In brief, Sporn and Roberts state that biochemical mechanisms by which cells and tissues respond to injury and initiate the repair process are now known to be highly relevant to the study of carcinogenesis. More recent evidence comes from studies which have shown the role of growth factors in the malignant process which are also expressed physiologically by cells that mediate inflammation and repair - namely platelets, macrophages and lymphocytes.

Use of antibodies in the inhibition of adhesion indicated that total inhibition by any single antibody could not be achieved. Therefore it remains to be seen whether a combination of antibodies could achieve this end. This was not possible through lack of time.

6.4 THE ROLE OF ENDOTHELIAL CELLS IN TUMOUR CELL SPREAD

In order to strengthen the interaction of the tumour cells with the endothelium, it was necessary for the tumour cells to spread. It seemed possible that the molecules

involved in the adhesion process might also be involved in promoting spreading. Hence, the molecules which had been extracted from the endothelial cell surface and the conditioned medium were tested for their spreading-promoting properties. The antibodies raised for testing in the cell attachment assay were also used to try and inhibit spreading. As for adhesion, CE and CEM promoted spreading of B16F10 cells. The antibodies which inhibited adhesion to CEM also inhibited spreading, but none of the antibodies tested inhibited spreading to CE.

From these results, it was concluded that the 187kD component from CEM, thought to be a fragment of Fn and the Vn/ATIII complex, promoted spreading as well as adhesion. On the other hand, spreading to CE could not be attributed to either Lm or the 37kD component, both of which had been shown to promote adhesion. It may be that molecules other than these proteins are involved in promoting spreading, or alternatively that Lm and the 37kD do promote spreading together with each other and/or with other components. One way of eliminating these possibilities would be to raise antibodies to the purified 37kD component and to use this in combination with anti-Lm to try and inhibit spreading.

6.5 THE ROLE OF THE ENDOTHELIAL CELLS IN PROMOTING TUMOUR CELL MIGRATION

Interest in the migration of tumour cells has been fuelled by its relevance to the process of metastasis, and

much research has been done in this area to reveal the molecules that tumour cells utilise in adherence and movement through tissues during invasion.

Migration follows spreading and so the molecules which had been shown to be adhesive and to promote spreading were tested in Boyden chambers for their migration-promoting properties. Neither of the cell derived extracts promoted migration, but Fn and Lm which had been used as positive controls did. One reason suggested was that the endothelial extracts caused the tumour cells to aggregate which would not allow them to migrate through the pores of the filter used in the Boyden chamber assay. In order to demonstrate the role of endothelial cells in promoting tumour cell migration it would be necessary either to purify the extracts or to use a different system for measuring migration. From the results presented in this thesis, the role of endothelial cells is to provide a barrier against the tumour cells. The role of these cells in promoting migration has not been shown. However, the molecules which are known to promote migration are found on the endothelial cells. It is therefore likely that the endothelium plays a role in promoting tumour migration.

6.6 CONCLUSION

In conclusion, it can be seen that the endothelial cells play a vital role in spreading and adhesion; their role in migration has not been proven in this study, but is likely. These preliminary results have therefore shown potential for

further studies in this area of investigation.

This study has indicated possible molecules which are involved in adhesion, spreading and possibly migration. The results suggest possible directions for *in vivo* studies and has contributed to knowledge in that it has narrowed the field so that future investigations may be confined to a small number of types of molecule. For future work on these molecules, it would be necessary to purify them using methods already outlined. The ultimate aim of further work would be in the testing of the purified molecules and monoclonal antibodies raised against them in *in vivo* studies. The antibodies could be used at different stages in extravasation - cell adhesion, spreading or migration - to see which step can be blocked in order to successfully prevent the tumour cells from forming metastases. It may be that the *in vitro* situation. However, the possible beneficial effects in the clinical situation which could ensue means that these results cannot be disregarded as they may be of help in evaluating future *in vivo* studies.

APPENDICES

APPENDIX 1 - METHODS FOR BUFFERS

1. Dulbecco's phosphate buffered saline (PBS) pH7.2

component	concentration g/l
NaCl	8.0
KCl	0.2
$Na_2HPO_4.2H_2O$	1.15
KH_2PO_4	0.2
$CaCl_2$	0.1
$MgCl_2.6H_2O$	0.1

Made up in distilled water and adjusted to pH7.2 with HCl.

2. Dulbecco's calcium magnesium free phosphate buffered saline (CMF-PBS) pH7.2

Same as above, but without the last two ingredients. Adjusted to pH7.2 with HCl.

3. Borate buffer 10x pH8.7

Component	Concentration g/l
H_3BO_3	6.2
$MgCl_2.6H_2O$	2.0
NaCl	58.4

Made up in distilled water and adjusted to pH8.7 with 1M NaOH.

4. Phosphate buffer x5 pH8.0

Solution	Component	Conc. g/l
A	$Na_2HPO_4.10H_2O$	179.0
B	$NaH_2PO_4.2H_2O$	78.0

Solutions A and B were made up in distilled water. Solution B was added to solution A to bring the pH to 8.0.

5. Citrate buffer x5 pH6.5, 4.5 or 3.5

Solution	Component	Conc. g/l
A	$Na_3C_6H_5O_7.2H_2O$	147.0
B	$C_6H_8O_7$	105.0

Solutions A and B were made up in distilled water. Solution A was added to B or B to A as appropriate to bring the pH to 6.0, 4.5 or 3.5.

6. Carbonate buffer pH11.0

component	Concentration g/l
Na_2CO_3	1.59
$NaHCO_3$	2.93

Made up in distilled water and the pH adjusted to 11.0.

7. Ortho-phenylenediamine

0.04% ortho-phenylenediamine and 0.012% hydrogen peroxide were dissolved in phosphate-citrate buffer, pH5.0.

8.Phosphate-citrate buffer, pH5.0

Solution	Component	Conc. g/l
A	$Na_2HPO_4.10H_2O$	21.0
B	$C_6H_8O_7$	28.3

9. Protocol for making up different percentage SDS containing polyacrylamide gel solutions

SOLUTION	POLYACRYLAMIDE CONC.		
	5%	10%	15%
30% acrylamide	5.0	10.0	15.0
1% bisacrylamide	7.8	3.9	2.6
1.5M tris-HCl pH8.7	7.5	7.5	7.5
distilled water	9.3	8.2	4.5
10% ammonium persulphate	0.2	0.2	0.2
10% SDS	0.3	0.3	0.3
TEMED	0.02	0.02	0.02

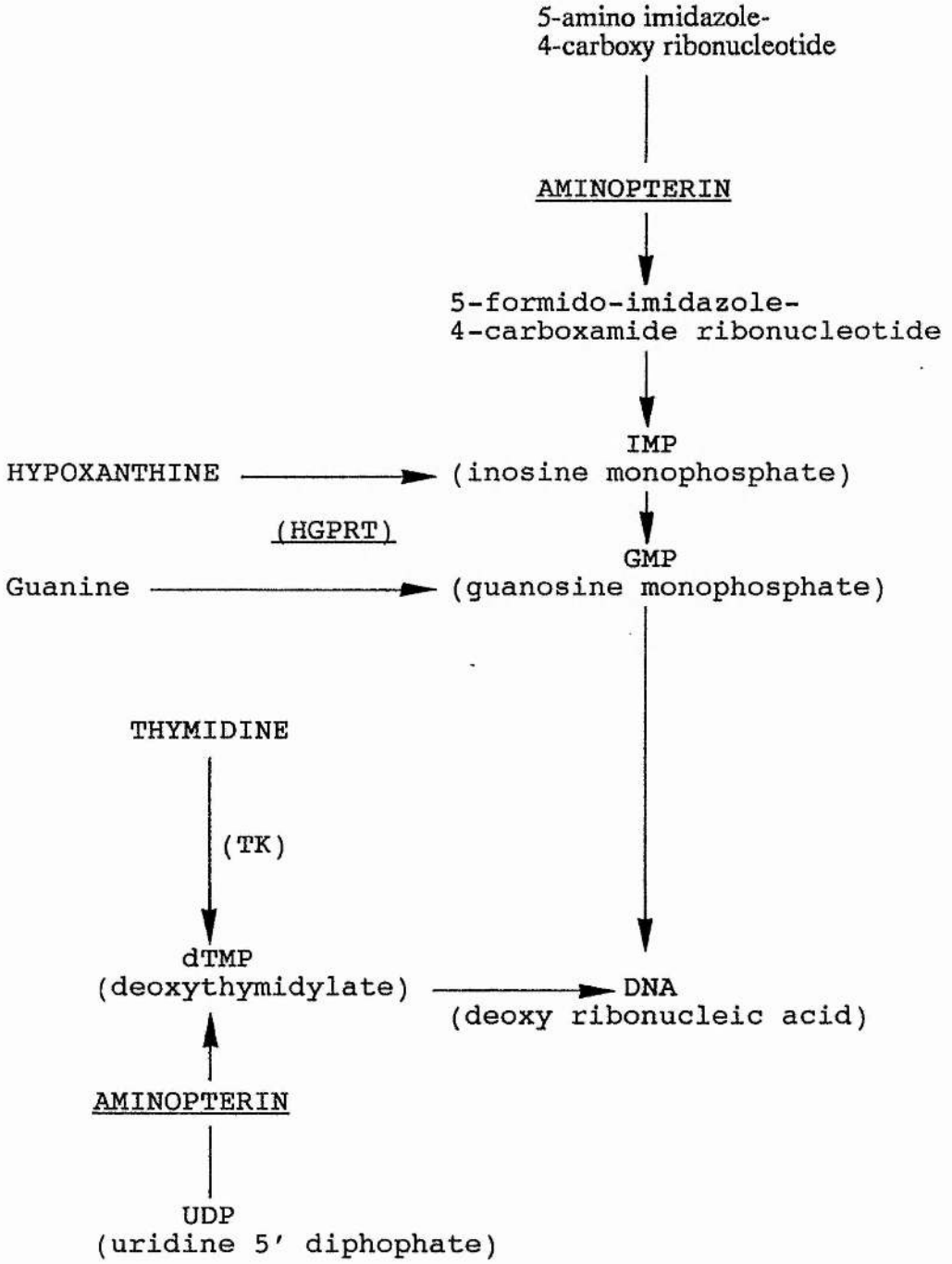
Volumes are given in ml/gel.

10. Protocol for staining the filters

SOLUTION	time (mins)
96% ethanol	2
70% ethanol	2
distilled water	2
Mayer's Haematoxylin	5
tap water	2
70% ethanol	2
100% ethanol	4

APPENDIX 2 - HYBRID SELECTION PROCEDURE

The following diagram shows the metabolic pathways relevant to hybrid selection in HAT medium. When the main systhetic pathway for guanosine is blocked by the folic acid antagonist aminopterin, the cell must use the salvage enzymes HGPRT (hypoxanthine guanine phosphoribosyl transferase) and TK (thymidine kinase). Cells lacking HGPRT (such as the JKAg8 cells) die in medium containing hypoxanthine, aminopterin and tymidine (HAT) since they are unable to use the salvage pathways. However, if these cells are fused with cells that contain HGPRT (such as spleen cells) they will survive in HAT medium.


5-amino imidazole-
4-carboxy ribonucleotide
AMINOPTERIN
5-formido-imidazole-
4-carboxamide ribonucleotide
IMP
HYPOXANTHINE
(inosine monophosphate)
(HGPRT)
GMP
Guanine
(guanosine monophosphate)
THYMIDINE
(TK)
dTMP
(deoxythymidylate)
DNA
(deoxy ribonucleic acid)
AMINOPTERIN
UDP
(uridine 5' diphophate)

APPENDIX 3 - WESTERN BLOTTING ADHESION MOLECULES

Blotting analysis of adhesive proteins: an evaluation of the technique using B16F 10 malignant melanoma cells

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The identification of adhesive molecules can proceed in several different ways, but one of the most convenient involves the adsorption of a putative adhesive molecule on to an artificial substrate (such as tissue culture plastic) followed by probing the adsorbed material with a suspension of cells. In the absence of protein in the suspending medium, most cells adsorb non-specifically to tissue culture plastic and thus any sites not occupied by the adsorbed adhesive molecules under test must be blocked with a non-adhesive agent such as albumin. The major problem of identifying an adhesive protein, however, is not so much in showing that cells adhere to it, but more in how to identify it from a complex mixture of molecules which may have been extracted from whole cells or multicomponent substrates. Within a mixture of molecules, some may block adhesion by competing for substrate adsorption with the adhesive molecule, while others may interact with the molecule in question to effectively neutralize its adhesive properties. Some of these problems may be overcome if the mixture can be fractionated and the fractions tested in an adhesion assay. In the original report on cell blotting ('bioautography') by Klebe *et al.* (1978), 50–100% (v/v) serum was electrophoresed on cellular acetate and a diffusion replica was made by overlaying on to a gel of 0.25% (w/v) type I collagen. Material which did not bind to the collagen was eluted by washing, and any adhesive molecules were then identified by probing with 4×10^7 CHO cells followed by fixing, and then staining with 0.1% (w/v) Toluidine Blue. Using this technique, two adhesive bands were identified in serum: one by its isoelectric point (approx. pI 4.8) and the other by its extremely large size (failing to run into the gel). The main limitation of this study was associated with restriction of the analysis to those molecules which bound to collagen under the conditions of the experiment. Hayman *et al.* (1982) improved upon this technique by first depleting plasma of albumin and immunoglobulin G, and then by separating the remaining proteins using SDS/polyacrylamide-gel electrophoresis (PAGE) under reducing conditions. A replica of the electrophoretic pattern was then made by diffusion blotting of the separated molecules on to a nitrocellulose filter according to the method of Bowen *et al.* (1980). After blotting, the filter was blocked with 5 mg of bovine serum albumin/ml, probed with NRK cells (10^7 cells

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; Fn, fibronectin; PBS, phosphate-buffered saline; CE, cell extract; BAE, bovine aortic endothelial; Ln, laminin; ELISA, enzyme-linked immunosorbent assay; 2-Me, 2-mercaptoethanol; TGS, 25 mM-Tris/192 mM-glycine/1% (w/v) SDS electrophoresis buffer; TGM, 25 mM-Tris/192 mM-glycine/20% (v/v) methanol blotting buffer.

in 50 ml), fixed and visualized by staining with 0.1% (w/v) Amido Black. Using this approach, Hayman *et al.* (1982) were able to identify two adhesive molecules in plasma, one of M_r 220000 and the other of M_r 70000 [corresponding to fibronectin (Fn) and vitronectin, respectively]. Since these reports, numerous laboratories have attempted to use the blotting technique to identify adhesive proteins, but with mixed success. In order to further the use of this technique we have carried out a systematic study of the method and examined its shortcomings as a technical procedure.

