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PLASMINOGEN ACTIVATOR OF THE BLOOD VESSELS IN TUMOURS AND IN CARRAGEENIN-INDUCED GRANULOMAS

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SUMMARY.—Fibrinolytic activity in tumours was studied by the fibrin slide technique. The tumour cells were inactive and fibrinolysis was seen only in areas with young blood vessels. In carrageenin-induced granulomas at 6 days the fibrinolytic activity was small and confined to mature veins, but from 7–14 days activity was high in zones containing young vessels supplying the terminal capillary buds; these latter showed no activity. In old fibrosed granulomas there was no fibrinolytic activity. The vascular permeability changes of inflammation (detected by the colloidal carbon technique) showed no correlation with fibrinolytic activity, and systemic injection of inflammatory agents had no effect on the fibrinolytic activity of the vessels. These findings are discussed in relationship to tumour vascularization.

THE growth of blood vessels into a tumour is an important aspect of the tumour/host relationship. The tumour blood supply appears to be a weak point in the tumour organization and the mechanisms of its production and control are imperfectly understood. On the one hand there is the hypothesis of O'Meara (1958) and his colleagues that tumours produce a " Cancer Coagulative Factor " (CCF) causing deposition of fibrin which is organized by the host to produce stroma and vessels: on the other hand Cliffton and Grossi (1955) showed by the fibrin plate technique of Astrup and Müllertz (1952) that fibrinolytic activity was present in a wide range of tumours.

In this paper we have used the fibrin slide technique of Todd (1959) to study the localization of the fibrinolytic activity in human and experimental tumours. As a model for study of the fibrous tissue stromal reaction produced by the host in response to tumour invasion we have used carrageenin-induced granulomas in rats. In these it was possible to determine the effects of age, size, and nature of vessels on their fibrinolytic activity. By employing the Pelikan ink method of Majno, Palade and Schoefl (1961) the relationship between vessel permeability and fibrinolytic activity could be determined, and the effects of inflammatory agents injected systemically (by the techniques of Cater and Taylor, 1966; Cater and Wallington, 1968; Oswald and Cater, 1969) could be observed.

MATERIALS AND METHODS

The fibrin slides were prepared by Method I of Todd (1959). Freeze-dried thrombin (Parke Davis " Thrombin Topical ") was diluted to 25 units/ml. saline plus streptomycin 20 units/ml. Two drops from a 19 gauge hypodermic needle were spread on a clean glass slide

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and before this was dry 8 drops of a 2 per cent solution of fibrinogen (Bovine plasma fraction I, Armour) in Veronal buffered saline, pH 7·2, was added. This was quickly spread by tilting the slide which was then left for 3 min. on a level surface for the fibrin to form. The slides were then kept in a moist chamber. Thick cryostat sections (16 μm.) of the fresh tumours or granulomas were placed on the fibrin slides and incubated in moist boxes at 37° for 1 hr (human tissues), 4 hr (rat), 4 or more hr (mouse). The tissues were then fixed in formalin vapour overnight followed by 4 or more hr in 10 per cent formol saline. They were stained with Harris haematoxylin and alcoholic eosin, and then mounted in DPX.

Duplicate sections were incubated on plasminogen-free fibrin-covered slides. The method of Lassen (1952) in which the plasminogen is destroyed by heating for 1 hr at 80°, we found unsatisfactory because the fibrin film was uneven and distorted. Instead, the plasminogen was removed by stirring 5 ml. of 2 per cent fibrinogen with 100 mg. of bentonite. The bentonite was removed by centrifugation and the process repeated (Brackman, 1965). This removed all the plasminogen and some fibrinogen—so the control fibrin slides were thin making them very sensitive to any fibrinolysis not due to plasminogen activation (*e.g.* preformed plasmin).

Operation specimens of human tumours were collected immediately after removal, transported on ice and stored at −180°. Transplanted hepatomas in August strain rats were processed immediately after the animals were killed. The granulomas were induced with 2 ml. of 0·5 per cent lambda carrageenin (W.B. 205) injected s.c. into the flank of White Wistar rats. The rats were taken at 6–14 days, anaesthetized with ether and given either saline or the inflammatory agent followed by Pelikan ink i.v. (5-HT, 5 mg. base/kg. was given i.p., 5 min. before the ink; or kallikrein 10 units/kg. i.m., 30 min. before the ink. The Pelikan ink was diluted 1 part ink to 4 parts 1 per cent gelatine in saline (5 ml. of dil. ink/kg.). The ink cleared from the circulation in about 30 min., as judged by the colour of the rat's eyes and feet. The rats were then killed in the CO_2 chamber and the tissues taken for section. In addition to the fibrin slides, duplicate sections were fixed and stained with Mayer's alum carmine, which showed up the labelled vessels clearly, whereas the H. and E. tended to mask the carbon label.

