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Experimental studies of the intravascular dissemination of ascitic V2 carcinoma cells in the rabbit, with special reference to fibrinogen and fibrinolytic agents[1, 2]

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Introduction

Cancer cells have been demonstrated in the blood of man and animals. A most significant event after the initial appearance of neoplastic cells in the host is their transport and successful growth at new sites, or metastasis formation, beyond the limits of local or radical eradication. This spread occurs by *lymphatic dissemination, hematogenous transport and direct implantation.*

In recent years, the frequency and importance of metastasis production by blood-borne cancer cells has received major attention, since the majority of patients may die of metastatic disease. The phenomenon of tumor metastasis has been reviewed extensively [19, 20, 23, 24, 25, 32, 36, 37, 50, 56, 59, 64, 78, 80, 82, 84].

This report will not reiterate what has been stated in these compilations. Rather, discussion will be limited to the mechanisms of production of metastasis by blood-borne cancer cells. A brief review of recent experimental findings of many investigators will be supplemented by a description of the author's personal observations of the *in vivo* behavior of tumor emboli and studies of the factors influencing the establishment and growth at new sites by these emboli [81, 82]. Special reference will be given to the role played by *fibrinogen* and *plasmin* in the formation and prevention of metastatic growth.

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[1] Supported by research grants from United States Public Health Service (C5319), Parke, Davis and Company, and American Cancer Society.

[2] Illustrated and demonstrated by a special 16 mm color motion picture film, available on request.

[3] Career Research Development Awardee, United States Public Health Service.

Production of metastases by blood-borne cancer cells occurs in three continuous stages: 1. The invasion of blood vessels by cancer cells, 2. the transport of tumor via established vascular pathways, and 3. the lodgement, attachment and growth of embolic cells at distant sites.

Before summarizing the present knowledge of these stages of hematogenous spread of malignant tumors, it is well to emphasize that the normal host possesses largely unexplored mechanisms of defense against the progressive growth and dissemination of cancer. The infrequent occurence of spontaneous remissions of a wide variety of human malignant tumors [14, 29, 70] and the rare cases in which cancer behaves as a chronic disease during several decades [57] indicate the dominant influence of unknown endogenous or host factors. That such spontaneous remissions may occur without the mediation of chemotherapy or radiation is well documented.

Several recent contributions to our understanding of entry of tumor cells into vascular pathways have dealt with such factors as tissue pressures [46, 83], decrease of mutual cohesiveness [12, 21, 22, 35, 52], ameboid activity [28, 43], and the interrelationship between tumor and surrounding cells [1, 26, 53, 54, 75, 77, 79].

Both clinical and experimental data clearly establish that tumor cell embolism is not invariably metastasis. The vast majority of embolic cancer cells perish within the blood. The death or survival of these cells is primarily determined by two factors: characteristics of the cells and host factors.

It should be reemphasized that both undissolved particles [76] and normal cells may also be demonstrated as emboli, such as the recovery of endothelial cells, megakaryocytes and various hematopoietic elements from peripheral blood [45], and the demonstration of emboli within the pulmonary vessels, especially after trauma. Illustrations of the latter are provided by the finding of bone marrow, bone, brain, liver, spleen, fat, skin, muscle, amniotic fluid and chorionic villi within pulmonary vessels. It is obvious, therefore, that embolism by these cells does not result in metastatic growth, and this is further indicative of the fact that the *biologic characteristics of embolic cells* are determinants of metastasis as are *physiological factors of the host.* Furthermore, *Stewart* [71] has reported that benign tumor cells may enter vascular channels. In the presence of sclerosing adenosis of the breast, he has observed cells "entering" the perineural lymphatics. Three examples of benign pigmented nevus cells were found in the peripheral sinuses of axillary lymph nodes [71]. It is possible that many proliferative lesions of low potency behave

in a manner similar to endometriosis but, unlike endometriosis, lack a physiologic function that calls attention to their presence.

This report is primarily concerned with the experimental studies, in the ear chamber and in mesenteric blood vessels of living animals, of the intravascular behavior of single embolic V2 carcinoma cells. This tumor was established in 1959, and modified through 70 consecutive intraperitoneal inoculations to grow in a predominately single cell ascitic form[1].

In 1902, *Schmidt* [65] published a monograph in which he presented photomicrographs showing the earliest stages of tumor metastasis. Many subsequent investigators have illustrated these events—the adhesion of cancer cells to vascular endothelium and their entrapment within thrombi.

The occurrence of thrombi and cancer cells, as well as the clinical association of thrombosis in certain neoplasms, especially of the lung, stomach, pancreas and hematopoietic system, is well recognized.

Past investigations have revealed the ability of cancer cells to stick to endothelium and their association with thrombi [81, 82]. The exact mechanisms are unknown, but apparently fibrin formation is a major factor. Recent experiments have revealed that anticoagulants or fibrinolytic agents may afford significant protection against metastasis formation by blood-borne cancer cells [2, 3, 16, 17, 18, 30, 39, 40, 82].

The subjects of *dormancy* and *differential* or *selective organ susceptibility* to metastasis, as well as the *precise fate* and graveyard of unsuccessful emboli are beyond the scope of the present presentation.

From the histologic examination of both human and animal material, the principal cellular events associated with the development of intravascular cancer cells have been described [82]. Briefly, embolic neoplastic cells generally lodge within capillaries and are promptly surrounded by delicate meshworks of fibrin and platelets. Within these intravascular micro-coagula, embolic tumor cells may grow. Scattered leukocytes are frequently found about dividing tumor cells, adherent to the endothelium and surrounded by thrombus. When intravascular growth, invasion of the capillary endothelium, and extension into the perivascular connective tissue occur, leukocytes, chiefly lymphocytes, may be more conspicuous. Within arterioles, tumor cells stick to the endothelium and grow, surrounded by thrombus. Endothelial invasion occurs after filling of the arteriolar lumen and extension into the capillaries. It is accompanied by a leukocytic reaction. Progressive growth occurs in the region of the capillaries within the perivascular connective tissue.

[1] Developed with the collaboration of J. Knaack, M. D. and E. A. Arnold, M. D.

Rabbit ear chamber experiments

Routine histologic studies capture but a single moment of the changing cellular, biochemical and biophysical processes of metastasis formation. Cells and blood vessels are static and fixed in stained sections. To investigate the living, dynamic pathology of cancer cells, blood vessels and endothelium in the ecology of the normal or altered host, trained, unnarcotized Laboratory Lop rabbits with special ear chambers are employed (Fig. 1). Single ascitic V2 carcinoma cells were injected into the central artery proximal to the transparent membrane (7 mm $\times$ 15μ), observed at high magnification and photographed simultaneously. The cinephotomicrographic records obtained are particularly valuable for the objective analysis of the cellular phenomena and comparison of their rates of change (Fig. 2 and 3).

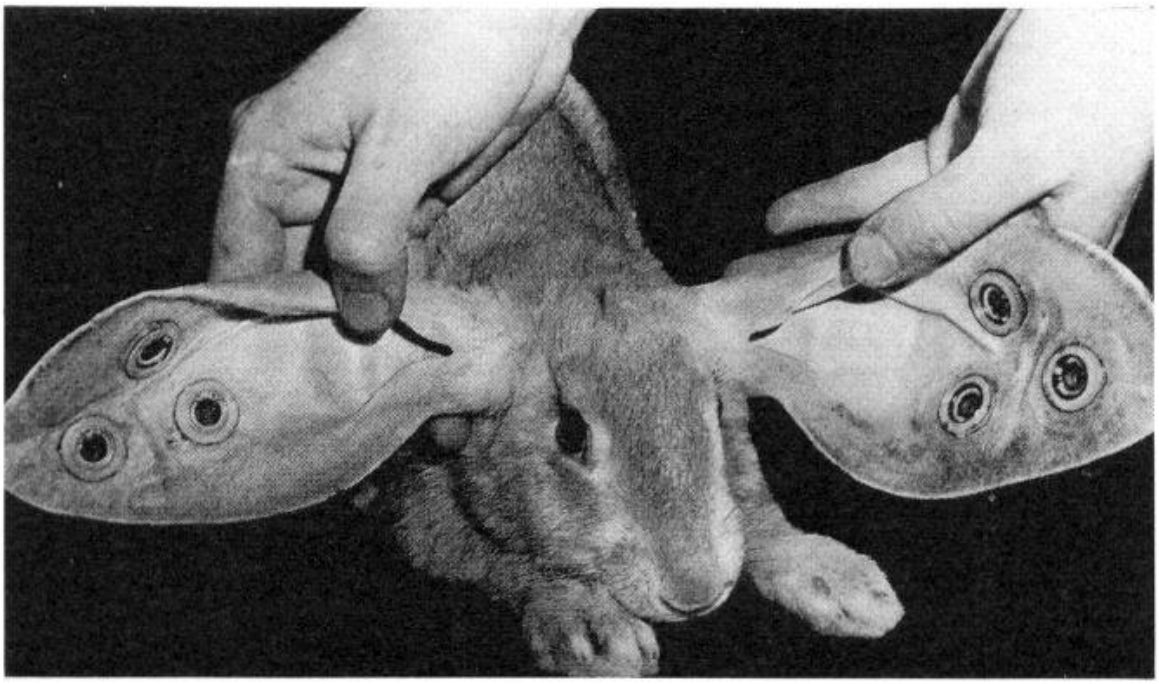

Fig. 1. The Laboratory Lop rabbit (bred at the Bar F Rabbitry, Perry Hall, Maryland) with three special plastic and glass ear chambers in each ear. Each chamber provides a thin (15 micron) layer of living, vascularized tissue, 7 mm in diameter, that can be observed microscopically at high magnification at frequent intervals in the unanesthetized rabbit. It is a convenient tool for study of the dynamics of pathologic change, especially those changes associated with intravascular phenomena and endothelium [15].

From observations of more than 300 rabbit ear chamber experiments, the following sequence of events has been substantiated [81, 82].

