INTERACTIONS BETWEEN MALIGNANCY AND COAGULATION

Anand David Purushotham MBBS, FRCS (Ed)

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Department of Surgery, Western Infirmary, Glasgow

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"to strive, to seek, to find, and not to yield"

Alfred, Lord Tennyson

SUMMARY

There is evidence from clinical and experimental studies of a two-way relationship between cancer and the haemostatic system. Human cancer is associated with activation of haemostatic mechanisms, whilst animal studies of coumarin effects on metastasis suggest that coagulation may be implicated in the metastatic process. Warfarin inhibits, whilst the warfarin-dependent coagulation factor complex II, IX, X enhances, pulmonary tumour seeding in an animal model of metastasising mammary carcinoma. The purpose of this study was to investigate the mechanisms responsible for this effect of tumour enhancement by the coagulation factor complex II (prothrombin), IX, X. Initially, preliminary experiments were performed to validate the experimental tumour model used by previous workers. In particular, the effect of the factor complex II, IX, X on tumour cell aggregability and the influence of the timing and dose of administration of the factor complex on pulmonary metastasis were studied. The results of these experiments validated the results obtained by previous workers.

In order to exclude the possibility that this effect of tumour enhancement by the factor complex is secondary to it's xenogeneic properties, a comparison was made between the prometastatic effects of human and rat coagulation factor complexes. The results of this experiment showed that both rat and human factor

complexes enhanced pulmonary tumour seeding to an equal degree.

In order to assess changes at the level of the pulmonary microcirculation on administration of tumour cells and the factor complex II, IX, X, a further study was performed to look at the passage of radiolabelled tumour cells through the pulmonary microcirculation. This study demonstrated a 20% increase in the number of tumour cells trapped in the lungs within the first hour of tumour cell and factor complex administration, when compared with the group that received tumour cells alone. These results suggest that an intravascular event may be responsible for the enhancement of metastasis previously demonstrated.

Further animal studies demonstrated that individual purified components of the factor complex II, IX, X, and various combinations thereof, enhanced pulmonary tumour seeding to a similar degree. This suggests that they may do so via a common pathway (factor II), involving activation of the coagulation system. Evidence for activation of coagulation was demonstrated by estimating plasma fibrinopeptide A, a highly sensitive indicator of activation of the coagulation system.

Based on these results, we hypothesised that factors II, IX, X, enhanced metastasis by encouraging tumour cell entrapment within a fibrin clot, in the pulmonary microcirculation. In a further experiment we demonstrated that fibrinolysis, using a "clot-lysis" dose of intravenous Streptokinase, reversed this effect

of tumour enhancement. Furthermore, intravenous Streptokinase inhibited pulmonary tumour seeding even in the absence of exogenous factor complex II, IX, X. This inhibitory effect of Streptokinase on tumour seeding may have clinical implications with regard to the prescription of antimetastatic therapy in cancer patients.

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
APC	Activated Protein C
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
DIC	Disseminated Intravascular Coagulation
FCS	Foetal Calf Serum
FDP	Fibrin(ogen) Degradation Products
FpA	Fibrinopeptide A
F344	Fischer 344
F10/DMEM	1:1 mixture of Hams' F10 and Dulbecco's Modified Eagles' Medium
HMW-kininogen	High Molecular Weight-kininogen
^{125}IUDR	^{125}I-iodo-2'-deoxyuridine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
t-PA	Tissue Plasminogen Activator
u-PA	Urokinase-type Plasminogen Activator

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DECLARATION

I declare that this thesis has been composed by myself, and that the work described herein was performed by me, or by myself in conjunction with others. The laboratory tests for analysis of Fibrinopeptide A, described in Chapter 5, were performed by Drs I McGregor and O Drummond, Headquarters Unit Laboratory, National Blood Transfusion Service, Edinburgh. The remaining tests of haemostasis described in this thesis were performed by Mr T Cavanagh and his colleagues, Department of Haematology, Western Infirmary. Preparation of microscopic slides illustrated in Chapter 1 were performed by Dr E Mallon, Department of Pathology, Western Infirmary. Statistical analysis of all the work described were supervised by Drs G Murray and J Findlay, Medical Statistics, University Department of Surgery, Western Infirmary. All other work described herein was performed entirely by myself. This work has not been presented at any previous application for a degree by myself or others.

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INTRODUCTION:

1. THE MECHANISM OF TUMOUR METASTASIS.

1.1 The metastatic process.

Metastasis has been defined as the "transfer of disease from one organ or part to another not directly connected with it. It may be due either to the transfer of pathogenic organisms or to transfer of cells as in malignant tumours" (Dorland, 1965). The major significance of metastasis in modern clinical medicine is in neoplastic disease. Malignant tumours differ by definition from benign tumours because of their capacity to metastasise. In clinical oncology, it is this dissemination of tumour cells, leading to widespread metastatic disease, that is responsible for most therapeutic failures. Understanding the underlying biology and behavioural aspects of cancer metastasis would help to formulate successful therapy in disseminated malignancy. The mechanisms responsible for tumour cell dissemination are complex and although recent research has provided considerable insight into the understanding of these mechanisms, much remains to be learned about this fascinating aspect of cancer biology.

Metastasis can occur either via the lymphatic channels or via the bloodstream (haematogenous). In the former case, regional lymph nodes are involved whilst in the latter, the tissue of distant organs such as liver,

lungs, brain or bone is invaded. Haematogenous metastasis results in parenchymal destruction leading to major organ dysfunction and ultimately death. Tumour cell spread can also take place by direct extension or by migration of cells trans-coelomically, but such routes of dispersion are secondary in importance to those involving lymphatic or haematogenous spread. Early theories of metastasis viewed metastasis as occurring primarily via the lymphatic system and only from there to the bloodstream (Halstead, 1907; Handley, 1922). This theory has been largely discredited since it is now well recognised that carcinomas frequently invade blood vessels directly and this has been demonstrated in pathological studies (Friedell et al, 1965). In addition, circulating cancer cells have been demonstrated in the blood stream of cancer patients even when there is no gross involvement of regional lymph nodes (Salsbury, 1975). It is now recognised that the lymphatic and venous systems are intimately interconnected and that cancer cells can invade the bloodstream independently of the lymphatics (Fisher & Fisher, 1967). It is therefore widely accepted that tumour metastasis via the lymphatics and the bloodstream occur in parallel and the pattern of this dissemination is dependent on the basic biological behaviour of the tumour cell line in question.

The development of a metastatic lesion represents the outcome of a series of sequential interactions between the tumour cell and the hosts defences. For a

metastasis to develop, the tumour cell has to successfully survive a variety of potentially lethal confrontations which might be regarded as part of the host's defence against metastasis.

During the process of haematogenous metastasis, tumour cells must invade the bloodstream at one point and emerge from it at another. There is sufficient evidence to suggest that this process occurs in a series of stages, each stage posing an obstacle to further passage of the tumour cell. Only a small proportion of tumour cells eventually survive this process to become metastases (Poste & Fidler, 1980). The important steps in metastasis are (a) Invasion of host tissue by tumour cells with penetration of blood vessels and/or lymphatics, (b) Detachment of tumour cells and the subsequent transport of tumour cell emboli in the circulation to distant organs, (c) Arrest of the tumour cells, that survive this process, in the microcirculation of target organs, (d) Adherence to endothelium/basement membrane of blood vessels and subsequent extravasation of cells, (e) Establishment of micrometastasis by proliferation of malignant cells and (f) Angiogenesis to form macrometastasis.

To produce detectable lesions, metastases must develop a vascular network and evade the host immune system. Once this has been achieved, tumour cells can invade host stroma, penetrate blood vessels, and enter the circulation to produce additional metastases. The relative importance of each step in limiting the number

of cells which eventually become metastases is uncertain, and probably varies under different circumstances.

Tumour cell invasion:

Several mechanisms regulate tumour cell invasion of host tissues. Tumour cells may pass between endothelial cells (Sindelar et al, 1975) or alternatively a group of cells may create a breach in the vessel wall (Locker et al, 1970). Rapidly proliferating tumours may by mechanical pressure, force cords of tumour cells along lines of least tissue resistance. However, even in the absence of pressure factors, many malignant tumours infiltrate host tissue (Easty & Easty, 1974; Mareel, 1983). In some tumours, cells may invade tissue by virtue of their own motility (Strauli & Haemmerli, 1984). In addition, many malignant neoplasms express higher levels of lytic enzymes such as Cathepsin B and plasminogen activator, than do benign tumours or corresponding normal tissue (Sloane et al, 1982; Sloane, 1990; Jones & DeClerck, 1982; Salo et al, 1983). Destruction of host tissue by these enzymes, aided by mechanical pressure caused by a rapidly growing tumour mass, probably facilitates invasion of host tissue by a malignant neoplasm.

To enter and leave the circulation, tumour cells penetrate the basement membranes surrounding blood vessels. Liotta and colleagues showed that the constituents of the extracellular matrix can be destabilised by degradative enzymes secreted by tumour

cells (Liotta et al, 1979, 1980; Liotta, 1984, 1986). Metastatic tumour cells can preferentially attach to the major collagen class of basement membranes, type IV collagen, and it has been shown that they often can secrete high amounts of a collagenase, specific for type IV collagen (Woolley, 1984). Other workers have shown that metastatic tumour cells can also produce high amounts of enzymes capable of cleaving the major proteoglycan of basement membranes (Nakajima et al, 1983).

Transport of tumour cells:

The entry of tumour cells into the circulation does not in itself constitute metastasis since most tumour cells that enter the circulation die (Fidler, 1970; Weiss, 1986). Using tumour cells radiolabelled with ^{125}I-5-Iodo-2'-deoxyuridine, Fidler showed that 24 hours after entry into the circulation, <1% of the cells are viable, and only <0.1% of all tumour cells in the circulation survive to produce metastases (Fidler, 1970). This study also showed that the greater the number of cells released by a primary tumour the greater the probability of metastases developing. Models in which tumour cells have been injected intravenously into animals, have invariably shown that all surviving tumour cells are situated in the lungs suggesting that tumour cells by and large are arrested at the first capillary bed that they reach (Fidler & Nicolson, 1976; Proctor, 1976). The intravascular phase of tumour metastasis is essentially

a phase of attrition, in which most circulating tumour cells die. To a great extent this is due to mechanical effects: the turbulence of blood flow and the mechanical shearing forces, especially during the first passage through a capillary bed (Sato & Suzuki, 1976), immunological attack by the host defences mainly NK (Natural Killer) cells, may also play a role (Fidler et al, 1976b; Weiss et al, 1974). Aggregation of tumour cells with one other, with platelets or with lymphocytes, enhances cell survival in the circulation (Updyke & Nicolson, 1986; Gasic, 1984; Fidler & Bucana, 1977).

Tumour cell adhesion and extravasation:

Tumour cell emboli that reach the microcirculation may be arrested here, and the ones that survive this process can interact with the cells of vascular endothelium. Adherence of tumour cells to vascular endothelium may occur secondary to mechanical blockage of a small vessel by clumps of tumour cells (Roos & Dingemans, 1979) or alternatively by stable adhesions between tumour cells and vascular endothelial cells (Dvorak et al, 1983). **In vitro**, tumour cells have been shown to adhere preferentially to the constituents of the basement membrane of the blood vessel rather than to the endothelial cells themselves (Nicolson et al, 1985). Platelets may facilitate this process via specific platelet glycoprotein receptors, Ib and the IIb/IIIa complex, which interact with both tumour cells and the

subendothelial matrix (Grossi et al, 1987). In addition, a platelet secreted protein, thrombospondin, may enhance binding of tumour cells to a platelet-fibrin clot or alternatively encourage increased mechanical trapping of tumour cells (Tuszynski et al, 1987). The trapping of tumour cells in the microcirculation, along with the formation of a platelet-fibrin clot may result in damage to the vascular endothelium, which would further encourage tumour cell adhesion to the vessel wall (Dvorak et al, 1983).

Following adhesion to the vascular endothelium, tumour cells penetrate into the extravascular matrix, partly by a process involving enzymatic degradation of the basement membrane as described earlier. The extravasation of malignant tumour cells into surrounding tissues of particular organs may also be due in part to selective organ preference (Nicolson et al, 1985). Host connective tissue can resist this invasion of tumour cells by producing protease inhibitors which can block the process of the enzyme-dependent invasion of the vascular basement membrane (Liotta, 1986; Woolley, 1984).

1.2 Metastatic Heterogeneity of Malignant Neoplasms

It has long been recognised that metastases results from the proliferation of a few "seeded" tumour cells in the favourable "soil" of selected organs (Paget, 1889).

The current view of the seed-and soil hypothesis (Fidler, 1991), consists of three main principles:

a. Different cells from the same neoplasm are heterogenous with regard to their metastatic properties.

b. The process of metastasis is selective although it contains variable elements

c. The production of metastases is dependent on the interaction between metastatic cells and various host factors.

Cells with different capacities to metastasise have been isolated from parent neoplasms (Fidler, 1973; Fidler & Balch, 1987). In 1977, Fidler and Kripke demonstrated that different tumour cell clones, each from an individual cell isolated from the parent tumour, varied quite dramatically in their capacity to produce lung tumour seeding following intravenous injection (Fidler & Kripke, 1977). In addition, it has been shown that human tumour cell lines of colon and renal cancer contain subpopulations of cells with widely varying metastatic properties (Fidler, 1986). Further, **in vitro** experiments have demonstrated organ-selective adhesion, invasion and growth, offering evidence in support of the "seed-soil" hypothesis. Circulatory anatomy and haemodynamic factors may also influence the spread of malignant cells, as indicated earlier (Weiss, 1980).

Equally, local immune defences might account for preferential metastatic growth in target organs (Reif, 1978). Finally, individual cells within a neoplasm may be exposed to different microenvironments, and these differences might be responsible for their varying metastatic potential (Fidler, 1991).

The metastatic process is thus a complex one, involving a series of obstacles, each of which a tumour cell has to overcome if it is to ultimately form a metastatic focus. It is clear that most tumour cells die during this process, but those which survive are potentially lethal to the cancer patient. Although most metastases found in clinical practice have been initiated before diagnosis, there are situations (for eg. during operations for cancer) where the prevention of metastasis may be clinically useful. It is therefore of interest to consider the influences which may modulate the metastatic process. In addition to those factors already discussed, one major influence is the haemostatic system with which the "intravascular" phase of the metastatic process is closely linked. The coagulation and fibrinolytic pathways influence this phase of the metastatic process to a considerable degree, as will be outlined later. In order to give a clear account of this interaction, it is first necessary to describe the role of the haemostatic system under normal circumstances.

2. THE HAEMOSTATIC SYSTEM

The haemostatic mechanism consists of a series of reactions whereby liquid blood is converted to solid clot. Under normal circumstances this mechanism is kept in balance by the fibrinolytic system, either or both being activated or inhibited in appropriate circumstances. Macfarlane (1964) and Davie & Ratnoff (1964) independently proposed the view that the coagulation mechanism is composed of a series of enzyme reactions which function as a biological amplifier, each factor in turn activating the next factor in the sequence by cleaving peptide bonds within it - the so-called "cascade" or "waterfall hypothesis". The main modulating influences on this process are specific inhibitors of activated coagulation factors, the fibrinolytic system, platelets and the vascular endothelium.

2.1 The Coagulation System:

Coagulation occurs when crossed-linked fibrin is formed. This is achieved via a sequence of reactions between plasma proteins termed "coagulation factors". The various factors in the sequence fall within two apparently separate systems only in as much as they are stimulated differently - the intrinsic and extrinsic systems. In the latter half of the coagulation cascade, these two systems join together to act via a common pathway, so that irrespective of what the initial stimulus is, the final outcome is the same. In recent

Table 1 **Coagulation Factors In Humans**

Traditional Name	Preferred Name	Plasma Conc.(ug/ml)	Amino Acids
Fibrinogen	Factor I	3,000.00	3,026
Prothrombin	Factor II	120.00	579
Tissue Factor	Factor III	----	263
Calcium	Factor IV	100.00	----
Pro-accelerin	Factor V	10.00	2,224
Pro-convertin	Factor VII	1.00	406
Antihaemophiliac Factor	Factor VIII	0.05	2,332
Christmas Factor	Factor IX	4.00	415
Stuart Prower Factor	Factor X	12.00	445
Plasma Thrombopl Ant	Factor XI	6.00	1,214
Hageman Factor	Factor XII	40.00	536
Fibrin-stabilizing factor	Factor XIII	20.00	2,680
Fletcher Factor	Prekallikrein	40.00	619
Fitzgerald Factor	HMW - Kininogen	70.00	626

HMW: High molecular weight

years it has become increasingly clear that these two systems are functionally interdependent.

The plasma proteins involved in the process of blood coagulation and those that modulate this process are listed in Table 1.

All the factors apart from factor VII, are normally present in their inactive zymogen form. Following activation by limited proteolysis, they exhibit increased enzymatic activity and act on the next factor in the sequence. Factor VII is present in the active form but can only bind to it's substrate Factor X after it reacts with Tissue Factor.

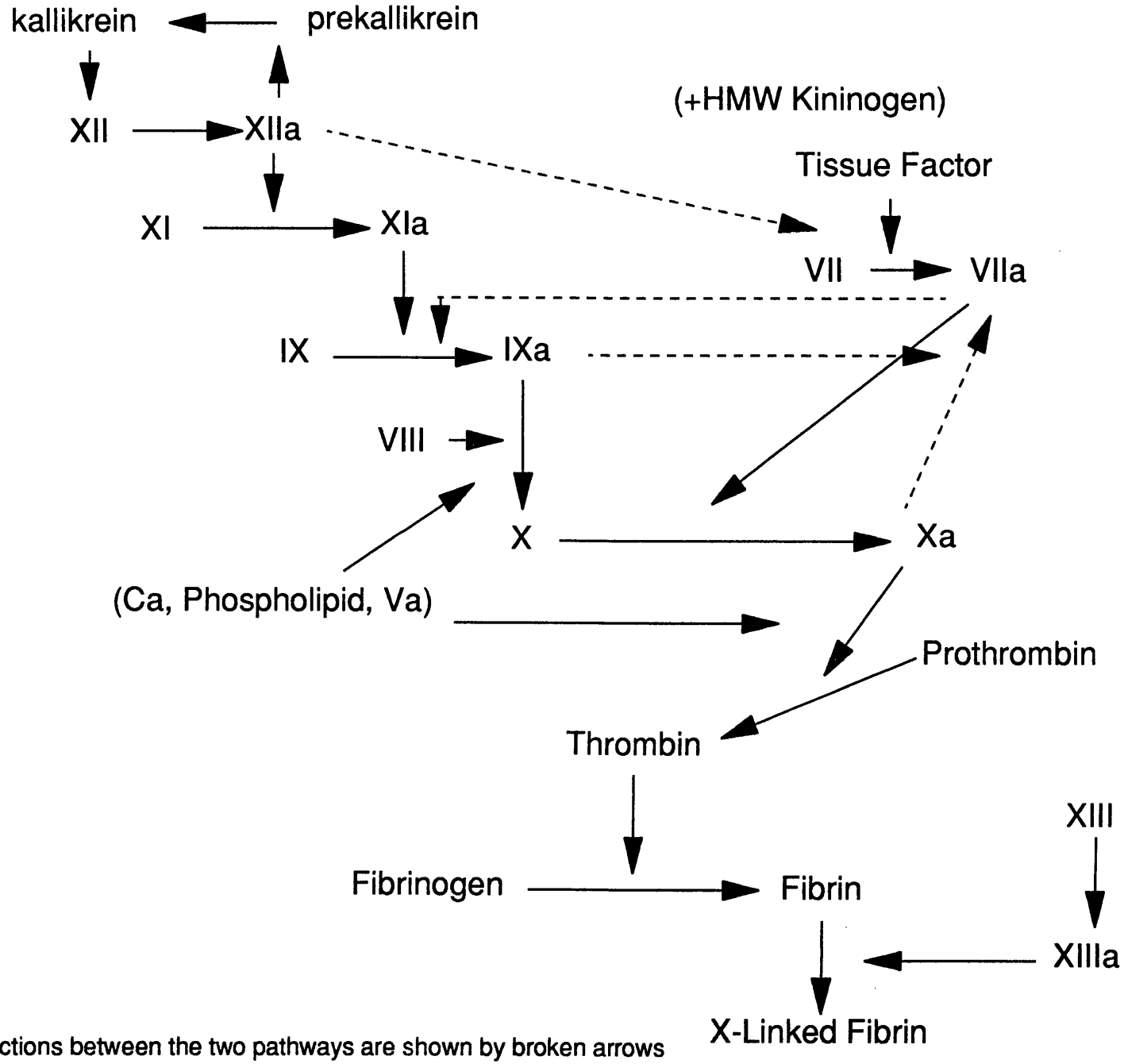
2.2 Pathways of Blood Coagulation:

The relationship between the enzymes making up the intrinsic and extrinsic pathways are illustrated in figure 1 together with the main interactions known to occur between the two systems. Amongst these complex relationships, the following are particularly noteworthy:

The Intrinsic System:

The stimulus to activation of the intrinsic system appears to be the exposure of plasma to a negatively charged surface - for eg. glass **in vitro**, and the collagen of damaged blood vessels **in vivo**. This system is initiated by the interaction between four plasma proteins - factor XII, Factor XI, prekallikrein and high molecular weight kininogen (HMW-kininogen). In the

Figure 1 Major reactions of the coagulation pathway



Note: Interactions between the two pathways are shown by broken arrows

presence of an anionic surface and kallikrein, Factor XII is converted to the two-chain alpha-factor XIIa. The optimal activation of factor XII requires prekallikrein, HMW-kininogen and an activating surface. Factor XIIa in turn activates prekallikrein to form kallikrein which is capable of reciprocally activating further surface-bound factor XII. This surface-bound factor XIIa proceeds to activate factor XI to factor XIa by limited proteolysis.

The mechanism by which a negatively charged surface triggers the intrinsic system is as yet unclear. It is proposed that the Factor XII zymogen changes in conformation rendering it vulnerable to proteolysis (Griffin, 1978). A detailed study, of the kinetics of the enzymatic reactions involving the activation of factor XII, concluded that reciprocal activation rather than autoactivation is the predominant mechanism involved.

Kallikrein is also responsible for releasing elastase from neutrophils during blood coagulation (Wachtfogel et al, 1983). It is recognised that elastase from granulocytes has the capacity to inactivate factor IX (Takaki et al, 1983). This is therefore an additional effect of kallikrein on the coagulation system.

The next step in the intrinsic system is the activation of factor IX by factor XIa. This step requires calcium ions (Amphlett et al, 1981) and results in the formation of IXa. Factor IXa, factor VIII, negatively-charged phospholipid (from platelets) and calcium ions form a complex, in which the enzymatic

activity of factor IXa is responsible for activating factor X to Xa.

The Extrinsic System

When tissue is damaged, tissue factor is released into the bloodstream. This is a lipoprotein consisting of apoprotein III and a mixture of phospholipids and when bound to Factor VII in the presence of calcium ions facilitates binding of Factor VII to it's substrate Factor X, which is then activated to Xa. The Factor VII/tissue factor complex is also capable of activating factor IX. (Osterud & Rapaport, 1977). Activated factor IX can also then activate factor X directly, as described above.

The Common Pathway:

The intrinsic and extrinsic pathways converge at the level of activation of factor X. The next stage is the conversion of prothrombin to form thrombin, a reaction catalysed by an enzyme complex termed prothrombinase - a combination of activated factor X, factor Va, calcium ions and negatively-charged phospholipid. Platelets contain 18-25% of the factor V found in blood (Tracy et al, 1982) and are the source of phospholipid. Thrombin is a potent activator of factor V, but factor Va is required for the generation of thrombin. It appears to act as a receptor for factor Xa.