RESULTS

Some difficulty was encountered in finding a suitable staining method which would show up zones of fibrinolysis without masking the histology of the tissues. The staining properties of fibrin vary with age (Lendrum, Fraser, Slidders and Henderson, 1962), and recently formed fibrin is difficult to stain. Using H. and E. the fibrin stained well with the eosin but lost most of the stain during dehydration. This difficulty was overcome by the use of alcoholic eosin. Zones with partial fibrinolysis, in which a pale pink meshwork of fibrin threads remained (Beneke and Hey, 1965) could be detected by using a light green filter. The zones of fibrinolysis then appeared as green, or grey areas contrasting sharply with the red colour of intact fibrin. A few drops of light green in a Petri dish of water made an easily adjustable colour filter. Ordinary black and white photography did not show up the fibrinolysis very clearly and the finite thickness of the fibrin film caused the tissue to sink into the zones of lysis so that it was in a different focal plane.

Fibrinolytic activity of human and animal tumours

Table I gives the details of the various human and animal tumours which were studied for their fibrinolytic activity. No activity could be detected in relation to the tumour cells. Fibrinolysis was seen only in and around mature vessels or at the site of the stromal reaction to an invasive tumour, where recently formed vessels were present.

Fig. 1 shows an adenocarcinoma infiltrating the muscular coat of the human colon. At the top of the figure is a relatively well differentiated zone of tumour and this shows no fibrinolytic activity. In the centre, anaplastic cancer cells are

actively invading muscle and have provoked a stromal reaction by the host. It is in these areas that fibrinolysis is present, and under higher magnification young

TABLE I.—*Details of Tumours Examined for Fibrinolysis*

No.	Species	Pathological diagnosis	Incubation time (hr)	Site of fibrinolysis
1 (Fig. 1)	Human	Well differentiated adeno-carcinoma infiltrating colon	1¼	Mature vessels and in small vessels of the stromal reaction to invading cancer cells
2	Human	Mucous-cell carcinoma of rectum	1½	At site of stromal reaction to invasive tumour. Where it has penetrated peritoneum, and there is no reaction, there is no lysis
3	Human	Non-malignant destruction of mucosa of colon by granulation tissue	1½	In the area of granulation tissue where many young vessels are present
4	Human	Infiltrating anaplastic carcinoma of terminal ileum	1½	At the site of stromal reaction to the invading tumour and in the vessels of the muscle coat
5	Human	Intestinal polyp, oedematous but non-invasive	1	In the polyp stalk only, round mature vessels
6 (Fig. 2)	Dog	Experimental osteosarcoma in the heart of a puppy	2½	Round the mature vessels in the heart muscle, and the small vessels at the edge of the tumour
7	Rats 4	Transplantable hepatomas, poorly differentiated	4	Young vessels at edge of tumour; in the granulomatous response to the tumour; in the mature vessels in the muscle

EXPLANATION OF PLATES

FIG. 1.—Adenocarcinoma in muscle coat of human colon. At the top, a well differentiated zone of tumour can be seen with no fibrinolytic activity. In the centre and at the bottom anaplastic cells have invaded the muscle and there are zones of lysis associated with the stromal reaction. Incubation 1¼ hr, Harris haematoxylin and alcoholic eosin (H. and AE.). ×30.

FIG. 2.—An osteosarcoma in the heart of a puppy. Fibrinolysis can be seen on the right associated with large vessels in the myocardium, and the edge of the tumour shows slight activity. Incubated 2½ hr. H. and AE. ×30.

FIG. 3.—Carrageenin granuloma in rat (6-day-old). Fibrinolysis is confined to the area round the large vessels. Proliferation of young vessels can be seen in this area. Incubated 4 hr. H. and AE. ×30.