After the slow infusion of trypan blue- or alcian blue-stained or unstained cells, the majority perished. In general, *unsuccessful emboli* were conspicuously less adhesive to other cancer cells and to endothelium. These cells generally emerged into venules and were often surrounded by

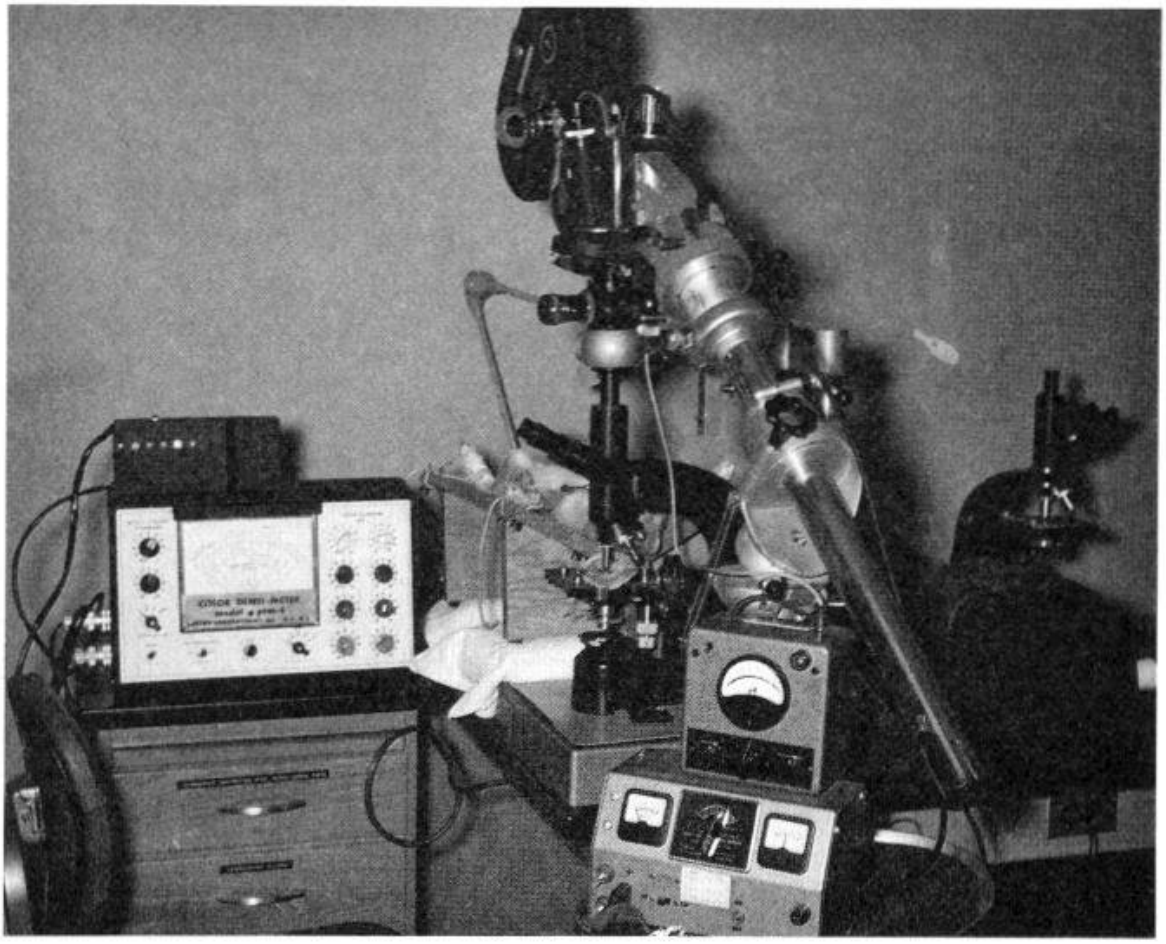

Fig. 2. The cinephotomicrographic equipment showing (left to right) the color analyzer with optical probe for red-blue-green direct analysis, Arriflex-16 motion picture camera with 400-foot magazine, Zeiss optical beam splitter with viewing eyepiece and photoelectric cell for measuring intensity of illumination, Leitz microscope, four-stage meter for photoelectric cell, voltage regulator for motion picture camera motor, and Leitz zeon illuminator. Results were generally recorded on color 16 mm motion picture film, Kodak's Commercial Ektachrome, Type 7255, at 24 frames per second.

leukocytes that formed small "white" thrombi within which they fragmented and vanished. A few were swept through the capillaries into larger venules and disappeared into the venous circulation beyond the limits of the ear chamber. This passage from the arterioles to the venules and fragmentation occurred within a period as short as 10–15 minutes. Their precise fate and mechanism of ultimate destruction is unknown. Biologic, cytologic and autoradiographic studies would be expected to reveal the exact nature and fate of such cells [7, 38, 66].

Among the few embolic V2 carcinoma cells destined to penetrate the endothelium and grow, there is a prominent tendency: 1. To stick together to form loose clumps of 3–6 or more cells, and 2. to stick to normal endothelium. Successful emboli generally stuck momentarily to the arteriolar endothelium, even in the absence of leukocytic sticking or during periods of rapid arteriolar flow. Endothelial adhesion was most

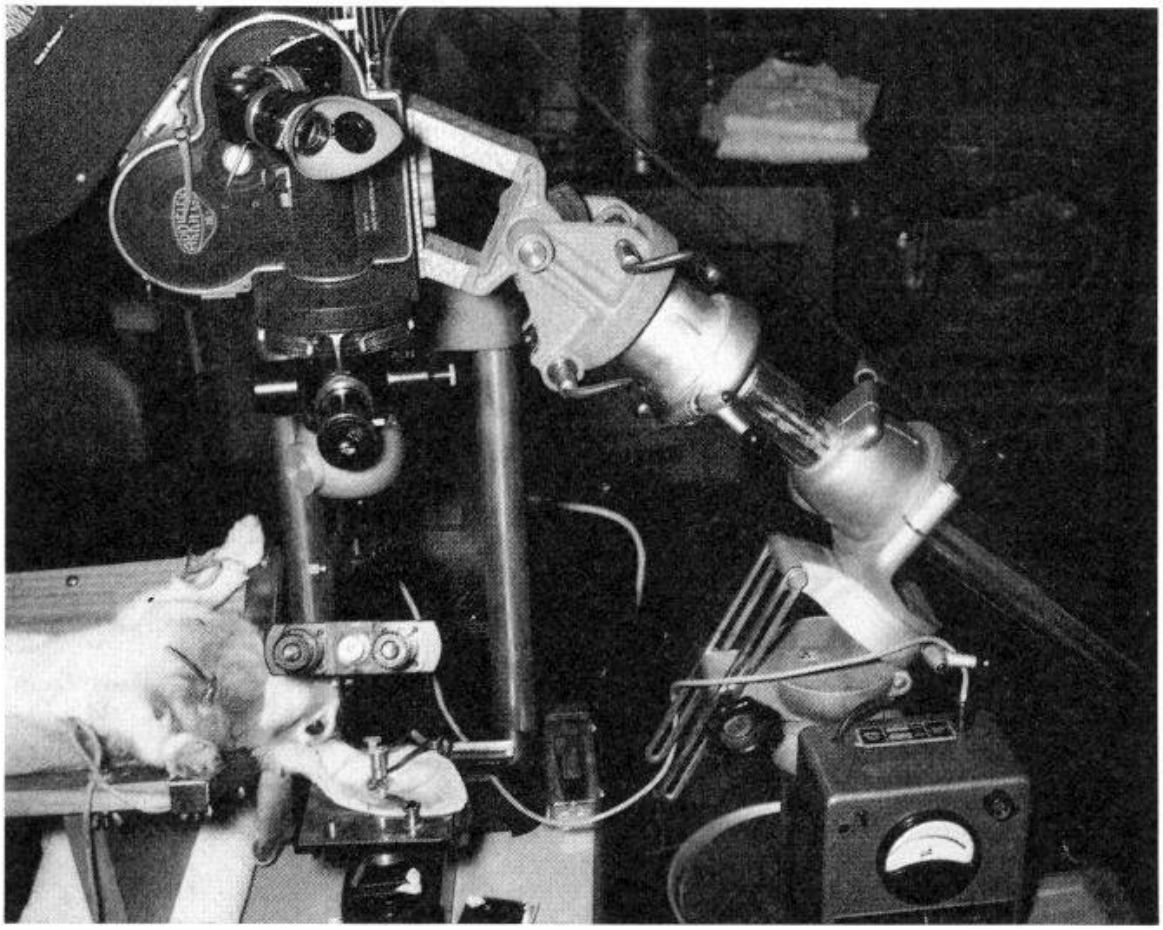

Fig. 3. Close-up view of cinephotomicrographic equipment. The Zeiss optical beam splitter is separated from the microscope objective by a light trap, and is attached to the Arriflex-16 motion picture camera. The camera is attached to a single-pillar, adjustable Linhoff stand via a Saltzman junior tripod head. No camera lenses are employed. The lenses employed for photography are the Leitz 3.2× projection eyepiece and the Leitz Ultrapac objectives, 6.5×11×, 32× and 50× (dry) without ring condenser and mount. The small diameter of these objectives permits their use with this ear chamber. Since cinephotomicrographic results are vastly more obvious when the entire motion picture records are reviewed, no single frame enlargements are herein presented.

prominent within capillaries, and up to the present time growth from single cancer cells into the ear chamber has been observed only within capillaries or venous capillaries. A small number of tumor cells that were clearly viable and had stuck to arteriolar as well as capillary endothelium were seen to traverse capillaries and enter the venous circulation. Rarely, arteriovenous shunts within the chamber provided a pathway for direct entry into the venous circulation. Within venules, carcinoma cells exhibited a similar tendency for endothelial adhesion. Further observations on the fate of these cells are required [7, 38, 66].

In the normal animal, the initial adhesion of embolic carcinoma cells to capillary endothelium observed *in vivo* in the rabbit ear chamber was independent of leukocytic sticking, capillary diameter, rate of flow or

vasomotor activity. It is apparent, therefore, that the *labile capillaries do not function as simple mechanical filters* of embolic carcinoma cells [81, 82, 85, 86]. Within 20 minutes, thrombi, composed predominately of fibrin, entrapped these cells. The precise behavior of such stained cells was revealed by prolonged observations and serial cinephotomicrographs.

The recovery and identification of cancer cells in the peripheral circulation of patients may be particularly difficult. The precise nature of the neoplastic cells studied in the microcirculation of the rabbit ear chamber clearly revealed their malignancy by virtue of their behavior: extraordinarily firm endothelial adhesion within capillaries or venous capillaries, prompt entrapment within a fine thrombus, principally composed of fibrin with sparse leukocytes and platelets, prompt endothelial penetration and growth within 2 ½ hours, progressive growth in the perivascular connective tissue, as well as within the blood vessel, and the ingrowth of numerous new vessels, or neovascularization, beginning as early as 18 hours after lodgement. The nature of the neoplasm was confirmed by routine histologic sections [81, 82].

Enhanced metastasis

In the experimental animal, many factors significantly increased metastasis production from blood-borne cancer cells, such as stress [82], cortisone [4, 82], pituitary growth and adrenocorticotrophic hormones [82], local trauma [32, 82], and bacterial products [31].

To illustrate the latter[1], ascitic V2 carcinoma cells were injected intravenously into rabbits that had received the prior intravenous injection (1–18 hours before tumor inoculation) of 10 micrograms of endotoxin from *E. coli*[2]. At autopsy 21 days later the frequency of pulmonary metastases was doubled. The most prominent increase in the number of metastases was observed in the extrapulmonary organs (EPM), especially the liver, spleen, adrenal and kidney (Table 1). Previous investigations have indicated that these organs are altered by endotoxin, especially the liver [51, 67].

Of significance in this and later experiments is the characteristic be-

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[1] Conducted in conjunction with Drs. J. H. Mulholland, J. Knaack, and E. A. Arnold.

[2] 10 micrograms of this endotoxin is approximately one-fifth the LD_{50} dose, and evokes the characteristic biphasic febrile response, thrombocytopenia, leukopenia, and leukocytic sticking and aggregation (observed in the rabbit ear chamber by Ebert and Koch-Weser [27] and confirmed in this laboratory). In addition, McKay and Shapiro [55] have demonstrated that there is a transient and prompt hyperfibrinogenemia in rabbits treated intravenously with endotoxin, followed by a slight fall in fibrinogen level [34].

havior of the ascitic V2 carcinoma to produce extrapulmonary metastases in a significant percentage of normal animals (Table 1). Thus, one may study the frequency and distribution of both pulmonary and extrapulmonary metastases simultaneously.