The formation of fibrin:

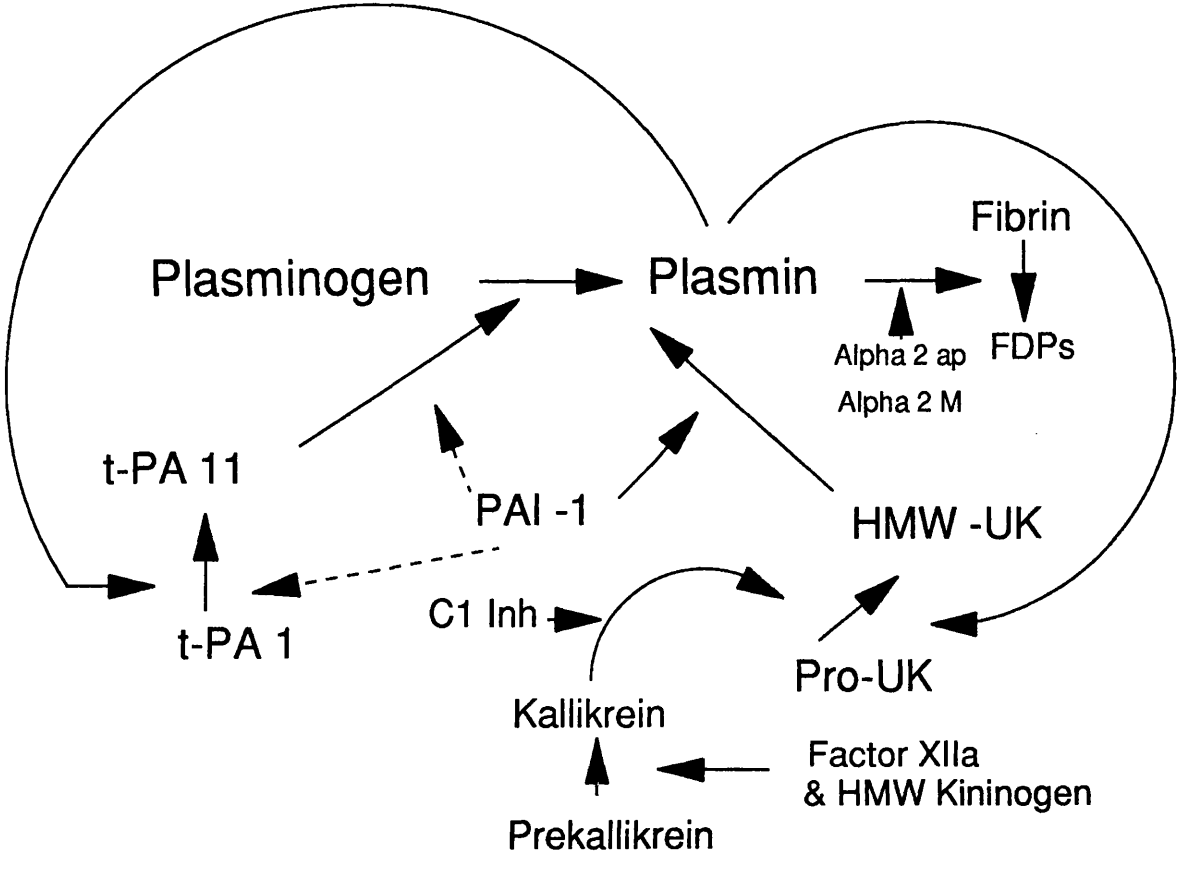
The coagulation cascade ends in the conversion of soluble fibrinogen to insoluble fibrin catalysed by thrombin. A fibrin network at the site of injury provides stability for a plug of platelets.

Fibrinogen is a dimer with two identical halves each consisting of 3 polypeptide chains (A alpha, B beta and gamma). Disulphide bonds also join the two half molecules through their A alpha and gamma chains near the amino-terminus. During the conversion of fibrinogen to fibrin, under the influence of thrombin, the short fibrinopeptides A and B are removed from the amino-terminal ends. This exposes the polymerisation sites by which the fibrin monomer units crosslink with each other (Doolittle, 1977). By the formation of covalent bonds between adjacent fibrin monomers, stabilised fibrin is produced, a reaction catalysed by calcium ions and activated factor XIII. Factor XIII is activated by limited proteolysis catalysed by thrombin. Not only does cross-linked fibrin possess an increased tensile strength, but it is also more resistant to fibrinolysis (Folk & Finlayson, 1977).

2.3 The fibrinolytic system: (Fig 2)

The formation of a fibrin clot and it's subsequent degradation is under the control of two closely interwoven mechanisms, the coagulation pathway and the fibrinolytic pathway. These two systems are in constant balance with each other and one or the other is

Figure 2 Major reactions of the fibrinolytic system



Alpha 2 ap: Alpha 2 antiplasmin

Alpha 2 M: Alpha 2 Macroglobulin

C 1 Inh: C 1 Inhibitor

tPA: Tissue plasminogen activator

PAI: Plasminogen activator inhibitor

HMW: High molecular weight

UK: Urokinase

Note: Broken arrows represent inhibitory influences

activated in appropriate circumstances.

The plasma proteins involved in the process of fibrinolysis and those that modulate this process are listed in Table 2.

Fibrinolysis involves the conversion of plasminogen to plasmin, an enzyme capable of destroying the fibrin clot. Plasminogen, a glycoprotein synthesised by liver parenchymal cells, is present in blood. Only a part of circulating plasminogen is available for binding to fibrin. The remainder exists in a reversible bound form to histidine-rich glycoprotein and to a minor extent to alpha2-antiplasmin.

Activation of plasminogen to plasmin occurs via one of two mechanisms: a) via tissue-plasminogen activator (t-PA) or alternatively b) via urokinase-type plasminogen activator (u-PA). The existence of a third proactivator, activated by kallikrein and a factor XII-dependent pathway and inhibited by C1-inhibitor, is postulated (Kluft, 1980; Kluft et al, 1984). In addition to the activator molecules, a series of inhibitors exist which interact with the activators via a complex system. The way this system functions is understood only in part and awaits further investigation.

t-PA is a protease synthesised by the endothelial cells of blood vessels (Kristensen et al, 1984). Fibrin adsorbs plasmin and t-PA onto itself, thereby bringing the necessary components of fibrinolysis in proximity with each other and also enhancing the effect of t-PA on plasminogen (Bachmann, 1987). In the absence of fibrin,

Table 2 **Components of the Fibrinolytic System**

Component	Plasma Conc (ug/ml)	Amino Acids
Plaminogen	200.000	796
t-pa	0.005	530
Urokinase	0.008	411
t-pa inhibitor I	0.050	379
t-pa inhibitor II	0.005	----
Alpha2 antiplasmin	70.000	452
Histidine-rich Glycoprotein	100.000	507
Alpha2 macroglobulin	2,500.000	1,451

t-pa: tissue plasminogen activator

t-PA is incapable of activating significant amounts of plasminogen.

u-PA is linked directly to the intrinsic system. Factor XIIa in conjuction with HMW-kininogen activates prekallikrein to form kallikrein. Kallikrein activates the proenzyme pro-urokinase to u-PA, which in turn activates plasminogen to form plasmin. Interestingly, this interacts closely with the activation of the intrinsic system in the coagulation pathway.

Both t-PA and u-PA are inhibited by plasminogen-activator inhibitor substances, PAI-1 and PAI-2 (Bachmann, 1987). These inhibitors of plasminogen-activators, particularly PAI-1, act by inhibiting the conversion of the weaker proenzyme form (t-PA 1) to the more active enzyme form (t-PA 2) and also by directly inhibiting the effect of t-PA on plasminogen.

Alpha2-antiplasmin, a glycoprotein synthesised by liver parenchymal cells, influences fibrinolysis by directly inhibiting plasmin.

Several links between coagulation and fibrinolysis have been identified, some of which have been outlined above. Activation of the contact system, gives rise to the formation of bradykinin from kininogen. Bradykinin is a potent inducer of t-PA release from the vascular endothelium (Prowse & Cash, 1984). Two other compounds formed during coagulation, thrombin and platelet-activating factor, are powerful inducers of t-PA release (Emeis & Kluft, 1985).

The role of Protein C:

Protein C is a vitamin K-dependent plasma protein that is converted to it's active form (APC) by thrombin. Thrombomodulin, a protein found in the plasma membrane of vascular endothelial cells, enhances the thrombin-catalysed conversion of APC. APC has two effects:

a. An anticoagulant effect produced by inactivating factors V and VIII (Marlar et al, 1981) and

b. An enhancing effect on fibrinolysis (Zolton & Seegers, 1973).

The role of Antithrombin III:

Antithrombin III is a major physiological inhibitor of thrombin and factor Xa and also inhibits factors IXa, XIa and XIIa to a lesser degree. Most of the anticoagulant effect of exogenous heparin is mediated through it's interaction and stimulation of antithrombin III. Heparin Cofactor II is another inhibitor of thrombin and it's activity is also stimulated by heparin. At high heparin concentrations, it is more active than antithrombin III. Other inhibitors of coagulation deserving mention are alpha-1-antitrypsin (inhibits factor XIa) and C1 inhibitor (inhibits C1, kallikrein, factors XIIA and XIa).

The role of Protein S:

Protein S is a vitamin-K dependent plasma protein but not a serine protease. It is chiefly synthesised in the liver and also by endothelial cells. 60% of the protein

S in plasma is found in a 1:1 stoichiometric complex with the C4B-binding protein, which regulates the activity of the C4 component of complement, and cannot react with protein C. The remaining 40% of protein S in plasma is in the free form and though having no direct inhibitory effects on factors VIII and V, is available to form a calcium-dependent complex with activated protein C on phospholipids exposed on the surface of activated platelets. Like other Vitamin K-dependent factors, total protein S is reduced in the newborn. This is compensated for by a marked reduction in C4B-binding protein so that a relatively normal level of free protein S is maintained.

Activated Protein C Inhibitor:
This is a single-chain glycoprotein with several features in common with Antithrombin III though immunologically different from it. It blocks the action of it's substrates (activated protein C and to a lesser extent thrombin and factor Xa), forming a 1:1 inhibitor:protease stoichiometric complex.

2.4 The Role of Platelets

Platelets form an integral part of the coagulation system. They participate in several aspects of haemostasis. Early in the process of haemostasis, platelets adhere to the collagen fibres of exposed subendothelium of the damaged vessel wall. This adherence is brought about by the interaction between

von Willebrand factor and platelet glycoprotein Ib or the IIb/IIIa complex or both (Zucker et al, 1977; Ruggeri et al, 1983). Following adhesion, platelets undergo a series of reactions beginning initially with a loose platelet-platelet attachment. This progresses to the "release-reaction" wherein platelets release a number of biochemically active mediators from granules within their cytoplasm. The dense bodies within platelets contain ADP, ATP, serotonin, calcium and pyrophosphate. The alpha granules contain the major secreted glycoprotein thrombospondin as well as, fibrinogen, platelet factor 4, beta-thromboglobulin, factor V, factor VIII and platelet-derived growth factor. The relevant functions of some of these substances, in haemostasis is outlined below:

1. Thrombospondin promotes and strengthens the inter-platelet bonds.
2. Platelet factor 4 binds to platelet receptors and augments aggregation and secretion.
3. Platelet-derived growth factor is a potent chemoattractant for monocytes, neutrophils, fibroblasts and smooth muscle cells, the cells that participate in the repair of the vascular injury.
4. The negatively-charged phospholipids situated in the plasma membrane of platelets, calcium ions, factor Va and factor VIIIa are essential for the crucial stages of the coagulation cascade as described earlier (activation of factor X and the conversion of prothrombin to thrombin). The thrombin thus generated stimulates

further platelet aggregation and release by positive feedback.

5. Fibrinogen is the principal interplatelet bridging molecule.

The role of ADP in platelet aggregation remains controversial. ADP can stimulate platelets and produce a change in conformation accompanied by a rise in cytosolic calcium. This may lead directly to the binding of fibrinogen. It has been suggested that there may be an enzyme with an external nucleotide binding site such that ADP acts only as an essential co-factor for the binding of fibrinogen. What is clear however, is that ADP is essential for platelet activation to occur.

Finally, platelets promote clot lysis through their ability to cause clot contraction (Carroll et al, 1981).

It is clear therefore that platelets have several roles to play and are essential for normal haemostasis.

2.5 Role of Vitamin K

This fat soluble vitamin was discovered in 1929. It is required for the synthesis of active forms of the factors VII, IX, X, and II (prothrombin), as well as the coagulation related proteins C, S and Z. Each of these proteins contain a series of gamma-carboxy glutamic acid residues, and the role of vitamin K is to act as a co-factor for the production of these residues from glutamic acid. The gamma carboxylated residues appear to bind to calcium ions, which in turn are responsible for binding the proteins to phospholipid bilayers. This

binding provides vital amplification of the reactions catalysed, essential to the rapid production of a fibrin clot. Exactly how this occurs is not known, but it is believed that binding to this kind of structure may enhance the probability of substrate and enzyme approaching each other close enough for catalysis to occur.

2.6 Measurements of the coagulation system.

There are several available tests to measure the haemostatic system. Some of these are outlined in Table 3. The overall function of the coagulation system is usually measured in terms of the time taken for a fibrin clot to form under defined conditions. The simplest tests, the bleeding and clotting times, are applied to whole blood, whilst the conditions for the other tests are more complex. The time taken for clot formation reflects the efficiency of the mechanism as a whole, though not as precisely as a direct measurement of the rate of fibrin formation would do. The thrombin time, activated partial thromboplastin time (APTT) and prothrombin time are the most commonly used measures. They reflect the activity of the final common pathway to fibrin formation, the intrinsic and the extrinsic pathways respectively. Measurements of the levels of individual factors can be performed using a variety of functional assays. They provide relatively limited information, as only very major reductions in the levels of individual factors are likely to cause changes in the

Table 3 **Tests of Coagulation**

Test	Factors Involved	Comments
APTT	I,II,V,VIII-XII	Intrinsic & Common
Clotting time	I,II,V,VIII-XII,Platelets	Crude test
Prothrombin time	I,II,V,VII,X	Extrinsic & Common
Thrombotest	II,VII,X ?IX	Oral anticoagulation
Thrombin time	I	Also FDP, heparin
Protamine sulphate	Fibrin monomers	Also Fibrinogen/FDP
FDP tests	Fibrinogen/FDP	Immunological method
Bleeding time	Platelets,Von Willebrand Fact	Number and function
Platelet aggregation	Platelet function	ADP/Ristocetin/other
FpA measurement	Fibrinogen	Highly sensitive

APTT: Activated Partial Thromboplastin Time
FDP: Fibrin(ogen) Degradation Products
FpA: Fibrinopeptide A
ADP: Adenosine diphosphate

efficiency of coagulation. Haemophiliacs, for example, have adequate haemostatic function for normal purposes if their level of factor VIII is greater than 30% of normal.

Immunological assays for the breakdown products of fibrin give a rough measure of the activity of the system. The standard assay cannot differentiate between the degradation products of fibrin and of fibrinogen. High levels of fibrin(ogen) degradation products (FDPs) may therefore reflect excessive activity in either the coagulation or the fibrinolytic system or both. A more sensitive method of monitoring haemostatic function is the measurement of specific products of individual reactions. An example of this is fibrinopeptide A (FpA), a 16 amino-acid peptide cleaved from the alpha chain of the fibrin molecule in the first stage of the formation of fibrin (Rickles and Edwards, 1983). It has a short half-life of about 3 minutes and therefore gives a picture of the level of activation of the coagulation system at one precisely defined point in time. The other tests used to measure specific alterations in the coagulation system are outlined in Table 3.

3. CANCER AND THE COAGULATION SYSTEM.

3.1 Evidence for altered coagulation in malignant disease.

The haemostatic system is altered significantly by malignancy. In 1865, Trousseau described an association between visceral malignancy and migratory thrombophlebitis (Trousseau, 1865). This was the first published report of evidence of a disturbance of coagulation in cancer patients. Since then, several workers have substantiated this evidence by demonstrating a disturbance in coagulation in patients with malignant disease in a variety of situations (Miller et al, 1967; Goodnight, 1974; Hagedorn et al, 1974; Sack et al, 1977; Bick, 1978; Nand et al, 1987). Evidence of thromboembolism is present in up to 50% of cancer patients at post-mortem and is related to the type of malignancy (Thompson & Rodgers, 1952). Thrombosis is a more common complication of pancreatic and gastric cancer than of prostatic or skin cancer (Dvorak, 1987). Furthermore, Goldberg et al (1987) demonstrated a significantly higher incidence of occult malignancy in patients with deep venous thrombosis. It is well recognised that patients undergoing surgery for cancer, postoperatively, have a 33% risk of deep venous thrombosis (Pineo et al, 1974; Negus et al, 1980), as compared to a risk of 10% in patients with benign disease.

Haemmorhage occurs in about 10% of cancer patients but half of these result from thrombocytopaenia due to chemotherapy (Belt et al, 1978). Local haemorrhage may result from tumour necrosis or direct invasion of blood vessels, while more generalised bleeding is due to bone marrow involvement or acute Disseminated Intravascular Coagulation (DIC) (Dvorak, 1987). Overall, thromboembolic and haemorrhagic complications are the second most common cause of death in cancer patients (Ambrus et al, 1975).

DIC, a disorder caused by generalised inappropriate activation of the coagulation system within the vasculature, results in diffuse haemorrhage due to a "consumptive coagulopathy". 8 - 15 % of cancer patients with laboratory abnormalities, develop DIC of clinical significance (Sack et al, 1977; Weick, 1978; Kies et al, 1980). However, these represent only the decompensated form of DIC (Goldsmith, 1984), with a much larger proportion of patients having evidence of compensated or overcompensated DIC.

Several studies have demonstrated subclinical abnormalities of haemostasis in cancer patients (Sun et al, 1979; Doni et al, 1984). In their series of 108 patients, Sun et al (1979) showed that 98% of their patients had one or more abnormal coagulation test. The abnormalities commonly seen were thrombocytosis (57%), hyperfibrinogenemia (46%), increased fibrin degradation products (68%) and thrombocytopenia (11%), clearly

illustrating that subclinical coagulopathy is frequently present in cancer patients.

Plasma Fibrinopeptide A (FpA) is a sensitive indicator of activation of coagulation. It is found to be elevated in many cancer patients and has been shown to correlate closely with disease stage and prognosis (Peuscher et al, 1980; Myers et al, 1981; Mombelli et al, 1982). Rickles et al,(1983) demonstrated that terminally ill patients show a persistently high level of FpA.

Various other coagulation abnormalities are seen in cancer patients. 1/3 of patients show decreased plasma Antithrombin III levels (Kies et al, 1980). This however, has not been corroborated in other studies (Miller et al, 1967; Rubin et al, 1980). Deficiencies of the coagulation factors II, VII, IX, X and XIII have also been demonstrated in patients with malignant disease (Miller et al, 1967).

Animal experimental work has shown altered coagulation in different tumour-bearing models. These include thrombocytopenia, hyperfibrinogenemia, an increase in fibrin degradation products, decreased fibrinolytic activity, leucocytosis and microangiopathic haemolytic anaemia (Hilgard et al, 1973; Rasche, 1974; Poggi et al, 1977; Hilgard, 1977; Chmielewska et al, 1980). Using thromboelastography, Raina et al (1985) showed significant acceleration of coagulation in tumour-bearing animals in an experimental model. They further observed, that complete resection of the tumour

restored coagulation to normal while partial resection or sham operation did not.

3.2 The role of fibrin and the fibrinolytic system in malignant disease.

Fibrin has been frequently demonstrated within and surrounding neoplasms. This was first demonstrated in transplanted tumours (Day et al, 1959) and was further confirmed in human tumours (O'Meara & Jackson, 1958; Hiramoto et al, 1960; Dvorak et al, 1981). The presence of fibrin in malignant tumours has attracted considerable attention and the relevance of it's presence has been subject to several interpretations, some of which are speculative with very little direct support. It has been believed to be a source of nutrition (Ambrus et al, 1982), a preventive barrier for the spread of tumour cells and a source of "angiogenesis factors" to facilitate the growth of blood vessels into the tumour (Dvorak et al, 1979). Fibrin is an early and consistent marker of tumour cell stroma. It is deposited within hours of tumour implantation and remains in the tumour throughout the period of tumour growth (Dvorak et al, 1984). It appears to have opposing effects in the development of metastasis. At the level of the primary neoplasm, fibrin may play a dual role in tumour growth and spread by a) promoting primary tumour growth and b) preventing cell shedding by forming a barrier.

There is considerable evidence that neoplastic cells secrete plasminogen activators at a higher rate and in

different ways from normal cells (Markus et al, 1983; Skriver et al, 1984). Some workers have suggested that this increased plasminogen activator (PA) production may be an essential step in the metastatic process though others have shown no correlation between PA production by tumour cells and metastatic potential (Wang et al, 1980; Whur et al, 1982; Eisenbach et al, 1985). An important study by Ossowski and Reich (1983) showed that injection of specific antibodies to human PA into a chick embryo significantly reduced the number of metastases arising from a transplanted primary tumour of human origin. In further animal experiments they showed that inhibition of the PA pathway by a polyclonal antibody drastically reduced the metastatic potential of various human tumour cell lines (Ossowski, 1988). On balance, the evidence suggests that PA by generating local proteolytic activity enables tumour cells to overcome the natural barrier of the vascular basement membrane permitting them to enter the circulation (Dano et al, 1985; Liotta, 1986).

Circulating tumour cells may form aggregates with platelets and fibrin which facilitate their trapping within the microcirculation of target organs, thereby enhancing their metastatic efficiency (Crissman, 1984). The earlier view that fibrin destroys trapped tumour cells (Iwasaki, 1915), has now been abandoned. Contrary to this view, the current belief is that fibrin may facilitate adhesion of tumour cells to vascular

endothelium by acting as an intercellular "glue" (Francis, 1989).

When it was realized that fibrin may be involved in tumour growth and spread, several workers began investigating the effect of fibrinolytic modulation in experimental animal tumour models. The results were however conflicting (Grossi et al, 1961; Cliffton & Agostino, 1962; Tanaka et al, 1977; Giraldi et al, 1977; Turner & Weiss, 1981).

In summary, the results of all these studies would suggest that fibrin may have several roles to play (some of them opposing), in tumour growth and progression:

1. To encourage local tumour growth by facilitating angiogenesis and perhaps serving as a nutritional source.
2. To form an effective barrier to tumour invasion of the vascular basement membrane.
3. To form a mesh with platelets to facilitate trapping of cells in the microcirculation of target organs encouraging tumour cell adhesion to vascular basement membrane, thereby facilitating the metastatic process.

The relative importance of these various actions under clinical conditions is unknown at present.

3.3 The role of platelets in malignant disease:

Platelets appear to be closely associated with the development of malignant disease. 30-60% of cancer patients have thrombocytosis (Sun et al, 1979; Rickles & Edwards, 1983b) and 4-11% have thrombocytopenia

(Hagedorn et al, 1974; Sun et al, 1979). The aggregation of platelets around tumour cells was first described in 1903 (Schmidt). This was confirmed by Wood et al (1958; 1961) in their classic cinemicrographic studies of the fate of injected tumour cells in the blood stream. In their experiments, they demonstrated an association between platelet thrombus formation and tumour cell deposition. Platelet-tumour cell emboli have often been proposed to facilitate the entrapment of tumour cells in the microcirculation (Jones et al, 1971). Crissman et al however suggest that tumour cell-induced platelet aggregation occurs after the initial arrest has occurred (Crissman et al, 1985). Electron microscopy has demonstrated a massive aggregation of platelets at the site of tumour emboli after the initial arrest of intravenously injected tumour cells. Platelet disaggregation commences after 3 hours and tumour cells attach themselves to the vascular endothelium by short pseudopodia (Sindelar et al, 1975). It would seem that one of the ways platelets function in the metastatic process is by stabilising the initial adhesion between tumour cells and endothelium. Gasic et al (1973) studied platelet-tumour cell interactions in mice and showed that tumour cell lines produced platelet aggregation both **in vitro** and **in vivo**. They also demonstrated the presence of membrane associated platelet-aggregating material in these tumours (Gasic et al, 1973). Subsequent studies demonstrated that platelet-aggregating material of cultured murine tumours is

spontaneously shed in the form of membrane vesicles (Pearlstein et al, 1979). This platelet-aggregating material contains sialic acid and requires complement for it's activation. It is inhibited by trypsin, adenosine, indomethacin, phospholipase and cyclic AMP (Pearlstein et al, 1980; Hara et al, 1980(b); Karpatkin & Pearlstein, 1981; Bastida & Ordinas, 1988; Cavanaugh et al, 1988). Adhesion of the tumour cell-platelet aggregate to disrupted vascular endothelium leads to the formation of a dense fibrin layer and firm adhesion to the subendothelial layers.

Several **in vitro** studies have shown that platelets contain a growth-promoting factor for tumour cells (Kohler & Lipton, 1977; Eastment & Sibrasku, 1978; Hara et al, 1980). Cowan & Graham (1981) demonstrated that 48 different tumours grown in culture were stimulated by platelet-derived factor. The significance of this remains unclear since platelet-derived growth factor may may stimulate normal fibroblasts, smooth muscle cells and glial cells **in vitro** (Kohler & Lipton, 1974; Ross et al, 1974; Westermark & Wasteson, 1976). In addition, it has been postulated that platelets enhance tumour cell adhesion to extracellular matrix via specific platelet glycoprotein receptors, Ib and the IIb/IIIa complex which interact with both tumour cells and the subendothelial matrix (Grossi et al, 1987). Finally, implantation of tumour cells may be encouraged by platelet-derived vascular permeability activity (Donati & Poggi, 1980).