Preparation of adhesive proteins

Fibronectin (Fn) was either prepared from bovine blood by affinity chromatography on gelatin or it was purchased from Sigma. For affinity chromatography, plasma prepared from 50 ml of fresh blood (treated with 15 ml of standard acid/citrate/dextrose anticoagulant) was diluted 1:1 with phosphate-buffered saline (PBS), pH 7.2, and applied to a gelatin column (Biorad) equilibrated with PBS. Bound material was eluted with 50 mM-sodium acetate buffer, pH 5.0, containing 1 M-sodium bromide and dialysed against PBS before being sterile filtered and stored at 4°C.

Cell extract (CE) was prepared from cloned bovine aortic endothelial (BAE) cells (the gift of Dr. Jean R. Starkey, Montana State University, MT, U.S.A.). Briefly, six roller bottles (growth area 750 cm^2) were seeded with 6.5×10^6 BAE cells in 117 ml of Eagle's complete minimal essential medium as described by Elvin & Evans (1984), but further supplemented with endothelial cell growth factor (5 ng/ml) and insulin (0.013 i.u./ml). On reaching confluence (3 days), each bottle culture was washed three times with 100 ml of 150 mM-NaCl in 20 mM-Tris (pH 7.5), and extracted by rolling for 30 min at 37°C with 10 ml of washing medium containing 2 mM-EDTA. The extracts were then pooled, centrifuged (30 min, 1500 *g*), concentrated against flake polyethylene glycol, and stored at 4°C. Some experiments used this crude extract, but for others the crude material was adjusted to 75 mM-NaCl and pH 8.3 immediately after extraction, and then applied to a column of Q Sepharose (Pharmacia). Bound material was eluted with 500 mM-NaCl in 20 mM-Tris (pH 8.3) and then applied to a column of Sepharose G200 (Pharmacia). A high M_r (>600000) eluting in the void volume was found to have adhesive-promoting activity.

Laminin (Lm) was purchased direct from Sigma. Protein concentrations were determined by a modification of the Lowry method (Larson *et al.*, 1986).

Cell probing of dot-blots

Briefly, protein samples (about 1 μl) were dotted directly on to a nitrocellulose filter (BA85; Schleicher and Schuell, Inc.), blocked with 10% (w/v) bovine serum albumin, and then incubated with B16F10 cells (minimum 5×10^5 per cm^2 of filter) suspended in serum-free complete minimal essential medium. After 2 h the filter was washed with 10 ml of PBS per cm^2 of filter) dispensed from a syringe through a 19-gauge needle, and the remaining cells were then fixed with 70% (v/v) ethanol and stained with Mayer's haemalum. The sensitivity of the cell probing assay was tested on 10-fold serial dilutions of Lm, Fn and CE, and the results were compared with detection by enzyme-linked immunosorbent assay (ELISA) using a phosphatase-labelled second antibody detection system. Using this ELISA-based approach, the adhesive molecules were detectable down to 50 ng, 5 ng and 150 ng, respectively. Using the cell probing method, recognition of Lm (as judged by adhesive cells) seemed to be as sensitive as using ELISA (50 ng), whereas the detection of Fn was more sensitive since as little 0.5 ng applied to the filter promoted adhesion. Like the results with Lm, cell probing of CE was as sensitive as ELISA (150 ng). Although these results are dependent on the nature of the antibodies employed, they nevertheless provide an indication of the relative sensitivity of the cell probing method for identifying adhesive molecules. Interestingly, in the original study of Klebe *et al.* (1978), the serum molecule of pI 4.8 with adhesive activity was not readily detectable on Coomassie-Blue-stained gels, and thus cell probing may have considerable sensitivity over this general staining method for the recognition of adhesive molecules.

The effects of various electrophoresis and electroblotting reagents on cell probing were also tested using the dot-blotting procedure. As summarized in Table 1, 0.625% (w/v) SDS in PBS with or without 10% (v/v) 2-mercaptoethanol (2-Me inhibited the adhesion of B16F10 cells to dot-blots of Lm 250 ng), Fn (275 ng) or CE (75 ng). The inhibitory effect of SDS on adhesion was also seen when the dot-blots were treated with 25 mM-Tris/192 mM-glycine/1% (w/v) SDS electrophoresis buffer (TGS). The inhibitory effects of SDS and/or 2-Me were not overcome by additional washing with 25 mM-Tris/192 mM-glycine/20% (w/v) methanol blotting buffer (TGM) before cell probing, and it would thus seem likely that they result from the denaturing effects of SDS and/or 2-Me on the proteins tested. Regardless of the mechanism involved, these preliminary studies suggest that fewer problems might be expected with cell probing if the electrophoretic separation of putative adhesion molecules is performed in the absence of SDS and 2-Me.

Electrophoresis and electroblotting

SDS/PAGE was performed on 7.5% (w/v) slab gels under both non-reducing (0.625%, w/v, SDS in the sample) and reducing conditions (0.625%, w/v, SDS plus 10%, v/v, 2-Me). Flat bed agarose electrophoresis of non-denatured material was carried out over 2 h at 200 V using 1% (w/v) gels prepared in Tris/acetate buffer, pH 7.8. Electroblotting (Western blotting) was performed from both types of gels in a Transblot system (Biorad), using TGM at pH 8.3 as the running buffer for SDS/PAGE (Towbin *et al.*, 1979) and methanol-free TGM for agarose gels. Electroblots were blocked with 10% (w/v) BSA in PBS for 30–60 min at 37°C and then probed with B16F10 melanoma cells as described above. Protein bands in gels and blots were identified by staining with 0.02% (w/v) Coomassie Blue and 0.1% (w/v) Amido Black, respectively.

When Lm (20 μg), FN (22 μg) and CE (6 μg) were subjected to SDS/PAGE under reducing and non-reducing

Table 1. *Effects of electrophoresis and blotting agents on adhesion*

Proteins were pretreated with the various reagents under conditions similar to those in which they would be used for electrophoresis or electroblotting. The results are expressed on a semi-quantitative scale in which the reaction with each protein under control conditions (PBS) was taken as 5+.

Reagent	Fn (275 ng)	Lm (250 ng)	CE (75 ng)
PBS	5+	5+	5+
SDS	1+	—	—
SDS + TGM wash	—	—	—
SDS + 2-Me	—	—	—
SDS + 2-Me + TGM wash	—	—	—
1.5 M-Tris	5+	5+	4+
TGS	1+	1+	—
TGM	5+	5+	3+

Fig. 1. *Adhesion of B16F10 cells to electroblots*

Samples of Lm (*a*), Fn (*b*) and CE (*c*) were electrophoresed in agarose, electroblotted on to nitrocellulose and probed with B16F10 melanoma cells as described in the text. Note that the adhesive activity of Fn did not resolve into a sharp band like Lm, and that the adhesive activity of CE (known to be of high M_r) remained at the origin.

conditions, electroblotted and then probed with B16F10 cells, the results did not always indicate adhesive activity. Electroblots of Lm and purified CE, for example, failed to show any adhesive activity, while electroblots of Fn were adhesive only under non-reducing conditions. All three of these molecules are adhesive at the concentrations employed in electrophoresis as shown by standard dot-blot assays. Nevertheless, these results were essentially as expected from our preliminary studies based on dot-blots, which identified inhibitory effects of SDS and/or 2-Me on adhesion. Consequently, we next used non-denaturing conditions to test for adhesive activity after electroblotting of electrophoretically separated proteins. Fig. 1 shows an electroblot of Lm, Fn and crude CE electrophoresed in agarose under non-denaturing conditions and probed with B16F10 cells. The adherence activity of Lm is seen to reside in a sharp band, whereas that for Fn is more diffuse. Adherence of B16F10 cells to CE was seen to be localized over the origin, suggesting that this extract contained material which behaved differently to both Lm and Fn under these experimental conditions.

In our hands, the use of non-denaturing electrophoresis provides more consistent results on subsequent cell probing than does denaturing electrophoresis. Although it is difficult to resolve mixtures of proteins by M_r using agarose electrophoresis, this limitation should be overcome through the use of non-denaturing PAGE (Bryan, 1977) and considerable benefits may also accrue from the use of isoelectrofocusing. In general, electroblotting seemed to be without significant effect on subsequent cell adhesion. There thus appears to be little advantage in the use of diffusion blotting for the transfer of adhesive molecules, although this method was employed originally by both Klebe *et al.* (1978) and Hayman *et al.* (1982). We conclude that cell probing of electroblotted molecules after separation by electrophoresis can be a sensitive method for detecting adhesive substances, particularly if non-denaturing conditions are employed. Clarification of the role of CE in the adhesion of malignant melanoma cells to the endothelium during the process of blood-borne metastasis is currently under study.

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Bowen, B., Steinberg, J., Laemmli, U. K. & Weintraub, H. (1980) *Nucleic Acids Res.* **8**, 1–20.
Bryan, J. K. (1977) *Anal. Biochem.* **78**, 513–519
Elvin, P. & Evans, C. W. (1984) *Eur. J. Cancer Clin. Oncol.* **20**, 107–111
Hayman, E. G., Engvall, E., A'Hearn, E., Barnes, D., Pierschbacher, M. & Ruoslahti, E. (1982) *J. Cell Biol.* **95**, 20–23
Klebe, R. J., Hall, J. R., Naylor, S. L. & Dickey, W. D. (1978) *Exp. Cell Res.* **115**, 73–78
Larson, E., Howlett, B. & Jagendorf, A. (1986) *Anal. Biochem.* **155**, 243–248
Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4353

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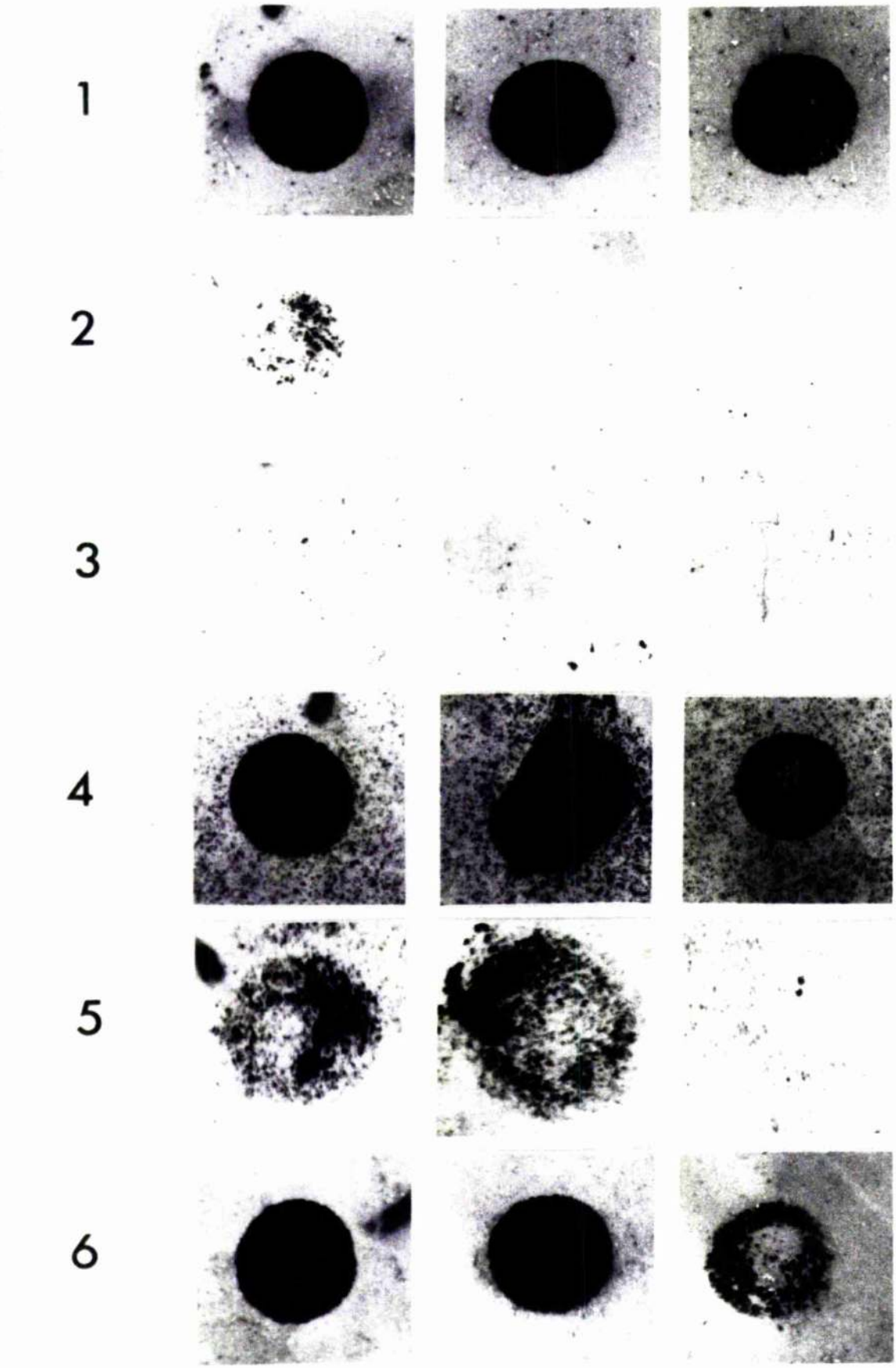
Test panel of the effect of electrophoresis and electroblotting reagents on adhesion

Reagent		TEST MOLECULE (amount blotted) Fn	Lm	CE	CEM
1.	PBS	5+	5+	5+	5+
2.	SDS	1+	-	-	1+
3.	SDS + 2-Me/ SDS + 2-Me + WBB wash	-	-	-	-
4.	1.5M tris-HCl	5+	5+	4+	4+
5.	EBB	1+	1+	-	2+
6.	WBB	5+	5+	3+	3+

See paper for details on concentrations and abbreviations. The photograph shows the effects of electrophoresis and Western blotting reagents on B16F10 cell adhesion to Fn, Lm and CE1. CE1 is the same as CE.