Table 1. Effect of Endotoxin[1] on metastasis[2]

	Total E.P.M.	Liver	Spleen	Kidney	Adrenal	Heart	I.P.	S.C.	I.M.
Controls	15/25								
	60%	0%	4%	8%	12%	12%	24%	20%	30%
Endotoxin	44/48								
	92%	31%	19%	29%	35%	15%	50%	56%	65%

[1] E. Coli Endotoxin, 10μg
[2] V2 Carcinoma in Ascitic fluid

Diminished metastasis

Numerous agents exert a protective effect against the formation of metastases from blood-borne tumor cells. The role of two *endogenous* factors, anticoagulants and fibrinolytic agents (plasmin) will be emphasized since these may be endogenous regulators [2, 3, 16, 17, 18, 30, 39, 40, 82].

The intimate association between embolic tumor cells and thrombi has been illustrated in tissue sections and by *in vivo* microscopy [81, 82]: It has been shown that only a relatively small number of embolized tumor cells are capable of metastasis formation, and that a prime factor in the formation of a metastasis may be the adhesion of the embolized cells to the vascular endothelium. It has further been demonstrated *in vivo* that formation of intravascular thrombi about the adhesive tumor cells occurs promptly. Histologically, these thrombi appear to be predominately fibrin-like masses containing sparse platelets and a small number of leukocytes.

On the basis of these findings implicating the clotting mechanisms as a factor in metastasis formation, experiments have been conducted with various anticoagulants, such as heparin or dicumarol, and with fibrinolytic agents such as plasmin [2, 3, 16, 17, 18, 30, 39, 40, 82]. In each instance, a decrease in metastasis formation was observed.

Mesenteric experiments: Formation and Lysis of Thrombi[1]

Additional experiments were conducted to permit direct *in vivo* observation and cinephotomicroscopy of the events that occur at the moment

[1] Joint experiments with J. H. Johnson, M. D.

and site of initial vascular invasion, or the entry of carcinoma cells into the blood, and to record the direct effects of fibrinolytic enzymes on cancer cells and thrombi related to these cells. The ascitic V2 carcinoma cell of the rabbit was employed, diluted with Morgan and Parker's Mixture 199 immediately after harvesting from the peritoneal cavity. The tumor cell suspension was filtered through a Millipore filter to remove all particles greater than 20 microns in diameter and maintained at 37° C in a thermostatically controlled water bath until injection. Tumor cells were used within one hour after harvest.

With a smooth, uniform thrust, the tip of a glass needle (30–60 microns in diameter) was inserted into an arteriole in the direction *opposite* the blood flow [47]. Observations and cinephotomicrographs were made with a Leitz dissecting microscope and an Arriflex-16 motion picture camera. A square-ended, fused quartz-rod transilluminator supplied light (*Knisely*) (Fig. 4).

The filtered tumor cell suspension was slowly injected until the formation of the thrombus containing tumor cells was achieved that totally blocked the lumen. No attempt was made to deliver a standard dose of tumor. Generally, the dose did not exceed 2 ml. When a micro-cannula was introduced into a small artery, and tumor cells injected, three distinctive types of thrombi appeared [48, 63]. First, the firm *injury thrombus* (or hemostatic plug), composed predominately of platelets, formed almost simultaneously about the locus of entry of the needle through the vessel wall. These thrombi developed, sealed the defect in the vessel wall, and did not further enlarge.

When V2 carcinoma cells were slowly infused, the promptness with which carcinoma emboli aggregated and were enmeshed within a tenacious fibrin-like matrix was remarkable. *Tumor thrombi* formed gradually and continued to enlarge as long as cells were injected into the vessel. These thrombi were characteristically much softer and more delicate than those of the injury type. Both injury and tumor thrombi were devoid of erythrocytes.

The precise mechanism whereby the tumor thrombus is formed is unknown [5]. The thromboplastic properties of carcinoma cells, as well as those possessed by many normal cells, are well established [41, 49]. Cancer cells, especially those of the V2 carcinoma, are richly endowed with such thromboplastic components. From human tumors, *O'Meara, Thornes* and associates have defined recently cancer coagulative factors (CCF) [13, 60, 61, 62, 72]. This is a thromboplastic material reported to differ from normal tissue thromboplastin in three major respects: 1. Marked thermolability, 2. prompt diffusability, and 3. great concentra-

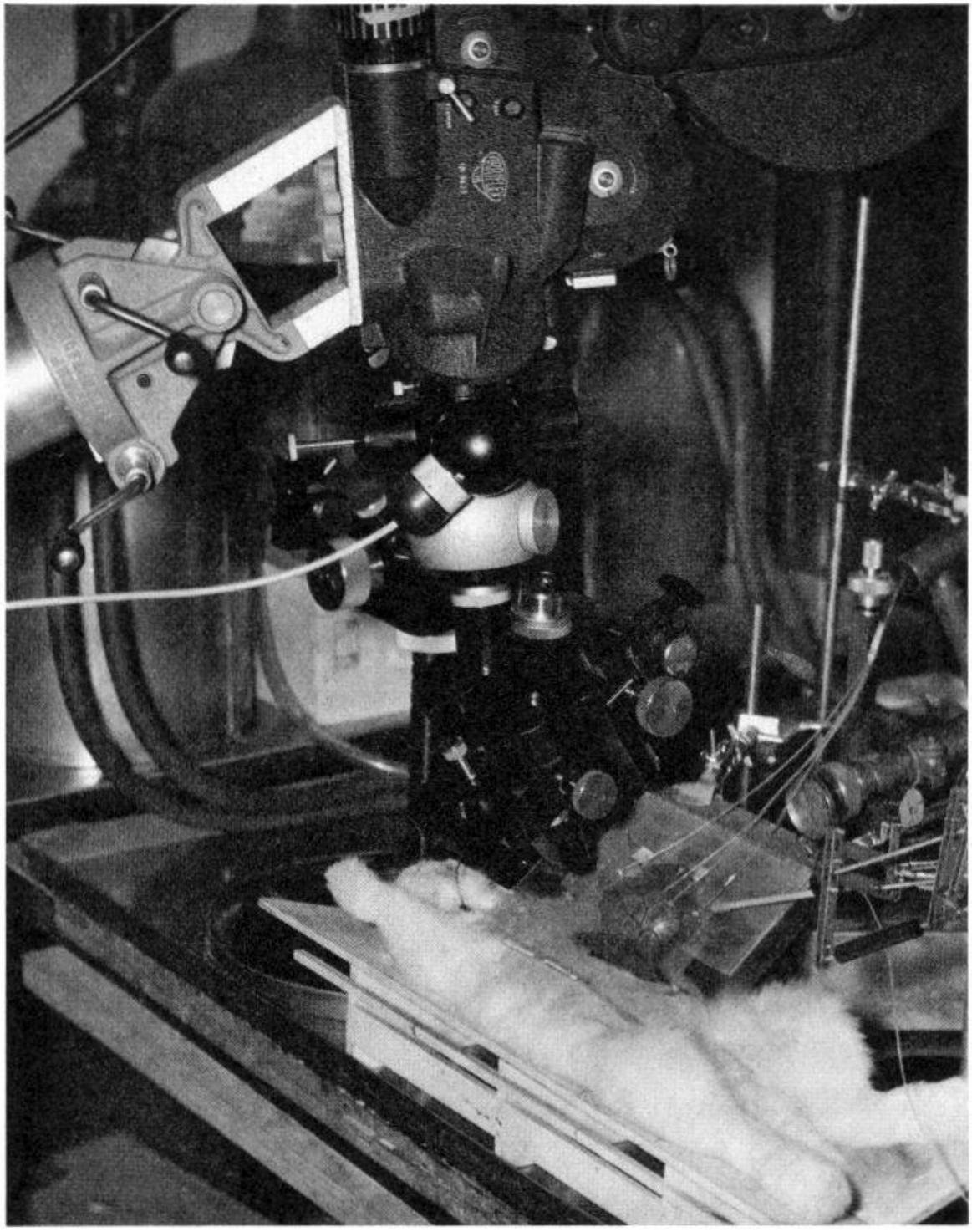


Fig. 4. Rabbits were anesthetized with sodium pentobarbital. A small polyethylene tube was inserted into the trachea to provide a continuous flow of oxygen (2 to 3 liters/minute for optimal oxygenation when respiratory movements were reduced or stopped. Through a laporatomy incision, a selected loop of small intestine was gently delivered onto a warm, moistened plastic sheet, attached to a balsam board [47, 48]. Minute entomological pins were carefully inserted through the serosal surface and into the balsam board. A thin piece of absorbent paper was placed over the intestine and moistened with warmed mammalian Ringer's solution (37°C). On the right, note the Singer micromanipulator that carried the glass needle and permitted the insertion in small mesenteric arteries (30–100 microns). The Leitz microscope is positioned over the mesentery. Above the left eyepiece, the Arriflex-16 motion picture camera is connected to an optical beam splitter and a photoelectric cell. The microscope and camera are separated by a light trap.

tions, at times being in excess of 16,000 times that of normal cells. Of further interest is their investigation that indicates that the CCF may be fractionated into several components, including a mucopolysaccharide and a protein. Its thromboplastic abilities are inhibited *in vitro* by streptokinase-activated plasminogen or low molecular weight dextran (10,000), but apparently not by either plasminogen or plasminogen activators alone, such as streptokinase or urokinase [13, 72, 74].

The delicate and friable *red cell thrombus*, last of the three to form, appeared 15–20 minutes after the tumor thrombus had developed, and injection of carcinoma cells had ceased.

As controls, 15 rabbits received an intra-arterial injection of tumor cells and were studied 1 to 4 hours *after* formation of thrombi. No loosening or lysis of any thrombi was observed.

Three enzyme preparations were employed.

1. Human urokinase-activated plasminogen (UK-PL), Lots X9000 and EX1919, obtained from Parke, Davis and Company, Detroit, Michigan. Vials contained 500–630 RPMI units of fibrinolytic activity, 300 units of caseinolytic activity, 800 units of activator activity, and 33-37 mg protein. Each preparation was dissolved in 5 per cent dextrose in water.