Based on the evidence presented above, various antiplatelet agents have been used in animal tumour systems to prevent metastatic disease. The results of these studies have not been consistent (Wood & Hilgard, 1972; Gasic et al, 1973; Gordon et al, 1979, Zacharski et al, 1982).

Therefore it appears that platelets play an important role in malignant disease. Tumour cells are capable of aggregating and activating host platelets thereby promoting metastasis. In animal experiments, platelet-aggregating activity correlates well with the metastatic potential of tumour cells. Tumour cell survival may be enhanced by platelet-derived growth factors. Platelets may encourage tumour cell adhesion to the extracellular matrix. The full significance of all these findings is as yet unclear.

3.4 Procoagulant activity of tumour cells.

Tumour procoagulant activity can be detected in centrifuged cell-free supernatants or cell-free ascitic fluid from various tumour cell lines (Dvorak et al, 1983a). Tumour cell procoagulant can be further categorised into three subgroups:

1. Tissue-Factor-like procoagulants
2. Factor X activators
3. Miscellaneous procoagulants

1.Tissue factor activity in cancer cells:

Tissue Factor was first identified in 1973 by Gralnick and Abrell from buffy coat cells in patients with acute progranulocytic leukemia and is considered to be the main cause of intravascular coagulation seen in this condition. Since then, it has been identified in a variety of malignant tumours including lymphomas, adenocarcinomas and other leukemias (Sakuragawa et al, 1977; Mussoni et al, 1986; Zacharski et al, 1986). It should be noted that tumour cells shed plasma membrane vesicles containing procoagulant activity (Dvorak et al, 1983a). Mononuclear cells are a further source of procoagulant in both normal patients and patients with malignant disease (Edwards et al, 1981; Schwartz & Edgington, 1981; Semararo, 1988), although monocytes from cancer-bearing animals exhibit more tissue factor activity than monocytes from controls (Lorenzet et al, 1983). Tissue factor activates the extrinsic pathway of the coagulation cascade by increasing the activity of Factor VIIa which in turn catalyzes the conversion of Factor X to Xa (Dvorak et al, 1983a).

2. Factor X-activating activity

Gordon et al (1975) compared the procoagulant activities of normal and malignant tissues and showed that tumours, in addition to exhibiting tissue factor, also contained a procoagulant, which unlike tissue factor, did not require factor VII for it's activity. This substance termed "Cancer Procoagulant" has the capacity to

activate factor X directly. Gilbert and Gordon (1983) demonstrated a strong positive correlation between the metastatic potential of B16 mouse melanoma variants and cancer procoagulant. Similar cancer procoagulant activity has been shown to occur in cells from different experimental tumours (Curatolo et al, 1979; Hilgard & Whur 1980). Procoagulant activity in benign melanocytic lesions is dependent on Factor VII and can be blocked by concanavalin A, a tissue factor inhibitor. In contrast to this, extracts as well as isolated cells from malignant melanoma lesions exhibit procoagulant activity which is Factor VII independent and is inhibited by cysteine protease antagonists but not by concanavalin A (Gordon et al, 1979; Donati et al, 1986). These findings would suggest that normal tissue contains tissue factor and that malignant transformation is responsible for cancer procoagulant production.

3. Miscellaneous procoagulants:

In addition to tissue factor and factor X-activators, other tumour-associated procoagulants have been described though they have not been confirmed (Lawrence et al, 1952; Boggust et al, 1963; Svanberg, 1975).

Finally, patients with metastatic disease show low levels of Antithrombin III and Protein C, the two major natural anticoagulants produced by the liver (Rubin et al, 1980; Honegger et al, 1981; Rodeghiero et al, 1984).

3.5 Summary of evidence and hypothesis.

The foregoing evidence indicates the presence of complex interactions between haemostasis and cancer. In summarizing the salient points, we are especially interested in features of this relationship which may suggest ways of exploiting or inhibiting relationships described, so as to interfere with pathophysiology in ways which may have clinical potential.

To summarize:

1. Influences of cancer on the haemostatic system:

It is evident from clinical and animal studies that cancer activates the coagulation system. Activation of platelets have also been demonstrated. The effects of cancer on haemostasis correlate with disease progression and this influence may partly contribute to developing pathophysiology, although this is not proven. Finally, tumour cells have been shown to have relevant stimulatory effects on platelets, the coagulation system and fibrinolysis.

2. Influences of the haemostatic system on coagulation:

Several theories have been offered for the presence of fibrin within and surrounding primary neoplasms. These are mainly speculative and lack supportive evidence. Evidence for platelets and fibrin deposits around tumour cells in the microcirculation is more convincing. It impicates fibrin and platelets in the "intravascular" phase of the metastatic process. Furthermore, evidence

that plasminogen activator generates local proteolytic activity to enable tumour cells overcome the natural barrier of the basement membrane permitting them to enter the circulation, supports the hypothesis that fibrinolysis contributes to the metastatic process.

3. Haemostasis and metastasis:

The most convincing and potentially relevant area of evidence is that fibrin and platelets play a role in the "intravascular" phase of the metastatic process. Manipulation of the haemostatic system in animal studies have shown an alteration in the pattern of metastatic development. Anticoagulant therapy has been successful in inhibiting cancer metastasis in several animal studies and in a small number of clinical studies as well. It has been suggested that coumarin anticoagulants inhibit metastasis by virtue of their anticoagulation properties (Agostino et al, 1966; Ryan et al, 1969). These workers showed that it was possible to achieve a dramatic reduction in pulmonary seeding of intravenously injected tumour cells by pretreating the animals with anticoagulants. Wood in 1974 reported that such tumour cells often became surrounded by a mass of adherent fibrin and platelets when they lodged on the vascular endothelium and that this phenomenon was abolished by anticoagulant therapy. The hypothesis that developed as a result of these studies is that anticoagulants inhibit metastasis by interfering with clot formation around

intravascular tumour cells thereby preventing tumour cell adhesion to the vessel wall (Brown, 1973).

Retrospective clinical studies have suggested that there might be a beneficial effect on survival in patients on warfarin who develop cancer. However, the number of cases reported is small and further confirmatory work is required before any conclusions can be drawn (Forman, 1979; Michaels, 1974; Annegers & Zacharski, 1980). The well conducted prospective randomised study by Zacharski and colleagues in the Veterans Administration hospitals of the USA (Zacharski et al, 1979; 1984) showed no detectable benefit of anticoagulation for any category of cancer patient apart from small cell lung cancer. In this sub-group of patients, survival in the warfarin-treated group was approximately double that in the group that did not receive treatment with warfarin. The main criticism of this trial is that the patients admitted into it were mostly suffering from advanced disease and as such were unlikely to benefit from antimetastatic therapy which essentially ought to be beneficial in patients whose disease has not yet metastasised widely.

McCulloch & George (1987), showed that warfarin inhibits metastasis, whilst injection of the warfarin-dependent coagulation factors II, IX, X enhance metastasis in an animal model. Injection of factor VII alone (also warfarin-dependent) had no effect on tumour metastasis. Important areas of doubt remain as to the exact mechanism(s) by which coagulation factors II, IX,

X produce tumour enhancement. These must be eliminated if further studies are to go from this model to potential clinical level.

The aim of this study was to investigate this phenomenon of tumour enhancement, in a similar animal model. In order to identify the mechanism(s) involved, we set out to investigate the following possibilities:

1. The observed effect of tumour enhancement by the human coagulation factor complex II, IX, X in this animal model, may be directly related to it's xenogeneic properties. It is proposed that a comparison of the prometastatic effects of human and rat coagulation factor complexes II, IX, X be made.
2. It is not unreasonable to assume that in order to enhance pulmonary tumour seeding, the coagulation factor complex II, IX, X, may be responsible for an increase in the number of tumour cells physically trapped in the pulmonary microcirculation. This would theoretically increase the number of potentially metastatic cells in the lung, increasing the likelihood of a greater number of metastases thereby developing. It would be possible to detect the number of tumour cells trapped in the pulmonary microvasculature by studying the passage of intravenously injected radiolabelled tumour cells, through the lung.
3. The role of individual factors II, IX and X on tumour enhancement needs to be investigated. These factors are closely linked to each other in the coagulation cascade

and it is possible that any one of them might be solely responsible for the effect of tumour enhancement.

4. Administration of exogenous coagulation factor complex II, IX, X may cause activation of the coagulation system, resulting in the formation of intravascular fibrin clots. These microvascular fibrin clots may encourage entrapment of tumour cells, thereby enhancing metastasis. In order to investigate this possibility, highly sensitive tests of activation of coagulation need to be employed. It is believed that estimation of plasma FpA is the most sensitive indicator of activation of coagulation.

Furthermore, it would be interesting to investigate whether destruction of fibrin by fibrinolytic therapy has any effect on tumour metastasis.

CHAPTER 1

VALIDATION OF PREVIOUS WORK AND THE ANIMAL MODEL OF METASTATIC CARCINOMA.

INTRODUCTION

The foregoing review has outlined persuasive evidence that malignancy affects the coagulation system in a diverse and profound manner. Clinical and animal experimental evidence has been outlined suggesting a beneficial effect of anticoagulation on the metastatic process. McCulloch and George (1987), have shown that Warfarin inhibits and the Warfarin-dependent factor complex II, IX, X enhances pulmonary tumour seeding in their animal model. In order to investigate this phenomenon of tumour enhancement by the coagulation factor complex II, IX, X, it was initially necessary to validate the results of previous workers. The most obvious and simple way of doing this is by adaptation of the existing animal model. This has the twin advantages of comparibility and elimination of extraneous variables.

Areas requiring validation:

1. Tumour model and the method of detection of pulmonary metastases because of the possibility of a phenotypic drift in the tumour cell line resulting in a possible change in the nature of the lung deposits.
2. Use of human coagulation factor complex II, IX, X because of the limited nature of the previous work: the

results related to the dose and timing of administration of the factor complex are unknown.

3. Direct effect of the factor complex preparation on tumour cell aggregation because this may affect metastasis and has not been previously considered.

The original reasons for selecting this tumour model still apply. These are outlined below:

The tumour-host system selected requires the following important properties in order to represent the situation in human cancer as closely as possible:

a. The tumour should metastasise reliably and spontaneously from the primary site.

b. It should resemble human carcinoma as closely as possible in terms of it's histological origin, morphology and behaviour.

c. It should be possible to grow the tumour cells **in vitro**.

d. The tumour should be syngeneic with the host animal and should not elicit a significant immune response in the host.

e. It should be possible to effectively evaluate changes in the haemostatic system in the host animal.

f. Administration of intravenous tumour cells should give measurable and reproducible results.

The rat mammary carcinoma cell line used was the $Mtln_3$ clone of the 13762 NF mammary carcinoma. It has a high rate of spontaneous metastasis to the lung and resembles human breast cancer both morphologically and

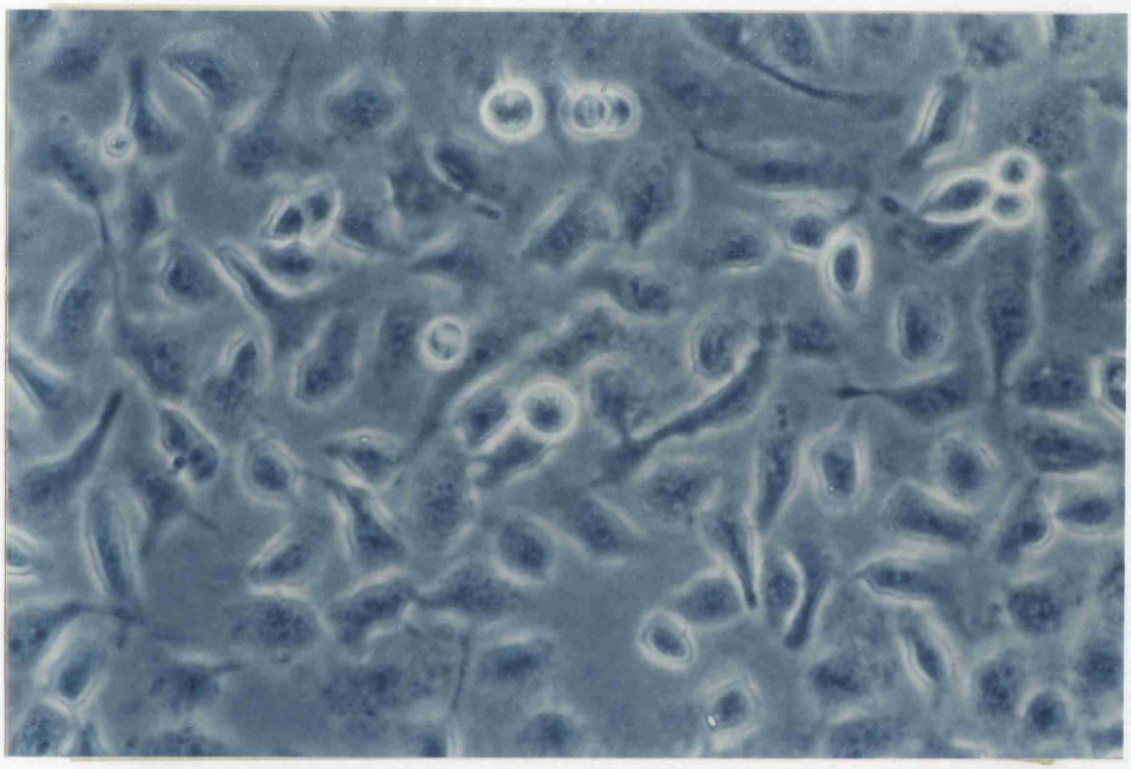


Figure 3 Morphology of $Mtln_3$ rat mammary carcinoma cells in culture - x 625 magnification.

in it's propensity to metastasise to regional lymph nodes (Neri et al, 1982). The tumour cells have been shown to be syngeneic with the Fischer 344 rat, in which the parent tumour was raised and do not elicit a significant cellular or humoural immune response during prolonged growth in this host (North & Nicolson, 1985). The cloned line can be easily maintained in culture, though repeated passage must be avoided in order to retain it's metastatic potential (Neri & Nicolson, 1981).

Metastatic tumour model.

Animals:

The animals used throughout this study were female Fischer 344 (F344) inbred rats (Olac Ltd, Bicester, England). Animals were housed 3 to a cage and were fed a standard laboratory diet (CRM diet, Labsure, Cambridge, England), and tap water with a chlorine content of 7 mg/l. No measures were taken to avoid coprophagy. All animals used in these experiments were normal and healthy according to visual observations and to the results of routine microbiological testing for infection. The mean weight of the animals at the time of use was 140g.

Tumour cells:

The tumour cells used in these studies were a clone of rat mammary carcinoma designated $Mtln_3$ (Figure 3), originally derived from the 7,12-dimethylbenz (a)

anthracene-induced adenocarcinoma 13762 (Segaloff, 1966). This clone was derived from the parent tumour by Neri and Nicolson (Neri et al, 1982), and was characterized as being of high metastatic potential. Frozen cultures of these cells were used to establish the cell line **in vitro** at the Department of Oncology, University of Glasgow. Cells were cultured in 75 cm^2 tissue culture flasks (Gibco, Paisley, Scotland), in equal parts of Hams' F10 and Dulbecco's Modified Eagle's Medium (F10/DMEM), with 10% foetal calf serum (FCS) but without antibiotics. Cultures were maintained at 37^oC in equilibrium with 2% CO_2 in air. Cultures were passaged when they approached confluence by using Ca^{++} and Mg^{++} free phosphate buffered saline followed by 0.25% Trypsin (Gibco, Paisley, Scotland). Cells were passaged a maximum of six times between thawing and use, to minimise the problems of phenotypic drift (Neri & Nicolson, 1981). Cultures were regularly tested for contamination with Mycoplasma, using Hoechst stain 33258.

For the purpose of inoculation, the tumour cells were trypsinised, washed three times in F10/DMEM by centrifugation at 200g for 5 minutes, and finally resuspended in F10/DMEM. Cells were then counted using a Coulter model ZM counter(Coulter Ltd, Luton, England). A standard suspension containing 10^4 cells per 0.5ml was prepared. Animals were inoculated with this tumour cell suspension by the intravenous route.

The method selected for the detection of pulmonary metastases in our studies relies on the formation of easily detectable, discrete tumour nodules, on the pleural surface of the lungs.

Validation of Method for Identifying Lung Metastases

Estimation of pulmonary tumour seeding by serial histological section is prohibitively time consuming for repeated use. For studies of metastasis which would necessarily involve the use of large numbers of experimental animals, a rapid and reliable alternative method is required. A simple method which has proved reliable in other tumour/host studies, is that described by Wexler (Wexler, 1966). This technique is well validated and involves inflating the lungs via the trachea with a 15% solution of Indian Ink in distilled water, excising them and bleaching them for 48 hours in Fekete's solution, a bleaching fixative containing alcohol, acetic acid and formaldehyde. This reveals surface pulmonary metastases as white nodules against a black background (Figure 4). Conventional histology was used to assess the accuracy of this method in detecting tumour deposits (Figures 5 a & b).

A pilot study was performed to validate:

a. the dose of $Mtln_3$ cells previously used (10^4)

b. the optimum time of sacrifice (17 days)

c. Wexler's method

(McCulloch & George, 1987).

Figure 4 Rat lungs with multiple subpleural deposits prepared by the method of Wexler

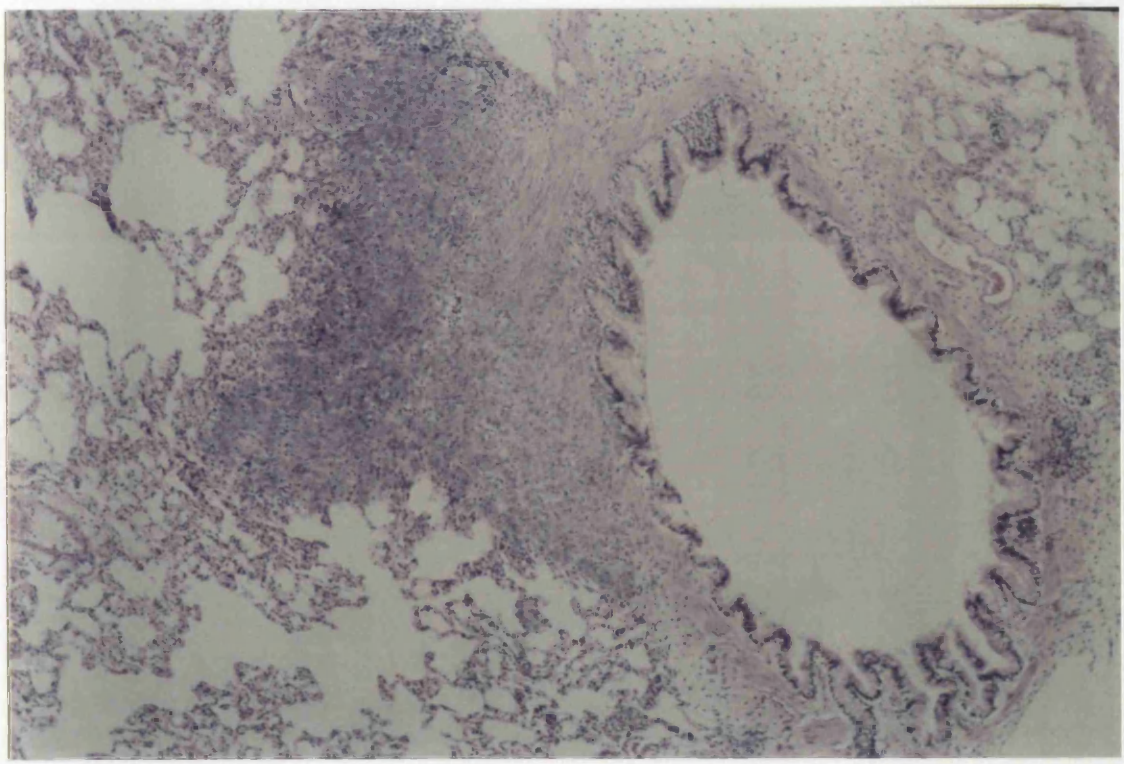


Figure 5(a) Histology of pulmonary tumour deposit:
Peribronchial tumour deposit - x 62.5 magnification

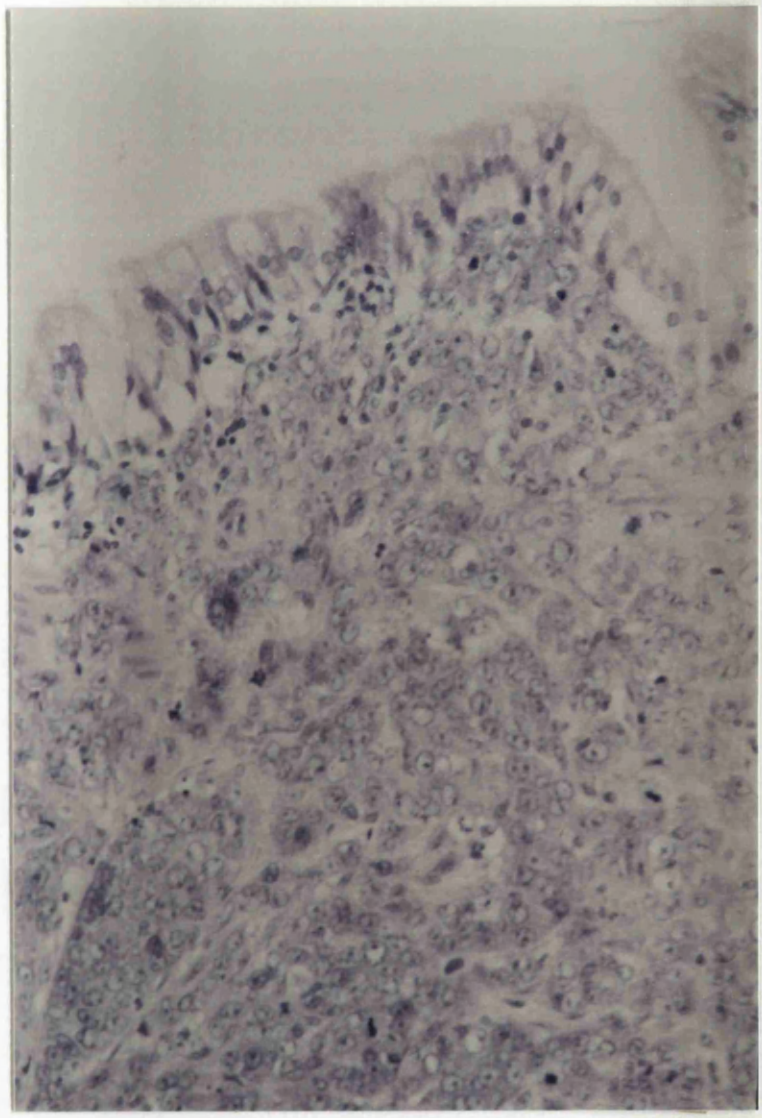


Figure 5(b) Histology of pulmonary tumour deposit: Tumour underlying bronchial epithelium - x 625 magnification

EXPERIMENT:

12 female Fischer 344 rats, 6-8 weeks old were given intravenous injections of 10^4 $Mtln_3$ rat mammary carcinoma cells suspended in 0.5 mls of F10/DMEM, under chloral hydrate anaesthesia. The animals were sacrificed at 17 days afer injection and in 6 animals, pulmonary tumour nodules were assessed by the method of Wexler as described above. In the remaining 6 animals, the lungs were excised, and stored in 10% formalin/saline solution and subjected to serial sectioning with conventional haematoxylin and eosin staining. Full autopsy was carried out on all animals and samples of any tissue suspected of containing metastases were submitted for histological examination.

RESULTS:

Table 4 shows the number of pulmonary tumour nodules detected by the two methods. There was no statistical difference in the estimated number of tumour nodules detected by the two methods used and the inter-animal variation was very similar in the two groups (Mann-Whitney, $p = 0.5752$). No tumour deposits were seen in any of the other organs submitted for histology.