EFFECT OF AGENTS ON ADHESION



APPENDIX 4 - GEL DRYING PAPER

Use of a rapid method for dehydrating polyacrylamide gels after electrophoresis of cell adhesion molecules

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SDS polyacrylamide-gel electrophoresis (PAGE) is frequently used for separation and molecular mass estimation of protein components in various biological extracts. One of the major problems encountered with this technique is associated with identification and quantification of minor protein components in the extract. In addition, long-term storage of wet gels for future reference often causes problems due to storage space and the bleaching of dye from stained proteins. At present gels are stored either wet in destaining solution or are more commonly dehydrated on to filter paper using a commercial drying apparatus. Although the latter method is convenient for storage of large numbers of samples, dehydration is time consuming and gels are often susceptible to cracking or distortion during the drying process, especially if they are thicker than 1.5 mm (Hames, 1987).

In this report, a simple and inexpensive procedure is described for rapidly extracting up to 90% of the water from gels before final dehydration by vacuum aspiration. Advantages of this method are that thick gels do not crack, are less susceptible to distortion and in addition a greater number of gels can be processed much quicker than has been possible in the past.

We have been using this technique for the identification of endothelial cell proteins which promote adhesion and spreading of B16 melanoma cells (Ferro *et al.*, 1988). Cells grown in roller bottles for 3 days were washed with calcium–magnesium-free phosphate-buffered saline (CMF-PBS) and then the surface proteins were extracted by rolling for 10 min at 37°C with 25 ml of 2 mM-EDTA in CMF-PBS to give cell extract (CE). Control bottles containing medium without cells were similarly treated to give cell-free extract (CFE). The extracts were dialysed against distilled water, concentrated against flake polyethylene glycol, re-dialysed against 2 mM-EDTA, filtered through a Millipore filter (pore size 0.22 μm) and stored at 4°C until used.

Protein solution (40 μl; 100 μg of CFE/well and 10 μg of CE/well) was separated by SDS/PAGE on 2.5 mm × 80 mm × 120 mm slab gels under reducing conditions as previously described (Ferro *et al.*, 1988).

Gels were placed into about 500 ml (1:2, w/v) aquacide III slurry (Behring Diagnostics) in distilled water, and left at room temperature until the dimensions decreased from 2.5 mm × 80 mm × 120 mm to approximately 1 mm × 50 mm × 90 mm (this represents a 16-fold decrease in gel volume). Rates of dehydration of the gels were related to the acrylamide concentrations. Gels with concentrations of 5, 7.5 and 10% (w/v) took about 60, 90 and 120 min, respectively, to reduce to the stated dimensions (with removal of 90% of water). The dehydrated gels were then rinsed in distilled water and placed on to filter paper (Whatman no. 2) in a gel drier (Pharmacia) and covered with cling film. A hot-air blower was positioned about 150 mm above the gels to aid the drying process. The gels were left for a further 3 h to dry completely and then placed under a 1 kg weight overnight before being labelled and stored permanently in polythene bags.

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; CMF-PBS, calcium–magnesium-free phosphate-buffered saline; CE, cell extract; CFE, cell-free extract.

Fig. 1. *A comparison of the band patterns of CE (tracks 2–3, 7–8) and CFE (tracks 4–5, 9–10) on (a) a wet 10% (w/v) SDS/PAGE gel and (b) the same gel after aquacide reduction and vacuum dehydration*

Identical gels took over 48 h to process to a thin film in the drier without prior dehydration in aquacide and were susceptible to cracking. Reducing the gels to these dimensions means that four gels can be dried down simultaneously compared with only one without prior aquacide dehydration.

An additional advantage of this procedure, apart from the rapid processing time, is that weakly stained bands previously not visible can be clearly identified after aquacide dehydration. This is due to the fact that reduction in size results in diffuse bands becoming concentrated and compacted in a small area.

For our particular application, the band patterns of both CE and CFE initially appeared to be the same when run on a conventional 10% gel (Fig. 1*a*) even though in a dot-blot adhesion assay (Ferro *et al.*, 1988) CE promoted adhesion of the B16 melanoma cells whereas CFE did not. On further treatment of the gels with aquacide, minor bands became visible including a high-molecular-mass (above 200 kDa)

component in CE but not in CFE (Fig. 1*b*), thus explaining the difference in adhesion properties of the two extracts.

It is possible to accurately assess molecular mass from the dried gels since the slurry of aquacide causes dehydration to occur evenly across the gel surface. Hence there is no distortion of the gels. Furthermore, aquacide dehydration prevents the gels from cracking during subsequent vacuum drying.

In our hands, the use of aquacide in pre-dehydration of gels has enabled us to identify and quantify by scanning densitometry, minor protein components associated with cell adhesion and to make more accurate molecular mass assessments of protein mixtures. The technique has also reduced the time involved in processing and has given us a space conserving permanent record of our data. We believe that the technique will have many further uses including applications in autoradiography.

We are grateful to the Association for International Cancer Research for their support of this study.

Ferro, V. A., D'Arrigo, C., Ogden, D. & Evans, C. (1988) *Biochem. Soc. Trans.* **16**, 144–146

Hames, B. D. (1987) in *Gel Electrophoresis of Proteins — A Practical Approach* (Hames B. D. & Rickwood, D. eds.), pp. 48–49, IRL Press, Oxford

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APPENDIX 5 - EFFECT OF ANTIBODIES ON ADHESION TO FIBRONECTIN AND LAMININ

The antibodies (at a dilution of 1:100 of the concentration shown in brackets) which inhibited adhesion to fibronectin were the monoclonals anti-FN1 (250ug/ml) and anti-FN2 (1390ug/ml) and the polyclonals anti-FN (18mg/ml) and anti-BAE EXT (3.2mg/ml).

Anti-LM (at a dilution of 0.001mg/ml)inhibited adhesion to laminin.

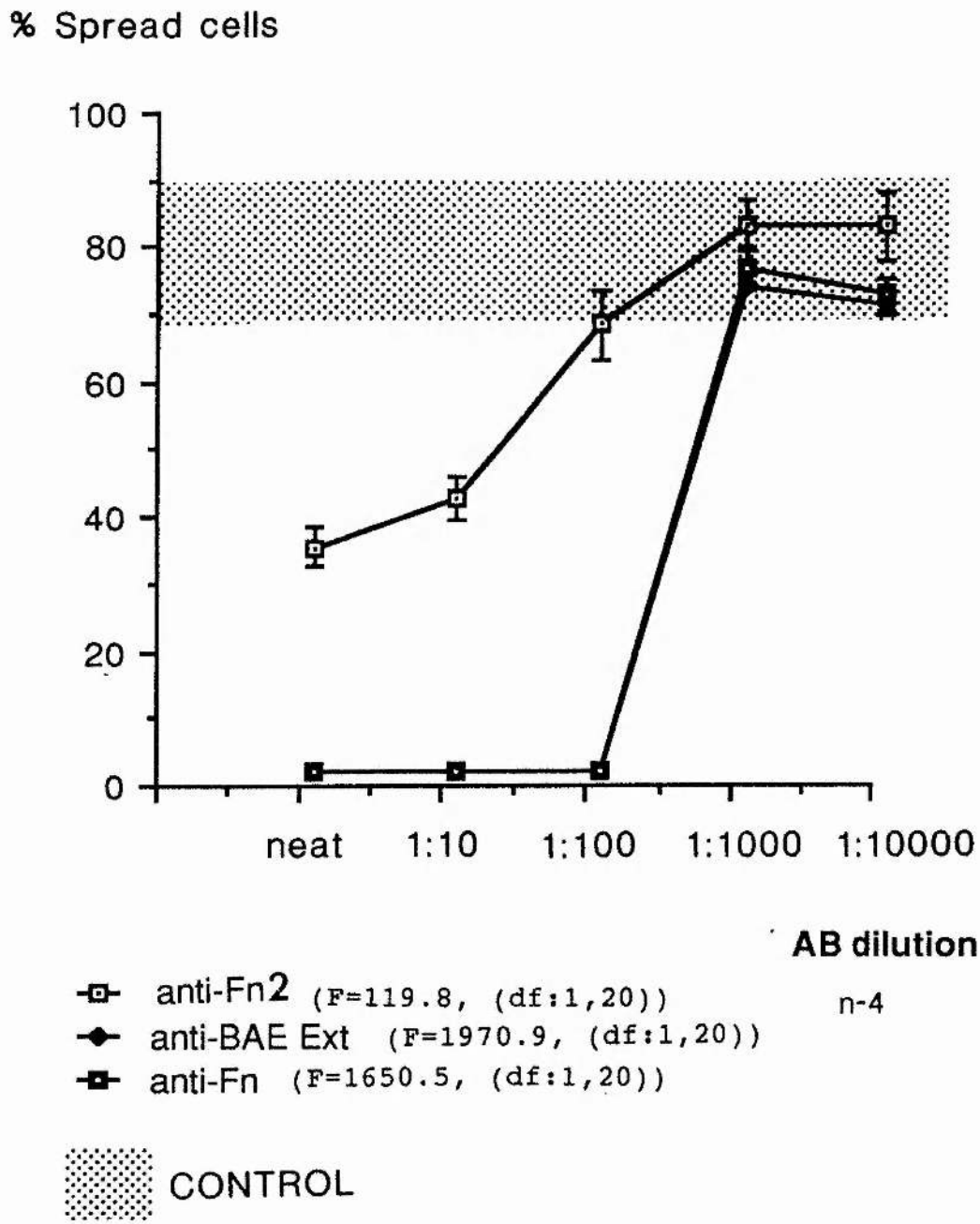
APPENDIX 6 - EFFECT OF ANTIBODIES ON SPREADING TO FIBRONECTIN AND LAMININ

Fibronectin

The following antibodies show a significant inhibition of cell spread to 40ug/ml Fn: anti-FN2 (0.14mg/ml), anti-BAE EXT (0.032mg/ml) and anti-FN (0.18mg/ml) - the F ratios are shown on the following graph. The table shows the response for the antibodies tested.