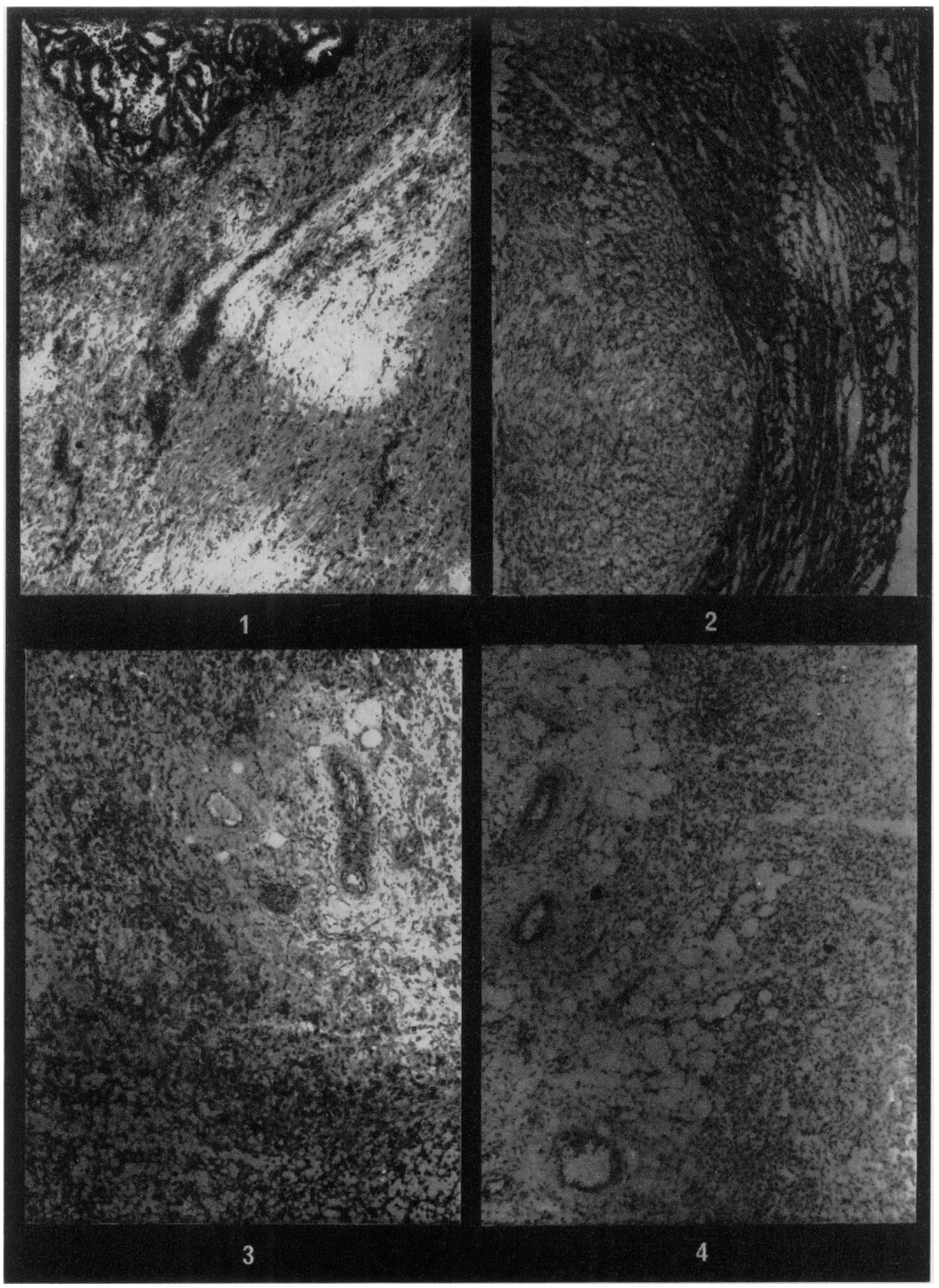
FIG. 4.—The same granuloma as Fig. 3 but incubated on plasminogen-free fibrin. No areas of lysis can be seen. Incubated 4 hr. H. and AE. ×30.

FIG. 5.—Granuloma (8-day-old) from a rat injected with Pelikan ink 1 hr before autopsy. Some capillary labelling can be seen at the edge of the zone of lysis (see arrows) but none is present within the lytic zone. Incubated 4 hr. H. and AE. ×30.

FIG. 6.—An 8-day-old granuloma from a rat given kallikrein (2 units/kg. i.m.) 30 min. before ink injection. Extensive labelling (see arrows) can be seen within the lytic area. Incubated 4 hr. H. and AE. ×30.