DISCUSSION:

This preliminary study, confirmed that the tumour model chosen was suitable for our purpose, namely to investigate influences on metastasis in an animal model. The tumour seeded readily and in a discrete manner in

Table 4 **Validation of Wexler's method**

Number of pulmonary deposits

Pathology	Wexler's Method
15	14
8	11
19	6
16	11
12	19
7	8

Mann - Whitney: p = 0.5752

the lungs, following intravenous injection of a small dose of tumour cells. The likelihood of a major disturbance of the circulatory or coagulation systems, occurring as a consequence of injection of these cells is remote. The method proposed for the identification of pulmonary tumour nodules was also successfully validated. Previous work in 8 other model syestems (Wexler, 1966) has shown that excellent correlation exists between the number of surface tumour nodules detected by this method and the total lung tumour burden estimated by more rigorous but laborious methods. A discrepancy between the true and estimated numbers of pulmonary tumour nodules may arise in three ways: tumours may grow in a fashion which prevents accurate identification and counting; there may be a variable relationship between the total lung tumour burden and the surface tumour nodules; artefacts may arise which indicate the presence of a tumour deposit where none exists. The first two of these possibilities may be discounted in the experiments described above, where the pattern of tumour growth was observed. The tumour nodules formed were well-circumscribed and discrete and easily identifiable. Also, the proportion of deposits on the pleural surface was consistent as judged by histological studies and artefact formation did not appear to be a source of serious error when Wexler's method was applied to this tumour/host system. Direct comparison was made in these studies between quantitative estimates of tumour burden based on

histology and those obtained using Wexler's method. The results of these studies indicate that Wexler's method of identifying surface pulmonary tumours is adequate for estimation of major changes in the lung burden of $Mtln_3$ tumour with various treatments.

Human Coagulation Factor Complex II, IX, X.

A heat treated concentrate of human coagulation factor complex II, IX, X, prepared from pooled plasma by cryoprecipitation and supernatant adsorption with DEAE cellulose, was obtained from Dr RJ Perry of the Protein Fractionation Centre, Edinburgh, UK.

Previous experiments have shown that a dose of six units of prothrombin and factor X, and seven units of factor IX reconstitutes coagulation in a fully warfarinised rat for approximately 12 hours. This is the dose of Factor Complex that has previously been used in the studies wherein the effect of enhancement of tumour metastasis has been demonstrated (McCulloch & George, 1987).

Studies to validate previous work.

EXPERIMENT 1.

Pilot studies to verify the enhancing effect of human coagulation factors II, IX, X on pulmonary tumour seeding. These studies also assessed whether this effect of tumour enhancement is dependent on the concentration or time of administration of the factor complex II, IX, X.

Seven groups of 4 female Fischer 344 rats each, 6-8 weeks old were used. All animals were injected intravenously with 10^4 $Mtln_3$ cells as described above. Additional treatments were then commenced as follows:

Group A: These control animals received no additional treatment.

Group B: These animals received one injection of Factor Complex II, IX, X (6:7:6 Units), at the same time as tumour cell injection (t=0)

Group C: These animals received one injection of Factor Complex II, IX, X (1/2 of the dose administered in Group B animals) (t=0)

Group D: These animals received one injection of Factor Complex II, IX, X (1/10 the dose administered in Group B animals) (t=0)

Group E: These animals received one injection of Factor Complex II, IX, X (6:7:6 Units) (t=2hrs)

Group F: These animals received one injection of Factor Complex II, IX, X (6:7:6 Units) (t=4 hrs)

Group G: These animals received one injection of Factor Complex II, IX, X (6:7:6 Units) (t=6 hrs)

Animals were sacrificed at 17 days and pulmonary seeding assessed by the method of Wexler as previously described.

RESULTS:

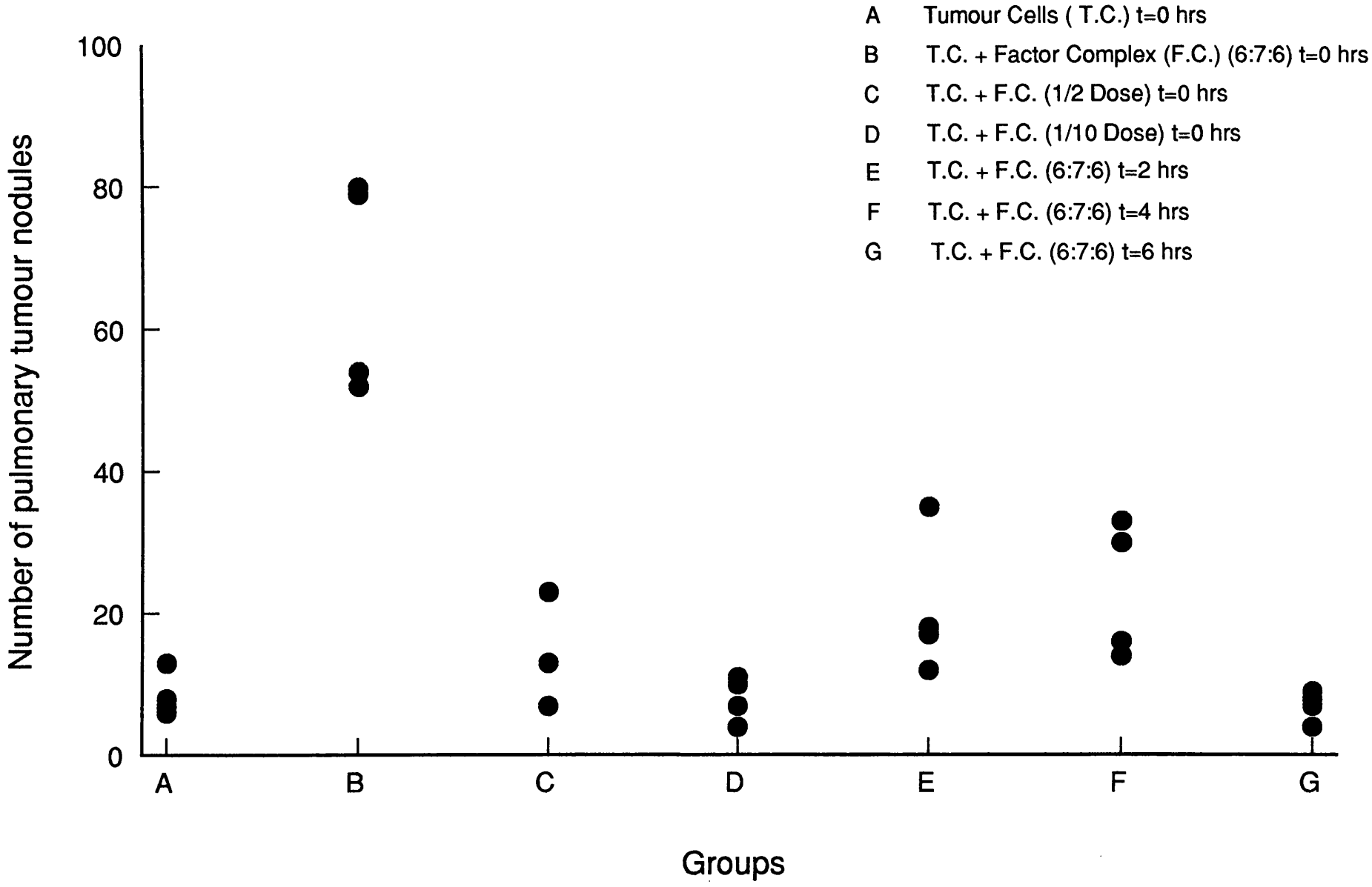
The dose and time of administration of the Factor Complex II, IX, X used in earlier experiments was validated since either a 50 % reduction in the dose or a 2 hour delay in the time of administration eliminated the marked enhancing effect on pulmonary tumour seeding seen with the "standard" timing and dosage (Figure 6) (Groups B vs C, p=0.03; Groups B vs E, p=0.03, Mann-Whitney, Bonferoni correction for multiple comparisons).

EXPERIMENT 2.

Pilot study to determine the effect of Factor Complex II, IX, X on aggregability of $Mtln_3$ tumour cells **in vitro**.

$Mtln_3$ cells were grown in culture as described above, trypsinised, washed and resuspended in F10/DMEM without FCS at a concentration of 10^5 cells/ml. Five such suspensions of cells were stirred very gently and to four of them, Factor Complex II, IX, X (6:7:6 Units/10^4 cells) was added, the fifth cell suspension acting as a control. For 4 hours thereafter, the cell suspensions were gently stirred. At several intervals (0, 1/2, 1, 2 and 4 hours) multiple samples of cell suspensions were assessed for viability and cell aggregagation using a haemocytometer and trypan blue exclusion.

Figure 6 Factor Complex/Tumour Cells - Dose & Time Response



RESULTS:

Aggregates of cells were monitored (Table 5) depending on whether the majority of cells seen, occurred singly (1), or were seen in aggregates of two (2), three (3) or four (4). Aggregability of $Mtln_3$ cells remained unaltered when Factor Complex II, IX, X was added to cell suspensions. Viability of cells gradually declined at an equal rate over a period of 4 hours in both experimental and control groups.

DISCUSSION:

The aim of this study was to validate the work performed by previous investigators, wherein the effect of tumour enhancement by factor complex II, IX, X, has been demonstrated. As a result of our studies we established the following:

1. Human Coagulation Factor Complex II, IX, X, in a dose of 6:7:6 units respectively, administered as a single bolus injection intravenously, enhances pulmonary tumour seeding in this animal model.
2. The dose used is near to the lowest practicable, as a 50% reduction in dosage eliminates the tumour enhancing effect.
3. Similarly, a delay of 2 hours or more in the time of administration of the factor complex relative to inoculation of the tumour cells eliminates this effect.
4. Factor Complex II, IX, X does not affect the aggregability of $Mtln_3$ cells.

Table 5 **Effect of Factor Complex on Cell Aggregability**

GROUPS	Time of Sampling (Hrs)				
	0	1/2	1	2	4
A	2 + a	2 + a	3 + a	2 + b	3 + c
B	3 + a	2 + a	3 + b	2 + b	2 + c
C	2 + a	1 + a	2 + a	3 + a	4 + b
D	3 + a	2 + a	2 + a	3 + b	2 + c
E	1 + a	3 + a	4 + b	2 + b	1 + c

A Control - Tumour Cells

B-E Cells + Factor Complex

a > 95 % viable b > 75 % viable c > 50 % viable d < 50 % viable

In order to ensure consistency in our work, for the purposes of all our principal experiments, the dose of factor complex II, IX, X used was 6:7:6 units and this was administered as a single intravenous bolus, at the same time as tumour cell inoculation. This animal model was considered suitable for the purposes of our study on the basis of these experiments.

Chapter 2

STUDIES TO DETERMINE WHETHER THE XENOGENEIC NATURE OF THE FACTOR COMPLEX IS RESPONSIBLE FOR THIS EFFECT OF TUMOUR ENHANCEMENT.

INTRODUCTION:

The results of the work performed thus far, have validated previous work and confirmed that the factor complex II, IX, X, when administered intravenously at the same time as the tumour cells, enhanced metastasis in an animal model. Several questions however, remained unanswered. In the previous experimental work, human factor complex II, IX, X was administered to the animals. It could be argued that the effect of metastatic enhancement thereby produced, could in some way be related to the xenogeneic nature of the factor complex used. We regarded this unlikely, in view of the fact that previous workers had shown that administration of human coagulation factor VII, did not affect pulmonary tumour seeding to any significant degree and also because control groups for foreign protein (bovine serum albumin) were used in all the experiments (McCulloch & George, 1988).

Despite this, we could not entirely exclude the possibility, that the effect of tumour enhancement seen in our experiments was secondary to the xenogeneic nature of the factor complex preparation. It was therefore essential to investigate this in greater detail by comparing human factor complex and rat factor

complex II, IX, X with regard to their effects on pulmonary tumour seeding.

Rat factor complex II, IX, X, is not available commercially and to our knowledge no attempt had been made to prepare it.

A preliminary experiment was designed to attempt to prepare syngeneic rat factor complex II, IX, X. The method adopted was a modification of the standard method used to prepare human factor complex II, IX, X (Ahmad et al, 1989).

Preparation of syngeneic rat factor complex II, IX, X

The preparation of rat factor complex II, IX, X involves three principal stages:

1. Method of blood collection

Female Fischer 344 rats, 10-12 weeks old were used for this experiment. The animals were anaesthetised by halothane induction and chloral hydrate administered intra-peritoneally. A laparotomy was then performed. The abdominal aorta was cannulated and the animals were exsanguinated. Blood was collected in plastic bottles containing 6% sodium citrate in a ratio of 9:1 v/v and protease inhibitors (1 mM benzamidine-hydrochloride 1 mM phenylmethylsulfonylfluoride, 2.4 uM soybean trypsin inhibitor and 100 uM aprotonin). Following centrifugation at 2100 g for 15 minutes at 4^{o}C, fresh plasma was collected.

2. Barium citrate precipitation

1 M Barium chloride was added dropwise to the plasma containing all the inhibitors and the mixture stirred slowly for 1 hour at 4°C. The precipitate was collected by centrifugation at 5000 g for 20 minutes at 4°C and the supernatant discarded. The precipitate was resuspended in 0.1 M sodium chloride and 10 mM barium chloride. The washed precipitate was again collected by centrifugation and resuspended in 20 mM Trisphosphate buffer containing 1 U/ml heparin, 1 mg/ml soybean trypsin inhibitor and 10 mM benzamidine hydrochloride.

3. Ammonium sulphate elution

The mixture was placed in an ice bath and 2 M ammonium sulphate was added dropwise with continuous stirring for 1 hour. The supernatant was recovered after centrifugation at 10,000 g for 30 minutes. This was dialysed overnight at 4°C against 50 volumes of 0.15 M sodium chloride, 10 mM sodium phosphate and 1 mM benzamidine.

The resulting solution was assayed for Factors II, IX and X (Quick's one-stage assay) and found to contain a concentration ranging from 140% - 1000% of these factors. Note: 100% normal activity = [1 unit (ml plasma)$^{-1}$]. The ratios of the Factors II, IX, X varied from 21:10:7 to 11:6:8. For the purposes of this experiment, the batch used had a ratio of 11:6:8. Each rat received a dose of 1 ml of concentrate containing 11

units of Prothrombin, 6 units of Factor IX and 8 units of Factor X.

Experiment to compare the effect of human and rat factor complexes II, IX, X on pulmonary tumour seeding:

Three groups of Fischer 344 rats, 8 per group, 6-8 weeks old, were used in this experiment. All animals were given tail vein injections of 10^4 $Mtln_3$ rat mammmary carcinoma cells in 0.5mls of F10/DMEM prepared and administered as previously described. At the same time additional treatments were begun as follows:

Group A: These control animals received no additional treatment.

Group B: These animals received one injection of rat factor complex II, IX, X at the same time as tumour cell injection (t=0).

Group C: These animals received one injection of human factor complex II, IX, X at t=0..

The dose of human factor complex was similar to that used in previous experiments (6:7:6 Units).

Animals were sacrificed at 17 days and pulmonary seedings assessed by the method of Wexler, as described previously (Chapter 1).

Comparison of the different groups was made by Kruskall-Wallis and Mann-Whitney tests.

RESULTS:

The number of pulmonary metastases in animals treated with rat factor complex (Group B, median 233) and with human factor complex (Group C, median 226) was significantly higher than that detected in the control group A, (median 25) ($p< 0.001$). There was no significant difference between the experimental groups B and C ($p = 0.71$) (Figure 7). Rat factor complex and human factor complex enhanced pulmonary metastases to a similar degree.

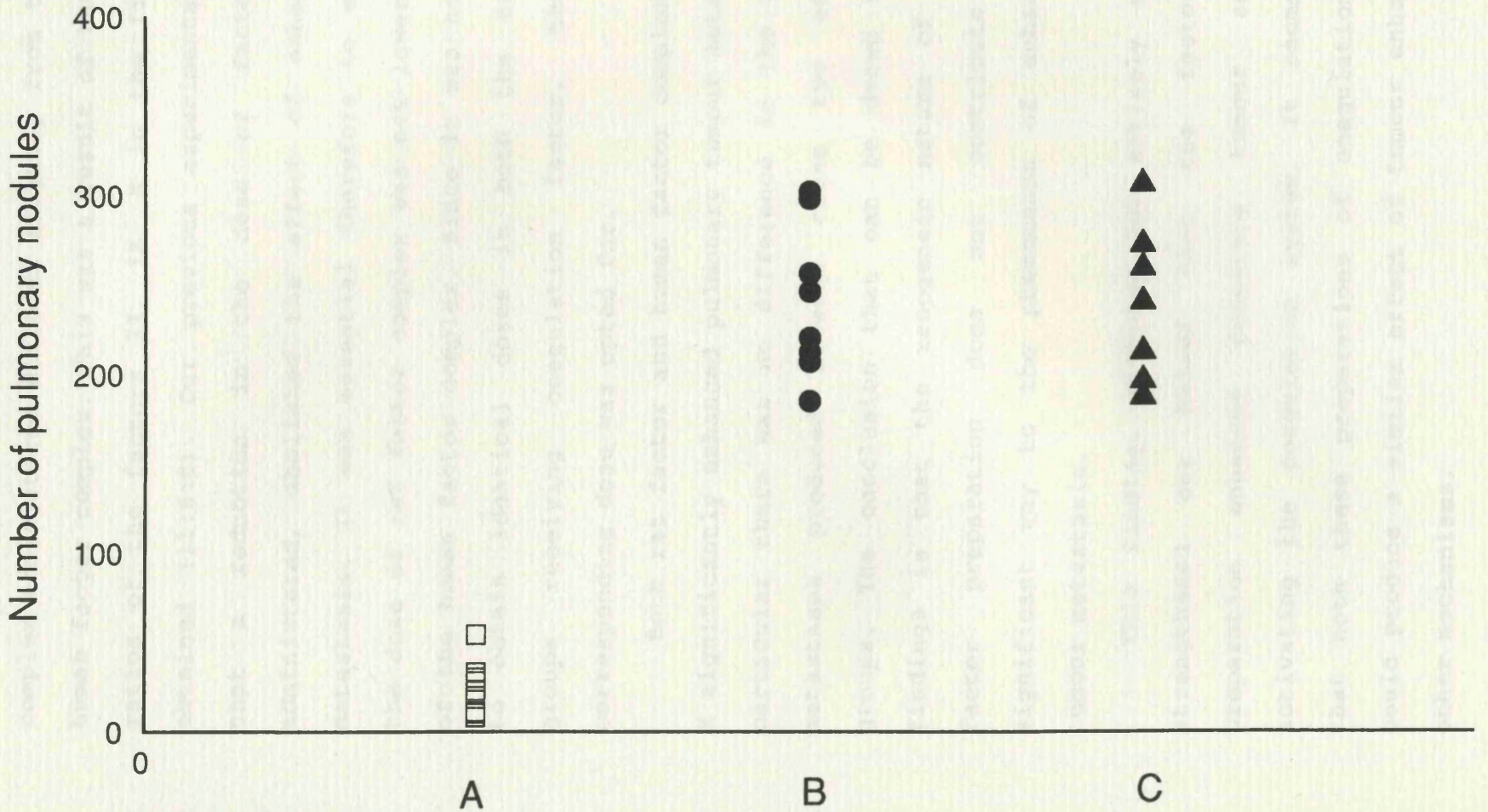
DISCUSSION:

In our previous studies we have demonstrated the effect of enhancement of pulmonary tumour metastasis by human factor complex II, IX, X in a rat model. It might be argued that the injection of foreign protein (xenogeneic) might have been responsible for producing this effect. The aim of this study was to investigate this possibility. To achieve this, it was necessary to prepare rat factor complex II, IX, X from syngeneic animals and compare the effect of this preparation and human factor complex II, IX, X, on pulmonary tumour metastasis.

A method was devised to prepare rat factor complex II, IX, X which was based on the standard method of preparing human factor complex II, IX, X. This was modified to suit our requirements. Analysis of the resultant preparation showed it to contain factors II, IX, X in very high concentration. The dose of rat factor

Figure 7

Effect of rat factor and human factor complex on pulmonary tumour seeding



complex administered differed slightly from that of the human factor complex. This was a result of the different ratios of the factors II, IX, X in the final sample obtained (11:6:8). Our previous experiments suggested that a reduction in the dose of factor complex administered, abolished the effect of enhancement of metastasis. It was essential therefore to ensure that the dose of rat factor complex was not lower than that of the human factor complex. Since it was not possible to obtain identical doses in both the experimental groups receiving coagulation factor, the nearest corresponding dose was opted for.

Both rat factor and human factor complexes II, IX, X significantly enhanced pulmonary tumour metastasis. In particular there was no difference in the numbers of metastases produced between these two experimental groups. The conclusion that can be drawn from these findings is that the xenogeneic nature of the human factor preparation does not contribute in any significant way to the phenomenon of enhancement of tumour metastasis.

This finding, although not entirely surprising, strengthened our belief that the factor complex preparation enhanced pulmonary tumour seeding by activating the coagulation system. It seems unlikely that both these preparations of coagulation factors, could produce a similar effect of tumour enhancement by other mechanisms.

We have so far confirmed that the factor complex II, IX, X enhances pulmonary tumour seeding in our animal model and that this effect is unrelated to the xenogeneic nature of the human coagulation factor preparation. Having established this, we hypothesised that this effect of enhanced pulmonary tumour seeding was secondary to an actual physical increase in the number of tumour cells trapped within the microcirculation. This formed our next line of investigation.

CHAPTER 3

STUDIES TO DETERMINE WHETHER THE EFFECT OF TUMOUR ENHANCEMENT BY FACTOR COMPLEX II, IX, X IS SECONDARY TO INCREASED PULMONARY ENTRAPMENT OF TUMOUR CELLS

INTRODUCTION:

From the preceding chapters, it is clear that intravenous injection of factor complex II, IX and X with tumour cells enhances pulmonary tumour seeding. The exact mechanism by which this effect occurs is unclear. The greatest known barrier to tumour cells in the metastatic process is the intravascular phase. Changes in the intravascular phase are likely to be an important determinant of changes in metastasis. Our earlier experiments suggest that the metastatic enhancement seen in our experiments is a direct consequence of changes in the intravascular phase. The most plausible explanation for this effect of tumour enhancement is an increased trapping of tumour cells in the microcirculation. It was therefore necessary to establish whether an increase in physical trapping of tumour cell emboli in the pulmonary microcirculation actually occurred.

To investigate this phenomenon, radiolabelled tumour cells were injected under different conditions and their passage through the the lung assessed.

Radiolabelling of tumour cells:

Several conditions must be satisfied if labelled cells are to be used for the study of metastasis:

(a) The radioactive label should be firmly bound to the cell while it is viable.
(b) On death of the cell, the label must not be re-utilized but be rapidly excreted from the body.
(c) The label must not alter the biological behaviour of the tumour cell.
(d) The label must exhibit sufficient radioactivity to allow **in vivo** detection of relatively few cells.
(e) Injected tumour cells must be radiolabelled uniformly.

A homogenous population of $Mtln_3$ cells grown **in vitro**, labelled with ^{125}I-iodo-2'-deoxyuridine (^{125}IUDR) and injected into Fischer rats, fulfilled the criteria specified above. This analogue of thymidine is incorporated exclusively into the DNA of proliferating cells and is released only after cell death. Unlike thymidine, ^{125}IUDR is hardly re-utilized (Huges et al 1964; Commerford 1965). After cell death, ^{125}IUDR is released into the blood stream and rapidly degraded in the liver. The product of degradation is largely excreted in the urine.

Prior to proceeding with the proposed study, it was necessary to establish the following:
1. The dose range within which ^{125}IUDR had no effect on tumour cell growth **in vitro**.

2. The dose of ^{125}IUDR that satisfied criterion (1) and also produced measurable radioactivity after tumour cells were injected into rats.
3. The capacity of ^{125}IUDR to affect tumorigenecity of tumour cells.
4. The uniformity of uptake of isotope by the cells.

To this end, the following pilot studies were performed to evaluate the radiotoxicity of ^{125}IUDR on $Mtln_3$ mammary carcinoma cells.

1. Pilot studies to assess a suitable non-cytotoxic dose of ^{125}IUDR.

(a) Clonogenic Assay.
(b) Chemosensitivity Assay (microtiter assay).

(a) Effect of ^{125}IUDR on clonal growth rate (Fidler IJ, 1970).

16, 25cm^3 flasks, each containing the same number of cells in 5 mls of F10/DMEM were left to incubate overnight at 37^{o}C in equilibrium with 2% CO_2 in air. 24 hours later, ^{125}IUDR was added to the flasks in a dose range of 0.001 - 5.0 microcuries/ml (0.001, 0.005, 0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5, 0.7, 1.0, 2.0, 3.0, 4.0, 5.0), with one flask acting as a control. The cells were exposed to ^{125}IUDR for 24 hours. The medium was then removed and the cells trypsinised, washed three times and resuspended in 10 mls of medium/flask. The cells in the control group (untreated flasks), were

counted and then diluted down to 100 cells/ml. 5 mls of this cell suspension (500 cells), were plated out into each of 4 petri dishes. Cells from the isotope-treated flasks were diluted and plated out in an identical manner to the control flask. The petri dishes were incubated at 37°C in an atmosphere of 2% CO_2 in air for 10 days. The medium was then removed and the clones washed with PBS, fixed in methanol and stained with crystal violet. Colonies were then counted.

(b) Chemosensitivity (microtiter) Assay: (Plumb JA et al, 1989).