The effect of active antibodies inhibiting cell spread to Fn

	% no. of cells spread ± SD				
antibody	neat	1:10	$1:10^2$	$1:10^3$	$1:10^4$
a-FN1 (250ug/ml)	80.2 ± 4.3	75.0 ± 4.1	72.1 ± 6.1	69.8 ± 2.5	66.9 ± 2.5
a-FN2 (1.4mg/ml)	33.3 ± 2.9	40.6 ± 3.1	66.1 ± 5.2	80.7 ± 3.7	80.6 ± 5.1
a-BAE EXT (3.2mg/ml)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	71.7 ± 0.35	68.9 ± 1.4
a-FN (18mg/ml)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	74.5 ± 3.1	70.4 ± 2.5


% Spread cells
100
80
60
40
20
0
neat
1:10
1:100
1:1000
1:10000
AB dilution
anti-Fn2 (F=119.8, (df:1,20))
anti-BAE Ext (F=1970.9, (df:1,20))
anti-Fn (F=1650.5, (df:1,20))
n-4
CONTROL

Laminin

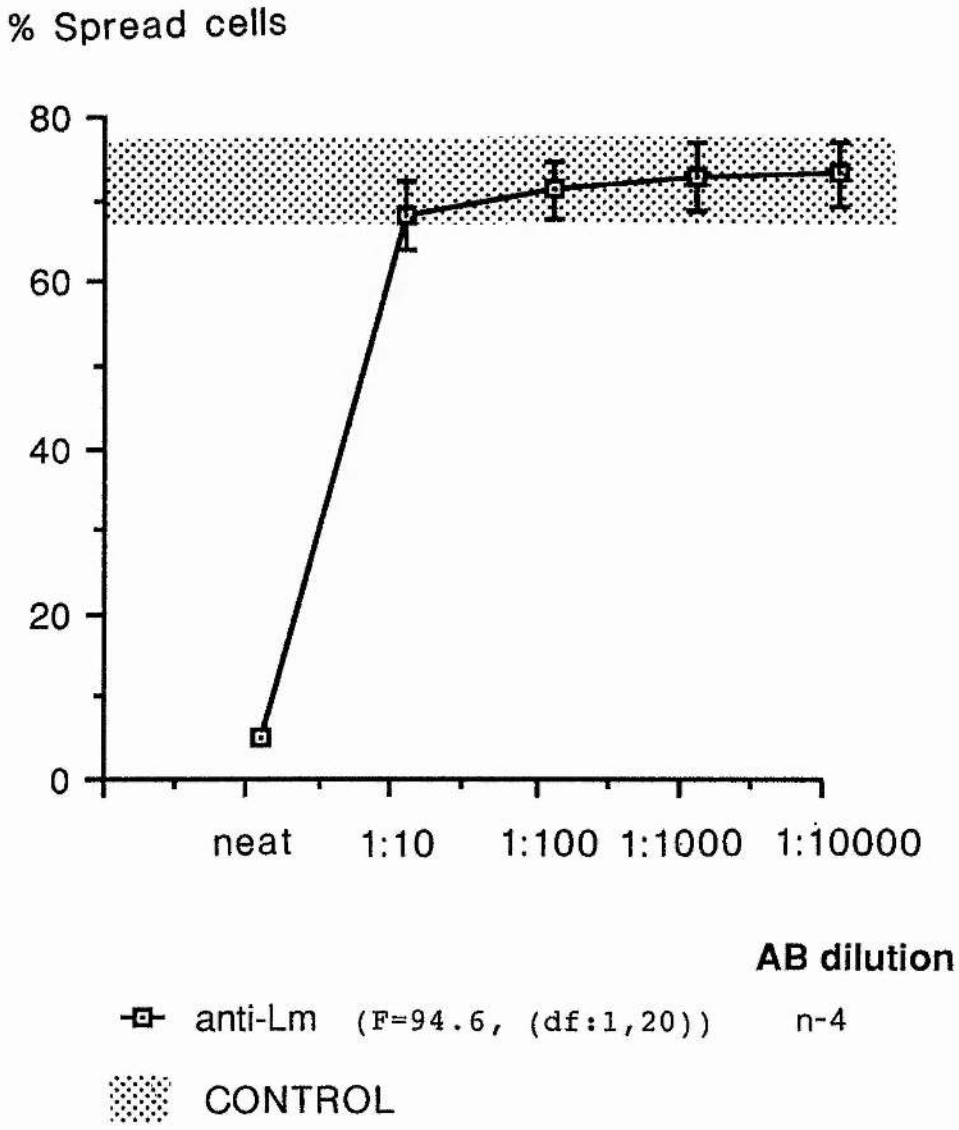
A comparison was made of spreading on Lm in the presence and absence of antibodies which revealed that anti-LM (1mg/ml) showed a significant inhibition of cell spread to 30ug/ml Lm. The F ratios are as shown on the following graph. The following table shows a summary of the response for the antibodies tested.

The effect of anti-LM in inhibiting cell spread to Lm

n=4

	% no. of spread cells ± SD				
antibody	neat	1:10	$1:10^2$	$1:10^3$	$1:10^4$
a-LM (p) (1mg/ml)	3.1 ± 0.5	66.2 ± 4.1	69.2 ± 3.4	70.6 ± 4.2	71.0 ± 4.1
a-BAE EXT (p) (3.2mg/ml)	84.3 ± 4.1	82.6 ± 3.5	81.1 ± 3.1	79.0 ± 4.5	79.2 ± 4.1
a-FN (p) (18mg/ml)	84.2 ± 3.4	81.3 ± 2.7	80.5 ± 4.2	78.8 ± 3.9	78.9 ± 4.1

The neat concentration of the antibody is shown in brackets.


% Spread cells
80
60
40
20
0
neat
1:10
1:100
1:1000
1:10000
AB dilution
anti-Lm (F=94.6, (df:1,20)) n-4
CONTROL

APPENDIX 7 - EFFECT OF ANTIBODIES ON MIGRATION TO FIBRONECTIN AND LAMININ

The method as described in Part 5.2.1 was used to produce a haptotaxis dose response curve with the test protein. The peak protein concentration obtained from the dose response curve was used to set up more chambers. Before incubating the chamber with cells, 500ul of 3% BSA was placed in the lower chamber and 200ul in the upper chamber for 30min at 37^0C in order to block any adhesive sites on the filter. The BSA was aspirated and replaced with 500ul of diluted antibody in the lower chamber and 200ul PBS in the upper chamber for 1hr at 37^0C. The chambers were then washed twice with PBS before incubating with cells and staining as described in Part 5.2.1.

Fibronectin

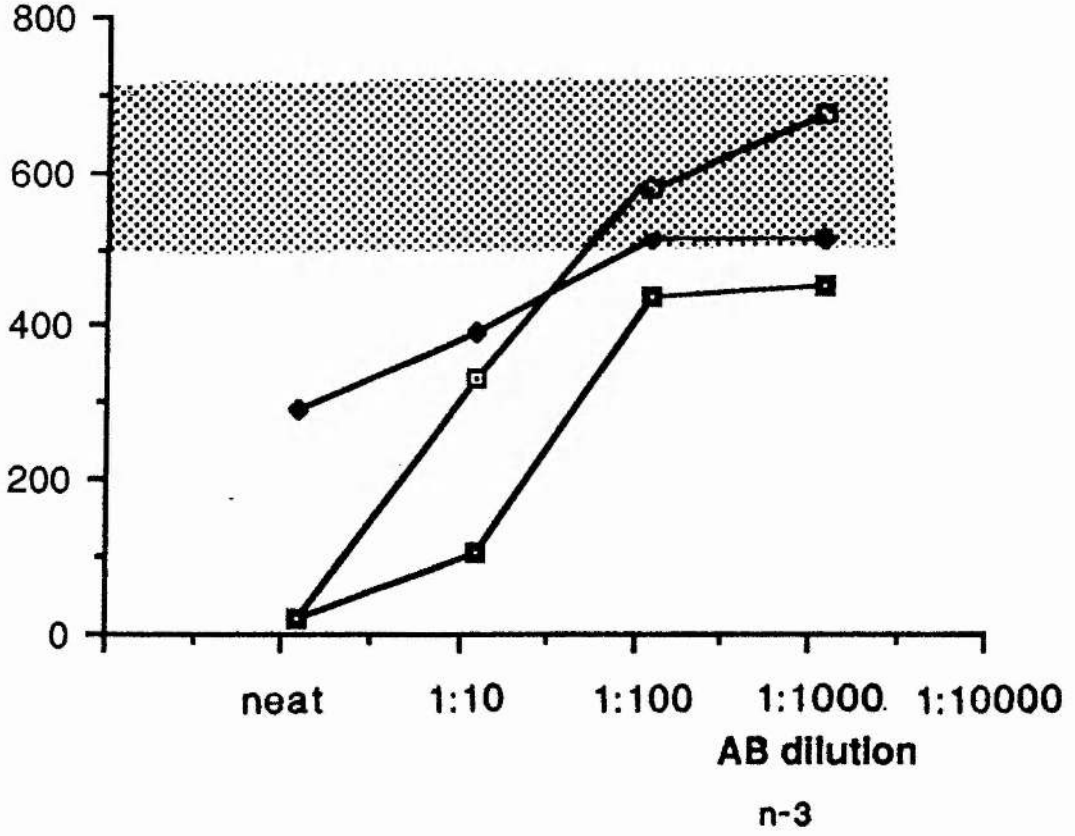
The antibodies which showed a significant inhibition of cell migration to 30ug/ml of Fn were the polyclonals anti-FN (18mg/ml) and anti-BAE EXT (3.2mg/ml) and the monoclonal anti-FN2 (1.39mg/ml). These were the same antibodies which inhibited spreading to Fn. The following table shows the response of these antibodies.

The effect of active antibodies in inhibiting migration to Fn.

	Cells moved/cm^2 ± S.D.			
antibody	neat	1:10	1:100	1:1000
a-FN1 (250ug/ml)	556.8 ± 19.0	577.3 ± 12.5	577.3 ± 23.2	575.0 ± 27.0
a-FN2 (1.4mg/ml)	0.0 ± 0.0	313.6 ± 13.2	579.5 ± 29.5	580.0 ± 28.0
a-BAE EXT (3.2mg/ml)	272.7 ± 12.6	370.5 ± 15.2	493.0 ± 19.0	495.0 ± 14.5
a-FN (18mg/ml)	0.0 ± 0.0	84.1 ± 6.5	420.0 ± 19.0	431.8 ± 23.4

The following graph shows the effect of these antibodies in inhibiting migration to Fn.

No of cells moved/cm^2



n-3

CONTROL

anti-Fn2 (F=227.9, (df:1,16))

anti-BAE Ext (F=176.6, (df:1,16))

anti-Fn (F=534.8, (df:1,16))

Laminin

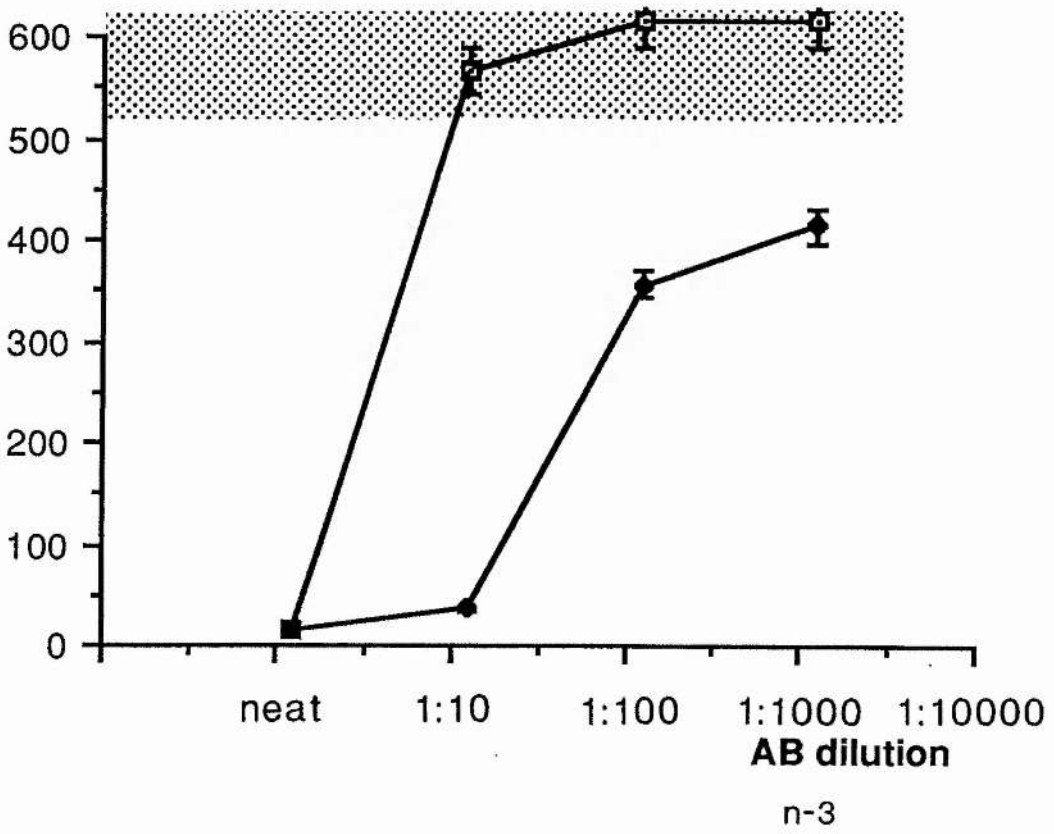
The antibodies which showed a significant inhibition of cell migration to 40ug/ml of Lm were anti-LM (1mg/ml) and anti-FN (18mg/ml). The following table summarise the responses. Anti-LM inhibits spreading and migration to Lm; anti-FN inhibits migration only.

The effect of active antibodies in the inhibition of migration to Lm. n=3

	Cells moved/cm^2 ± S.D.			
antibody	neat	1:10	1:100	1:1000
a-Fn (18mg/ml)	0.0 ± 0.0	551.0 ± 22.0	598.7 ± 25.2	599.0 ± 23.6
a-Lm (1mg/ml)	0.0 ± 0.0	20.8 ± 2.4	341.2 ± 12.1	399.0 ± 16.8

The following graph shows the effect of these antibodies in the inhibition of migration to Lm.