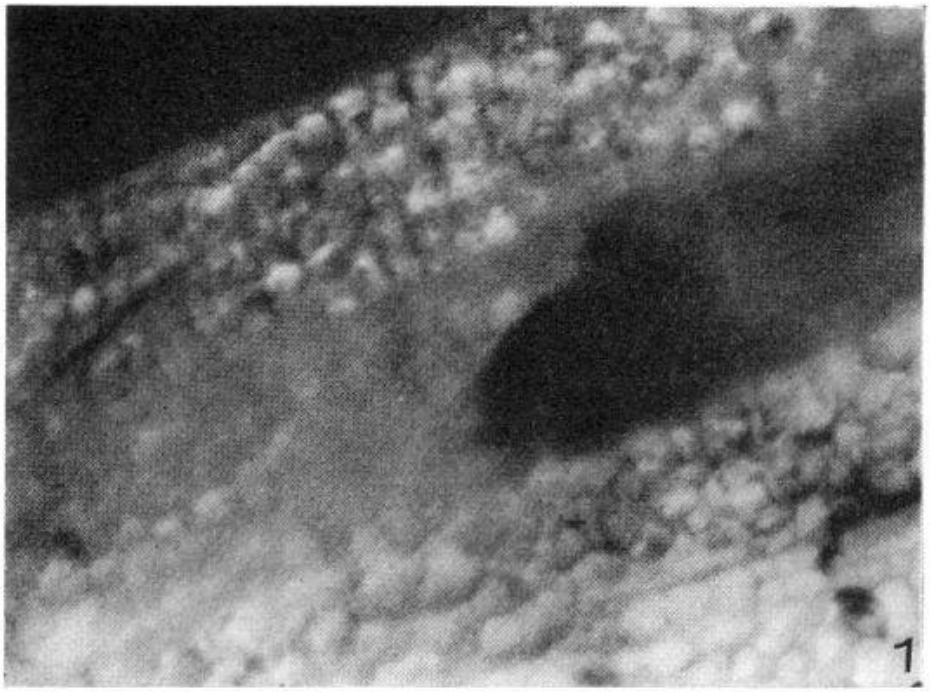
2. Streptokinase-activated human plasminogen (SK-PL), Lot C-1679, obtained from Merck, Sharp and Dohme, West Point, Pennsylvania. Each vial of enzyme containing 50,000 MSD units of fibrinolytic activity was dissolved in 0.85 per cent NaCl.

3. Human urokinase (UK), Lot 4, obtained from the Michigan Department of Health, Lansing, Michigan, contained 98 MDH units of activator activity in solution and was employed without dilution.

Fibrinolytic agents (UK-PL, SK-PL or UK) were introduced into the artery through the same needle system 15–90 minutes after the formation of the tumor thrombus. No differences in response were apparent within this interval of time.

When UK-PL was employed in 35 experiments, the lysis was almost instantaneous. Initially, the red cell thrombus was seen to loosen, fragment and break away. Next, the tumor thrombus softened, loosened, oscillated back and forth in the artery, broke away and was swept downstream (Fig. 5). The injury thrombus was far more resistant and the thrombi of this type could never be completely dissolved. In some preparations, it was thought that they were reduced in size by the infusion, but some recognizable components of the injury thrombus always remained. The amount of UK-PL required to remove the red cell throm-

Fig. 5. Four consecutive single frames (1–4) enlarged from 16 mm color motion picture showing the sequential lysis of a tumor thrombus capped by a small red cell thrombus. The diagrams clarify relationships that are easily seen during projection of the film.



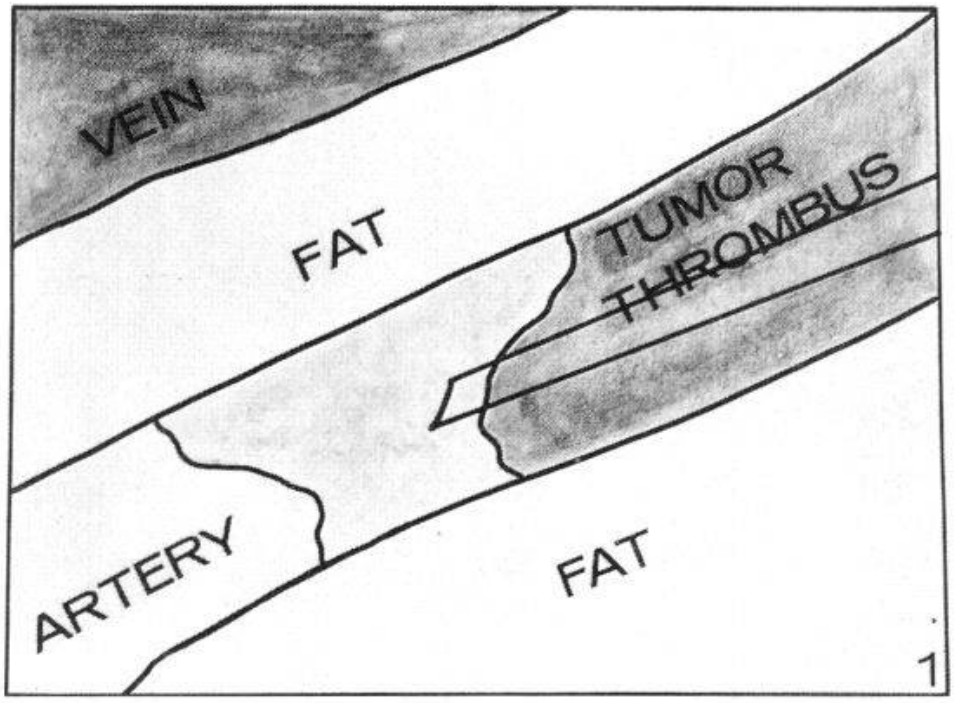


Fig. 5a) shows the thrombi as they appeared at the beginning of the injection of UK-PL.

bus could not be calculated as this usually occurred instantaneously upon contact of UK-PL with the thrombus. The average dosage of UK-PL required for the complete removal of the tumor thrombus was approximately 28 RPMI units per kilogram.

If the dosage of UK-PL exceeded 165 units per kilogram, hemorrhage

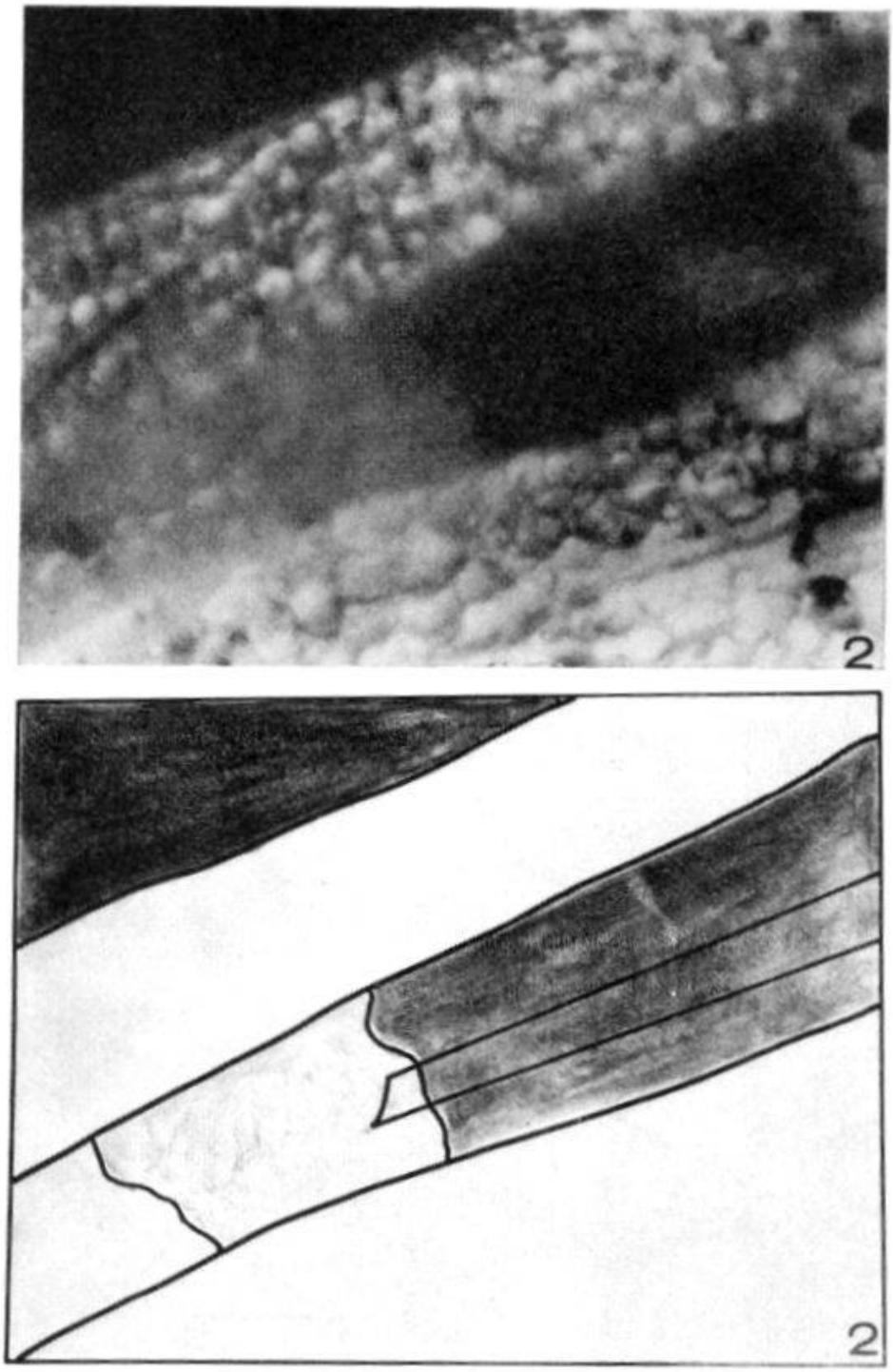


Fig. 5b) the red cell thrombus has vanished 1/60 of a second later. The tumor thrombus has loosened and moved slightly to the left.

would usually occur around the needle-puncture site and petechiae could be seen developing in the capillary bed supplied by the artery into which the injections were being made.

The pattern of action of SK-PL or UK preparations upon tumor thrombi was different from that of UK-PL. It was frequently several minutes before the first effects of the SK-PL or UK could be seen. The thrombus then seemed to dissolve gradually and to melt away completely