This assay relies on the ability of live but not dead cells to reduce a water-soluble yellow dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a water-insoluble purple formazan product.

Adherent cells were plated out at a concentration of 10^3 per well in 200ul of medium in 96-well microtiter plates (Linbro; Flow Laboratories, Irvine, Scotland) and allowed to attach and grow for 2 days. The medium was then removed from the wells and replaced with either 200ul of fresh medium (Control), or 200ul of medium containing ^{125}IUDR at different concentrations ranging from 0.001 - 3.0 uc/ml (0.001, 0.005, 0.01, 0.03, 0.05, 0.07, 0.1, 0.5, 1.0, 3.0). Cells were incubated with isotope for 24 hours after which the medium was replaced with isotope-free medium. The medium was replaced at 24 hour intervals for a further 2 days. On day 4 following isotope addition, the cells were fed with 200ul of fresh

medium containing acid buffer. MTT (50ul) in PBS was added to the wells and the plates incubated for a further 4 hours. Medium and MTT were then removed from the wells, and the formazan crystals were dissolved in 200 ul of DMSO. Absorbance was recorded in an enzyme-linked immunosorbent assay plate reader (Model 2550 enzyme immunoassay plate reader; Bio-Rad Laboratories Ltd., Watford, England), at a wavelength of 570nm. The first and last rows of 8 wells which contained medium and PBS were used to blank the plate reader.

RESULTS:

(a) Clonogenic Assay

The number of colonies seen with each dose of isotope are shown in Figure 8. The control group showed a mean of 280 colonies. In groups treated with doses of 0.07 uc isotope per ml of medium and above, there was clearly a marked reduction in the number of colonies formed, whereas, doses of 0.05 uc/ml of medium and below, did not affect clonal growth. This would suggest that doses of ^{125}IUDR greater than 0.05 uc/ml of medium, are toxic to cells and interferes with cell survival and growth while at doses less than this, cells survive and grow adequately to form colonies.

(b) Chemosensitivity (Microtiter Assay).

At the end of the first 24 hours, wells with isotope at doses greater than 0.07 uc/ml of medium, showed a

Figure 8

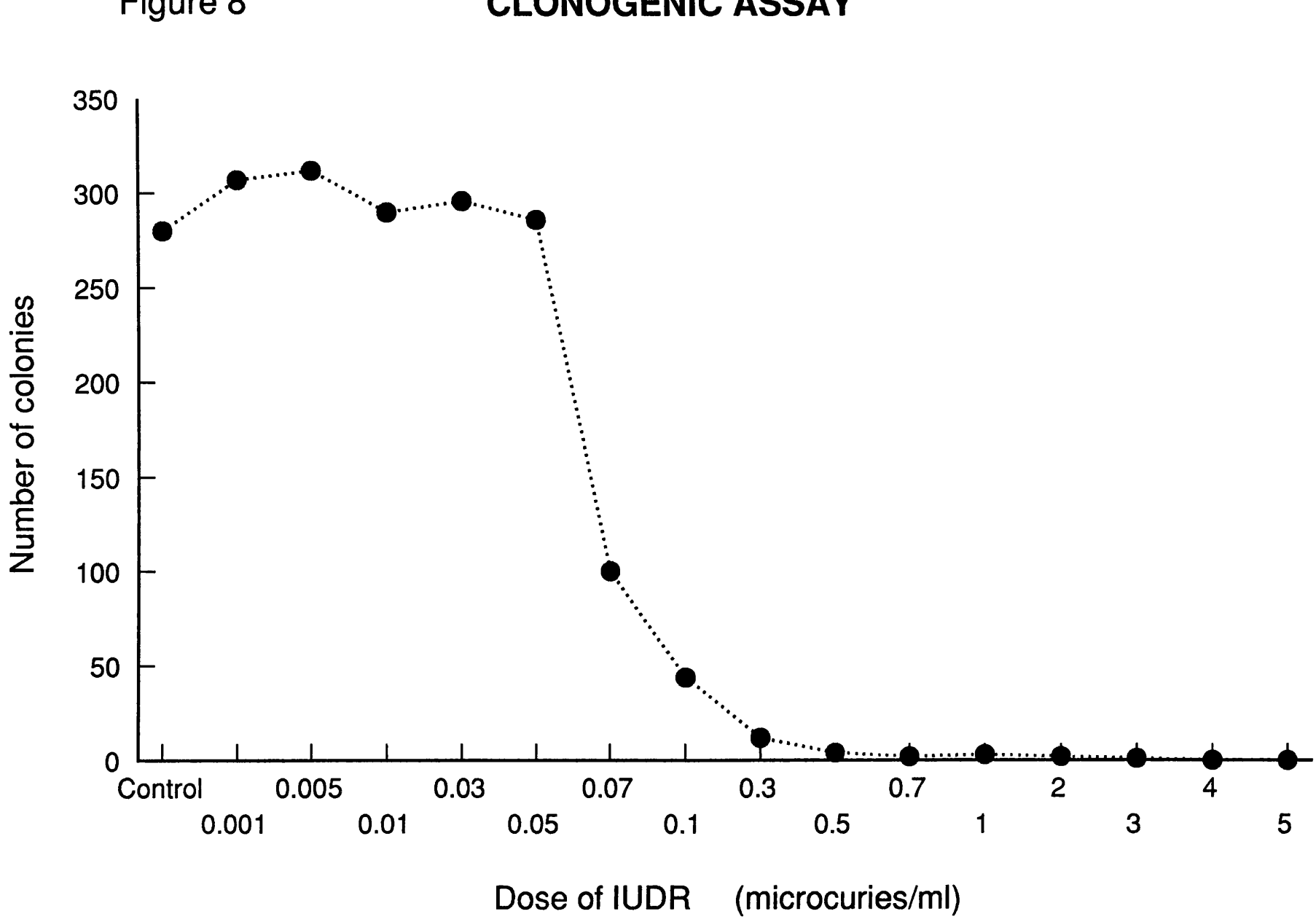

CLONOGENIC ASSAY
Number of colonies
350
300
250
200
150
100
50
0
Control
0.001
0.005
0.01
0.03
0.05
0.07
0.1
0.3
0.5
0.7
1
2
3
4
5
Dose of IUDR (microcuries/ml)

Figure 9 **Results of Microtiter Assay - Day 1**

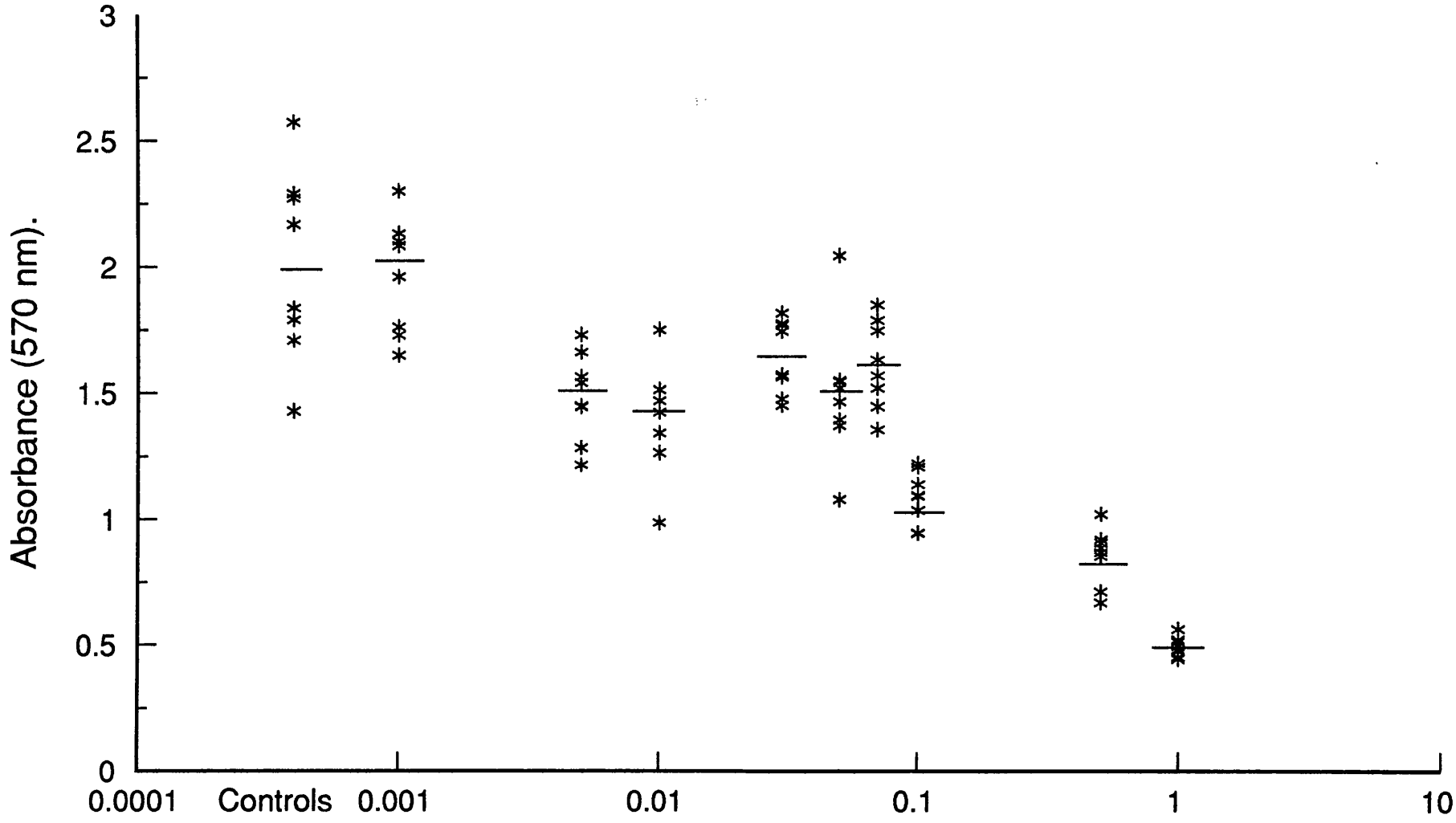
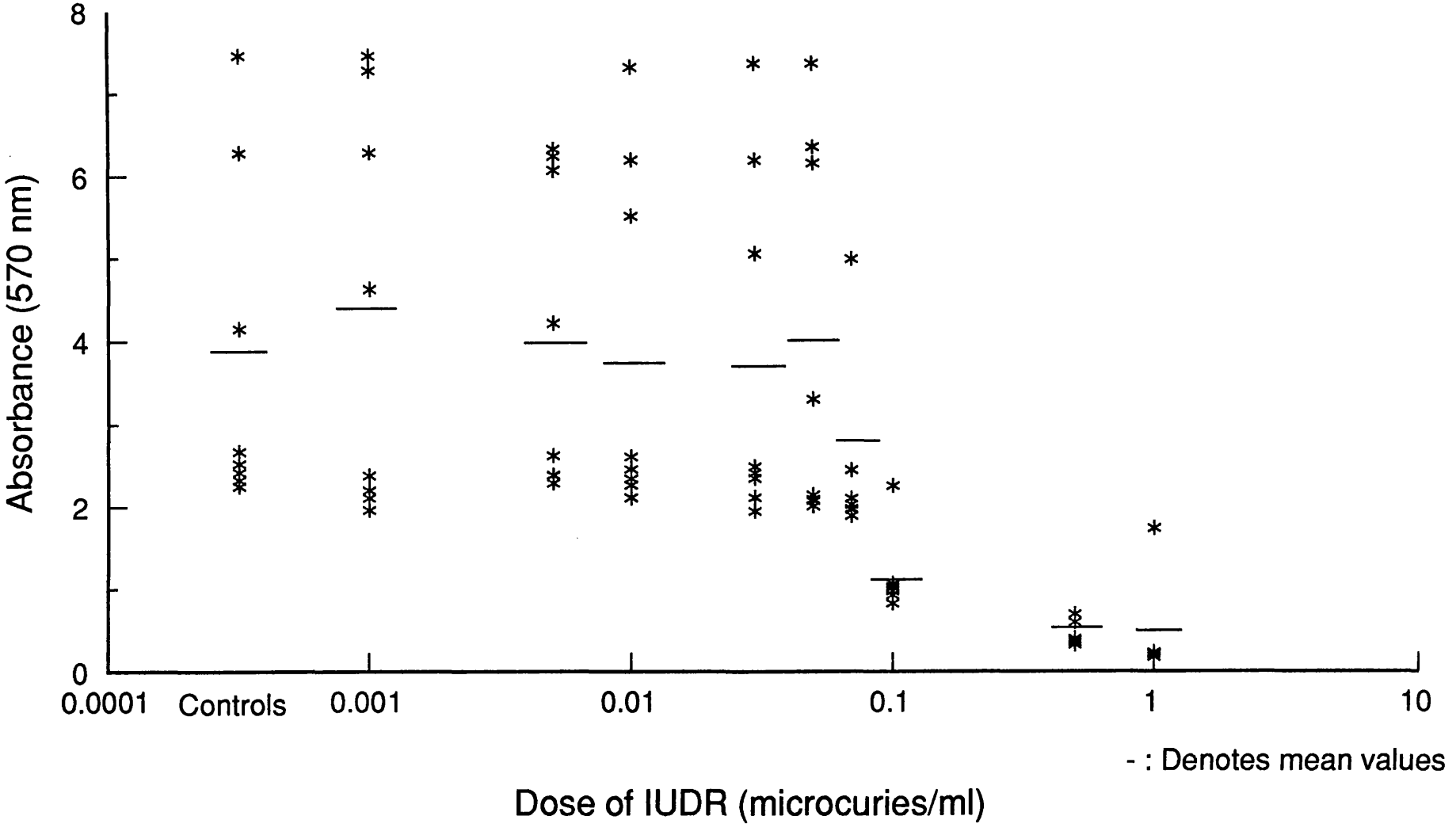


Figure 10 **Results of Microtiter Assay - Day 2**



decrease in the number of live cells as shown by absorbance (Figure 9). This was further confirmed at the end of 48 hours (Figure 10). Doses below 0.07 uc/ml, did not affect cell survival and proliferation. Any increase in the concentration of isotope above 0.07 uc/ml, correspondingly decreased the number of surviving cells.

Based on the above two studies, 0.05 uc/ml of medium was considered to be an appropriate dose of ^{125}IUDR, for the purpose of labelling $Mtln_3$ cells.

2. Pilot study to determine dose of injected isotope that will produce adequate measurable lung radioactivity.

$Mtln_3$ cells were grown in culture in F10/DMEM at 37^{o}C with CO_2 in air, in separate flasks (4), and radiolabelled by incubating them with ^{125}IUDR, at a dose of 0.05 uc/ml of medium for 24 hours. Serial samples of cell suspensions (10^4, 10^5, 10^6, 10^7) were counted in triplicate on a gamma counter (1282 Compugamma, LKB, Wallac, Milton Keynes, England).

RESULTS:

The background count was 40. Mean activity in the cell suspensions ranged from 52 cpm with 10^4 cells to 35,931 cpm with 10^7 cells (Table 6). We anticipated using radiolabelled cells to detect pulmonary seeding expecting to find only a small fraction of injected cells within the lungs after one or more hours. An

Table 6 **Radiolabelling of different doses of cells**

Tumour Cells	Gamma Count (Mean)
10^4	52
10^5	392
10^6	3,727
10^7	35,931

Background count - 44

activity which was clearly detectable above baseline even after 95% reduction was therefore desirable. Based on these criteria, in order to be able to produce measurable radioactivity in the lungs, a dose of 10^6 cells radiolabelled with ^{125}IUDR at a dose of 0.05 uc/ml of medium was thought to be appropriate for use in the main study.

3. Pilot study to assess tumorigenicity of radiolabelled $Mtln^3$ cells **in vivo**.

For the purpose of the main study, it was essential to assess if tumorigenecity of the $Mtln_3$ cells was affected by radiolabelling them with ^{125}IUDR.

The **in vitro** studies previously described, established the dose of ^{125}IUDR appropriate for labelling of $Mtln_3$ cells under innocuous conditions. However, cells exposed to toxic levels of radiation may undergo several normal divisions before mitotic arrest. Therefore, the clonal growth rate may not be an absolute index of radiotoxicity. Obviously, a further study was necessary to demonstrate that similar doses of unlabelled and labelled $Mtln_3$ cells would yield a similar number of tumours **in vivo**.

MATERIALS AND METHODS:

Two groups of female Fischer 344 rats, 6-8 weeks old, were used in this experiment. Under general anaesthesia, animals were given tail vein injections of $Mtln_3$ rat

mammary carcinoma cells suspended in 0.2 mls of F10/DMEM as outlined below:

Group A: These animals received one injection of 10^6 $Mtln_3$ cells radiolabelled with $^{125}IUDR$ (0.05 uc/ml)

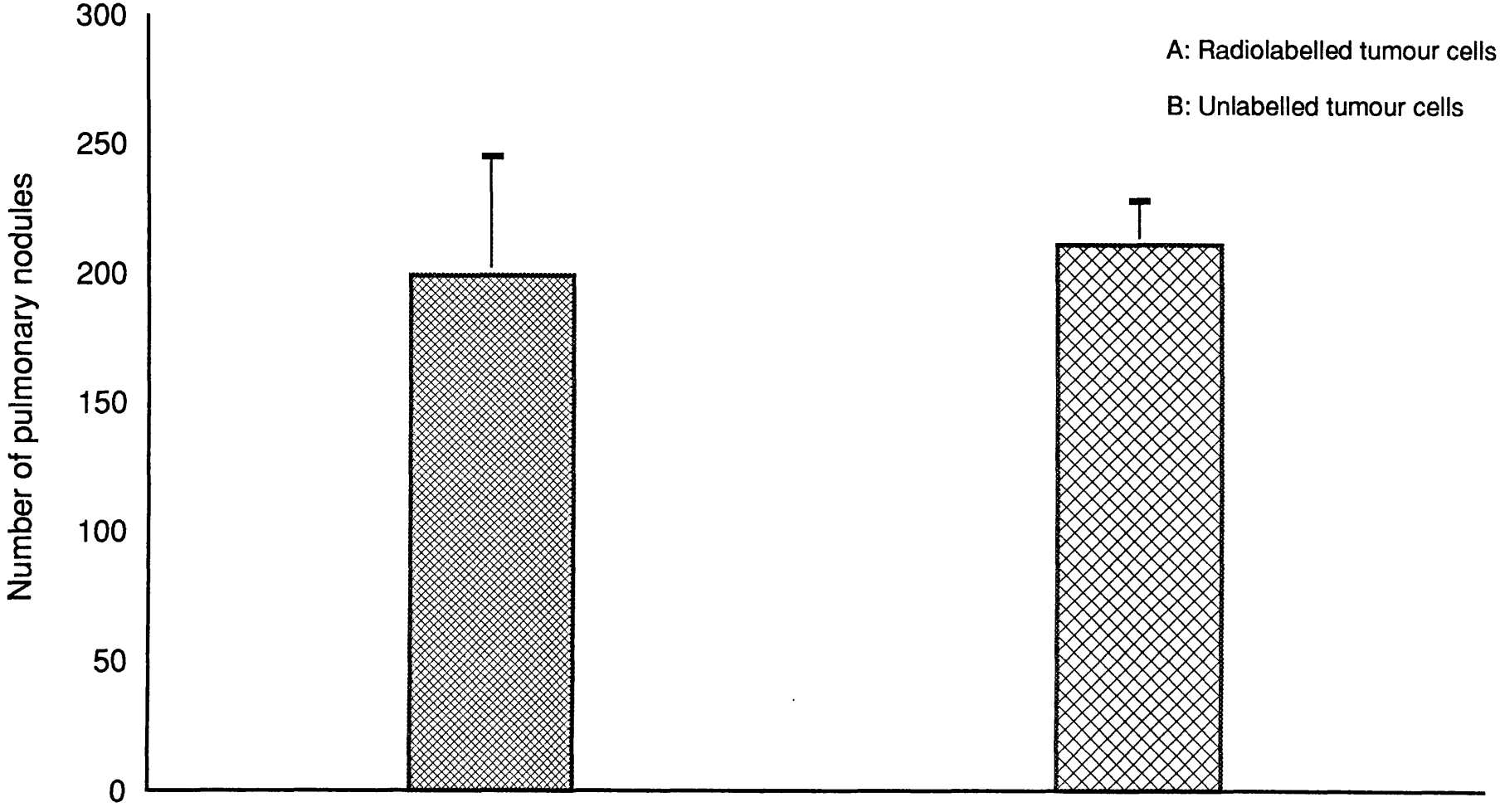
Group B: These animals received one injection of 10^6 unlabelled $Mtln_3$ cells.

Animals were sacrificed 17 days after tumour cell inoculation and pulmonary tumour seeding assessed by the method of Wexler (Chapter 1). Comparison of the two groups was made by the Mann-Whitney Test.

RESULTS:

In all previous experiments, an inoculum dose of 10^4 cells was used. However, as shown earlier, this dose would have been unsuitable to ensure measurable radioactivity in the main experiment. Hence for the purpose of this study a dose of 10^6 $Mtln_3$ cells was opted for. As expected, this produced a far greater number of pulmonary metastases than that seen in previous experiments, where a lower inoculum dose was used. In some of the lungs, in both groups, confluence of metastases was seen. However, all metastases that could be identified clearly were counted. Statistically, there was no significant difference between the two groups, in the number of metastases seen, (Figure 11, p= 0.6650, Mann-Whitney).

Figure 11 **Tumorigenecity of radiolabelled cells**



p = 0.6650, Mann - Whitney

Figure denotes median and maximum values

This showed that labelling $Mtln_3$ cells with $^{125}IUDR$ at a dose of 0.05 uc/ml of medium, did not affect their capacity to produce pulmonary metastases.

4. To ensure uniform uptake of isotope by the cells, a simple **in vitro** study was performed.

MATERIALS AND METHODS:

$Mtln_3$ cells were grown in 10 separate flasks. Cells were incubated with $^{125}IUDR$ at a dose of 0.05 uc/ml for 24 hours. Following trypsinisation, the labelled cells were washed several times to remove non-bound isotope and then resuspended in F10/DMEM. Different doses of inocula, ranging from 10^4 - 10^7, one dose per flask, in 1 ml of medium, were monitored in a gamma counter and radioactivity measured.

RESULTS: (Table 7)

This experiment demonstrated uniform uptake of $^{125}IUDR$ by $Mtln_3$ cells grown in culture in different flasks. It further confirmed that at inoculum doses below 10^5 of cells, measurable radioactivity was too low to be considered for use in the proposed main experiment.

As a result of the above 4 pilot studies, the following criteria were established:

1. A satisfactory labelling dose of $^{125}IUDR$ that was non-cytotoxic to $Mtln_3$ cells was 0.05 uc/ml of medium.

Table 7 **Uptake of isotope by tumour cells**

Tumour Cells	Gamma Count (Mean)
10^4	44
10^5	357
2×10^5	703
3×10^5	1,117
5×10^5	1,747
10^6	3,464
2×10^6	6,527
3×10^6	9,637
5×10^6	17,342
10^7	32,658

Background count - 40

2. The appropriate inoculum dose of cells, labelled with ^{125}IUDR at this dose of 0.05 uc/ml of medium, that would reliably produce measurable radioactivity, was 10^6.
3. Radiolabelling $Mtln_3$ cells with ^{125}IUDR did not affect tumorigenecity **in vivo**.
4. Uniform uptake of the isotope by $Mtln_3$ cells was noted to occur, **in vitro**.

Experiment to determine whether enhancement of tumour metastasis is due to increased pulmonary entrapment of tumour cells.

MATERIALS AND METHODS:
$Mtln^3$ cells were cultured in $75cm^2$ tissue culture flasks in F10/DMEM with FCS, at 37^oC, in equilibirium with CO_2 in air. When cells had reached their phase of exponential growth, ^{125}IUDR was added to the flasks at a dose of 0.05 uc/ml of medium. The flasks were incubated for a further 24 hours. Cells were then trypsinised and washed several times to remove unbound isotope. Cell suspensions were diluted to yield an inoculum dose of 10^6 in 0.2 mls of F10/DMEM. Representative inoculum doses were monitored in a gamma counter.

Fischer 344 female rats, 6-8 weeks old, were given tail vein injections of 10^6 radiolabelled cells, under general anaesthesia. At the same time, additional treatments were begun as follows:

Group A: These control animals received no additional treatment.

Group B: These animals received one injection of bovine serum albumin (Sigma, Poole, UK), in a dose of 30 mg in 0.6 mls of F10/DMEM.