No of cells moved/cm^2



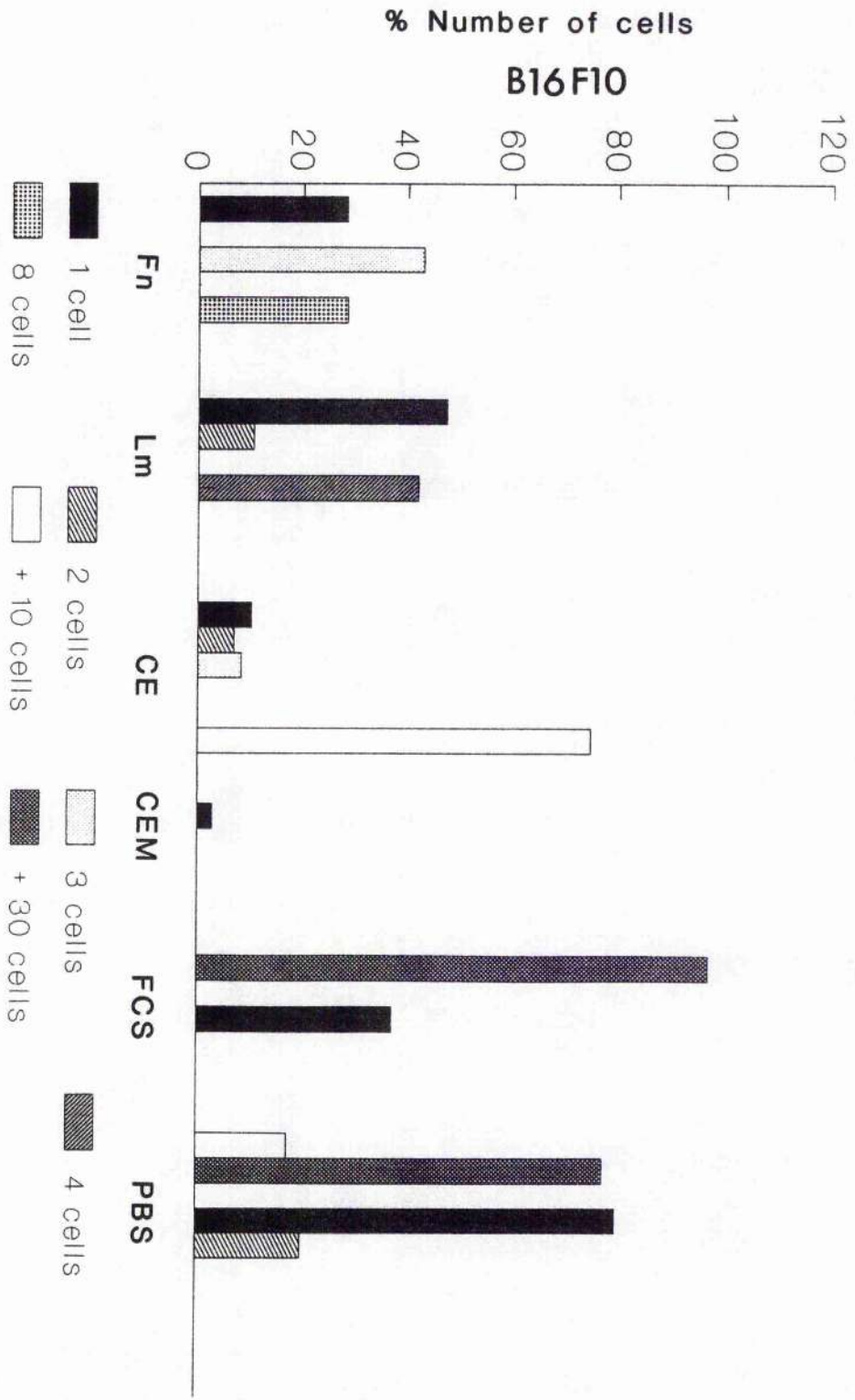
n-3

anti-Fn (F=89.9, (df:1,16))

anti-Lm (F=1177.8, (df:1,16))

CONTROL

APPENDIX 8 - AGGREGATION PROMOTING PROPERTIES OF VARIOUS PROTEINS



APPENDIX 9 - PROGRESSION OF CELL FRONTS

B16F10 cells and BAE cells were harvested as usual in EC medium with BAE supplements. Petri dishes were set up with a four-chambered silicon insert (Flexiperm, Heraeus) as shown in the photograph. In each dish B16F10 cells were seeded into two chambers at 0.64×10^4 cells/ml and BAE cells into the other two chambers at 1.6×10^4 cells/ml. The cells were allowed to grow for three days. The medium was aspirated and the rubber insert carefully removed. 5mls of medium was gently placed into the dish. The medium was changed every three days.

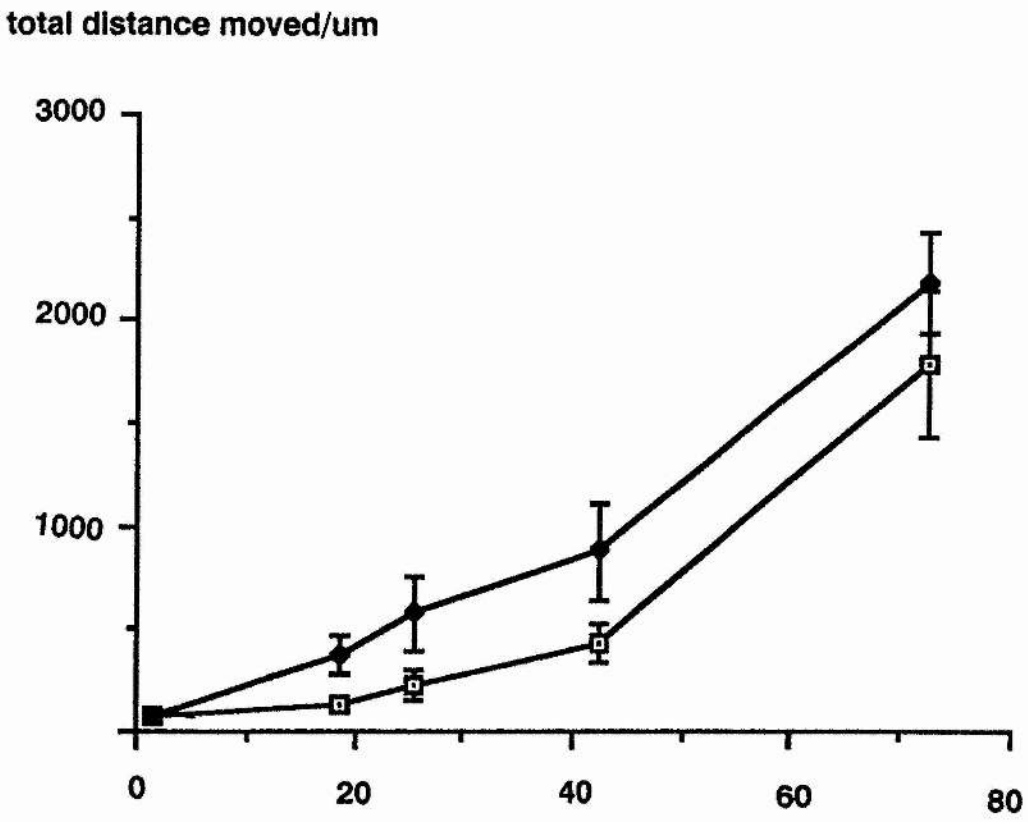
For microscopic observation of the progression of the cell fronts, a system was developed whereby a specific area on a dish could be located. A scratch mark on the side of the petri dish was matched up with a marking on the stage of the microscope. The vernier scale on the slide movement controls was then used to position and relocate chosen areas of each dish to provide information on the forward progression of cell fronts across the 3mm gap between the cell types caused by the rubber insert. The progression of B16F10 cell fronts across the gap was measured and compared in the presence and absence of BAE cells. Similarly the progression of the BAE cell fronts across the gap was measured and compared in the presence and absence of B16F10 cells. The following photograph shows the petri dish system used in this assay.


Flexipermᵀᴹ

B16F10 cell front progression (um)

Time (hours)	Total distance moved (um) BAE cells absent + S.D.	BAE cells present + S.D.
0	0.0 + 0.0	0.0 + 0.0
17	50.0 + 37.7	300.0 + 100.0
24	150.0 + 70.7	500 + 100.0
41	350.0 + 95.7	800 + 230.9
71	1700 + 355.9	2100.0 + 244.9

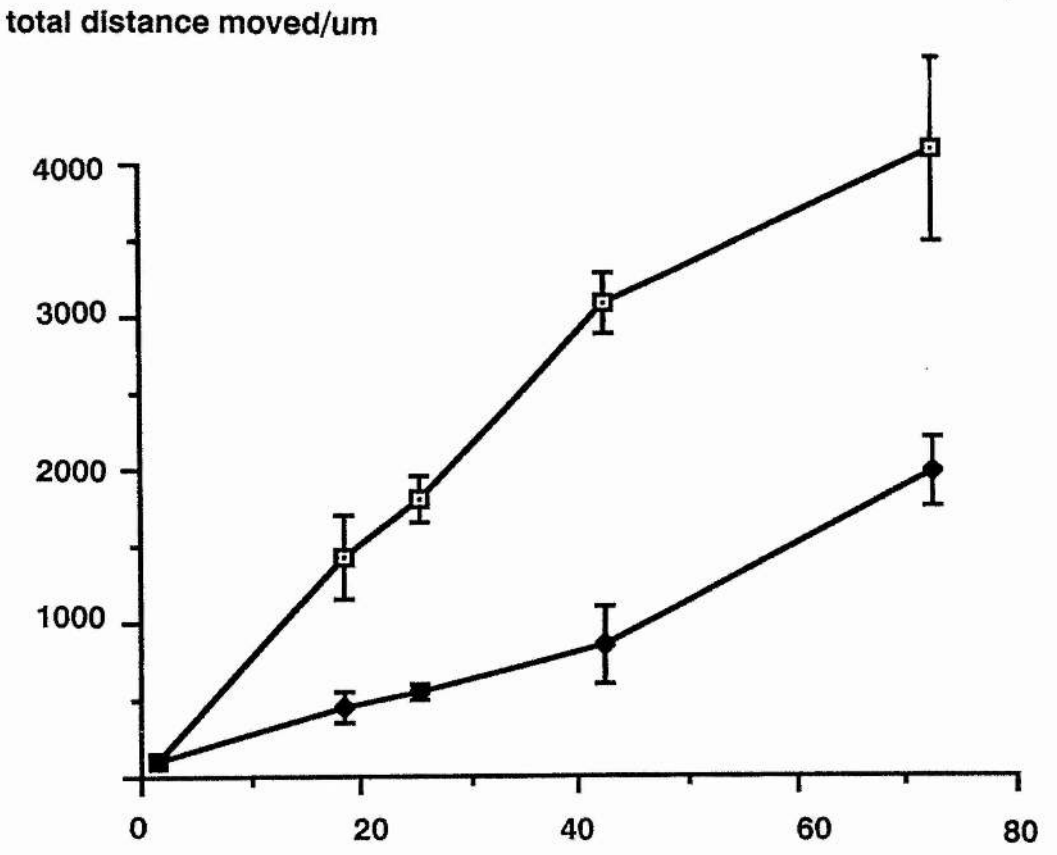
The following graph shows the B16F10 cell front progression in the presence and absence of BAE cells.


total distance moved/um
3000
2000
1000
0
20
40
60
80
time/hours
n-3
BAE absent
BAE present
(F=22.9, (df:1,20))

BAE cell front progression (um)

Time (hours)	Total distance moved (um) B16F10 cells absent ± S.D.	B16F10 cells present ± S.D.
0	0.0 ± 0.0	0.0 ± 0.0
17	1325.0 ± 275.0	350.0 ± 100.0
24	1700.0 ± 141.4	450.0 ± 41.4
41	2975.0 ± 200.0	750.0 ± 244.9
71	3975.0 ± 590.0	1875.0 ± 230.9

The following graph shows the BAE cell front progression in the presence and absence of B16F10 cells.


total distance moved/um
4000
3000
2000
1000
0
20
40
60
80
time/hours
n-3
B16 absent
B16 present
(F=211.4, (df:1,20))

In the presence of BAE cells, the B16F10 front across the gap increased significantly (compared with in the absence of BAE cells). The F ratio is shown on the graphs. In the presence of B16F10 cells, the BAE front across the gap increased significantly (compared with in tge absence of B16F10 cells). It is not possible to determine whether the results obtained were due to changes in cell proliferation or migration. A study using time-lapse photography might prove invaluable in this case.

REFERENCES

Alby, L. and Auerbach, R. (1984).
Proc. Natl. Acad. Sci. Vol. 81, p5739-5743.

Alexander, P. (1984).
Cancer Topics Vol. 4 (10), p116.

Ali, I.U. and Hynes, R.O. (1977).
Biochemica et Biophysica Acta. Vol. 471, p16-24.

Akiyama, S.K. and Yamada, K.M. (1985).
J. Biol. Chem. Vol. 260, p10402-10405.

Aplin, J.D. and Hughes, R.C. (1981).
J. Cell Sci. Vol. 50, p89-103.

Argraves, W.S., Pytela, R., Suzuki, S., Millan, J.L. and Pierschbacher, M.D. (1986)
J. Biol. Chem. Vol. 261, p12922-12924.

Asch, A.S., Barnwell, J., Silverstein, R.L. and Nachman, R.L. (1987).
J. Clin. Invest. Vol. 79, p1054-1061.

Auerbach, R. and Joseph, J. (1984).
In "The Biology of Endothelial Cells", p394-400
ed. Jaffe, E.A.,
The Hague, Nighoff.

Avnur, Z. and Gieger, B. (1984).
Cell Vol. 25, p121-132.

Balian, G., Click, E.M., Crouch, E.D., Davidson, J.M. and Bornstein, P. (1979).
J. Biol. Chem. Vol. 254, p1429-1432.

Barlow, D.P., Green, N.M., Kurkinen, M. and Hogan, B.L.M. (1984).
EMBO J. Vol.3, p2355-2362.