FIG. 7.—An 8-day-old granuloma from a rat given 5-HT (5 mg. base/kg.) i.p. 5 min. before ink injection. Again, labelling (see arrows) can be seen within the lytic area. Incubated 4 hr. H. and AE. ×30.

FIG. 8.—A 13-day-old granuloma from a rat injected with in 1 hr before being killed. It is just possible to see a few labelled capillaries (see arrows) at the edge of the horseshoe shaped zone of lysis. Incubated 4 hr. H. and AE. ×30.



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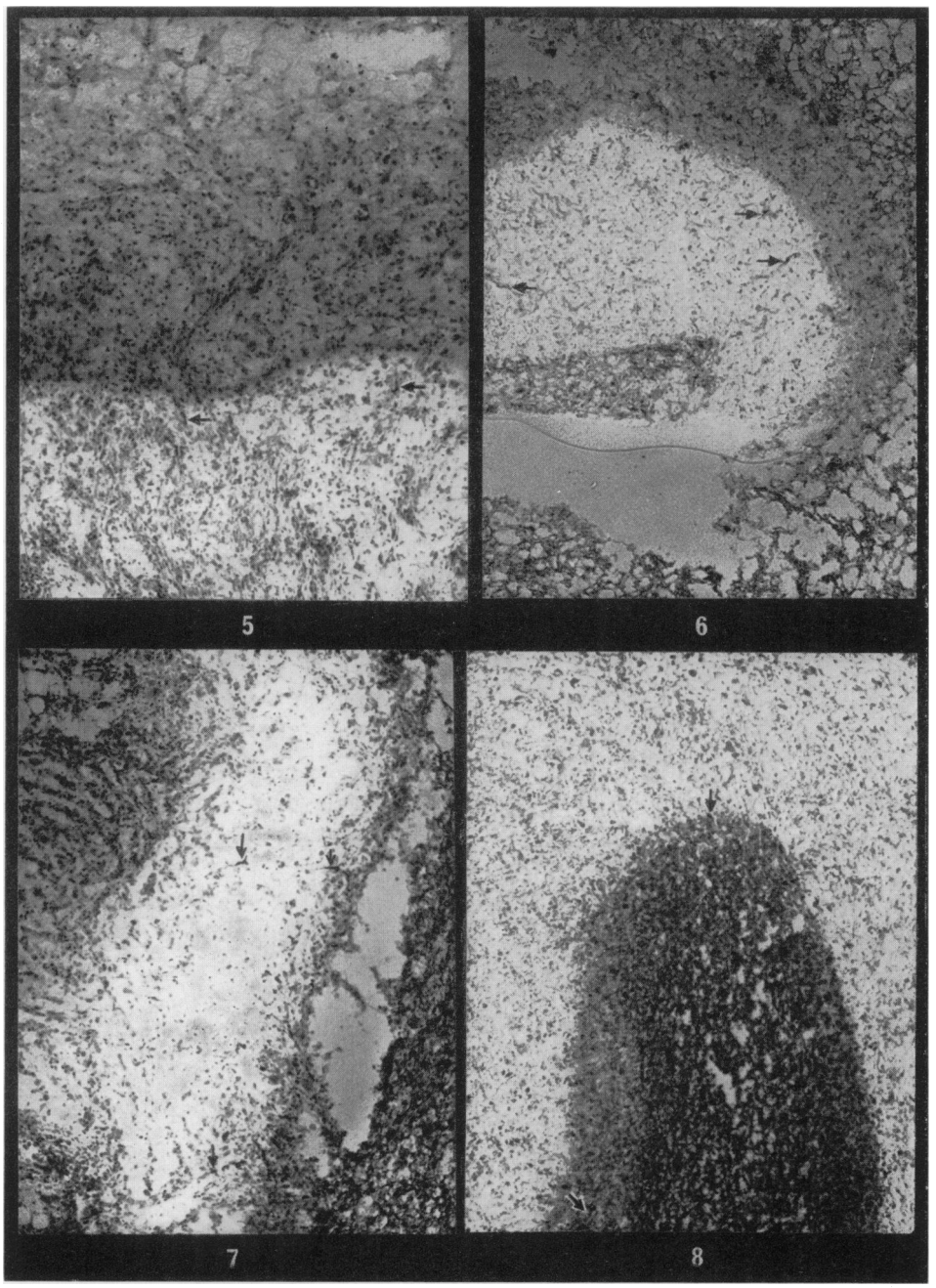

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vessels can be seen in these zones. In plasminogen-free control slides no lysis was seen, indicating that the fibrinolysis is produced by the presence of plasminogen activator.