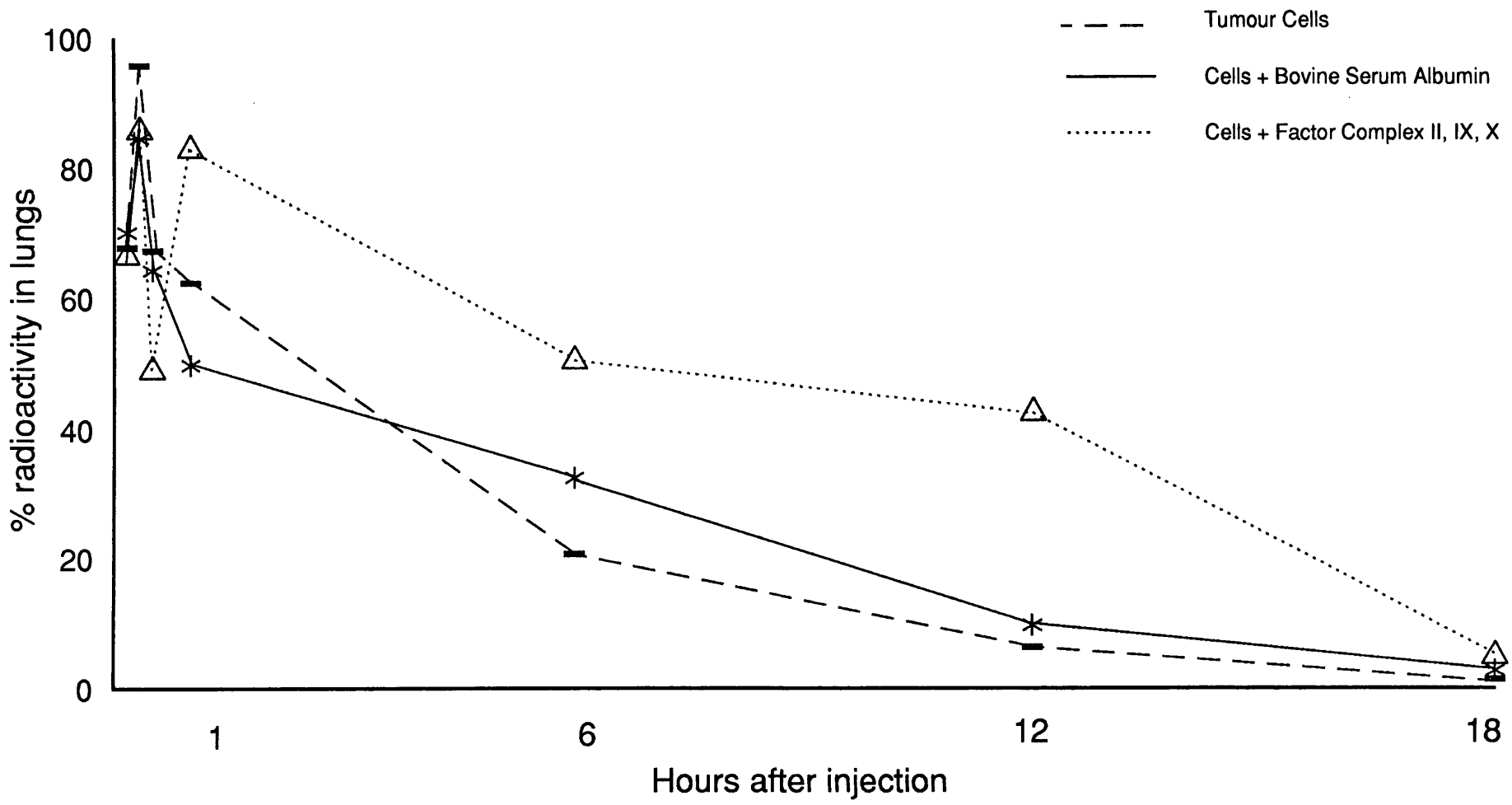
Group C: These animals received one injection of human factor complex II, IX, X.

The origin and dosage of the factor complex used are described in Chapter 1. The dose of bovine serum albumin used was selected so as to contain the same protein concentration as the factor complex preparation in Group C rats. Both preparations were passed through a 0.2 um filter before injection, for sterilization and removal of any particulate material which might embolise in the pulmonary microcirculation.

At the following intervals after injection, three animals per group were exsanguinated, their lungs removed immediately and placed in individual containers containing 70% ethanol: 5, 10, 30 minutes, 1, 6, 12 and 18 hours.

Lung radioactivity was measured in a gamma counter and expressed as a percentage of the total amount of radioactivity injected. Each sample was counted twice. Comparison of the three groups was made by analysis of variance and Student's t tests.

Figure 12 **Pulmonary Entrapment of Radiolabelled Tumour Cells**



RESULTS:

In all groups, an initial peak and trough of activity was noted in the first 30 minutes which was followed by an exponential decline with time (Figure 12). At 1 hour, the activity in the factor-treated rats (Group C) was 82.84% This was significantly higher than that in the control (Group A, 62.34%) and that in rats treated with bovine serum albumin (Group B, 50.10%, $p < 0.001$ on analysis of variance and Student's t tests, Figure 12). This significant difference persisted at all times thereafter. At 1 hour, but not thereafter, the difference between Groups A and B reached statistical significance ($p = 0.018$).

DISCUSSION:

We have already established that factor complex II, IX, X, enhances metastasis in an animal model. We hypothesized that this effect was probably secondary to an increase in the physical trapping of tumour cell emboli in the pulmonary microcirculation. The aim of this experiment was to assess this phenomenon in our animal model using radiolabelled tumour cells. ^{125}IUDR was regarded as a suitable isotope for this purpose, based on previous studies (Fidler, 1970), wherein the fate of radiolabelled tumour-cell emboli have been studied.

^{125}IUDR at a dose of 0.05 uc/ml of medium, labelled $Mtln_3$ cells satisfactorily in a uniform manner, was non-cytotoxic, and did not affect tumorigenecity **in vivo**.

Within the first 10 minutes of injection of tumour cells, there was a sharp rise in measured radioactivity in all experimental groups. This is thought to be due to trapping of tumour cells on first passage through the pulmonary circulation. In addition, some tumour cells that escape entrapment on first passage recirculate and may arrest in the pulmonary capillary bed subsequently. (Fidler, 1970). At the end of one hour the radioactivity measured in the lungs of animals administered factor complex II, IX, X along with tumour cells was significantly higher than that in the animals receiving cells alone or cells with bovine serum albumin, a difference that persisted till the end of the experiment at 18 hours. After one hour measured radioactivity showed an exponential decline with time indicating death of the tumour cells.

^{125}IUDR is incorporated exclusively into the DNA of proliferating cells, is released only on breakdown of cell DNA following cell death, and is hardly re-utilized (Huges et al, 1964; Commerford, 1965). Therefore the radioactivity measured in the lungs is a direct indication of the number of live tumour cells trapped in the pulmonary microcirculation at any given time. The increase in measured radioactivity in the lungs of animals receiving factor complex II, IX, X along with tumour cells can be attributed to increased pulmonary entrapment of tumour cells. This may be a contributory factor in producing the effect of enhancement of metastasis in our animal model. The exact reason for

this phenomenon of increased tumour cell entrapment is unclear. Several possibilities however exist.

Utilizing both electron microscopy and fibrin-specific immunofluorescence, it has been demonstrated that shortly after injection, tumour cells arrest in pulmonary capillaries and are surrounded by aggregated platelets and non-cross-linked fibrin (Jones et al., 1971; Chew and Wallace, 1976; Sindelar et al., 1975). Maximum numbers of thrombi are seen 1-3 hours after injection and these progressively decrease over 18 hours. Thrombus dissolution commences after 3 hours and tumour cells are then seen attaching themselves to the vascular endothelium. The formation of this thrombus, consisting of aggregated platelets and non-cross-linked fibrin around tumour cell emboli, is thought to encourage tumour cell arrest and adherence to the vascular endothelium. It is possible that factor complex II, IX, X may have an effect on platelet aggregability, thereby enhancing the formation of aggregates of platelet thrombi around tumour cell emboli. Alternatively, injection of this complex of factors may directly activate the coagulation system leading to the formation of a fibrin clot. Wood (1958) originally hypothesised that fibrin clot encourages adherence of tumour cells to vascular endothelium.

Whatever the mechanism involved, it is clear that a greater number of tumour cells are trapped in the pulmonary microcirculation on administration of the factor complex II, IX, X. We have already demonstrated

that the xenogeneic nature of the factor complex preparation is not responsible for this phenomenon of tumour enhancement. What is unclear at this stage is whether any one of the three factors II, IX and X is solely responsible for this observed effect. This formed the basis of our next study.

CHAPTER 4

STUDIES TO IDENTIFY WHICH COMPONENT OF THE FACTOR COMPLEX II, IX, X, IS RESPONSIBLE FOR ENHANCEMENT OF TUMOUR METASTASIS.

INTRODUCTION:

The results of the foregoing studies suggest that the enhancement of tumour metastasis seen with factor complex II, IX, X is secondary to increased trapping of tumour cells in the pulmonary microcirculation, this effect commencing within the first hour of injection. It has also been shown that this effect of enhancement of metastasis is unrelated to the xenogeneic nature of the factor complex, since rat factor complex II, IX, X produces a similar effect. It was clearly important to investigate which component of the factor complex II, IX, X was responsible for this effect of tumour enhancement. Identification of the coagulation factor(s) responsible, would be a step further in contributing to the understanding of the complex relationship between this aspect of the coagulation system and tumour metastasis. Also, should one specific factor of the factor complex II, IX, X be implicated, then therapeutic manipulation of the factor concerned in an attempt to reduce tumour metastasis, would be an attractive proposition.

Factors II, IX and X are very similar in their structural and chemical properties. The amino acid sequence around the active site in these enzymes has

considerable homology with that of the pancreatic serine proteases trypsin, chymotrypsin and elastase (Davie et al, 1979). The active site itself involves serine, histidine and arginine residues linked by hydrogen bonding, in a manner which allows the hydroxyl oxygen atom of the serine residue to act as a strong nucleophile (Blow et al, 1969). The factors are normally present in plasma in an inactive zymogen form. Limited proteolysis activates the zymogen by producing a new carboxy terminal amino acid. This results in a conformational change with a great increase in enzymatic activity. Active sites are found in the constant carboxy terminal region of about 250 residues, which is common to all these factors. Activation does not result in significant reduction in the size of these molecules, except in the case of factor II (prothrombin), whose molecular weight decreases by 50 percent following activation.

As a result of the similarity between these three factors, they have, until recently, been difficult to separate out in their purified forms.

The aim of this study was to assess what effect administration of purified factors II, IX and X, given individually and in various combinations, would have on pulmonary tumour seeding.

MATERIALS AND METHODS:

It was essential initially to obtain pure preparations of individual factors II, IX and X. These were obtained

from Enzyme Research Laboratories, Michigan, U.S.A. Purification was achieved by the method of Ahmad et al (1989), who originally devised the method of successfully separating these three factors. Their method is briefly outlined. Initially, blood is collected in 3.8% sodium citrate with protease inhibitors. This is then centrifuged and fresh plasma obtained. Barium chloride is then added to the plasma and the factors II, IX and X are precipitated out, washed and resuspended in a buffer solution containing protease inhibitors. Ammonium sulphate is then added to the mixture and the supernatant recovered after centrifugation. This is then dialysed overnight. Finally, the supernatant is applied to an antibody affinity sepharose-gel column and the factors II, IX and X are separated individually. Factors II and IX were homogenous as judged by 10% SDS-PAGE gels and showed no reduction upon incubation with 2-mercaptoethanol. Factor X was homogenous on SDS-PAGE gels and showed total reduction upon incubation with 2-mercaptoethanol.

EXPERIMENT:

8 groups of female Fischer 344 rats, 4 per group, 6-8 weeks were used in this experiment. A suspension of $Mtln_3$ cells was prepared, as previously described. The animals were given tail vein injections of 10^4 cells under general anaesthesia. At the same time, additional intravenous injections were administered as follows:

Group A: These control animals received no additional treatment.

Group B: These animals received an injection of Factor II.

Group C: These animals received an injection of Factor IX.

Group D: These animals received an injection of Factor X.

Group E: These animals received a combination of Factors II and IX.

Group F: These animals received a combination of Factors IX and X.

Group G: These animals received a combination of Factors II and X.

Group H: These animals received a combination of Factors II, IX and X.

The dose of the administered factors was similar to that used in the previous experiments (6:7:6 Units).

Animals were sacrificed 17 days after injection and pulmonary seedings assessed by the method of Wexler as previously described (Chapter 1). Comparison of the various groups was made by analysis of variance and Kruskal-Wallis tests.

RESULTS:

The number of pulmonary nodules detected in animals treated with factors II, IX and X, given individually

and in various combinations (Groups B-H), was significantly higher than that in the control group A ($p = 0.012$) (Figure 13). The median number of metastases in "treated" animals ranged from 131 - 270 compared with a median value of 13 in untreated controls. No significant difference was found between the individual groups B-H on statistical analysis ($p = 0.119$). Factors II, IX and X administered individually and in combination enhanced pulmonary metastasis to a similar degree.

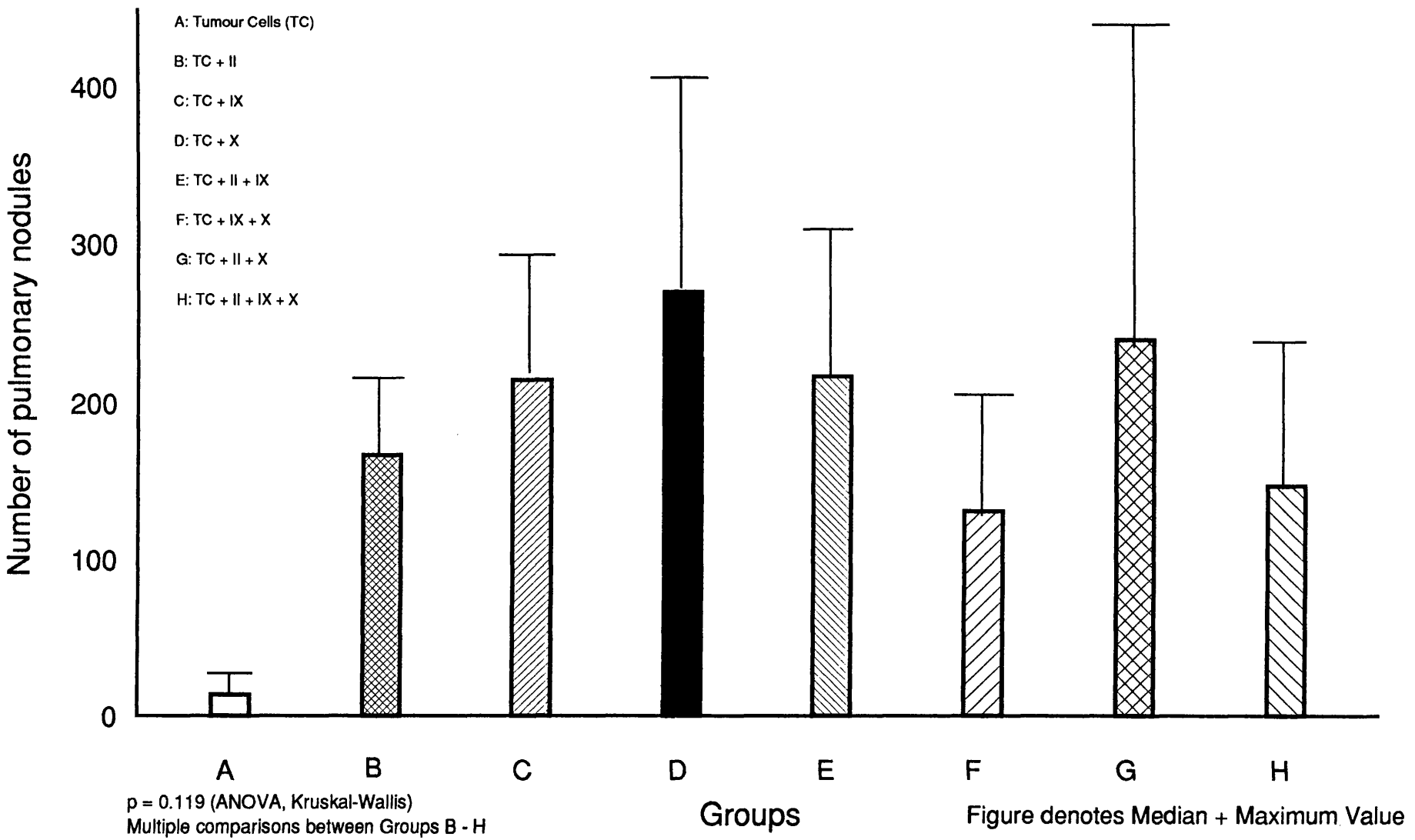
DISCUSSION:

The role of the factors II, IX and X in the coagulation pathway is depicted in figure 1 (Introduction). In the intrinsic system, activation of factor X by activated Factor IX, leads to activation of factor II to form thrombin. Thrombin acts on fibrinogen to form fibrin.

This experiment demonstrates the effect of factors II, IX and X on pulmonary tumour seeding, when administered individually and in various combinations. All three factors produce a significant enhancement of tumour seeding, irrespective of whether they were given individually or in various combinations. In particular, there was no difference in the numbers of metastases produced between the individual treatment groups, suggesting that the factors individually and in combination enhanced metastasis to a similar degree. This suggests that they may do so via a common pathway, possibly related to activation of the coagulation

Figure 13

Effect of Individual Factors on Pulmonary tumour seeding



p = 0.119 (ANOVA, Kruskal-Wallis)
Multiple comparisons between Groups B - H

Figure denotes Median + Maximum Value

system, ultimately leading to the formation of a fibrin clot.

The presence of fibrin, in thrombi surrounding tumour cell emboli, arrested in pulmonary capillaries, has been discussed in Chapter 3. Some studies have demonstrated an association between tumour cells and fibrin. Sindelar and colleagues (1975) however, showed no evidence of fibrin deposits at the intravascular sites of tumour arrest. They demonstrated that platelet aggregates formed the thrombus which surrounds arrested tumour cell emboli. Chew and Wallace (1976) emphasise that both non-cross-linked and cross-linked fibrin may be present in very small amounts and may be easily missed. What is not clear is whether fibrin formation has any effect on the process of embolization and subsequent growth of tumour cells. It is possible that fibrin, may form an amalgam with platelet aggregates and may aid in tumour cell arrest and adherence to vascular endothelium.

If fibrin does play an important role in the promotion of metastasis, then activation of the coagulation system must take place for fibrin to be formed. This study has shown that the factors II, IX and X enhance metastasis to an equal degree. It is possible that activation of factor II (prothrombin), via the intrinsic system, might be the common pathway involved. This would lead to an increased formation of thrombin and thereby fibrin. We have previously demonstrated that the factor complex II, IX, X, encourages trapping of

tumour cells in the pulmonary microcirculation (Chapter 3). This entrapment of tumour cells may be secondary to activation of coagulation, resulting in the formation of a fibrin clot, which by encouraging lung trapping of tumour cells could facilitate enhancement of tumour seeding.

CHAPTER 5

STUDIES TO DETERMINE WHETHER METASTATIC TUMOUR ENHANCEMENT OCCURS SECONDARY TO ACTIVATION OF THE COAGULATION SYSTEM.

INTRODUCTION:

The studies performed thus far have suggested that the factor complex II, IX, X, enhances pulmonary tumour seeding by encouraging trapping of tumour cells in the pulmonary microcirculation. This effect of tumour enhancement is seen when the factors are administered individually or in various combinations. This has led us /to believe that factors II, IX and X produce this effect of tumour enhancement by acting via a common pathway. It is hypothesised that the factor complex activates the coagulation system, resulting in the formation of a fibrin clot. This fibrin clot would encourage tumour cell entrapment resulting in the enhancement of tumour burden previously seen in our experiments. This study was designed to investigate whether administration of factor complex II, IX, X resulted in activation of the coagulation system.

Immunological assays for the breakdown products of fibrin have been available for a number of years. These give a rough measure of the activity of the coagulation system. High levels of fibrin(ogen) degradation products (FDP) reflect excessive activity in either the coagulation system or the fibrinolytic system, or both. These assays however, do not pin-point which system is

activated. A more sensitive way of monitoring haemostatic function is the measurement of specific products of individual reactions. Recently assays have been established to measure specific short-lived reaction products of both thrombin-mediated conversion of fibrinogen to fibrin and plasma-mediated fibrinolysis. Fibrinopeptide A (FpA) is a 16 amino-acid peptide cleaved from the alpha chain of the fibrin molecule in the first stage of the formation of fibrin (Rickles & Edwards, 1983). Elevation of plasma FpA is regarded to be a sensitive indicator of activation of the coagulation system (Introductory Chapter). Since FpA is excreted in the urine, urinary FpA can be measured in urine collected over a period of time.

Measurement of Urinary Fibrinopeptide A:

Measurement of urinary FpA seems an attractive proposition, since in theory it gives us an overall picture of activation of the coagulation system over a period of time, unlike estimation of plasma FpA which can only provide a "snapshot" of activity at one fixed time.

Preliminary experiments were performed wherein suprapubic catheterisation of the rat's urinary bladder was attempted. This resulted in significant contamination of the urine sample with blood. This method of urine sampling was regarded as being unsuitable for the purpose of our experiment, since any

contamination of samples with blood will give an erroneously high level of FpA.

A further series of experiments were hence performed to ensure that bladder catheterisation *per urethra* was feasible in the rat, and that adequate volumes of urine were obtainable over a period of an hour, to facilitate FpA estimation. It was also important to ensure that the samples of urine were not contaminated with blood since this would invalidate the results.

Pilot study to establish method of urine collection and estimation of urinary FpA.

MATERIALS AND METHODS:

8 female Fischer 344 rats, 6-8 weeks old were used in this study. Under chloral hydrate anaesthesia animals were administered a thrombogenic dose of Factor Complex II, IX, X (200 U/Kg), intravenously via the tail vein. The animals were then catheterised per urethra using a 4FG polythene catheter (Portex Limited, Kent, UK). To ensure adequate flow of urine, the animals were initially administered an intravenous injection of 1 ml of 0.9% saline to load their circulation. This was followed 20 minutes later by a slow intravenous injection of Frusemide (Hoechst UK Ltd, Hounslow, Middlesex), 1mg/kg body weight. Urine was collected in plastic bottles and snap-frozen. The animals were sacrificed at the end of the experiment by cervical

dislocation. Samples were sent to the Headquarters Laboratory, Scottish Blood Transfusion Service, Edinburgh, for analysis (Dr I McGregor)

The method of analysis of urinary FpA is similar to the method used to assay rat plasma FpA (Drummond et al, 1991). 1/2 of each sample was diluted in assay buffer (0.05m tris [hydroxymethyl] methylamine, 0.1% ovalbumin) and assayed separately.

RESULTS:

The results of this pilot study are shown in Table 8. Estimation of urinary FpA to assess activation of the coagulation system was deemed unsatisfactory for the following reasons:

1. All 8 samples showed microscopic contamination with blood, presumably traumatic in origin.
2. FpA is unstable if urine is stored at -40^{o}C for long periods, although addition of buffer with ovalbumin enhanced stability.
3. Canine experiments performed simultaneously in Edinburgh to assess the value of estimation of urinary FpA showed poor correlation between plasma Fpa and urinary FpA over short term experiments (McGregor I, personal communication). This is thought to be due to the fact that excreted intact FpA represents only 0.8% of total generated FpA, the rest being degraded by peptidase. Based on these results we decided to assess activation of the coagulation system by assaying plasma FpA.

Table 8 **Analysis of Rat Urinary Fibrinopeptide A (FpA)**

Sample	FpA Conc. ng/ml (untreated)	FpA Conc. ng/ml (buffer diluted)	Presence of blood in sample
A	Insufficient	Insufficient	+++
B	> 1000	>2000	+++
C	Insufficient	>2000	+++
D	Insufficient	82	+++
E	2.5	49	+++
F	Insufficient	Insufficient	++
G	2.7	>2000	+++
H	3.9	25	+

\+ minimum contamination
++ moderate contamination
+++ severe contamination

Measurement of Plasma FpA:

Method of sampling plasma:

Under general anaesthesia, female Fischer rats were subjected to a midline laparotomy. The abdominal aorta was identified and cannulated. Blood was collected in plastic tubes containing anticoagulant solution in a final concentration of Heparin 1000 U/ml and Trasylol 1000 Kallikrein Inactivator U/ml in a 0.15 molar solution of sodium chloride, in a ratio of 1 volume of anticoagulant solution to 9 volumes of blood. The samples were centrifuged at 2100 g for 15 minutes at 4^{o}C. The fresh rat plasma thus obtained was snap-frozen and stored at -70^{o}C prior to analysis of FpA.