Barnes, D.W. and Silnutzer, J. (1983).
J. Biol. Chem. Vol. 258, p12548-12553.

Barsky, S.H., Rao, C.N., Williams, J.E. and Liotta, L.A. (1984).
The Journal of Clinical Investigations. Vol. 74, p843-848.

Basara, M.L. and McCarthy, J.B. (1985).
Cancer Research Vol. 45, p2487-2494.

Baserga, R. and Saffiotti, U. (1955).
Archives of Path. Vol. 59, p26-33.

Bennett, J.S., Hoxie, H., Lecitman, S., Valaire, G. and Clues, D. (1983).
Proc. Natl. Acad. Sci USA Vol. 80, p2417-2421.

Bhakdi, S., and Roth, M. (1981)
J.Immun. Vol. 127, p576-580.

Birchmeier, C., Kreis, T.E., Eppenberger, H.M., Winterhalter, K.H, and Birchmeier, W. (1980).
Proc. Natl. Acad. Sci. Vol. 77 (7), p4108-4112.

Birdwell, C., Gospodarowicz, D. and Nicolson, G. (1978).
Proc. Natl. Acad. Sci. USA Vol. 75 (7), p3273-3277.

Bohnsack, J.F., Tenner, A.J., Laurie, G.W., Kleinman, H.K., Martin, G.R. and Brown, E.J. (1985).
Proc. Natl. Acad. Sci. Vol. 82, p3824-3828.

Bonthron, D.T., Handin, R.I., Kaufman, J., Wasley, L.C., Orr, E.C., Mitzock, L.M., Ewenstein, B., Loscalzo, J., Ginsburg, D. and Orkin,S.H. (1986).
Nature Vol. 324, p270-273.

Bowen, B., Steinberg, J., Laemmli, U.K. and Weintraub, H. (1980).
Nucleic Acids Res. Vol. 8, p1-20.

Boyden, S.V. (1962).
Journal of Experimental Medicine. Vol. 115, p453.

Brem, S., Gullino, P.M. and Medica, D. (1977).
Science Vol. 195, p880.

Brown, S.S., Malinoff, H.L., Wicha, M.S. (1983).
Proc. Natl. Acad. Sci. Vol. 80, p5927-5930.

Brown, P.J. and Juliano, R.L. (1986).
J. Cell Biol. Vol. 103, p1595-1603.

Browning, P.J., Ling, E.H., Zimmerman, T.S. and Lynch, D.C. (1983).
Blood Vol. 62, p281.

Brunson, K.W., Nicolson, G.L. and Beattie, G. (1978).
Nature Vol. 272, p543-545.

Brunson, K.W. and Nicolson, G.L. (1979).
J. Supramol. Struct. Vol. 11, p517-528.

Butcher, E.C., Scollay, R.G. and Weissman, I.L. (1979).
J. Immun. Vol. 123 (5), p1996-2003.

Carlson, T.H., Babcock, T., Atencio, A.C., Levinson, C. and Mora, H.B. (1988).
J. Biol. Chem. Vol. 263, p2187-2194.

Carr, I., McGinty, F. and Norris, P. (1976).
J. Pathol. Vol. 118, p91.

Carr, I., Carr, J. and Drecher, B. (1981).
Invasion Metastasis Vol. 1, p34-53.

Carter, R.L. (1984).
In "Invasion: experimental and clinical implications".
eds. Mareel, M.M. and Calman, K.C.,
Oxford University Press, Oxford.

Carter, R.L. (1978).
In "Secondary Spread of Cancer", p1.
ed. Balwin, R.W.
Academic Press, London.

Charbit, A., Clement, J-M. and Hofnung, M. (1984).
J. Mol. Biol. Vol. 175, p395-401.

Chen, L.B., Summerhayes, I. and Hsieh, P. (1976).
J. Supramol. Struct. Vol. 12, p139-150.

Chen, W.T. and Singer, S.J.(1980).
Proc. Natl. Acad. Sci. Vol. 77 (12), p7318-7322.

Chen, Y.H., Carey, G.D. and Woodruff, J.J. (1983).
J. Immun. Vol. 129, p1911-1916.

Cheresh, D.A. and Spiro, R.C. (1987).
J. Biol. Chem. Vol.268, p17703-17711.

Cheung, E. and Juliano, R.L. (1985).
J. Cell Physiol. Vol. 124, p337-343.

Cheung, E. and Juliano, R.L. (1987).
J. Cell Physiol. Vol. 133, p118-121.

Chew, E.G., Josephson, R.L. and Wallace, A.C. (1976).
In "Fundamental Aspects of Metastasis", p121-150,
ed. Weiss, L.,
North-Holland Publ, Amsterdam.

Chiquet, M. and Fambrough, D.M. (1984).
The Journal of Cell Biol. Vol. 98, p1937-1946.

Chiquet-Ehrismann, R., Mackie, E.J., Pearson, C.A. and Sakakura, T. (1986).
Cell Vol. 47, p131-139.

Chiquet-Ehrismann, R., Kalla, P., Pearson, C.A., Beck, K. amd Chiquet, K. (1988).
Cell Vol. 53, p383-390.

Choay, J., Petitou, M., Loreau, J.C., Sinay, P., Casu, B. and Gatti, G. (1983).
Biochemical and Biophysical Research Communications Vol. 116 (2), p492-499.

Cobb, R.A. and Steer, H.J.W. (1987).
Br. J. Exp. Path. Vol. 68, p461-474.

Cody, R.L. and Wicha, M.S. (1986).
Exp. Cell Res. Vol. 165, p107-116.

Cole, W.H. (1973).
Surg. Gynecol. Obstet. Vol 137, p853-871.

Coligan, J.E. and Slayter, H.S. (1984).
J.Biol Chem Vol. 289 (6), p3944-3948,

Crissman, J., Hatfield, J. and Schaldenbrand, M. (1985).
Laboratory Investigations Vol. 53 (4), p470-478.

Curtis, A.S.G. (1973).
Prog. Biophys. Mol.Biol. Vol. 27, p317-386.

Curtis, A. (1987).
Journal of Cell Science Vol. 87, p609-611.

Damsky, C., Wylie, D. and Buck, C.A. (1979).
J. Cell Biol. Vol. 80, p385.

Damsky, C.H., Knudsen, K.A. and Buck, C.A. (1982).
J. Cell Biol. Vol. 18, p1-13.

DeClie, J.E. and Martin, G.S. (1987).
Mol. Cell Biol. Vol. 7, p371-378.

Del Regato, J.A. (1978).
In "Pulmonary metastais", p104-113,
eds. Weiss, L. and Gilbert, H.A.,
Hall, Boston.

Dietrich, C.P., Nader, H.B. and Straus, A.H. (1983).
Biochemical and Biophysical Research Communications Vol. 111 (3), p865-871.

Dipasquale, A. and Bell, P.B. (1974).
J. Cell Biol. Vol. 62, p198-214.

Dingemans, K.P. (1974).
J.Natl Canc. Inst. Vol. 53, p1813.

Dixit, V.M., Grant, G.A., Santoro, S.A. and Frazier, W.A. (1984).
J. Cell Biol. Vol. 259, p10100-10105,

Dixit, V.M., Haverstick, D.M., O'Rourke, K.M., Hennessy, S.W., Grant, G.A., Santoro, S.A. and Frazier, W.A. (1985)
Proc. Natl. Acad. Sci. USA. Vol. 82, p3472-3476.

Doolittle, R.F. (1984)
Ann. Rev. Biochem. Vol. 53, p195-229.

Dziadek M., Paulsson M. and TimplR. (1985)
EMBO J. Vol. 4 (10), p2513-2518

Easty, G.C. and Easty, D.M. (1984).
In "Invasion: Experimental and Clinical implications",
eds. Mareel, M.M. and Calman, K.C.,
Oxford University Press, Oxford.

Eccles, S.A. and Alexander, P. (1974).
Br. J. Cancer Vol. 30, p42-49.

Edelman, G.M. (1983).
Science Vol. 219, p450-457.

Edwards, J.G., Robson, R.T. and Campbell, G. (1987).
Journal of Cell Science Vol. 87, p657-665.

Farr, A.G. and Nakane, P.K. (1981).
J. Immunol. Methods Vol. 47, p129-144.

Ferro, V., D'Arrigo, C.D., Ogden, D. and Evans, C.W. (1988).
Biochem. Soc. Trans. Vol. 16, 144-146.

Ferro, V.A., Aiton, J.F. and Cramb, G. (1989).
Biochem. Soc. Trans. in press.

Fidler I.J. (1973).
Eur. J. Cancer Vol. 9, p223

Fidler, I.J. and Nicolson, G.L. (1976).
J. Natl. Cancer. Inst. Vol. 57, p1199-1202.

Fidler, I.J. and Kripke, M.L. (1977).
Science Vol. 197, p893-895.

Fidler, I.J. (1978).
Cancer Res. Vol. 38, p2651-2660.

Fidler, I.J., Gersten, D.M. and Hart, I. (1978).
Adv. Canc. Res. Vol. 28, p149-250.

Fidler, I.J. and Nicolson, G. (1981).
In "Cancer Biology Reviews", Vol. 2, p171-234,
eds. Marchalonis, J.J., Hanna, M.G. and Fidler, I.J.
Plenum Press, New York and Basel.

Fitzgerald, L.A., Poncz, M., Steine, B., Rall, S.C., Bennett, J.S. and Phillips, D.R. (1987).
Biochemistry Vol. 26, p8158-8165.

Folkman, J., Merler, E., Abernethy, C. and Williams, G. (1971).
J. Exp. Med. Vol. 133, p275.

Folkman, J. (1974).
Adv. Cancer Res. Vol. 19, p331-358.

Folkman, J. (1975).
In " Cancer, A Comprehensive Treatise", p355-388,
ed. Becker, F.F. Vol. 3,
Plenum Press, New York and London.

Folkman, J. and Haudenschild, C. (1980).
Nature Vol. 288 p551-556.

Fujimura, Y.,Titani, K., Holland, L.Z., Roberts, J.R., Kostel, P., Ruggeri, Z.M. and Zimmerman, T.S. (1987).
J. Biol. Chem Vol. 262, p1734-1739.

Gailit, J. and Ruoslahti, E. (1988).
J. Biol Chem. Vol. 263, p12927-12932.

Gallatin, W.M., Weissman, I.L. and Butcher, E.C. (1983).
Nature Vol. 304, p30-34.

Gartner, K.T., Waltz, W.A., Aiken, M., Starr-Spires, L. and Ogilvie, M.L. (1984)
Biochemical and Biophysical Research Communications Vol. 124 (1), p290-295.

Gartner, K.T. and Bennett, J.S. (1984).
J. Biol. Chem. Vol. 260, p11891-11894.

Gastpar, H. (1977).
J. Med. Vol. 8, p103-114.

Gehlsen, K.R., Dilher, L., Engvall, E. and Ruoslahti, E. (1988).
Scienc Vol. 241, p1228-1229.

Geiger, B. (1983).
Biochemica et Biophysica Acta Vol. 737, p305-341.

Ginsburg, D., Handin, R.I., Bonthron, D.T., Doulan, T.A., Burns, G.A., Latl, S.A. and Orkin, S.H. (1985a).
Science Vol. 228, p1401.

Ginsberg, M., Pierschbacher, M.D., Ruoslahti, E., Marguerie, G. and Plow, E. (1985b).
J. Biol. Chem. Vol. 260 (7), p3931-3940.

Glucksman, A. and Cherry, C.P. (1964).
In "Dysplasia carcinoma *in situ* and microinvasive carcinoma of the cervix uteri", p351-378,
ed. Gray, L.A.,
Thomas, Springfield, Illinois.

Goding, J.W. (1986).
In "Monoclonal antibodies: principles and practice", p121-125,
ed. Goding, J.W.,
Academic Press, London.

Goodman, S.L., Vollmers, H.P., Imhol, B.A. and Birchmeier, W. (1983).
In "Fibronectin",
ed. Mosher, D.
Academic Press, London.

Gospodarowicz, D., Greenburg, G. and Birdwell, C.R. (1978).
Cancer Res. Vol. 38, p4155-4171.

Graf, J., Iwamoto, Y., Sasaki, M., Martin, G.R., Kleinman, H.K., Robey, F.A. and Yamada, Y. (1987).
Cell Vol. 48, p989-996.

Grinnell, F. and Hays, D.G. (1978).
Exp. Cell Res. Vol. 115, p221-229.

Haagenson, C.D. (1972).
In " The Lymphatics in Cancer",p50-54,
ed. Haagenson, C.D.,
Saunder, Philadelphia.

Hand, R., Thor, A., Schlom, J., Rao, C.N. and Liotta, L.A. (1985).
Cancer Research Vol. 45, p2713-2719.

Hanna, N. (1985).
Biochemica et Biophysica Acta Vol. 780, p213-226.

Hart, I.R. and Fidler, I.J. (1980).
Cancer Res. Vol.40, p2281-2287.

Hart, I.R. (1981).
In " Cancer biology reviews", Vol.2, p29-58,
eds. Marchalonis, J.J., Hanna, M.G. and Fidler, I.J.
Marcel Dekker, New York.

Hatcher, V.B., Fadl-Allah, N., Levitt, M.A., Brown A., Margossian, S.S. and Gordon P.B. (1986).
J. of Cell Phys. Vol. 128, p353-361.