Fig. 2 shows a deposit of osteosarcoma in the heart of a 5-week-old puppy. Dr. L. N. Owen produced this tumour by injecting the puppy *in utero* (3 weeks before birth) with a culture of osteosarcoma cells. On the right of Fig. 2 the 2 light areas are zones of fibrinolysis surrounding normal vessels in the heart muscle. There is no fibrinolysis associated with the tumour except for slight activity at the edge of the tumour where there are numbers of small vessels. The plasminogen-free control slides showed no fibrinolysis.

In transplantable hepatomas in August Strain rats, the tumour cells showed no fibrinolysis, activity was confined to areas where there were vessels in the tumour, or in muscle infiltrated by tumour, or in areas where the tumour had invoked a granuloma-like response in the surrounding tissues.

Fibrinolytic activity of carrageenin-induced granulomas in the rat

6-*day old granulomas.*—The granulomas examined 6 days after injection of carrageenin showed very little fibrinolytic activity except around mature vessels—as seen in Fig. 3. (No activity was seen in plasminogen-free control slides Fig. 4.) There was a great deal of fibrin in the central mass of carrageenin and the capillaries had only just begun to proliferate. Labelling of the vessels after injection of Pelikan ink was very slight and confined to terminal capillary loops. Injection of 5-HT i.p. or kallikrein i.m. did not increase the labelling, but in this experiment ink was injected before the inflammatory mediator, and this was not as satisfactory as the technique under Methods in which the ink was injected after the inflammatory mediator.

The large amount of fibrin seen in the 6-day-old granulomas suggested that carrageenin was causing bleeding into the injection mass. A thrombin time test was set up using the method of Hardisty and Ingrams (1965) with or without the addition of carrageenin.

TABLE II.—*Effect of Carrageenin on the Thrombin Time of Human Plasma*

Quantity of Carrageenin (g./ml.)	Thrombin time
0	15 sec.
5×10^{-6}	19 sec.
5×10^{-5}	75 sec.
5×10^{-4}	7 min.
5×10^{-4} plus Protamine sulphate 10×10^{-4} g./ml.	38 sec.

Therefore, the solution of carrageenin which was injected into the rats had a high anti-coagulant activity.

8-*day-old granulomas.*—The appearance of the 8-day-old granulomas differed markedly from the 6-day-old granulomas. Large confluent areas of fibrinolysis were seen in all the sections examined. These areas were associated with recently formed vessels invading the granuloma. The fibrin, so much in evidence in the 6-day-old granulomas, had completely disappeared. In rats which had received Pelikan ink i.v., but no inflammatory agent the vessel labelling was sparse and

confined to the terminal capillary buds at the edges of the zones of lysis. The rats treated with the inflammatory mediators (5-HT or kallikrein) before injection of Pelikan ink showed labelling of the vessels within the zones of lysis (*i.e.* the vessels supplying the terminal capillary buds). This means that the vessels responsible for fibrinolysis are those which are very sensitive to low concentrations of circulating inflammatory mediators. However, the extent and intensity of fibrinolysis was not increased by the injection of 5-HT or kallikrein.

Fig. 5 is a section of a carrageenin granuloma in a rat injected with Pelikan ink, i.v., but given no inflammatory mediator. There is very little carbon labelling of the vessels within the zone of lysis at the bottom, but a few labelled capillaries can be seen at the edge of this zone. However, in a rat given kallikrein 30 min. before the ink many labelled vessels are present in the zone of fibrinolysis, see Fig. 6.

The same pattern can be seen in Fig. 7, which is taken from a rat injected with 5-HT i.p. 5 min. before the ink.

The plasminogen free control slides showed no lysis whatsoever even in areas where the vessels were labelled.

10–13-*day-old granulomas*.—These granulomas differed little in appearance from the 8-day-old rats. Fibrinolysis was of the same intensity and was associated with vessels which were labelled with ink after the injection of inflammatory mediators. Fibrosis gradually increased as the granulomas aged and these fibrosed areas lost their fibrinolytic activity. In one 13-day-old granuloma, a great deal of fibrosis was seen and the sections showed no fibrinolytic activity—this granuloma was small, probably because of a small innoculum of carrageenin, which was organized more quickly than normal.