Method of analysis:

Samples of rat plasma were analysed at the National Science Laboratory, Scottish National Blood Transfusion Service, Edinburgh by a method previously reported by them (McLaughlin et al 1991; Drummond et al 1991).

Prior to proceeding with the main study, it was essential to establish whether our method of sampling blood was suitable for the estimation of FpA. A pilot study was hence performed, to assess:

a. if adequate controls could be obtained

b. if a rise in FpA could be demonstrated on administering a thrombogenic stimulus to the rat

c. what would be the most suitable time of sampling blood after administration of a thrombogenic stimulus to the animal.

Pilot study to validate method of sampling of blood to facilitate detection of plasma FpA:

EXPERIMENT:

4 groups of female Fischer 344 rats 4 per group, 6-8 weeks old, were used for the purpose of this experiment. Under general anaesthesia the following procedures were performed:

Group A: These animals received no treatment and had blood sampled for estimation of FpA (Control).

Group B: These animals received an intravenous injection of a thrombogenic dose of Factor Complex II, IX, X (200 U/Kg) and had blood sampled at 15 minutes after Factor Complex injection.

Group C: These animals received 200 U/Kg of Factor Complex II, IX, X intravenously and had blood sampled at 30 minutes.

Group D: These animals received 200 U/Kg of Factor Complex II, IX, X intravenously and had blood sampled at 60 minutes.

Samples of plasma were prepared and stored as described above.

RESULTS:

This study showed that this method produced reliable control levels of plasma FpA (Table 9). Secondly, a thrombogenic dose (200 U/Kg) of Factor Complex II, IX, X produced a detectable rise in plasma FpA. Finally, for the purpose of our experiment, a suitable time of sampling blood for estimation of FpA appeared to be 30 minutes after factor complex injection.

Having established that we could reliably assay rat plasma for FpA, using the above method of blood sampling, we proceeded in our main experiment to look for evidence of activation of the coagulation system by estimating FpA under different experimental conditions.

EXPERIMENT: Study to determine whether low-dose factor complex II, IX, X, causes activation of coagulation as detectable by estimation of FpA.

MATERIALS AND METHODS:

4 groups of female Fischer 344 rats, 10 per group, 6-8 weeks old were used. Under terminal anaesthesia, the following procedures were carried out:

Group A: These control animals received no treatment and had blood samples taken for estimation of FpA.

Group B: These animals received an intravenous injection of $Mtln_3$ cells (10^4) and had blood samples taken at 30 minutes after injection.

Group C: These animals received $Mtln_3$ cells (10^4) and

Table 9 **Analysis of Rat Plasma Fibrinopeptide A**

(Thrombogenic dose of factor complex administered)

Sample	Fibrinopeptide A (ng/ml)
A1	42.0
A2	41.6
A3	51.0
A4	18.4
B1	400.0
B2	126.0
B3	195.0
B4	320.0
C1	2,050.0
C2	216.0
C3	730.0
C4	230.0
D1	970.0
D2	232.0
D3	150.0
D4	255.0

Group A: Controls
Group B: Sampled at 15 minutes
Group C: Sampled at 30 minutes
Group D: Sampled at 60 minutes

Factor Complex II, IX, X (6:7:6 Units) intravenously and had blood sampled at 30 minutes after injection.

Group D: These animals received an intravenous injection of Factor Complex II, IX, X (6:7:6 Units) and had blood sampled 30 minutes after injection.

Rat plasma was prepared and stored for analysis of plasma FpA as described above.

Comparison of the different groups was made using the Mann-Whitney test with Bonferoni correction for multiple comparisons.

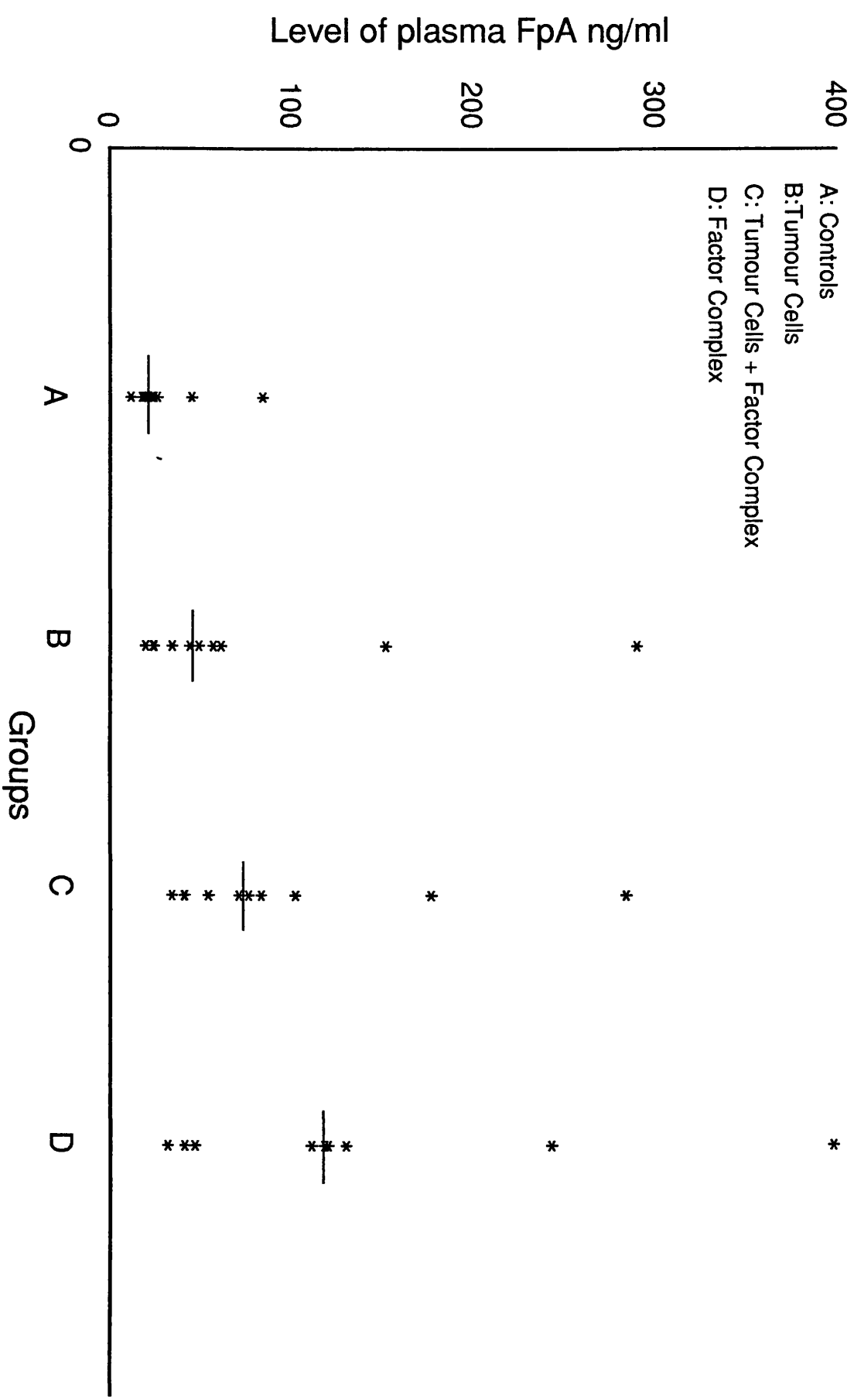
RESULTS: (Figure 14)

The levels of FpA obtained in the control group A were consistent (Median = 23.20). Animals administered factor complex showed a significant rise in plasma FpA when compared with controls (Groups A Vs D, $p = 0.0080$). Administration of tumour cells alone did not give rise to a significant increase in the level of FpA (Groups A Vs B, $p= 0.22$). Administration of tumour cells with factor complex caused a rise in detectable FpA similar to that seen in the group receiving factor complex alone (Groups A Vs C, $p=0.0192$; Groups B Vs C, $p= 0.3691$).

DISCUSSION:

This study was designed to assess whether activation of coagulation occurred on administration of tumour cells

Figure 14 **Analysis of Rat Plasma Fibrinopeptide A (FpA)**



or factor complex II, IX, X. Immunological assays for the breakdown products of fibrin have been available for years. These give a rough measure of the activity of the coagulation system. High levels of fibrin(ogen) degradation products (FDP) reflect excessive activity in either the coagulation system or the fibrinolytic system or both. These assays do not however pin-point which system is activated. A more sensitive method of monitoring haemostatic function is the measurement of specific products of individual reactions. Elevation of FpA is regarded to be a sensitive indicator of activation of the coagulation system. It suffers however from the disadvantage that it only provides a "snapshot" of activity at one fixed time, the time of sampling. Since FpA is excreted in the urine, we believed that an estimation of urinary FpA over a period of one hour would provide a more accurate assessment of the level of activation of the coagulation system.

The method of collection of urine suprapubically and per urethra suffered two main disadvantages:

1. Contamination with blood, probably traumatic in origin.
2. Inadequate volumes for assay despite a fluid challenge and diuretics.

As a result of these twin problems, this method was abandoned for the more feasible method of assaying plasma FpA.

Estimation of plasma FpA posed a few problems in interpretation. We were conscious that the information

obtained from this assay only provided us with an indication of activity of the coagulation system at one fixed point in time. Our preliminary studies suggested that the most suitable time of sampling blood for assay of FpA was 30 minutes after tumour cell injection. Having established this, we discovered that in all our experiments of FpA estimation occasional values showed wide variation within the same group. This made the results slightly more difficult to interpret. By analysis of all the data collected however, we were able to define clear and consistent differences between the study groups.

Our results showed that factor complex administered either alone or along with tumour cells, caused a significant rise in the level of plasma FpA. We also noted that tumour cell administration caused a small rise in the level of FpA. This however did not reach statistical significance. The marginal rise in the level of FpA detected on administration of tumour cells may be related to the procoagulant properties that tumour cells possess. Previous work performed to study the procoagulant properties of the $Mtln_3$ cell line has shown that this tumour cell line does possess a small procoagulant effect (Personal communication, P McCulloch, 1991). This would explain the marginal rise in FpA seen in this study.

Our studies thus far have demonstrated that in this animal model, coagulation factors II, IX, X when administered with tumour cells activate coagulation and

encourage tumour cell entrapment within the pulmonary microcirculation. Our working hypothesis is that for this process to take place, fibrin clot formation must take place. The controversy surrounding the role of fibrin in the metastatic process has already been discussed (Chapter 3). Further investigation is now required to assess whether fibrin clot formation is responsible for this effect of tumour enhancement.

CHAPTER 6.

DOES THE EFFECT OF ENHANCEMENT OF TUMOUR METASTASIS BY FACTOR COMPLEX II, IX, X REQUIRE THE FORMATION OF A FIBRIN CLOT?

INTRODUCTION:

The studies completed thus far have identified some of the possible mechanisms by which Factor Complex II, IX, X enhances pulmonary metastasis in our tumour/host system. Firstly, a greater number of tumour cells are trapped in the pulmonary microcirculation within the first hour of injection. During this first hour, some activation of the coagulation system does appear to occur, since there is a demonstrable rise in plasma Fibrinopeptide A. It seems likely that Factor Complex II, IX, X, when injected with tumour cells, activates the coagulation system thereby encouraging the arrest of circulating tumour cells in a mesh of platelets and fibrin, resulting in the formation of a fibrin clot.

The hypothesis that intravascular fibrin clot formation contributes to the metastatic process is controversial. We have already demonstrated that the effect of enhancement of tumour metastasis by Factors II, IX, X, in our model, may be mediated via a common pathway in the coagulation cascade involving the most distal of these factors ie. factor II (Prothrombin), perhaps ultimately leading to the formation of a fibrin clot. Using electron microscopy and fibrin-specific immunofluorescence, previous workers have demonstrated

the presence of tumour cells associated with platelet-fibrin thrombi within the pulmonary microcirculation within 1-3 hours of intravenous injection (Jones et al., 1971; Chew & Wallace, 1976). Assuming the hypothesis that fibrin clot formation is required to facilitate tumour progression is correct, then treatment with fibrinolytic enzymes or enhancement of endogenous fibrinolytic reactions might have a beneficial effect. Preliminary evidence in patients with small cell carcinoma of the lung treated with a combination of chemotherapy and urokinase suggests that this might indeed be the case (Calvo et al, 1985). Other workers investigated this possibility by administering Arvin, a snake venom, to animals in a similar tumour/host system (McCulloch & George, 1988). They found that a reduction in the ability of animals to form fibrin clots had no discernible effect on metastasis. However, it may be argued that the amount of fibrin required to form metastasis is very small and in the experiments using Arvin, complete defibrination of the animals was not achieved. Indeed, complete defibrination cannot be achieved and this has to be borne in mind when interpreting results of defibrinating experiments. The alternative method of removing fibrin is to lyse fibrin clot once it has formed. This can be successfully achieved using fibrinolytic agents. An experiment was therefore designed to study the effect of fibrinolysis on pulmonary tumour seeding.

For the purpose of our experiment, we used Streptokinase (Hoechst, UK) to achieve fibrinolysis. Streptokinase is produced by beta haemolytic streptococci and is antigenic and immunogenic in humans. This drug has been purified over the years and has been shown to be a valuable and effective thrombolytic agent. Streptokinase activates the intrinsic fibrinolytic system by the formation of a linkage compound between Streptokinase and the proactivator-plasminogen molecule. This complex possesses activator properties and brings about the conversion of plasminogen into the fibrinolytic enzyme plasmin. Plasminogen adsorbed on the clot is also activated. This results in lysis of the fibrin clot.

Assays to assess cytotoxicity of Streptokinase:

Prior to proceeding to study the effect of Streptokinase on pulmonary tumour seeding in our experimental model, it was essential to establish whether Streptokinase had any cytostatic or cytotoxic effect on the $Mtln_3$ tumour cell line. Two **in vitro** assays were performed to look at this possibility:

1. Clonogenic assay
2. 4-hour Chromium release assay

1. Clonogenic assay:

$Mtln_3$ cells were grown in culture in F10/DMEM with FCS in $75cm^3$ flasks. When confluence was attained,

Streptokinase was added to the flasks in a range of different doses: 25,000, 75,000, 100,000 and 250,000 Units (4 flasks per dose) with a further fifth group acting as the untreated control. The cells were incubated for 12 hours at 37°C in equilibrium with 2% CO_2 in air. The cells were then trypsinised, washed, and 200 cells from each flask were plated out in petri dishes containing medium, and left incubated for 10 days. Colonies were then counted after staining with crystal violet.

2. 4-hour Chromium release assay:

$Mtln_3$ cells were radiolabelled with $Chromium^{51}$ and exposed to doses of Streptokinase similar to that used in the clonogenic assay. After 4 hours, percentage cytotoxicity in each group was calculated using the formula:

$$C = \frac{\text{experimental sample - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

RESULTS:

1. Clonogenic assay:

The mean number of colonies in the experimental groups ranged from 142-156, with 152 colonies in the control (Table 10). Streptokinase did not have a cytostatic effect on $Mtln_3$ cells.

Table 10

The effect of Streptokinase on Tumour Cells

Clonogenic assay

Streptokinase Dose (Units)	0	25,000	75,000	100,000	250,000
Number of Colonies	152 (C)	147	156	142	137

C: Control

2. 4-hour Chromium release assay:
The mean percentage cell death was 2.9%. Streptokinase did not have a significant cytotoxic effect on $Mtln_3$ cells.

The results of these **in vitro** studies demonstrated that Streptokinase had no significant direct cytotoxic or cytostatic effect on the $Mtln_3$ tumour cell line.

EXPERIMENT: Studies to determine the effect of fibrinolysis on the enhanced pulmonary tumour seeding observed with Factor Complex II, IX, X.

8 groups of female 344 Fischer rats, 6 per group, 6-8 weeks old were used in this experiment. Under general anaesthesia, animals were given tail vein injections of a 0.5 ml suspension of 10^4 $Mtln_3$ cells in F10/DMEM. Additional treatment was then commenced as follows:

Group A: These animals received no additional treatment.

Group B: These animals received an intravenous injection of bovine serum albumin at the same time as tumour cell injection (t=0).

Group C: These animals received an intravenous injection of Factor Complex II, IX, X at t=0.

Group D: These animals received an intravenous injection of Factor Complex II, IX, X at t=0 and a further injection of intravenous Streptokinase at t=15 minutes.

Group E: These animals received the Factor Complex at t=0 and Streptokinase at t=30 minutes.

Group F: These animals received the Factor Complex at t=0 and Streptokinase at t=60 minutes.

Group G: These animals received the Factor Complex at t=0 and Streptokinase at t=90 minutes.

Group H: These animals received the Factor Complex at t=0 and Streptokinase at t=300 minutes.

The doses of coagulation factor complex II, IX, X and bovine serum albumin used were similar to that used in earlier experiments (Chapter 1). The dose of Streptokinase administered was 30 000 U/Kg body weight. This was based on previous animal experimental work wherein clot lysis was achieved **in vivo** (Lyden et al, 1990). All three preparations were passed through a 0.2um filter before injection for sterilization and removal of particulate material that might embolise in the pulmonary circulation.

The laboratory tests used to monitor the animal's coagulation were the thrombin time and the prothrombin time.

Animals were sacrificed at 17 days after injection and pulmonary seeding assessed by the method of Wexler as previously described (Chapter 1). Comparison of the different groups was made by the Mann-Whitney test using Bonferoni's correction for multiple comparisons.

RESULTS: (Figure 15)

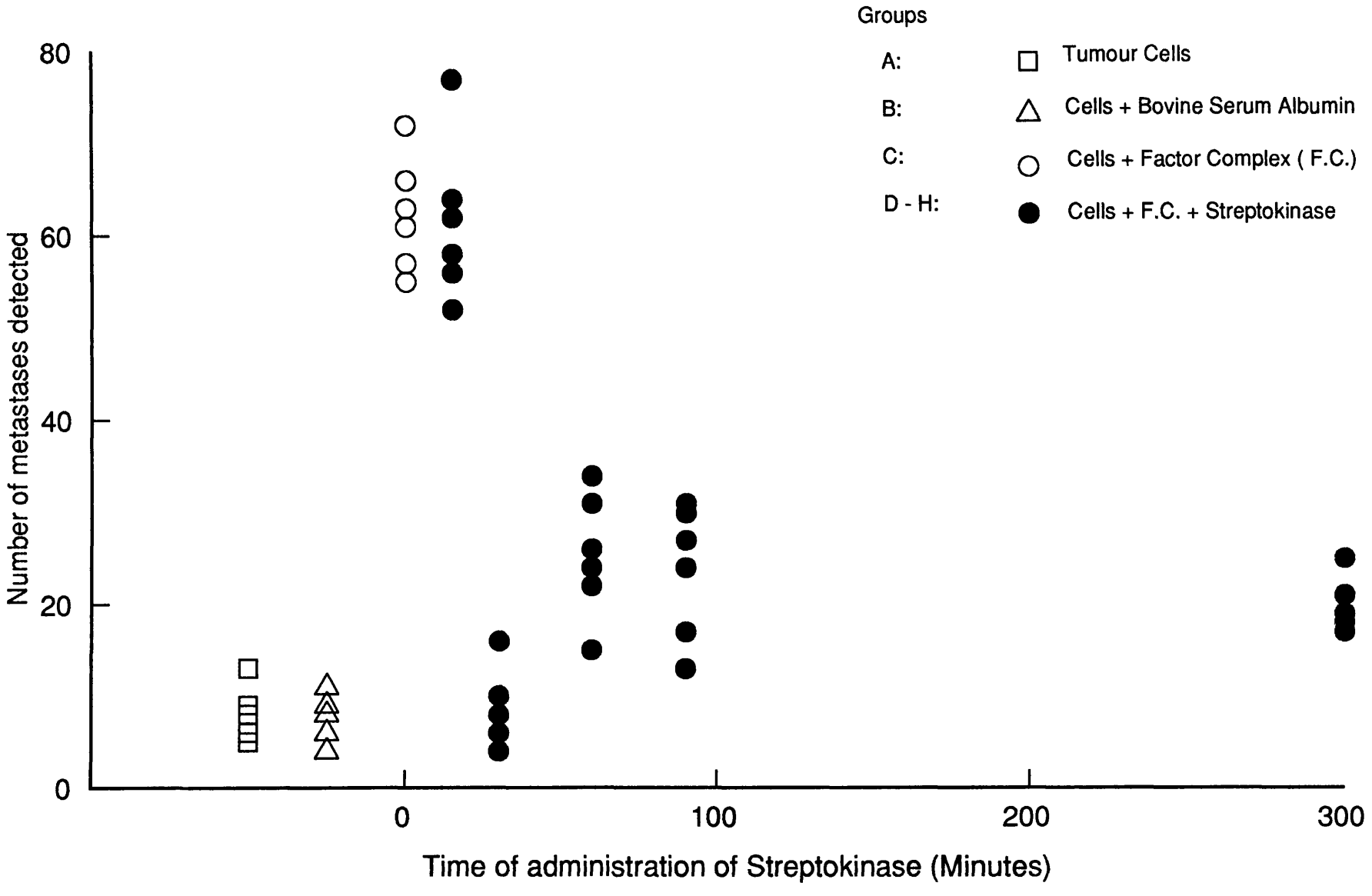
Serial measurements of the thrombin time and prothrombin time showed that at all time intervals ranging from 5 minutes to 24 hours after injection of Streptokinase, there was no significant alteration of coagulation in the animals.

As expected, animals that received factor complex II, IX, X, along with tumour cells (Group C), showed a significant enhancement of pulmonary tumour seeding (median = 62) when compared with control animals (Group A) that received tumour cells alone (median=7; p=0.025). Streptokinase administered 30 minutes after tumour cell injection returned metastases to control levels (median= 9, p=0.025 Group C vs E). In the remaining Groups F-H, Streptokinase was still effective in reducing pulmonary tumour seeding, although when administered at 300 minutes after tumour cell injection this effect was less marked (Group H, median = 19, p=0.40, group C Vs H). Interestingly, this effect of reduction in tumour seeding was not noted when Streptokinase was administered 15 minutes after tumour cell injection (Group D).

DISCUSSION:

Our earlier studies had led us to believe that the enhancement of tumour metastasis seen with factor complex II, IX, X, was secondary to increased pulmonary trapping of tumour cells which occurred in the first hour after tumour cell injection. Factors II, IX, X,

Figure 15 **EFFECT OF STREPTOKINASE ON PULMONARY TUMOUR SEEDING**



appear to bring about this effect of tumour enhancement via a common pathway via factor II (Prothrombin) involving activation of the coagulation system. Based on these findings, we concluded that in order to facilitate enhancement of tumour metastasis the formation of a fibrin clot would probably be required. In order to investigate this possibility, the above experiment was designed to study the effect of fibrinolysis on the tumour metastasis. We worked on the hypothesis that if formation of a fibrin clot is required for the effect of enhancement of metastasis to take place, then fibrinolysis should reverse this effect.

A schematic representation of the fibrinolytic system is shown in Figure 2 (Introductory Chapter). The serine protease plasmin is essential for the dissolution of the fibrin thrombus. The plasma concentration of it's inactive precursor plasminogen, greatly exceeds the amount required for physiological fibrinolysis; it is counterbalanced by rapid inhibitors in the fibrinolytic system that are normally present in the circulation. Endogenous plasminogen is activated through the release of tissue-type plasminogen activator (t-PA) into the circulation. t-PA is constantly secreted by vascular endothelial cells but immediately neutralised by a specific inhibitor, plasminogen activator inhibitor (PAI-1). Under normal circumstances, any circulating plasmin is rapidly neutralised by alpha2-antiplasmin, a specific plasmin inhibitor that forms a stoichiometric

1:1 complex with plasmin that is devoid of protease or esterase activity. The rate of complex formation is strongly dependent on the availability of free lysin-binding sites and a free active site in the plasmin molecule. Only when the circulating alpha2-antiplasmin level is reduced can plasmin circulate freely and exert a systemic proteolytic effect. Free plasmin will degrade not only fibrin, but also fibrinogen and plasma proteins, producing the so-called plasma proteolytic state.

Currently there are several thrombolytic agents in clinical use. Streptokinase and urokinase belong to the first generation of such agents. Produced by beta-haemolytic streptococci, streptokinase is antigenic and immunogenic in humans.

Preliminary **in vitro** studies showed that Streptokinase was not cytotoxic to the $Mtln_3$ cell line. Administration of Streptokinase **in vivo** showed no significant alteration in the coagulation of the experimental animals. It should be remembered that although fibrinolytic agents ultimately prevent the accumulation of thrombus, they have no effect on the clotting process itself except at very high doses when breakdown products of fibrinolysis begin to interfere with the reactions involved. The normality of the coagulation tests was therefore not an unexpected finding but rather a reassuring indication that the dose used was not excessive.