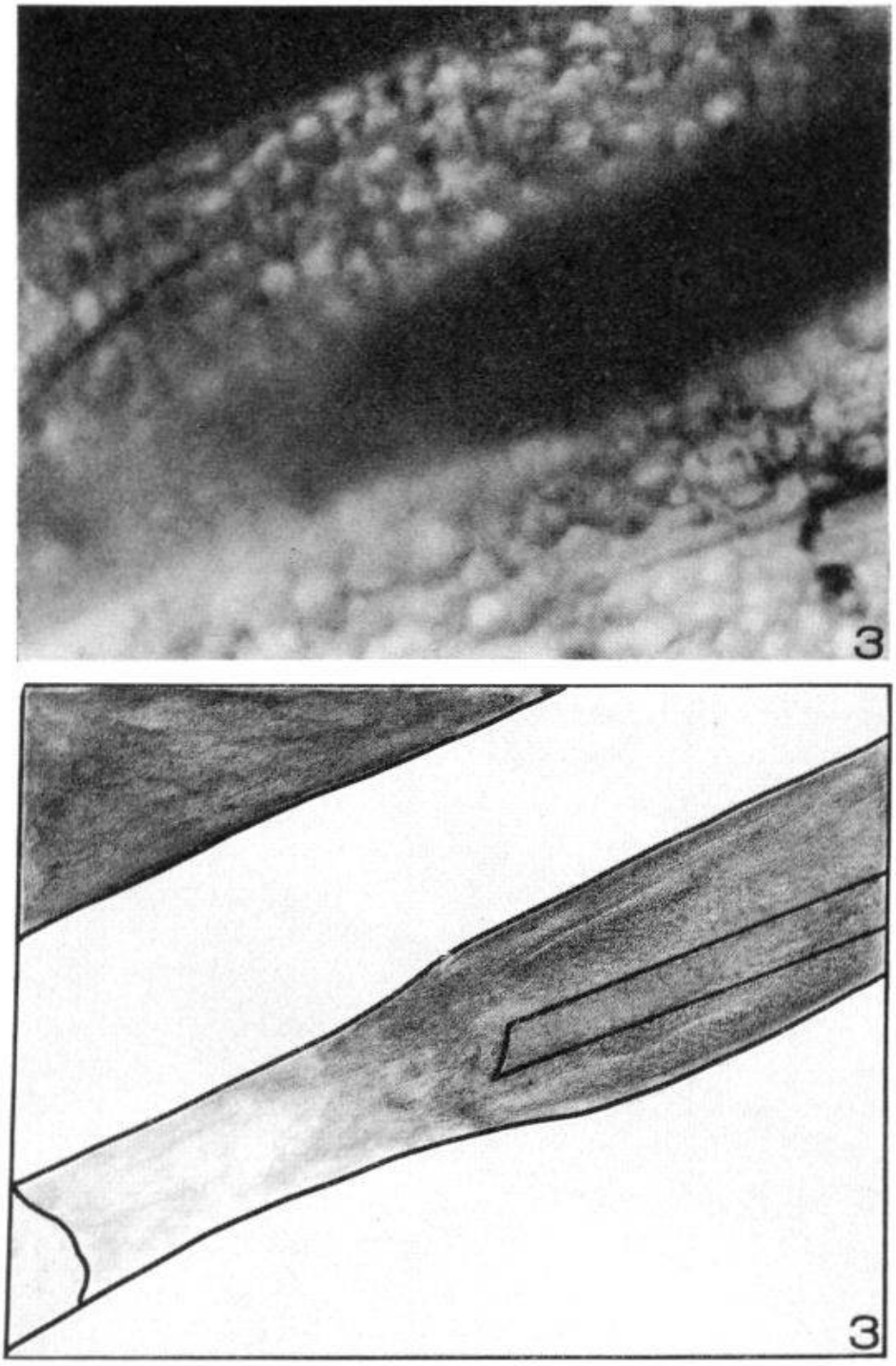


Fig. 5c) the tumor thrombus has been fragmented and swept away to the left. The oncoming arterial blood is seen to the right.

over a period of 20–25 minutes. The dosage (SK-PL) required before a response (lysis) was produced was 18,000–20,000 MSD units per kilogram. The dose of UK necessary to achieve lysis requires additional material.

SK-PL and UK were not observed to have a significant effect on the injury thrombus at the site of needle-puncture.

When fibrinolytic agents were administered 15–90 minutes after for-

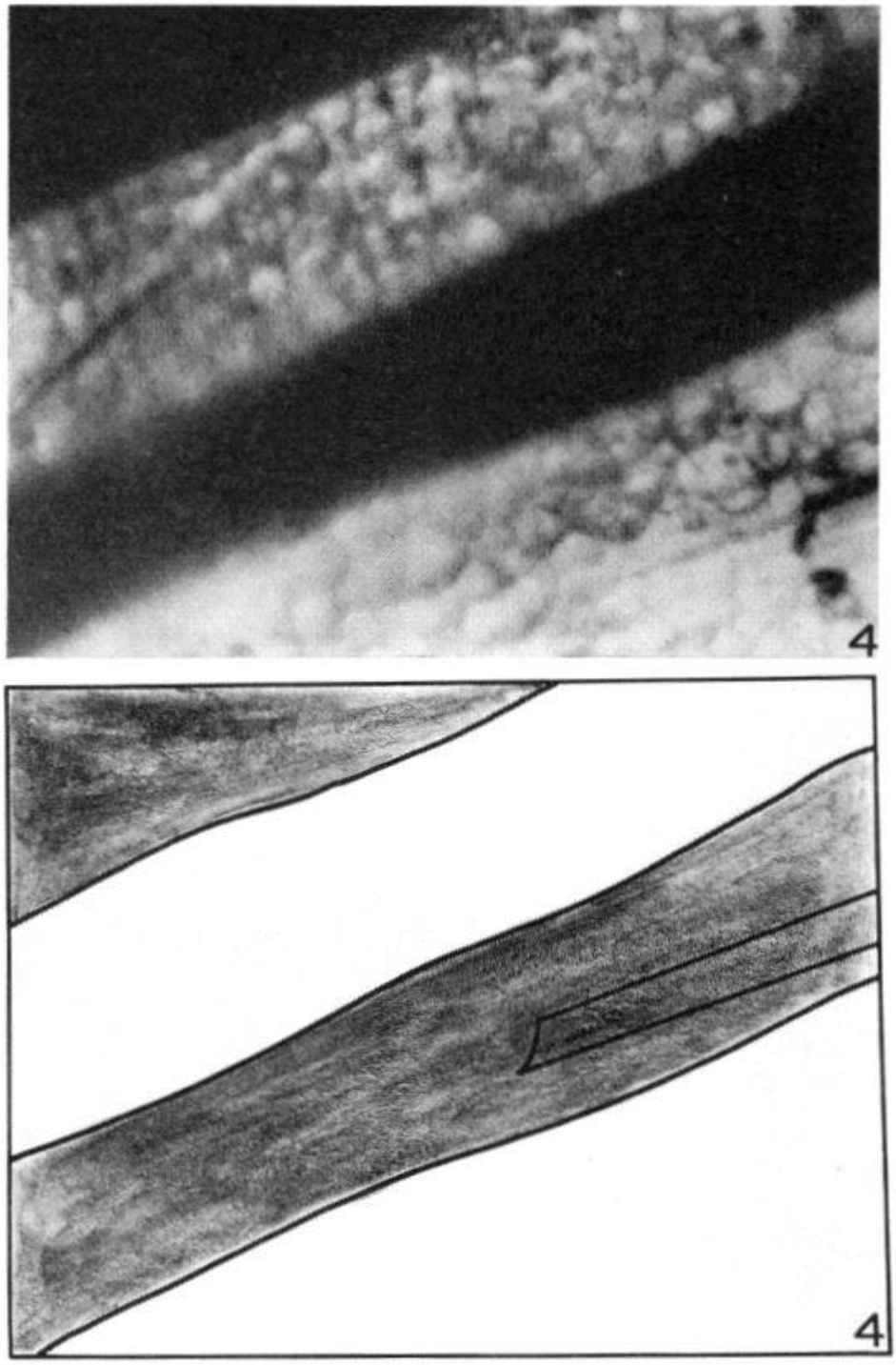


Fig. 5d) free blood flow has been restored in the artery. The entire sequence occurred in 1/15 of a second.

mation of the tumor thrombus, the red cell thrombus immediately disintegrated and was dislodged. Next, the tumor thrombus loosened, softened, and was swept away, most rapidly with the UK-PL infusion, less promptly with the SK-PL or UK. Most important may be the inability of either fibrinolytic agent to produce appreciable alteration in the injury thrombus. It remained as a firm hemostatic plug, capable of preventing bleeding even when the needle was withdrawn.

The observation that injury thrombi are not appreciably altered by these fibrinolytic enzymes requires further study. These thrombi are composed predominately of platelets that are known to contain factors that may enhance or diminish fibrinolysis [44]. *Murray* has described two different forms of fibrin, urea-soluble and urea-insoluble [58]. The type of fibrin within injury thrombi may be quite different from that in tumor thrombi.

Localization of fibrinogen and plasmin[1]

To provide direct evidence for the participation of rabbit fibrinogen and human plasmin (UK-PL) in the phenomena described, these proteins were iodinated using Iodine-125 [8]. Additional experiments employed I^{125}-labeled human albumin. Autoradiography was chosen as a suitable technic because if its relative simplicity compared to other methods of demonstrating fibrin or plasmin. I^{125}- was chosen as the isotope for the labeling because it possesses the advantage of a relatively short yet practical half-life, 60 days, and emits energy at a level of 35 KEV, compatible with good autoradiographic resolution [33].

To check these procedures, the following method analyses were employed: 1. Labeling efficiency was established for each preparation. 2. Electrophoresis showed that the iodinated proteins had the same migratory behaviour as non-iodinated reference samples. It was also found that the radioactivity migrated with the experimentally tagged protein. Control areas from the strips which were eluded for counting showed no activity above back-ground while the region corresponding to the reference contained significant isotopes to give counts 20–25 times that of background. 3. *In vivo* analysis of fibrinolytic activity of the iodinated plasmin showed comparable activity of uniodinated plasmin prepared from corresponding lots.

Fresh ascitic V2 carcinoma cells in ascitic fluid were harvested under sterile conditions and filtered through a 25 gauge needle. This fluid was then diluted with Morgan and Parker's Mixture 199 to a concentration of approximately 2×10^5 cells per ml. Additional experiments were carried out with cell-free ascitic fluid as a control. Animals were injected intravenously with 10 ml of either tumor cells in ascitic fluid plus Morgan and Parker's Mixture 199, or cell-free ascitic fluid and Mixture 199. Each group promptly received radioactive rabbit fibrinogen, administered intravenously. Animals were killed and autopsied at intervals of one minute, one hour, two hours, four hours, six hours, or 24 hours after

[1] Conducted with E. A. Arnold, M. D. and J. Knaack, M. D.

injection. Sections were taken from each of the lobes of the lung, thyroid, liver, spleen, kidney, adrenals, pancreas and gastric wall for autoradiography. At the time of autopsy, blood specimens were taken for electrophoresis to establish the stability of the iodine-protein complex.

Autoradiograms prepared from these animals injected with tumor followed by fibrinogen showed striking localization of grains over tumor thrombi in the vessels of the lung (Fig. 6, 7). In many instances grain localization is associated even with a single cell (Fig. 8) while at other times the activity is related to small clumps of cells. The radioactivity, judged by grain localization, was mainly within the capillaries, but occasionally was found within small venules or arterioles. This activity was found at all time periods investigated. No progression of the labeling was found from the earliest to the later periods, although quantitation is difficult.

Control animals injected with either Mixture 199 or ascitic supernatant (cell-free) displayed no grain localization in the lungs. Grains in these animals followed a random distribution within the blood vessels. One incidental finding of interest was in the thyroid of animals. Thyroid colloid of control animals showed a marked concentration of grains, whereas tumor-bearing animals killed at the same interval had little or no grain activity in the thyroid (Fig. 9).

In similar experiments, plasmin-injected animals displayed the same type of labeling as fibrinogen-treated animals receiving tumor. Rare thrombi which showed no malignant cell component were found either in tumor-bearing or control animals and these displayed some localization, as might be expected. In these experiments, it was found that the localization of grains occurred with considerably greater frequency in animals killed at the earlier time periods as contrasted to those killed at 24 or 48 hours.

As a control to the prompt localization of fibrinogen and plasmin about tumor emboli, iodinated albumin was injected into animals. This followed a random distribution and was not localized about tumor emboli.