Fig. 8 shows an area of fibrinolysis in a 13-day-old granuloma in a rat injected with Pelikan ink but no inflammatory mediator. There is a little labelling of capillaries at the edge of the zone of fibrinolysis but none in the fibrinolytic area. No lysis was seen in the plasminogen-free control slides.

DISCUSSION

Todd (1964a) reported plasminogen-activator around malignant tumours and other causes of chronic inflammation. We have found this activator localized to the stromal reaction set up by the host, and there was no fibrinolysis in the tumours except where they were invaded by the host blood vessels. Weiss and Beller (1969) reported that the tumour cells of endometrial carcinoma had no fibrinolytic activity but the venous channels in the stroma were very active. However, they found fibrinolytic activity in the tumour cells of squamous cell carcinoma of the cervix invading the uterus, but Astrup, Henrichsen, Tympanidis and King (1967); Henrichsen and Astrup (1967) showed that vaginal epithelial cells are fibrinolytically active. Therefore, the carcinoma cells had not lost this function of the normal cells. Haustein (1969) found fibrinolytic activity in the blood vessels of the stroma of skin tumours, but not in the tumour cells themselves, except in the case of a malignant melanoma.

Williams (1957) injected 5 ml. of 1 per cent carrageenin into guinea-pigs and found that invasion of the carrageenin mass by fibroblasts and capillaries occurred 5 days after injection. We injected 2 ml. of 0·5 per cent carrageenin into rats and found at 6 days a few carbon labelled capillary buds were to be seen; it thus

seems likely that invasion by vessels had just begun. At this stage we found fibrinolysis only round mature veins. In the 8-day-old granulomas many more recently formed vessels were present. They would have developed from the capillary buds—they are supplying the terminal loops with blood—and are probably 3 or 4 days old. They can conveniently be termed " supply capillaries ". It is these vessels which are producing plasminogen activator and it is these vessels which show increased permeability and become labelled with carbon when 5-HT is given i.p. or kallikrein is given i.m. *i.e.* when inflammatory mediators reach these vessels via the blood stream. The terminal capillary buds leak plasma and are labelled with carbon even in the absence of inflammatory agents, but are not fibrinolytically active. Increasing the permeability of the " supply capillaries " by inflammatory mediators did not increase the fibrinolytic activity. This confirms the observations by Todd (1964*a*, *b*) that in tissues rendered acutely inflamed by local injections of xylene or 5-HT the fibrinolytic activity was not increased. It is also interesting that Cliff (1963) in an EM study of sprouting capillaries in rabbit ear chambers found no sign of fibrinolytic activity, but noted in the older capillaries a pronounced endoplasmic reticulum and golgi apparatus suggestive of a secretory role.

The general picture presented by the 8-day-old granuloma persists up to day 13; during this time, more and more carrageenin is invaded by vessels and fibrous tissue, the larger capillaries remain fibrinolytically active and remain sensitive to circulating inflammatory agents. When fibrosis reaches a certain stage the fibrinolytic activity appears to be lost. This may be due (i) to maturation of the vessels so that their lytic activity is decreased. At 14 days the collagen content of a carrageenin granuloma has almost reached its maximum (Benitz and Hall 1959) or (ii) depletion or exhaustion of the store of plasminogen activator in the vessels. Pandolfi, Bjorlin and Nilsson (1969) believed that continual inflammation provided a stimulus for release of plasminogen activator from vessels and eventually depleted them. They found total fibrinolytic activity was lower in chronically inflamed tissues. However, 14 days seems a short time to cause complete depletion of activator from the vessel walls. Also, the results of Pandolfi *et al.* (1969) could be explained if the chronically inflamed tissues contained a lot of mature fibrous tissue.