As seen in earlier experiments, factor complex II, IX, X, enhanced pulmonary tumour seeding. Streptokinase administered 15 minutes after tumour cell and factor complex injection did not affect this phenomenon of tumour enhancement. The absence of this effect in Group D animals can be explained by the pharmacodynamics of Streptokinase which has a very short circulating half-life (Grierson & Bjornsson, 1987). By assessing the fate of radiolabelled tumour cells we have previously shown that between 30 and 60 minutes after tumour cell injection, there is a sharp rise in the number of cells trapped within the pulmonary microcirculation (Chapter 3). In the experimental group D, by the time this event of pulmonary entrapment takes place, most if not all the administered Streptokinase would have left the circulation and thereby would have been unable to have any effect on fibrin clot formed as a result of tumour cell entrapment.

Streptokinase administered 30 minutes after the initial injection of tumour cells and factor complex, returned metastases to control levels, thereby abolishing the effect of tumour enhancement. This effect of tumour burden reduction was still present, although to a lesser degree, when Streptokinase was administered intravenously at 300 minutes. These results suggest that the phenomenon of enhancement of tumour metastasis with factor complex II, IX, X, requires the formation of a fibrin clot. Destruction of the fibrin clot with fibrinolytic agents reverses this effect.

This intriguing result raises an important question: does fibrinolytic therapy have any effect on pulmonary tumour seeding in the absence of the exogenous factor complex II, IX, X in our animal model? Should fibrinolysis inhibit metastasis, it might have a role to play in the management of the cancer patient.

CHAPTER 7.

STUDIES TO DETERMINE THE EFFECT OF FIBRINOLYSIS ON PULMONARY TUMOUR SEEDING.

INTRODUCTION:

The presence of fibrin in malignant tumours has attracted considerable attention and the significance of it's presence has been subject to several interpretations. Fibrin is an early and consistent marker of tumour cell stroma. It is deposited within hours of tumour implantation and remains in the tumour throughout the period of tumour growth (Dvorak et al, 1984). Circulating tumour cells may form aggregates with platelets and fibrin which facilitate their trapping within the microcirculation of target organs, thereby enhancing their metastatic effeciency (Crissman, 1984). Currently it is believed that fibrin may facilitate adhesion of tumour cells to vascular endothelium by acting as an "intercellular" glue (Francis, 1989).

Our previous experiments have strongly suggested that Factor Complex II, IX, X enhances pulmonary tumour seeding by increasing tumour cell entrapment within a fibrin clot and this effect of tumour enhancement can be reversed by activation of the intrinsic fibrinolytic system on administration of a "clot-lysis" dose of intravenous Streptokinase.

This intriguing result raises two important questions:

1. Does Streptokinase reduce pulmonary tumour seeding in a dose-dependent manner?
2. Does fibrinolytic therapy have any effect on pulmonary tumour seeding in the absence of exogenous coagulation factor complex II, IX, X?

The answer to the second question should bring our model closer to the situation that is seen in clinical cancer patients.

EXPERIMENT 1: Studies to determine whether the effect of Streptokinase on pulmonary tumour seeding is dose-dependent.

MATERIALS AND METHODS:

5 groups of female Fischer 344 rats, 6 per group, 6-8 weeks old were used in this experiment. Animals received a single tail vein injection of a 0.5 ml suspension of 10^4 $Mtln_3$ cells in F10/DMEM. Additional treatment was then commenced as follows:

Group A: These animals received no additional treatment.

Group B: These animals received an intravenous injection of Factor Complex II, IX, X at the same time as tumour cell injection (t=0 minutes).

Group C: These animals received Factor Complex at t=0 minutes and an intravenous injection of Streptokinase (30,000 U/Kg), 30 minutes after tumour cell injection (t=30)

Group D: These animals received Factor Complex at t=0

minutes and Streptokinase (15,000 U/Kg) at t=30 minutes.

Group E: These animals received Factor Complex at t=0 minutes and Streptokinase (3,000 U/Kg) at t=30 minutes.

The dose of Factor Complex II, IX, X used was similar to that used in previous experiments (6:7:6 Units).

Animals were sacrificed at 17 days and tumour nodules assessed by the method of Wexler (Chapter 1).

RESULTS:

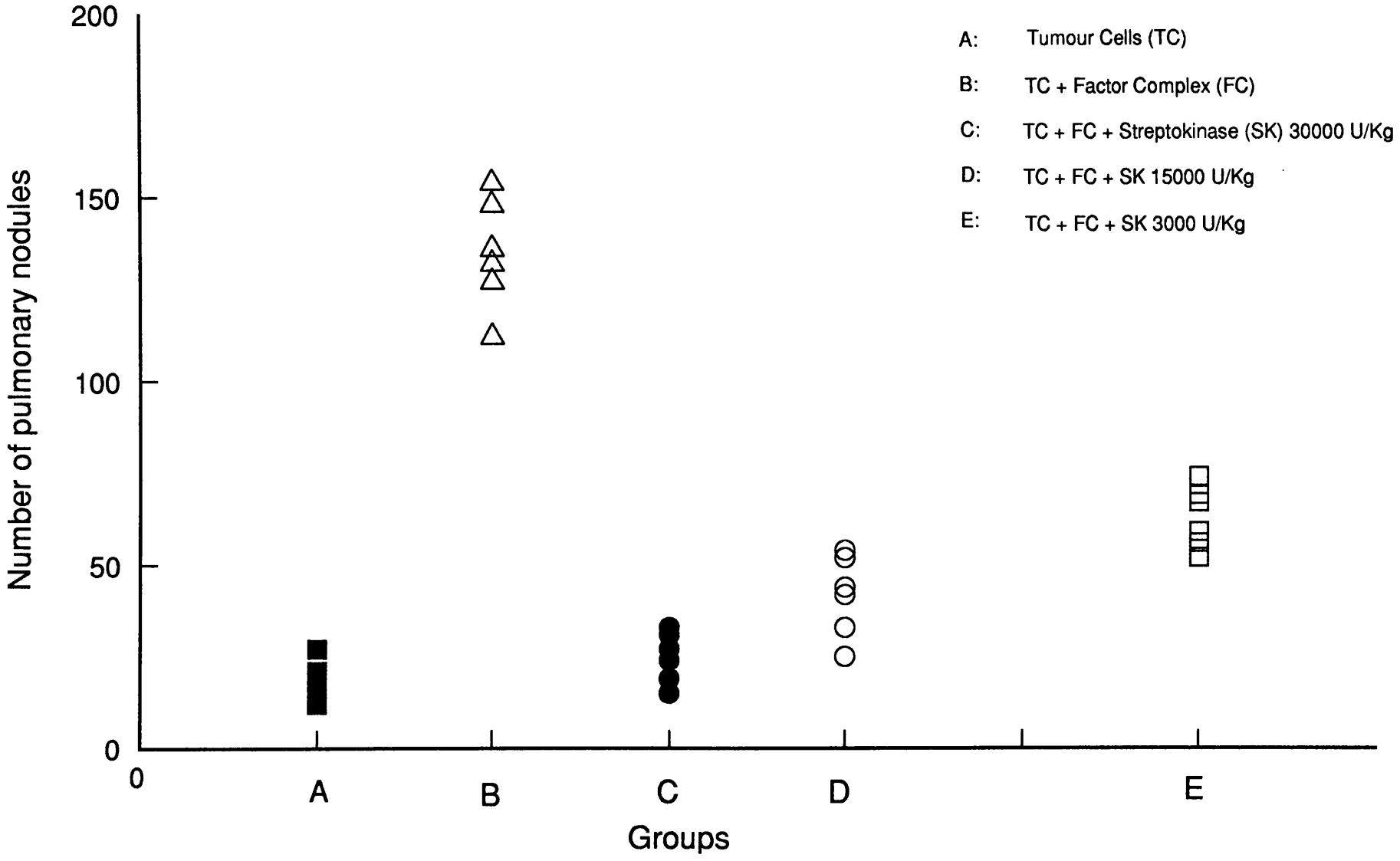
As expected, Factor Complex II, IX, X, enhanced pulmonary tumour seeding when compared with controls (p=0.0204, Groups A vs B). Streptokinase significantly reduced pulmonary tumour seeding in all the remaining groups in a dose-dependent manner, effective even at $1/10^{th}$ the standard "clot-lysis" dose (3000 U/Kg, Groups B vs E, median 134 vs 63 seedlings; p=0.0204) (Figure 16).

EXPERIMENT 2: Studies to determine the effect of Streptokinase on pulmonary tumour seeding in the absence of exogenous coagulation factor complex II, IX, X.

MATERIALS AND METHODS: 2 groups of 12 female Fischer 344 rats, 6-8 weeks old, were used in this experiment. Under general anaesthesia, animals were given tail vein

Figure 16

STREPTOKINASE - DOSE RESPONSE



injections of a 0.5 ml suspension of 10^5 $Mtln^3$ cells. Additional treatment was then commenced as follows:

Group A: These animals received no additional treatment.

Group B: These animals received a single intravenous injection of Streptokinase in a dose of 30,000 U/kg, 30 minutes after tumour cell injection.

Animals were sacrificed at 17 days and pulmonary nodules assessed by the method of Wexler.

Comparison of the 2 groups was made using the Mann-Whitney test.

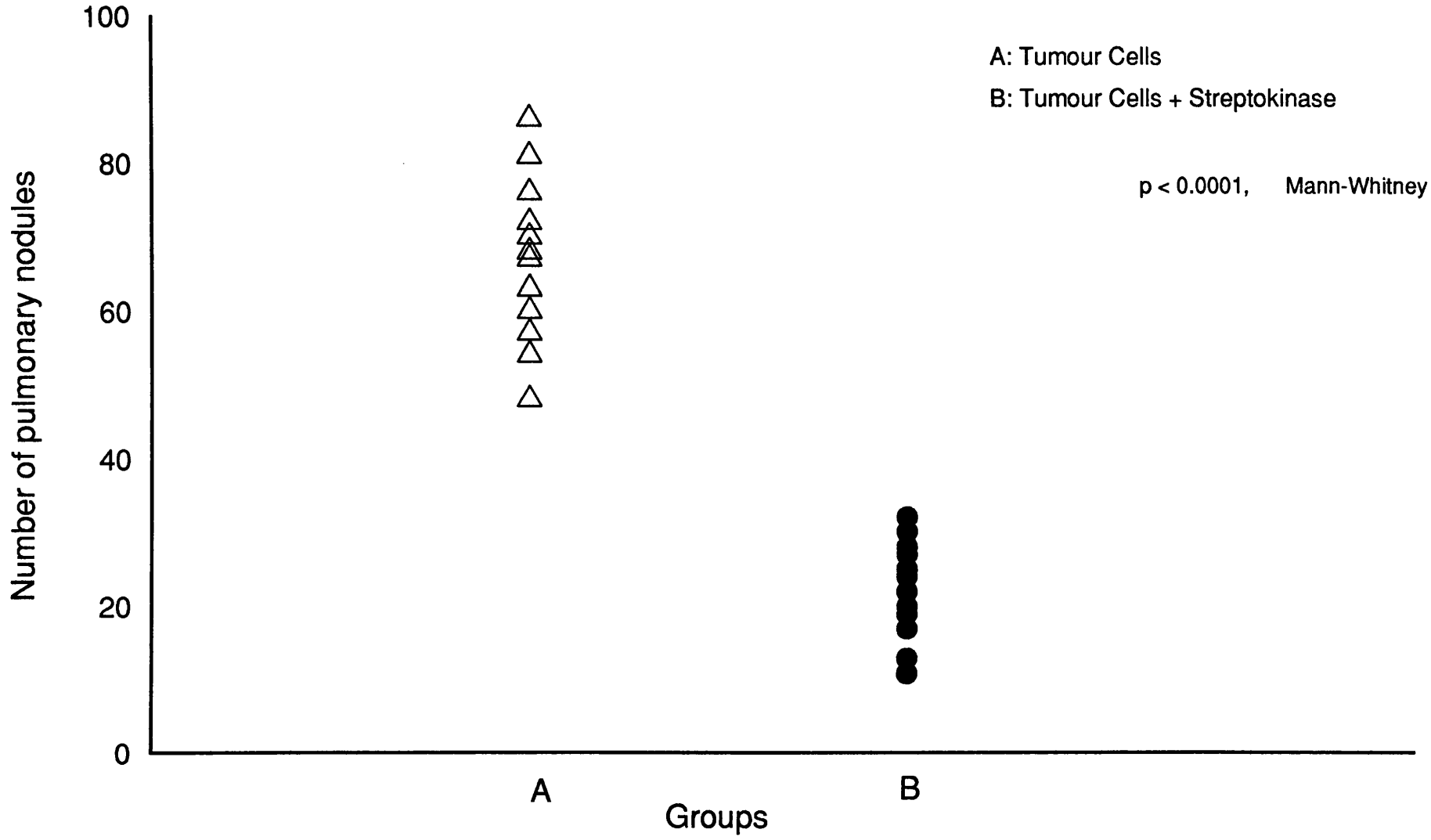
RESULTS:

Intravenous Streptokinase, administered 30 minutes after tumour cell injection, caused a threefold decrease in the number of pulmonary tumour nodules seen (median 23.0 seedlings in the Streptokinase-treated group vs 67.5 in untreated controls; $p<0.0001$) (Figure 17).

DISCUSSION:

In our previous experiments we showed that factor complex II, IX, X enhanced pulmonary tumour seeding and that intravenous Streptokinase administered in a "clot-lysis" dose of 30,000 U/Kg, 30 minutes after tumour cell injection, abolished this effect of pulmonary tumour enhancement. The purpose of this study was to assess whether this effect of Streptokinase is "dose-dependent". The results of the first experiment demonstrate that the effect of Streptokinase in reducing

Figure 17 **FIBRINOLYSIS AND TUMOUR METASTASIS**



pulmonary tumour burden is dose-related, the maximal effect being achieved using a "clot-lysis" dose of 30,000 U/Kg. It is worth noting that even $1/10^{th}$ of the "clot-lysis" dose of Streptokinase produced a significant reduction in tumour cell burden. This would be of considerable significance in the clinical situation.

The results of the second experiment are striking. Even in the absence of factor complex II, IX, X, a "clot-lysis" dose of Streptokinase produced a dramatic reduction in tumour cell burden, when administered as an intravenous bolus, 30 minutes after tumour cell injection. This finding raises interesting questions as to whether there is a role for Streptokinase in the management of cancer patients.

It is believed that during resection of a primary tumour, malignant cells are shed into the bloodstream. Some of these cells may get trapped in the microcirculation of target organs. Provided the "soil" is favourable, some of these tumour cells develop into metastasis. The "no-touch" technique in the resection of colo-rectal cancer, as advocated by Turnbull (1967) was based on the principle of avoiding tumour cell shedding at the time of surgery. This technique has largely been abandoned because of poor reproducibility, but the theory underlying it has not been convincingly refuted.

Tumour cells may therefore be shed into the circulation intra-operatively and trapped within a mesh of platelets and fibrin at the level of the capillary

bed of target organs. Fibrinolytic agents administered peri-operatively would facilitate destruction of this fibrin clot, thereby preventing tumour cell seeding. Anti-metastatic therapy has been tried with a varying degree of success (Zacharski et al, 1982, 1984; Zacharski, 1981, 1986b), but adequate controlled studies on a suitable population have not been performed. Previous work by White et al (1976), using urokinase to facilitate fibrinolysis in human cancer, suggests that prevention of implantation of circulating malignant cells onto vascular endothelium, is likely to improve patient survival. The practical benefits of antimetastatic measures is difficult to evaluate because the metastases prevented are likely to be relatively few compared to those which have already occurred before surgery. Large prospective studies would therefore be necessary to determine the value of antimetastatic therapy, but if a simple, safe treatment were available, such studies might be justified.

Fibrinolytic agents are attractive in theory. There are however a few disadvantages. Streptokinase is antigenic and immunogenic in humans. Tissue-plasminogen activator (t-PA), produced by recombinant DNA technology (Pennica et al, 1983), would be a more suitable alternative. The risk of haemorrhage with the use of fibrinolytic agents peri-operatively should also be considered. Our experiments have shown that a single "clot-lysis" dose is sufficient to inhibit metastasis. Monitoring of coagulation showed no systemic disturbance

of the coagulation system. Furthermore, low-dose Streptokinase was also effective in reducing tumour seeding in the model incorporating factor complex treatment. Whether this remains the case in the absence of this treatment is not yet known.

Further work needs to be performed to investigate the role of other fibrinolytic agents with regard to inhibiting tumour metastasis.

Final Discussion

The individual studies presented in this thesis have been fully discussed in the relevant chapters and this final section therefore represents an overview of the interaction between coagulation and cancer, incorporating the findings of the present work.

The evidence accumulated from previous studies, and discussed in the introductory section of this work, suggests a two-way interaction between cancer and the coagulation system. On the one hand, evidence exists for activation of the coagulation system in patients with cancer and for the ability of tumour cells to cause such activation. On the other hand, there is evidence from animal studies and a few human clinical studies to suggest that coagulation plays an important role in the metastatic process, and that the potential for influencing metastasis exists. Given these facts, it is easy to speculate that tumours promote their own metastasis by virtue of their activation of the coagulation system. If this is true, it raises the possibility of therapeutically interfering with the coagulation system in order to influence the metastatic process of malignant tumours, in the clinical situation.

Studies based on animal models are subject to many potential sources of error arising from the complexity of the variations between different species. As a result of this, it is rarely possible to exclude all influences other than those with which the experiment is primarily concerned. This series of experiments was no exception

and some of the possible sources of error in the individual experiments performed have been described and discussed in the relevant chapters. In these circumstances, the conclusions reached from individual experiments have to take into consideration other possible explanations and caution must be exercised in interpretation of results. All the experiments described in the foregoing chapters were repeated in order to ensure that the results obtained were reproducible. Equally the importance of mutually supportive findings from separate experiments cannot be overemphasised. Such independent confirmation increases the confidence with which conclusions are made and permits a greater accuracy in interpretation of findings, since two independent methods of determining the same result are much less likely to be in error than one method however often repeated. In the present series of studies, mutually supportive findings from separate experiments provided evidence for all the major points in our conclusions. The picture of the cancer/coagulation relationship is complex and subtle and the present studies have investigated only a few aspects of this complex relationship.

Our initial task was to validate the metastatic tumour model. Having achieved this, it was important to confirm the findings of previous workers, that the warfarin-dependent coagulation factor complex II, IX, X, enhanced pulmonary tumour seeding in this animal model. Studies critically analysing the dose and time of

administration of the factor complex were performed. The results of these studies validated the dose of factor complex previously used, and confirmed that the factor complex II, IX, X significantly enhanced pulmonary tumour seeding. The mechanism of tumour enhancement remained unclear. Several possibilities existed. The fact that human coagulation factors were producing this effect in an animal model raised the possibility of this effect being secondary to the xenogeneic nature of the factor complex. A comparison of the prometastatic effect of human and rat factor complexes showed that this effect of tumour enhancement was produced to an equal degree, suggesting that the xenogeneic nature of the human factor complex was not responsible for producing this effect of tumour enhancement.

Further questions could then be addressed. Previous work had left open the possibility that one of the three factors II, IX, X, was solely responsible for this effect of tumour enhancement. Until recently, the factors II, IX, X could not be obtained individually in a pure form. The ability to obtain these purified factors allowed us to demonstrate that all three factors when given either individually or in different combinations, produced an equal enhancing effect on pulmonary tumour seeding. The three factors involved occupy a central part in the coagulation cascade, the site where the extrinsic and intrinsic system meet to form the common pathway. The most distal of these three factors in the coagulation "cascade" is factor II

(prothrombin). Our working hypothesis at this stage was that the factor complex probably caused activation of the coagulation system via factors IX, X and II (in that order), resulting in the formation of a fibrin clot which by increasing tumour cell entrapment in a fibrin mesh within the pulmonary microcirculation, caused enhanced pulmonary tumour seeding.

To test this hypothesis, we needed to answer two questions:

1. Does the factor complex II, IX, X activate coagulation in this animal model?
2. Is there increased pulmonary entrapment of tumour cells on administration of the factor complex?

The study designed to answer the first of these questions was complex and difficult. Fibrinopeptide A (FpA) is a highly sensitive indicator of activation of coagulation. An attempt at analysis of rat urinary FpA proved unsuccessful as a result of contamination of samples with blood. Analysis of rat plasma FpA was more successful. This study demonstrated that factor complex II, IX, X when administered alone or along with tumour cells activated coagulation. There was a suggestion that tumour cells may on their own be responsible for an activation of coagulation, though in this study it was not shown to be of any significance.

In order to assess pulmonary entrapment of tumour cells, the passage of radiolabelled cells through the lungs was studied. The results of this study showed a 20% increase in the number of tumour cells trapped in

the pulmonary microcirculation, when administered along with factor complex II, IX, X.

If fibrin clot formation is required to facilitate increased tumour cell entrapment, then destruction of fibrin should result in abolition of this effect. Streptokinase, a thrombolytic agent when administered in a "clot-lysis" dose, succeeded in abolishing this effect of tumour enhancement. In order to be effective in our model, Streptokinase had to be administered 30 minutes after tumour cell injection. The results of this study supported our earlier hypothesis that fibrin clot formation is required to promote pulmonary tumour seeding. It also raised the possibility of considering the use of Streptokinase in clinical cancer patients.

A further final study required to be performed to answer the question: In the absence of exogenous coagulation factors II, IX, X, is Streptokinase effective in inhibiting pulmonary metastasis? The results of our study showed that Streptokinase inhibited pulmonary tumour seeding in this animal model even in the absence of exogenous coagulation factors.

There are several areas of our investigation which require further clarification. The association of fibrin and tumour cells within the microvasculature has not been conclusively proven in this model. Also, pathological evidence for intravascular dissolution of thrombus has not been demonstrated. These studies would entail extensive microscopic studies of changes in the pulmonary microvasculature under the experimental

conditions described earlier. In the absence of histological evidence for our hypothesis that Streptokinase reduces pulmonary tumour seeding by thrombolysis, we cannot exclude the possibility that Streptokinase may cause this effect via a completely different mechanism, although an overall view of the evidence presented makes this seem unlikely. Furthermore, we are aware that the behaviour of "artificial" metastases by intravenous tumour cell injection may be quite different from spontaneous metastases arising from a primary tumour. However, the animal model we used in all our experiments permitted us to examine the "intravascular" phase of the metastatic process in greater detail than would otherwise have been possible.

Our investigations have led us to an interesting conclusion: Streptokinase inhibits tumour seeding. Is this finding significant with regard to the practical management of clinical cancer patients? It is widely believed that during the resection of a primary tumour, malignant cells are shed into the circulation (Engell HC, 1955). Some of these circulating cells may arrest at the level of the microcirculation of target organs. Provided the "soil" is favourable, these cells may develop into metastatic deposits. Activation of coagulation, as a direct consequence of surgery, would theoretically encourage tumour cell entrapment at the level of the microcirculation of target organs. Indeed animal experimental models have shown that surgical

stress enhances tumour metastasis (Buinauskas P, et al, 1958). If tumour cells that are shed at the time of surgery are prevented from adhering to vascular endothelium and are kept in circulation, they will eventually die. This will reduce the number of potentially metastatic cells in the circulation. Fibrinolytic therapy administered in the peri-operative period would be theoretically effective in this respect. There are however a few potential disadvantages with fibrinolytic therapy that require serious consideration. These are the antigenicity and immunogenicity of Streptokinase and the potential risk of haemorrhage post-operatively. With regard to the latter problem, our animal work demonstrated two encouraging findings:

1. There was no evidence of an overall systemic disturbance of coagulation with the dose of Streptokinase used.
2. Streptokinase was effective in inhibiting pulmonary tumour seeding, even when administered at $1/10^{th}$ the "clot-lysis" dose.

The use of tissue plasminogen activator, obtained by recombinant DNA technology (Pennica et al, 1983), would avoid the former complication. Further animal experimental work needs to be performed to investigate the role of tissue plasminogen activator in inhibiting tumour seeding.

Most cancer patients die from tumour metastasis. The prevention of metastasis is therefore an important goal of cancer research. Evidence to date strongly

suggests that manipulation of the coagulation system can influence the metastatic process in favour of the host. A prospective clinical trial is now required to assess the role of antimetastatic therapy in clinical cancer patients. There are several practical difficulties in such a trial; for eg. potential complications arising in post-operative patients as a result of fibrinolytic therapy, potential antigen/antibody reactions if Streptokinase is to be used and the recognition that careful interpretation of results based on disease stage is essential, taking into consideration that some patients will have occult metastases at the time of surgery. These problems however, are not insurmountable. Despite these apparent difficulties, the potential benefits of such a trial could have an impact on the management of the patient with malignant disease.

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