Hayman, E.G., Engvall, E., A'Hearn, E., Barnes, D., Pierschbacher, M. and Ruosalahti, E. (1982).
The Journal of Cell Biol. Vol 95, p20-23.

Hayman, E.G., Pierschbacher, M.D., Ohgren, Y. and Ruoslahti, E. (1983).
Proc. Natl. Acad. Sci. Vol. 80, p4003.

Hayman, E.G., Pierschbacher, M.D., Suzuki, S. and Ruoslahti, E. (1985).
Experimental Cell Research Vol. 160, p245-258.

Hewitt, H.B. and Blake, E. (1975).
Br.J. Cancer Vol. 31, p25-35.

Hilgard, P., Schulte, H., Wetzig, G., Schmitt, G. and Schmidt, C.G. (1977).
Br. J. Cancer Vol. 35, p78-85.

Hirst, R., Horwitz, A., Buck, C and Rohrschneider, L. (1986).
Proc. Natl. Acad. Sci. Vol. 83, p6470-6474.

Horak, E., Darling, D.L. and Tarin, D. (1986).
JNCI Vol. 76 (5), p913-922.

Horwitz, A., Duggan, K., Greggs, R., Decker, C. and Buck, C. (1985).
The Journal of Cell Biology Vol. 101, p2134-2144.

Horwitz, A., Duggan, K., Buck, C., Beckerle, K. and Burridg,e K. (1986).
Nature Vol 320, p531-533.

Hynes, R.O. and Yamada, K.M. (1982).
J. Cell Biol. Vol. 95, p369-377.

Hynes, R.O. (1985).
Ann. Rev. Cell Biol. Vol. 1, p67-90.

Hynes, R. (1987).
Cell Vol. 48, p549-554.

Humphries, M.J., Akiyama, S.K., Konoriya, A., Olden, K. and Yamada, K.M. (1986).
J. Cell Biol. Vol. 103, p88a.

Ill, C.R. and Ruosalahti, E. (1985).
The Journal of Biological Chemistry Vol. 260, p15610-15615.

Iwamoto, Y., Graf, J., Sasaki, M., Kleinman, H.K., Greatorex, D.R., Martin, G.R., Robey, F.A. and Yamada, Y. (1988).
J. Cell Physiol. Vol, 134, p287-291.

Jaffe, E.A., Minick, R., Adelman, B., Necker, C.G. and Nachman, R. (1976).
J. Exp. Med. Vol. 144, p209-225.

Jaffe, E.A. and Mosher, D.F. (1978).
J. Exp. Med. Vol. 147, p1779-179125.

Jenne, D., Ferdinand, H. and Bhakadi, S. (1985).
Thromb. Res. Vol. 38, p401-412.

Jenne, D. and Stanley, K.K. (1985).
EMBO J. Vol 4 (12), p3153-3157.

Jones, P.A. and DeClerck, Y.A. (1982).
Canc Metast. Rev. Vol. 1, p289-317.

Juliano, R.L. (1987).
Biochemica et Biophysica Acta Vol. 907, p261-278.

Kawaguchi, T., Kawaguchi, M., Dulski, M.K. and Nicolson, G.L. (1985).
Invasion Metastasis Vol. 5, p16-30.

Kellie, S., Patel, B., Wigglesworth, N.M., Critchley, D.R. and Wyke, J.A. (1985).
Exp. Cell Biol. Vol. 165, p216.

Klagsburn, M., Knighton, D. and Folkman, J. (1976).
Cancer Res . Vol. 36, p110.

Kohga, S. and Tanaka, K. (1979).
Gann. Vol. 70, p615.

Kitten, G.T., Ogle, R.C., Von der Mark, K., Yamada, Y., Martin, G.R. and Kleinman, H.K. (1986).
J Cell Biol Vol. 103, p260a.

Klebe, R.J. (1974).
Nature (Lond) Vol. 250, p248-251.

Klebe, R.J., Hall, J.R., Naylor, S.L. and Dideey, W.D. (1978).
Exp. Cell Res. Vol 115, p73-78.

Kleinerman, J. and Liotta, L.A. (1977).
In "Cancer Invasion and Metastasis: Biological Mechanisms and Therapy", p135,
eds. Day, S.B., Laird, Myers, W.P., Stansley, P., Garathini, S. and Lewis, M.G.
Raven Press, New York.

Kleinman, H.K., McGoodwin, E.B., Martin, G.R., Klebe, R.T., Fietzek, P.P and Woolley, D.E. (1978).
J. Biol. Chem. Vol. 253, p5642-5646.

Kleinman, H.K., McGarvey, M.L., Hassell, J.R., Martin, G.R., Baron van Evercooren, A. and Dubois-Dalcq, M. (1984).
In " The Role of Extracellular Matrix Development", p123-143
Alan, R. Liss, New York.

Kleinman, H.K. (1988).
Proc. Natl. Acad. Sci. Vol. 85, p1282.

Kornblihtt, A.R., Vibe-Pederson, K. and Baralle, F.E. (1983).
Proc. Natl. Acad. Sci. USA Vol. 80, p3218-3222.

Knox, P. and Griffiths, S. (1980).
J.Cell Sci. Vol. 46, p97-112.

Knox, P. and Griffiths, S. (1982).
J. Cell Sci. Vol. 55, p301-316.

Knudsen, K., Rao, P., Damsky, C., Buck, C.A. and Damsky, C.H. (1981).
Proc. Natl. Acad.Sci. Vol. 78, p6071-6078.

Knudsen, K.A., Damsky, C.H. and Buck, C.A. (1986).
J. Cell Biol. Vol 18, p157-167.

Kramer, R. and Nicolson, G. (1979).
Proc. Natl. Acad. Sci. USA. Vol. 76 (11), p5704-5708.

Kramer, R.H. (1980).
Int. J. Cancer Vol. 26, p639-645.

Kramer R.H., Vogell K.G. and Nicolson G.L. (1982)
J. Biol. Chem Vol. 257 (5), p2678-2686

Kramer, R.H., Bensch, K.G. and Wong, J. (1986).
Cancer Research Vol. 46, p1980-1989.

Kuusela, P., Ruoslahti, E. and Vaheri, A. (1975).
Biochemica et Biophysica Acta. Vol. 379, p295-303.

Lackie, J.M. (1986).
In "Cell Movement and Cell Behaviour",
Allen and Unwin, London.

Lacovara, J., Cramer, E.B. and Quigley, J.P. (1984).
Cancer Res. Vol. 44, p1657-16663.

Laemmli, U.K. (1970).
Nature Vol. 227, p680-685.

Lan, W.C., Delikatny, E.J., Orr, F.W., Wass, J., Varani, J.and Ward, P.A. (1987).
Amer. J. Path. Vol. 104, p69-76.

Lane, D.A., Flynn, A.M., Pejler, G., Lindahl, U., Choay, J. and Preissner, K. (1987)
J. Biol. Chem. Vol. 261,p16343-16348.

Larson, E., Howlett, B. and Jagendorf, A. (1986).
Anal. Biochem. Vol. 155, p243-248.

Laug, W., DeClerck, Y. and Jones, P. (1983).
Cancer Res. Vol. 43, p1827-1834.

Lahav, J., Schwartz M.A. and Hynes, R.O. (1982).
Cell Vol. 31, p253-262.

Lawler, J.W., Slayter, H.S. and Coligan, J.E. (1978).
J. Biol. Chem. Vol. 253, p8609-8616.

Leivo, I. and Engvall, E. (1986).
J. Cell Biol. Vol. 103, p1091-1100,

Leptin, M. (1986).
Nature Vol 321 p728.

Leung, L.L.K. and Nachman, R.L. (1982).
J. Clin. Invest. Vol. 70, p542-549.

Levene, M.B., Haynes, H.A. and Goldwyn, R.M. (1982).
In "Cancer: Principles and practice of oncology", p1094-1123,
eds. Devita, V.T., Hellman, S. and Rosenberg, S.A.
J.B. Lippincott, Philadelphia.

Lindberg, R. (1972).
Cancer Vol. 29, p1446-1449.

Liotta, L.A., Kleinerman, J. and Saidel, G.M. (1976).
Cancer Research Vol. 36, p889-894.

Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I.R., Foltz, C.M. and Shakie, S. (1980).
Nature Vol. 248, p67-68.

Liotta, L.A., Goldfarb, R.H., Brindage, R., Siegal, G.P., Terranova, V.P. and Garbisa, S. (1981).
Cancer Res. Vol. 41, p4629-4636.

Liotta, L.A. (1986).
Cancer Res. Vol. 46, p1-7.

Locker, J., Goldblatt, P. and Leighton, J. (1970).
Cancer Res. Vol. 30, p1632-1644.

Mackie E.I., Thesleff, I. and Cliquet-Ehrissman, R. (1987).
J. Cell Biol. Vol. 105, p2569-2579.

Maier, A. and Mayne, R. (1987).
Am. J. Anat. Vol. 180 (3), p226-236.

Majno, G., Shea, S.M. and Leventhal, M. (1969).
J. Cell Biol. Vol. 42, p647-672.

Marcum, J.A. and Rosenberg, R.D. (1985).
Biochemica et Biophysica Acta Vol. 126, p365-372.

Martin, G.R. and Timpl, R. (1987).
Ann. Rev. Cell Biol. Vol. 3, p57-85.

McCarthy, J.B. and Furcht, L.T. (1984).
J Cell Biol. Vol. 98, p1474-1480.

McCarthy, J.B., Basara, M.L., Palm, S.L., Sao, D.F. and Furcht, L.T. (1985).
Cancer and Metastatsis Rev. Vol. 4, p125-152.

McCarthy J.B., Hagen S.T., and Furcht L.T. (1986)
J. Cell Biol. Vol. 102, p179-187

McMaster, G.K. and Zardi, L. (1982).
BBA Vol. 107, p608-617.

Mosher, D.F., Doyle, M.J. and Jaffe, E.A. (1982).
J. Cell Biol. Vol. 93, p343-348.

Nakajima, M., Irimura, T., DiFerrante, D.T., DiFerrante, N. and Nicolson, G.L. (1983).
Science Vol. 220, p611.

Netland, P.A. and Zetter, B.R. (1986).
Biochemical and Biophysical Research Communications Vol. 139 (2), p515-522.

Nicolson, G.L. and Winklehake, J.L. (1975).
Nature Vol 255, p230-232.

Nicolson, G.L., Winkelhake, J.L. and Nussey, A.C. (1976).
In "Fundamental Aspects of Metastasis", p291,
ed. Weiss, L.,
North-Holland Publishing Co, Amsterdam.

Nicolson, G.L. (1981).
Br. Soc. Cell Biol. Conference (Abstract),
Nottingham.

Nicolson, G.L. (1982).
Biochemica et Biophysica Acta Vol. 695, p113.

Nicolson, G.L. (1984).
Exp. Cell Res. Vol. 150, p3-22.

Nicolson, G.L. and Dulski, K.M. (1986).
Int. J. Cancer Vol. 38, p289-294.

Odermatt, E., Tamkun, J.W. and Hynes, R.O. (1985).
Proc. Natl. Acad. Sci. Vol. 82, p6571-6575.

Oppenheimer-Marks, N. and Grinnell, F. (1984).
Experimental Cell Research Vol. 152, p467-475.

Ozaki, T., Yoshida, K., Ushijima, K. and Hayashi, H. (1971).
Int. J. Cancer Vol. 7, p93.

Owens, M.R. and Miller, L.L. (1980).
Biochemica et Biophysica Acta Vol. 67, p30-39.

Papadimitriou, J.M. and Woods, A.E. (1975).
J. Path. Vol. 116, p65.

Paul, J.I. and Hynes, R.O. (1984).
J. Biol. Chem. Vol. 259, p13477-13488.

Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Deck, K. and Engel, J. (1987).
Eur. J. Biochem. Vol. 166, p11-19.

Pearlstein, E., Salk, P.L., Yogeeswaran, G. and Karpatkin, S. (1980).
Proc. Natl. Acad. Sci. USA Vol. 77, p4336-4339.

Petersen, T.E., Thogersen, H.C., Skorstengaard, K., Vibe-Pedersen, K., Sottrup-Jensen, L. and Magnusson, S. (1983).
Proc. Natl.Acad.Sci. USA Vol. 80, p137-141.

Philips, P., Steward, J.K. and Kumar, S. (1976).
Int. J. Cancer Vol. 17, p549-558.

Phondke, G.P., Madyastha, K.R., Madyastha, P.R. and Barth, R.F. (1981).
J. Natl. Can. Inst. Vol. 66, p643.

Pierschabacher, M.D., Ruoslahti, E., Sundelen, J., Lind, P. and Peterson, P.A. (1982)
J Biol Chem. Vol 257, p93-97.

Pierschbacher, M.D. and Ruoslahti, E. (1984).
Nature Vol. 30, p30-33.

Pikkarainen, T., Kallunki, R. and Tryggvason, K. (1988).
J. Biol. Chem Vol. 263, p6751-6758.

Poncz, M., Eisman, R., Heidenreich, R.,Silver, S.M. and Vilaire, G. (1987).
J. Biol. Chem Vol. 262, p8476-8482.

Poste, G. and Fidler, I.J. (1980).
Nature Vol. 283, p139-146.

Poste, G. and Nicolson, G.L. (1980).
Proc. Natl. Acad. Sci. USA. Vol.77, p399.

Poste, G., Doll J. and Fidler I.J. (1981)
Proc. Natl. Acad. Sci. Vol. 78, p6226-630.