Fig. 6. Autoradiograms illustrating the localization of radioactivity within tumor thrombi in pulmonary blood vessels of rabbits killed four hours after intravenous injection of fibrinogen I^{125} and ascitic V2 carcinoma cells. Hematoxylin and eosin. Magnification: ×600. ▶

Fig. 7. Autoradiogram of lung vessel containing V2 carcinoma cells in animal killed after intravenous injection of fibrinogen I^{125} and tumor cells. On the left, the cell nuclei are in the plane of focus. On the right, the overlying photographic emulsion, Kodak's NTB2, is in focus to illustrate the individual silver grains intimately associated with the tumor cells. Hematoxylin and eosin. Magnification: ×1000. ▶

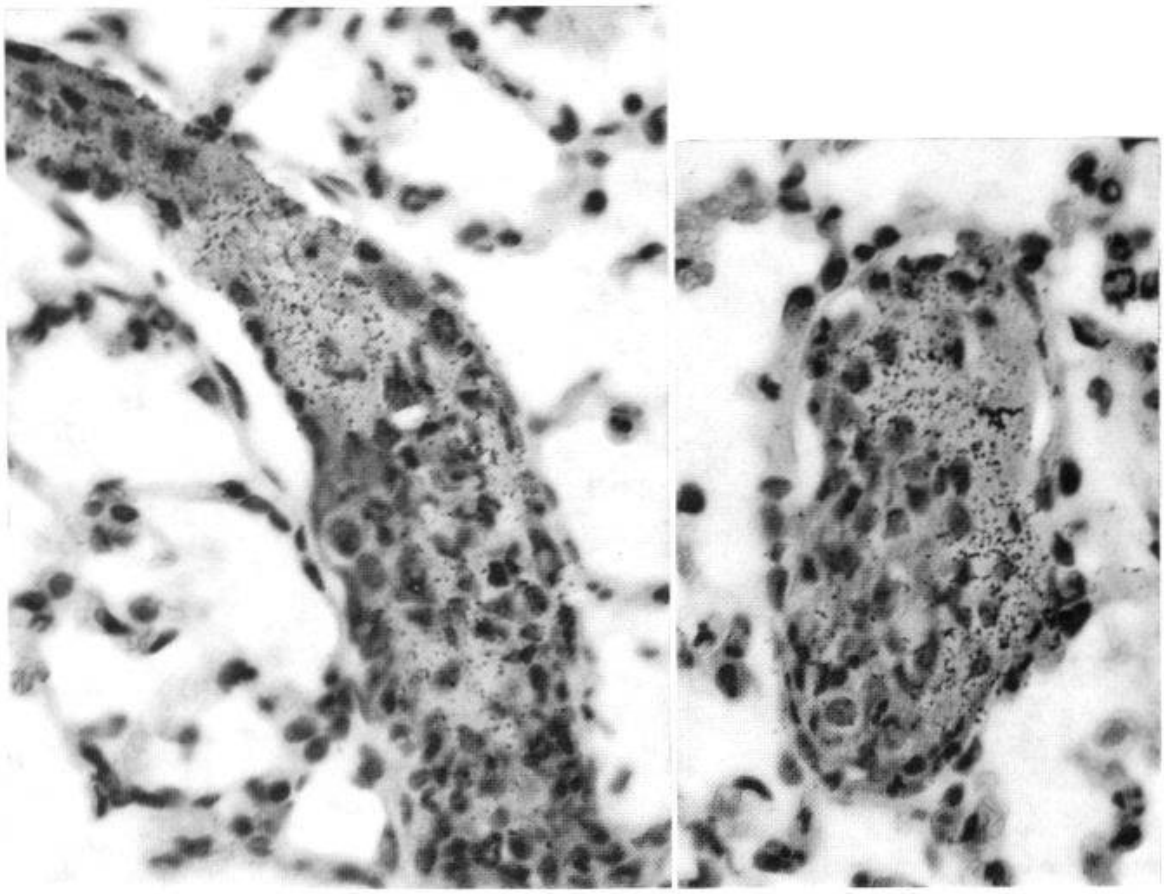


Fig. 6.

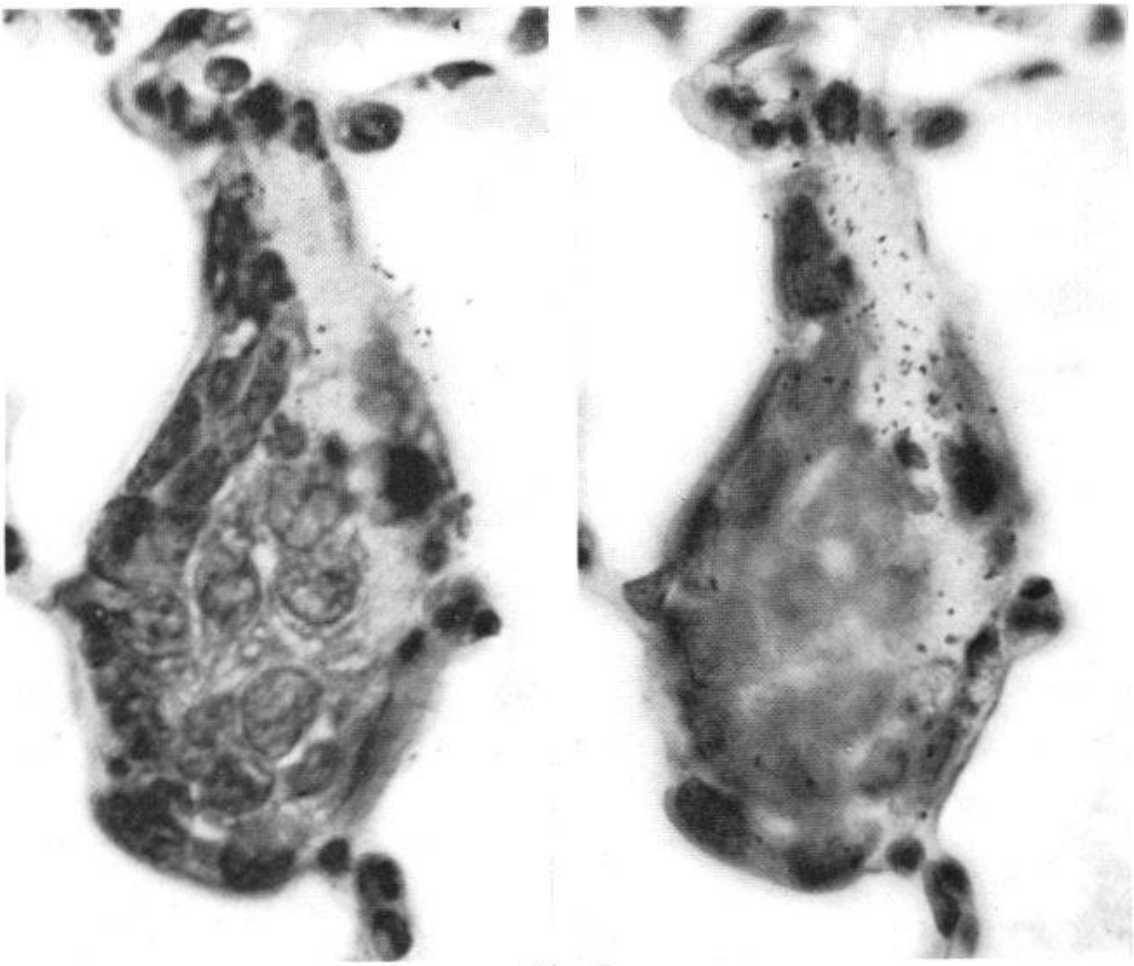


Fig. 7.

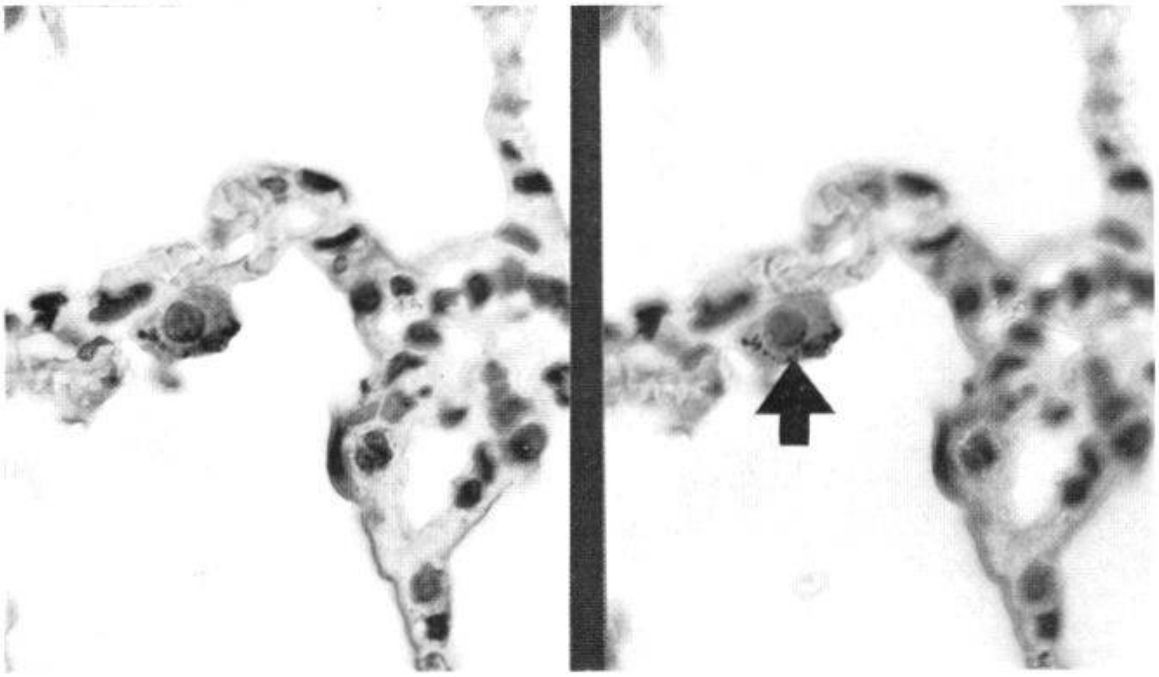


Fig. 8. Autoradiogram showing a single cell surrounded by silver grains within pulmonary blood vessel of rabbit killed 24 hours after intravenous injection of fibrinogen I^{125} and ascitic V2 carcinoma cells. Hematoxylin and eosin. Magnification: ×1000.