An important mechanism by which carrageenin induces the formation of fibrous tissue and vessels may be the accumulation of fibrin. Carrageenin is a sulphonated polygalactose and resembles heparin in its anticoagulant properties (Hawkins and Leonard, 1962, 1963). We found that 5×10^{-4} g./ml. (a solution 10 times more dilute than that injected into our rats) prolonged the thrombin time of human plasma from 15 sec. to 7 min. Thus the finding of fibrin in the 6-day-old granulomas is not surprising. Benitz and Hall (1959) found the fibrin content was greatest at 3 days after injection and then slowly declined until none was left by day 21. Large numbers of macrophages also accumulate and the carrageenin may also act like an artificial ground substance (Cater, 1961). We have used the carrageenin granuloma as a convenient experimental system for producing new blood vessels. These new vessels in the carrageenin-induced granulomas appear to respond to inflammatory mediators in the same way as the newly formed vessels in tumours (Cater and Wallington, 1968, Oswald and Cater, 1969).

O'Meara and others have shown that tumours are capable of producing a CCF, (O'Meara, 1958; O'Meara and Thornes, 1961; Thornes and O'Meara, 1961; Thornes and Martin, 1961; Boggust, O'Brien, O'Meara and Thornes, 1963;

Thornes, 1964; Glaser, Spink and O'Meara, 1965; Frank and Holyoke, 1968). This causes a layer of fibrin to form around the tumour which is demonstrable by the Picro-Mallory method (O'Meara, 1960). Other studies using a fluorescein label technique have demonstrated fibrin in a large range of tumours (Hiramoto, Bernecky, Jurandowski and Pressman, 1960). Day, Planinsek, Pressman (1959*a*, *b*), Back, Shields De Witt, Branshaw and Ambrus (1966), Spar, Bale, Goodland, Casarett and Michaelson (1960), Spar, Bale, Goodland and Izzo (1964) demonstrated the presence of fibrin in tumours and found there was uptake of labelled fibrinogen and plasmin by the tumours.

O'Meara (1964*a*, *b*) suggested that the fibrin layer was undissolved by the tumour since it lacked the fibrinolytic enzymes. Therefore it would undergo organization by the blood vessels of the host; and would thus be endowed with a blood supply.

The importance of this fibrin layer in tumour growth has been demonstrated by O'Meara and O'Halloran (1963*a*, *b*) and Muggleton, McClaren and Dyke (1964) who caused stasis or regression of a large proportion of tumours by the administration of protamine sulphate which was known to inhibit the coagulative effect of CCF. Christensen (1959) showed that rabbits with carcinoma of the testis, were less prone to metastases if they were infected with streptococci which produced streptokinase, a plasminogen activator. Grossi, Agostino and Cliffton (1960) demonstrated that fibrinolysin given to rats protected them against lung metastases of Walker carcinoma cells injected i.v. Larsen, Mogensen, Amris and Storm (1964) had some success in causing tumours to undergo stasis or regress by infusions of porcine plasmin in patients with various tumours. Although Dahl (1966) used this technique without success.

Despite the fact that fibrin seems to be an important factor in tumour growth; studies on the fibrinolytic activity of the blood of patients with cancer are conflicting. Raised blood fibrinolytic activity was reported by de Biase and Colangelo (1966) in numerous tumours of the jaws. Schmidt–Mattheisen (1966) also found raised levels in uterine myoma. Davidson, McNicol, Frank, Anderson and Douglas (1969) discovered a metastasis of giant cell carcinoma of the lung which had a very high fibrinolytic activity. In a general survey Montani (1966) found that blood taken from cancer patients had a high plasma clot opacity indicative of either high anticoagulant or fibrinogen levels in the blood. But Mohler, Kennedy and Brakman (1967) could find no evidence of increased activity of the blood in 5 patients with multiple myolomatosis; and Obuchi (1965) reported a lowering of the blood fibrinolytic activity in patients with lung cancer. So varied is the response of blood fibrinolysin levels to tumours, that Wierbitzky and Wierbitzky (1966) found that blood activity could not be satisfactorily used in early diagnosis of uterine cancer. And Schmidt–Mattheisen (1967) discovered that although a tumour may have high fibrinolytic activity, this is not always reflected in the blood. Later in 1967 Mann found that there was a diurnal fluctuation in blood plasmin levels.

Cliffton and Grossi (1955) showed by the fibrin plate technique of Astrup and Müllertz (1952); that small pieces of tumour tissue from a wide range of organs, had plasminogen activator present, and were thus fibrinolytically active. Yet in 1958 O'Meara showed that small pieces of tumour could coagulate plasma.

However, if the cancer coagulation factor is confined to the tumour cells and the fibrinolytic activity is produced by the endothelial cells of the vessel walls the apparent paradox can be resolved.

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