Preissner, K.T., Wassmuth, R. and Muller-Berghaus, G. (1985)
Biochem. J. Vol. 231, p349.

Preissner, K.T., Anders, E., Grulich-Henn, J. and Muller-Berghaus, G. (1988).
Blood Vol.71 (6), p1581-1589.

Pytela, R., Pierschabacher, M.D. and Ruoslahti, E. (1985a).
Cell Vol. 40, p191-198.

Pytela, R., Pierschbacher, M.D. and Ruoslahti, E. (1985b).
Proc. Natl. Acad. Sci. USA Vol.82, p5766-5770.

Quigley, J.P. (1979).
In "Surfaces of Normal and Malignant Cells", p247-285,
ed. Hynes, R.O.,
John Wiley and Sons, Chichester.

Rao, C., Margulies, I.M.K., Tralka, T.S., Terranova, V.P., Madri, J.A. and Liotta, L.A. (1982a).
The Journal of Biological Chemistry Vol. 257, p9740-9744.

Rao, N.C., Barsky, S.H., Terranova, V.P. and Liotta, L.A. (1983).
Biochemical and Biophysical Research Communications Vol. 111 (3), p804-808.

Raugi, G.J., Mumty, S.M., Abbot-Brown, D. and Bornstein, P. (1982).
J. Cell Biol. Vol. 95, p351-354.

Raz, A. and Lotan, R. (1981).
Cancer Res. Vol. 41, p3642.

Reich, R., Thompson, E.W., Iwamoto, Y., Martin, G.R., Deason, J.R., Fuller, G.C. and Miskin, R. (1988).
Cancer Res. Vol. 48, p3307-3312.

Rieber, M., Rieber, M.S., Urbina, C. and Lira, R. (1986).
BBRC Vol. 140 (2), p638-6433.

Riser, B.L., Varani, J., O'Rourke, K. and Dixit, V.M. (1988).
Exp. Cell Res. Vol. 174, p319-329.

Robertsm D.D., Sherwoodm J.A. and Ginsburgm V. (1987).
J. Cell Biol. Vol. 104,p131-139.

Robins, R.A. (1986).
Biochemica et Biophysica Acta Vol. 865, p289-305.

Rohrschneider, L. (1980).
Proc. Natl. Acad Sci. USA Vol. 77, p3514-3518.

Roos, E. and Dingemans, K.P. (1979).
BBA Vol. 560, p135-166.

Roth, J.A., Siverstein, M.J., and Morton, D.L. (1976).
Surgery Vol. 79, p669-673.

Ruosalahti, E. (1982).
Met. Enzymol. Vol.82 (46), p803-831.

Ruoslahti E. and Pierschbacher M.D. (1986)
Cell Vol. 44, p517-518.

Ruosalahti E. (1988a)
Biochemistry Society Meeting.
Nottingham.

Ruoslahti, E. (1988b).
Ann. Rev. Biochem. Vol. 57, p375-413.

Saga, S., Chen, W-T. and Yamada, K.M. (1988).
Can. Res. Vol. 48, p5510-5513.

Sage, H., Pritzl, P. and Bornstein, P. (1981).
Arterosclerosis Vol. 1, p427-442.

Salonen, E.M., Zitting, A. and Vaheri, A. (1984).
FEBS lett. Vol. 172, p29-32.

Salsbury, A.J. (1975).
Cancer Treatment Rev. Vol. 2, p55-72.

Santoro, S.A. (1987).
Exp. Cell Res. Vol. 173, p413-424.

Sasaki, M., Kato, S., Kohno, K., Martin, G.R. and Yamada, Y. (1987).
Proc. Natl. Acad. Sci. Vol. 84, p935-939.

Schwarzbauer, J.E., Tankum, J.W., Lemischka, I.R. and Hynes, R.O. (1983).
Cell Vol. 35, p421-431.

Selfton, B.M., Hunter, T., Ball, E.H. and Singer, S.J. (1981).
Cell Vol. 24, p165-174.

Shelton-Inloes, B.B., Titani, K. and Sadler, J.E. (1986).
Biochemistry Vol. 25, p3164-3171.

Sherbert G.V. (1982).
In "The Biology of Tumour Malignancy",
Academic Press, London.

Siegal, P.G., Barsky, S.H., Terranova, V.P. and Liotta, L.A. (1981).
Invasion Metastasis Vol. 1, p54-70.

Simonton, S.C., Basara, M.L., Barnes, D.W. and Furcht, L.T. (1985).
Lab. Invest. Vol. 52, p63.

Singer, S.J. (1979).
Cell Vol. 16, p675-685.

Situ, R., Lee, E.C., McCoy, J.P. and Varani, J. (1984).
J. Cell Sci. Vol. 70, p167-176.

Skorstengaard, J., Thogersen, H.C. and Petersen, T.E. (1984).
Eur. J. Biochem. Vol. 140, p235-243.

Skubitz, A.P.N., Charonis, A.S., Tsilibary, E.C. and Furcht, C.T. (1987).
Exp. Cell Res. Vol. 173, p349-369.

Skubitz, A.P.N., McCarthy, J.B., Charonis, A.S. and Furcht, L.T. (1988).
J Biol. Chem. Vol. 263, p4861-4868.

Sporn, M.B. and Roberts, A.B. (1986).
J. Clin. Invest. Vol. 78, p329-332.

Smalheiser, N.R. and Schwartz, N.B. (1987).
Proc. Natl. Acad. Sci. Vol. 84, p6457-6461.

Stamper, H.B. and Woodruff, J.J. (1977).
J. Immun. Vol. 119 (2), p772-780.

Steinman, C., Fenner, M., Parish, R.W. and Binz, H. (1984).
Int. J. Cancer Vol. 34, p407-414.

Streeter, P.R., Berg, E.L., Rouse, B.T.N., Bargartze, R.F. and Butcher, E.C. (1988).
Nature Vol. 331, p41-46.

Stringfellow, D.A. and Fitzpatrick, F.A. (1979).
Nature Vol. 282, p76-78.

Suzuki, S., Pierschbacher, M., Hayman, E.G., Nguyen, K., Ohgren, Y. and Ruoslahti, E. (1984).
J. Biol. Chem. Vol. 259, p15307-15314.

Suzuki, S., Argraves, W.S., Arai, H., Languino, L.R. and Pierschbacher, M.D. (1987).
J. Biol. Chem. Vol. 262, p14080-14085.

Suzuki, S. and Oldberg A. (1985).
EMBO J. Vol. 4 (10), p2519-2524.

Takada, Y., Huang, D. and Heuler, M.E. (1987).
Nature Vol. 326, p607-609.

Tamkun, J.W., Schwazbauer, J.E and Hynes, R.O. (1984).
Proc. Natl. Acad. Sci. Vol. 81, p5140-5144.

Tamkun, J.W., DeSimoie, D.W., Fonda, D., Patel, R.S. and Buck, C. (1986).
Cell Vol. 46, p271-282.

Tao, T., Matter, A., Vogel, K. and Burger, M.M. (1979).
Int. J. Cancer Vol. 23, p854-857.

Terranova, V.P., Rohrbach, D.H. and Martin, G.R. (1980).
Cell Vol. 22, p719-726.

Terranova, V.P., Liotta, L.A., Russo, R.G. and Martin, G.R. (1982).
Cancer Research Vol. 42, p2265-2269.

Terranova, V.P., Rao, C.N., Kalebic, T., Margulies, M.K. and Liotta, L.A. (1983).
Proc. Natl. Acad. USA Vol. 80, p444-448.

Terranova, V.P., Williams, J.E., Liotta, L.A. and Martin, G.R. (1984).
Science Vol 226, p982-985.

Terranova, V.P., Hic, S. Diflorio, R.H. and Lyall, M.R. (1986).
CRC Critical Reviews in Oncology/Haematology Vol. 5, p87-114.

Thesleff, I., Mackie, E., Vainio, S. and Chiquet-Ehrismann, R. (1987).
Development Vol. 101 (2), p289-296.

Timpl, R., Johansson, S., Van Delden, V., Oberbaumer, I. and Hook, M. (1983).
J. Biol. Chem Vol. 258, p8922-8927.

Tomasini, B.R. and Mosher, D.F. (1986).
Blood Vol. 68, p737-742.

Trinkaus, J.P. (1976).
In "The Cell Surface in Animal Embryogenesis and Development", p225-329, eds. Poste, G. and Nicolson, G.L.,
North-Holland, Amsterdam.

Tryggvason, K., Hoyhtya, M. and Salo, T. (1987).
Biochemica et Biophysica Acta Vol. 907, p191-217.

Tullis, J.L., Honeggar, H., Fleischaker, O., Hewitt, L., Kenney, D. and Chao F.C. (1981).
Cancer Vol. 48, p1177-1182.

Vaheri, A. and Ruoslahti, E. (1975).
J. Exp. Med. Vol. 142, p530-538.

Varani, J. (1982).
Cancer Met. Rev. Vol. 1, p17-28.

Varani, J., Fligiel, S.E.G. and Perona, P. (1985).
Int. J. Cancer Vol. 35, p559-564.

Varani, J., Dixit, V.M. Fligiel, S.E.G., McKeever, P.E. and Carey, T.E. (1986).
Exp. Cell Res. Vol 167, p376-390.

Vasiliev, J.M., Gelfand, I.M., Dominia, L.V., Zacharova, O.S. and Ljubinov, A.V. (1975).
Proc. Natl. Acad Sci. USA Vol. 72 p719-722.

Vincent, M.D. (1985).
In "The Molecular Basis of Cancer",
eds. Farmer, P.B. and Walker, J.M.,
Croon Helm, London.

Vollmers, H.P. and Birchmeier, W.(1983a).
Proc. Natl. Acad. Sci. USA. Vol. 80, p3729-3733.

Vollmers, H.P. and Birchmeier, W. (1983).
TIBS December, p2452-2455.

Van de Water, L., Destree, A.T. and Hynes, R.O. (1983).
Science Vol. 220, p201-204.

Von der Mark, K. and Kuhl, U. (1985).
Biochemica et Biophysica Acta Vol. 823, p147-160.

Ward, P.A., Hugh, T.E. and Chenoweth, D.C. (1979).
In "Chemical Messengers of the Inflammatory Process",
ed. Houck, J.C.,
Elsievier Press, Amsterdam.

Warren, B.A. (1974).
Thromb Diath. Haemorr Suppl. Vol. 59, p139-156.

Weber, K. and Osborn, M. (1969).
J. Biol. Chem. Vol. 244, p4406-4412.

Weigant, F.A.C., Blok, F.J., Defize, L.H.K., Linneman, W.A.M., Verkley,
A.J. and Boostra, J. (1986).
J. Cell Biol. Vol. 103, p87-94.

Weiss, L. (1976).
In "Fundamental Aspects of Metastasis",
North Holland, Amsterdam.

Weiss, L. (1977).
Int. J. Cancer Vol. 20, p87-92.

Weiss, L. (1980).
Int. J. Cancer Vol. 25, p385-392.

Weiss, L. (1985).
In "Principles of metastasis",
Acad. Press, London.

Wewer, U.M., Taraboletti, G., Sobel, M.E., Albrechtsen, R. and Liotta, L.A.
(1987).
Cancer Res. Vol. 47 (7), p5691-5698.

Willis, R.A. (1973).
In "The Spread of Tumours in the Human Body",
Butterworths, London.

Wilkins, J.A. and Lin, S. (1986).
J. Cell Biol. Vol. 102,p1085-1092.

Woods, A., Couchman, J.R., Johannson, S. and Hook, M. (1986).
EMBO J. Vol. 5, p665-670.

Wood, S. (1958).
Arch. Path. Vol. 66, p550-568.

Wood, S., Holyoke, E.D. and Yardley, J.H. (1966).
Canadian Cancer Conferens Vol. 4, p167-223.

Woodley, D.T., Rao, C.N., Hassell, J.R., Liotta, L.A., Martin, G.R. and Kleinman, H.K. (1983).
Biochemica et Biophysica Acta Vol. 761, p278-283.

Woodruff, M.F.A. (1980).
In "The Interaction of Cancer and Host. Its Therapeutic Significance", Grune and Stratton Inc., New York.

Yamada, K.M., Yamada, S.S and Pastan, I. (1976).
Proc. Natl. Acad. Sci. U.S.A. Vol. 73, p1217-1221.

Yamada, K.M. and Olden, K. (1978).
Nature Vol. 275, p179-184.

Yamada, K.M. (1983).
Ann. Rev. Biochem. Vol. 52, p761-799.

Yamada, K.M., Akiyama, S.K., Hasegawaa, T., Hasegawa, E., Humphries, M.J., Kennedy, D.W., Nagata, K., Vrushihara, H., Olden, K. and Chen, W-T. (1985).
J. Cell Biol. Vol. 28, p79-97.

Yamada, K.M., Hayashi, M., Hirano, H. and Akiyama, S.K. (1984).
In "The Role of Extracellular Matrix in Development", p89-121.
Alan R. Liss Inc., New York.

Yoffey, J.M. and Courtice, F.C. (1970).
In "Lymphatics and the Lymphomyeloid Complex",
Academic Press, London.

Young, W.C. and Herman, I.M. (1985).
J. Cell Sci. Vol. 73, p19-22.

Zamora, P.O., Danielson, K.G. and Hosick, H.L. (1980).
Cancer Res. Vol. 40, p4631-4639.

Zucker, A.M. (1979).
Blood Vol. 54, p8-12.