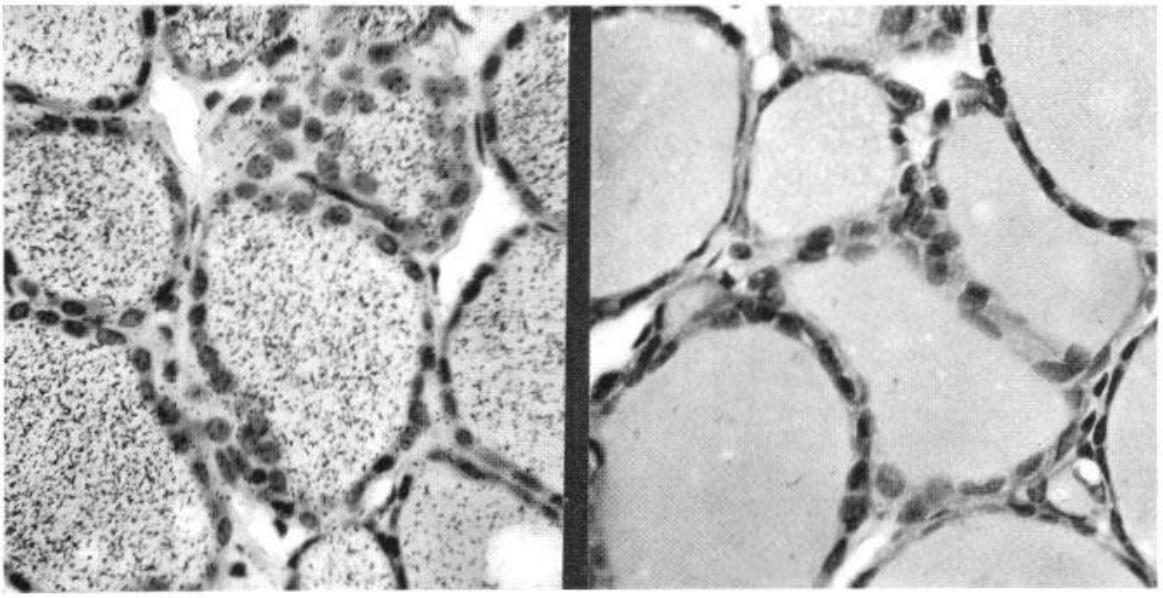


Fig. 9. Autoradiograms of thyroid (left) of control animal killed 4 hours after intravenous injection of fibrinogen I^{125} showing marked localization of radioactivity with follicles, whereas the right section from rabbit that received fibrinogen I^{125} and ascitic V2 carcinoma shows no appreciable radioactivity within the thyroid. This response may represent the result of the prompt and continued coating and precipitation of fibrinogen I^{125} about and within tumor thrombi in the lungs. See text. Hematoxylin and eosin. Magnification: ×400.

These data, in addition to routine histologic and in vivo studies, present further evidence that both fibrinogen (or fibrin) and plasmin may act by direct precipitation on the plasma membranes of embolic intravascular tumor cells or the tumor thrombus formed within vascular lumina, principally capillaries or venous capillaries. In contrast, no appreciable labeled proteins (rabbit fibrinogen or human plasmin, UK-PL) were found on leukocytes [6].

Fibrin within or about solid neoplasms has been studied by several other workers [9, 10, 24, 42, 61, 62, 68, 69].

Localization of I^{125}-labeled rabbit fibrinogen within established solid tumor V2 carcinoma was limited to 1. the reactive zone between the outer margin of tumor and the adjacent connective tissues of the host, and 2. the central zone of liquefaction necrosis.

Both intravenously injected ascitic V2 carcinoma cells and *spontaneous intravascular V2 emboli*, originating from the intraperitoneal growth of the ascitic V2 tumor, exhibited fibrinogen localization within the pulmonary blood vessels.

Relatively few of the embolic tumor cells within the lung were associated with labeled fibrinogen or plasmin. Although qualitative, this conforms with the evidence that only relatively few of the tumor emboli possess the prerequisite potential for metastasis formation: the majority perish. Those tumor cells that were incapable of localizing the labeled compounds showed little or no evidence of thrombus formation. It is apparent that there is a marked difference in the properties of the plasma membranes of those cells that adhere as opposed to those that do not adhere—and apparently perish.

Plasmin and metastasis[1]

With selected fibrinolytic agents, the prompt dissolution of these tumor thrombi within the blood vessels of the mesentery and the ear chamber prompted further investigations concerning their protective effect against metastasis formation in the rabbit. Numerous reports have revealed that if anticoagulants (heparin or dicumarol) or fibrinolytic agents are administered *before* the intravascular inoculation of transplantable animal tumor cells, the frequency of metastasis is significantly reduced [2, 3, 16, 17, 18, 30, 39, 40, 82].

In the light of the frequency of venous invasion encountered in resected primary tumors of the colon, stomach, lung and pancreas, among others, and the recovery of circulating tumor cells in patients such as these,

[1] Collaborative studies with E. A. Arnold, M. D. and J. Knaack, M. D.

and the fact that many of these patients later succumb to metastatic disease, experiments were designed to simulate a postoperative regime. The two fibrinolytic agents described above, UK-PL, 74 RPMI units per kilogram or SK-PL, 28,900 MSD units per kilogram, were administered intravenously 60-90 minutes *after* the intravascular inoculation of single V2 carcinoma cells in ascitic fluid. The number of both pulmonary and extrapulmonary metastases was significantly reduced (26–30%).

Plasmin (UK-PL) was administered one hour (50 RPMI units/kg) and again four hours (25 RPMI units/kg) *after* the intravenous inoculation of ascitic V2 carcinoma cells into the marginal ear vein of rabbits. Because of the prompt localization of radioactive plasmin, about embolic tumor cells, and the possibility that this agent might exert an effect on the cell membranes in altering them in a manner that might diminish their capability of successful lodgements and growth, in addition to its known fibrinolytic activity, radioactive plasmin (UK-PL) was likewise administered simultaneously one hour and four hours *after* the intravenous injection of tumor to a third group of animals.

These animals were autopsied 24 days after intravenous inoculation of the tumor, and the frequency and distribution of metastatic tumor determined. A significant reduction in the frequency of lung metastases was apparent, both with cold or regular UK-PL and with radioactive

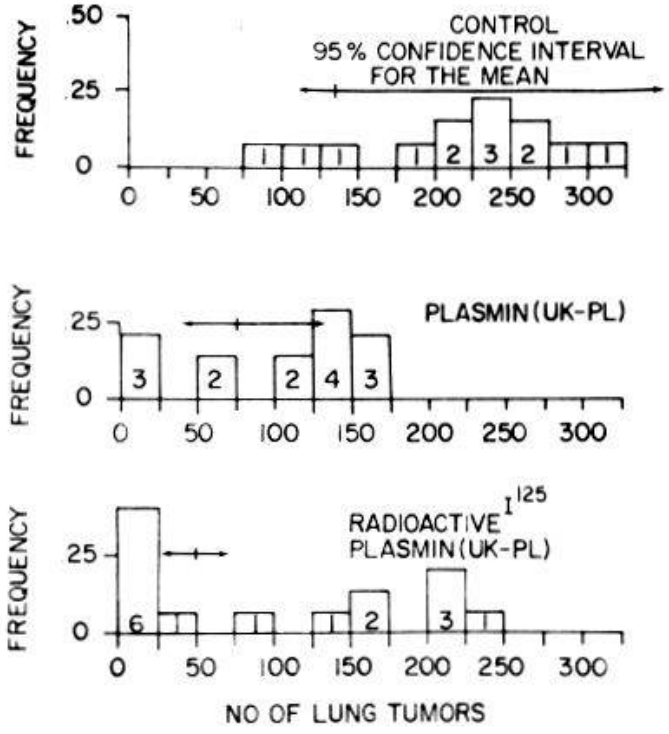


Fig. 10. Comparison of number of lung tumors in control (top graph), plasmin-treated (middle graph) and plasmin I^{125}-treated (bottom graph) rabbits. Results in figures 10, 11 and 12 analyzed by method of Beall [11] by Dr. Miles Davis. Level of statistical significance: control vs. UK-PL, $P < 0.05$; control vs. UK-PL^{125}, $P < 0.01$.

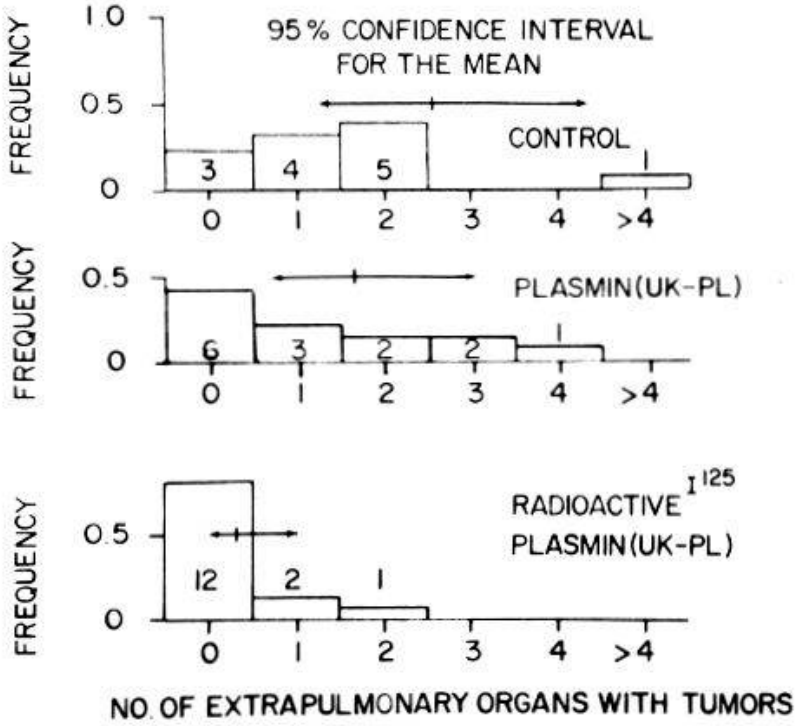


Fig. 11. Comparison of number of extrapulmonary organs with tumors in control (top graph), plasmin-treated (middle graph) and plasmin I^{125}-treated rabbits. Level of statistical significance: UK-PL vs. UK-PL^{I-125}, $P<0.05$; control vs. UK-PL^{I-125}, $P<0.01$.

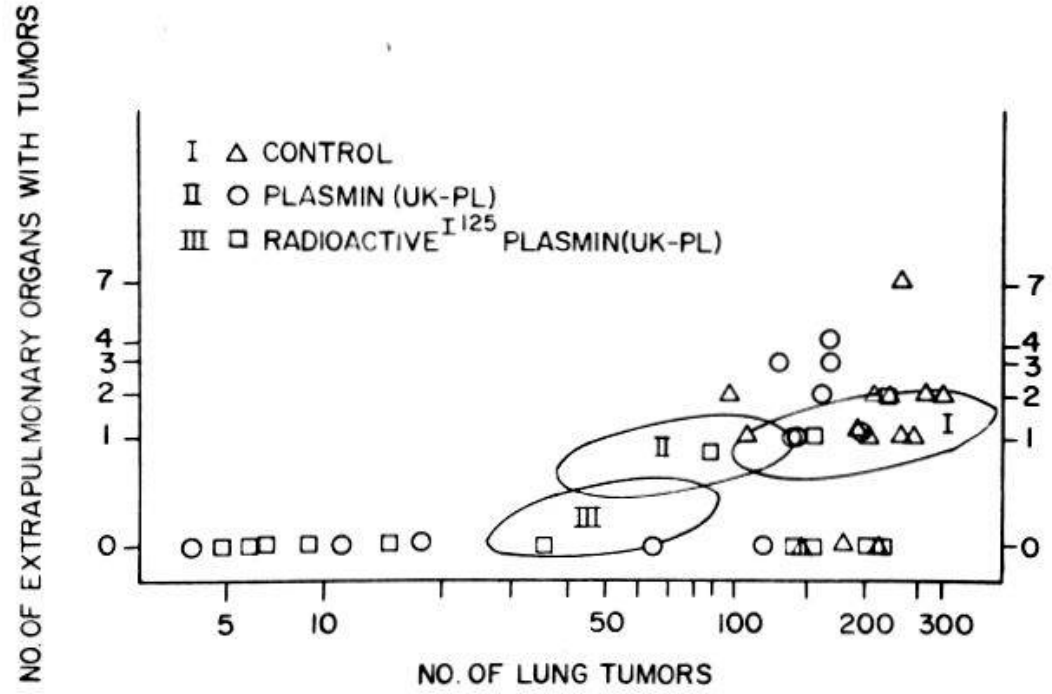


Fig. 12. Compilation of data from figures 10 and 11 illustrating the number of lung tumors vs. number of extrapulmonary organs with tumors in the three groups. The areas within the elipses represent the 95% confidence interval for the means of the groups.

UK-PL (Fig. 10). In addition, the extrapulmonary metastases were lower in the plasmin-treated group (Fig. 11, 12), especially in the group that received radioactive plasmin, due to both its ability to dislodge and destroy tumor thrombi and perhaps also its intimate coating of emboli where its radioactivity would be concentrated on the cell membranes.

These experiments require further elucidation employing non-radioactive iodinated plasmin, and attempts to determine any direct inhibitor effect that plasmin may have on embolic tumor cells, in the light of the report of *Thornes* that various fibrinolytic agents may inhibit *in vitro* the growth of HeLa cancer cells [73].

Summary

The biologic importance of the physical and chemical characteristics of cancer cells and the vascular endothelium has been emphasized. In the experimental animal, as in the human being, vascular endothelium represents the universal, dynamic host container of and barrier to blood-borne cancer cells. These are apparently only capable of metastasis production when they become adhesive to vascular endothelium.

During *in vivo* studies of single ascitic V2 carcinoma cells injected into the minute blood vessels of the rabbit ear chamber and mesentery, it was observed that within the mesentery cells aggregate promptly on entry, with fibrin deposition. Capillaries do not serve as simple mechanical filters since the adhesive properties of carcinoma cells enable them to remain securely attached without regard to blood flow rate or luminal size. Attached carcinoma cells, both in the mesentery and ear chamber, are generally surrounded by or enmeshed within thrombi, and capable of endothelial penetration within a few hours.

The prompt and selective localization of plasmin and fibrinogen labeled with I^{125} about embolic tumor cells has been noted. Plasmin has been observed to lyse rapidly both the red cell thrombus and the tumor cell thrombus with no appreciable alteration of the injury thrombus. Experiments are reported that illustrate the protective effect on metastasis formation of plasmin infused one hour or one hour and four hours *after* the intravenous injection of ascitic V2 carcinoma cells in the rabbit. Radioactive plasmin was found to provide a greater degree of protection than unlabeled or cold plasmin. Similar fibrinolytic agents may be effective and reasonably safe for clinical use immediately after surgical removal of tumors. A variety of investigations in this laboratory and elsewhere have revealed that a prerequisite for metastasis formation from blood-borne cancer cells is the ability of the tumor embolus to

stick to vascular endothelium. The non-adhesive cancer cells may perish promptly, apparently by disintegration and phagocytosis by mobile or fixed tissue macrophages.

It is apparent, then, that many host alterations, as well as individual characteristics of the embolic cancer cells, some known, others of which we are only dimly aware, await further evaluation.

Zusammenfassung

Es wird die Aufmerksamkeit auf die biologische Bedeutung der physikalischen und chemischen Eigentümlichkeiten der Krebszelle und des Gefäßendothels gelenkt. Sowohl beim Versuchstier als auch beim Menschen stellt das Gefäßendothel den ubiquitär vorhandenen dynamischen Wirt für die im Blut enthaltenen Krebszellen und zugleich die gegen sie gerichtete Schranke dar. Die Krebszellen sind offensichtlich nur fähig, Metastasen zu bilden, wenn sie dem Gefäßendothel anhaften.

Anläßlich von *in vivo*-Untersuchungen, in deren Verlauf in die kleinen Blutgefäße des Kaninchenohrs und des -Mesenteriums V2 Asciteskarzinomzellen injiziert wurden, konnte beobachtet werden, daß diese letzteren unmittelbar nach ihrem Eintritt in die Blutgefäße des Mesenteriums unter Fibrinablagerung aggregieren. Die Kapillaren wirken dabei nicht als einfache mechanische Filter, da die adhäsiven Eigenschaften der Krebszellen diese befähigen, unabhängig von der Geschwindigkeit des Blutstromes und der Größe des Gefäßlumens haften zu bleiben. Anhaftende Karzinomzellen im Mesenterium und Ohr sind meistens von Thromben umgeben oder in solche eingeschlossen und können in wenigen Stunden das Endothel durchdringen.

Es konnte eine rasche und selektive Lokalisation des mit I^{125} markierten Plasmins oder Fibrinogens um die embolischen Tumorzellen festgestellt werden. Plasmin löst sowohl die roten erythrocytenhaltigen Thromben als auch die Tumorzellthromben rasch auf, ohne eine nachweisbare Veränderung des Verletzungsthrombus zu bewirken. Es wird über Versuche am Kaninchen berichtet, welche die Schutzwirkung des 1 Stunde oder 1 und 4 Stunden *nach* intravenöser Injektion von V2 Asciteskrebszellen infundierten Plasmins gegenüber Metastasenbildung illustrieren. Radioaktives Plasmin zeigte einen höheren Schutzeffekt als nicht markiertes oder «kaltes» Plasmin. Entsprechend sollen fibrinolytische Substanzen sich bei klinischer Anwendung unmittelbar nach chirurgischer Entfernung von Tumoren als wirksam und ziemlich zuverlässig erweisen. Verschiedenartig angelegte Untersuchungen in unserem Laboratorium und anderswo zeigten, daß eine Vorbedingung für die Metastasenbildung

aus im Blut disseminierten Krebszellen in der Fähigkeit des Tumorembolus zum Anhaften am Gefäßendothel liegt. Die nicht anhaftenden Krebszellen können rasch zugrundegehen, offensichtlich durch Desintegration und Phagocytose durch bewegliche oder im Gewebe fixierte Makrophagen.

Es ist augenfällig, daß viele Veränderungen des Wirtes sowie individuelle Eigentümlichkeit der embolischen Krebszellen, von denen uns manche bekannt, andere nur undeutlich bewußt sind, weiterer Auswertung und Erforschung bedürfen.

Résumé

L'auteur soulève l'importance biologique des caractères physiques et chimiques des cellules cancéreuses et de l'endothélium vasculaire. Ce dernier représente chez l'animal en expérience ainsi que chez l'homme l'hôte universel et dynamique pour la cellule cancéreuse disséminée dans le sang et également une barrière contre elle. Les cellules cancéreuses ne sont capables de produire des métastases qu'en adhérant à l'endothélium vasculaire.

Des études «*in vivo*» de cellules séparées du carcinome ascitique V2 injectées dans de petits vaisseaux de l'oreille et du mésentère du lapin font observer que dans le mésentère les cellules s'agglomèrent dès leur entrée avec déposition de fibrine. Les capillaires ne sont pas de simples filtres mécaniques, étant donné que les caractères adhésifs des cellules cancéreuses les rendent capables de rester fortement attachées, sans égard à la vitesse du courant sanguin ou au calibre vasculaire. Dans le mésentère ou dans l'oreille, les cellules cancéreuses adhérentes sont généralement entourées de thrombes ou y incluses; elles sont capables de pénétrer à travers l'endothélium au cours de quelques heures.

On a constaté la prompte et sélective localisation de plasmine ou de fibrinogène marqués à l'I^{125} autour des cellules tumorales emboliques. La plasmine lyse rapidement les thrombes rouges contenant des érythrocytes ainsi que les thrombes tumoraux, sans qu'il soit possible de constater d'altérations appréciables du thrombus traumatique. Des recherches expérimentales sur le lapin ont illustré l'effet protecteur contre la formation de métastases à l'aide de la plasmine infusée 1 heure ou 1 et 4 heures *après* l'injection intraveineuse de cellules cancéreuses ascitiques V2. La plasmine radioactive démontre une protection supérieure à la plasmine non marquée ou «froide». Des agents fibrinolytiques similaires devraient également se trouver effectifs et assez sûrs pour l'emploi clinique après ablation chirurgicale d'une tumeur. De diverses investigations exécutées

dans notre laboratoire ou ailleurs ont révélé que la capacité du thrombue tumoral d'adhérer à l'endothélium vasculaire est une condition primairs pour la formation de métastases à partir de cellules cancéreuses dans le sang. Les cellules cancéreuses non adhésives peuvent périr immédiatement, notamment par désintégration et par phagocytose à l'aide de macrophages du tissu mobiles ou fixés.

Il est apparent que les altérations de l'hôte aussi bien que les caractères individuels des cellules cancéreuses emboliques, dont quelques-unes sont connues et d'autres plutôt supposées, demandent d'ultérieures recherches.

Riassunto

L'autore sottolinea l'importanza biologica dei caratteri fisici e chimici delle cellule cancerose e dell'endotelio vascolare. Quest'ultimo rappresenta nella cavia e nell'uomo l'ospite universale e dinamico per la cellula cancerosa disseminata nel sangue, ed ugualmente una barriera contro di essa. Le cellule cancerose non sono capaci di produrre metastasi che aderendo all'endotelio vascolare.

Studi «*in vivo*» di cellule ottenute dal carcinoma ascitico V2, e iniettate in minuscoli vasi dell'orecchio e del mesenterio del coniglio permettono di osservare che le cellule si agglomerano subito dopo la loro entrata nei vasi del mesenterio con deposizione di fibrina. I capillari non sono che semplici filtri meccanici, dato che il carattere adesivo delle cellule cancerose permette loro di rimanere fortemente accollate, indipendentemente dalla velocità della corrente sanguigna o del calibro vascolare. Nel mesenterio o nell'orecchio, le cellule cancerose aderenti sono generalmente attorniate da trombi o inchiuse da essi; le cellule cancerose sono atte a penetrare attraverso l'endotelio in qualche ora.

Si è constatata la pronta e selettiva localizzazione di plasmina o di fibrinogeno marcati allo I^{125} attorno alle cellule cancerose emboliche. La plasmina scioglie rapidamente i trombi formati dagli eritrociti e i trombi cancerosi, senza che si possano constatare alterazioni apprezzabili del trombo traumatico. Ricerche esperimentali sul coniglio hanno illustrato l'effetto protettivo contro la formazione di metastasi, ottenuto da infusioni di plasmina 1 ora o 1 e 4 ore *dopo* l'iniezione intravenosa di cellule cancerose ascitiche V2. La plasmina radioattiva dimostra una protezione superiore alla plasmina non marcata o «fredda». Simili agenti fibrinolitici dovrebbero rivelarsi efficaci e alquanto sicuri per l'applicazione clinica dopo ablazione chirurgica di un tumore. Diverse inchieste eseguite nel nostro laboratorio o altrove hanno rivelato che la proprietà del trombo canceroso di aderire all'endotelio vascolare, è una condizione primaria

per la formazione di metastasi a partire da cellule cancerose nel sangue. Le cellule cancerose non adesive possono perire immediatamente, per disintegrazione e fagocitosi, da parte di macrofagi dei tessuti, mobili o fissi.

Appare evidente che sia le alterazioni dell'ospite che i caratteri individuali delle cellule cancerose emboliche, delle quali talune sono conosciute, altre piuttosto supposte, necessitano di ulteriori ricerche.

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