THE SIGNIFICANCE OF FREE BLOOD IN LIQUID AND SOLID TUMOURS

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Summary.—Impregnation of the vasculature with ink was used to study micro-vascular changes induced in rats by liquid (ascites) and solid growth of W256 and Y-P388 tumour cells. Ascites fluid produced by both tumours was heavily blood-stained; the deep red colour of solid tumour deposits was similarly due to the presence of free blood. In both types of tumour growth this free blood was due to diapedesis of erythrocytes through tips of capillary sprouts which grow when neovascularization (angiogenesis) occurs in response to any suitable (non-neoplastic or neoplastic) stimulus. Ascites growth of these tumours induced profuse sprouting from the peritoneal capillaries; this sprouting, together with the " bleeding " it caused, were inhibited by local pre-irradiation of the peritoneal vasculature with X-rays before intraperitoneal inoculation of rats with the tumours. Similar angiogenesis with bleeding did not occur following inoculation with an allogeneic tumour in rats which had been previously immunized against the tumour. LI tumour cells (tumour cells lethally irradiated *in vitro* to destroy their proliferative integrity, but which remain metabolically active) also induced sprouts to grow in close proximity to the implanted LI cells, but heat-killed tumour cells caused no sprouting. The nature and significance of neovascularization of tumours and their so-called " haemorrhagic " growth are discussed.

IN MANY tumours, blood escapes from the vasculature of the tumour bed into the interstitium, giving rise to so-called " haemorrhagic " tumour growth. Marked leakage of blood into the peritoneum commonly occurs during liquid growth of certain transplantable rat and mouse ascites tumours. The interest of experimental oncologists in the presence of free blood in solid, and more particularly liquid, tumours seems to have centred on its inconvenience and interference with experimental procedures, rather than on its causation—a situation which is illustrated by an otherwise highly informative symposium (Miner, 1956) on the conversion of solid to liquid (ascites) tumours as tools in quantitative oncology. It is commonly stated or implied that such losses of blood into solid or liquid tumours are essentially haemorrhagic in nature, being caused by invasion of blood vessel walls by the growing tumour or by spontaneous rupture of newly-grown or remodelled tumour blood vessels which results in inherent weakness and increased fragility of capillaries and even larger conduits. Our dissatisfaction with these facile attempts to explain bleeding from the microvaculature of the tumour bed in solid tumours, and more particularly the blood in tumour ascites fluid, has prompted the studies of microvascular changes associated with bleeding into transplantable ascites tumours in the rat which are reported in this paper.

Our findings indicate that the free blood present in both solid and liquid forms of these tumours is primarily due to capillary sprouting (angiogenesis) stimulated by the presence of living tumour cells, and that leakage of blood from the

microvasculature of tumours is a normal manifestation of growth of capillary endothelium and is essentially comparable with that of granulation tissue.

MATERIALS AND METHODS

Female Carworth Farm strain (SPF) rats weighing 150–200 g body weight were used. The rats were caged on grids and allowed food and water *ad libitum*. A subline of the Walker (W256) carcinoma was passaged as an ascites tumour by intraperitoneal (i.p.) injection of rats with approximately 10^7 tumour cells. The ascites tumour fluid produced 4–5 days after injection was invariably heavily blood-stained; to prevent clotting it was collected in heparinized tubes for transplantation studies and contained 4–8 $\times$ 10^7 tumour cells/ml. Similarly, a subline of the Yoshida (Y-P388) sarcoma was used for transplantation in rats; its IP growth produced similarly a heavily blood-stained ascites fluid which contained 1·5–1·7 $\times$ 10^8 tumour cells/ml.

Perfusion of vasculature with India ink.—Rats were deeply anaesthetized by intra-thoracic injection with sodium pentobarbitone. The heart was exposed by opening the thoracic cavity without entering the peritoneal cavity, by removing the sternum and adjacent thoracic walls. About 50 ml of isotonic saline, containing 0·25% (w/v) sodium nitrate and pre-warmed to 37°, was slowly injected into the left ventricle to remove blood from the circulation. The vasculature was then perfused *via* the left ventricle with 20–30 ml pre-warmed 25% (v/v) India ink (Pelikan; Gunther Wagner) suspended in 2·5% gelatine in distilled water; 20 ml neutral formalin was then injected into the peritoneal cavity and the carcase was cooled at 1° for about 2 h. The abdomen was then opened in the midline, the skin was dissected off each flank and large squares of parietal peritoneum attached to the abdominal musculature of the flanks were removed; these were washed in saline and laid flat in Petri dishes between layers of gauze kept saturated with formalin. The diaphragm and omentum were similarly removed and fixed in formalin for 24 h. The tissues were then dehydrated, cleared in xylol, trimmed and mounted on large glass slides for examination (*en face*) and for microscopic study of the ink-impregnated vasculature with transmitted light. A portion of each cleared tissue was used to prepare 10-μm thick paraffin sections; these were stained with 0·5% aqueous light green or with haematoxylin–eosin. In one series of experiments the rats were injected i.v. with 0·5 ml shellac-free India ink (Batch C11/1413a, Pelikan; Gunther Wagner) and killed 2–4 h later after the circulation became

cleared of ink. The tumour ascites fluid was evacuated for examination, and pieces of peritoneum, omentum and diaphragm were removed, fixed in formalin and prepared as whole mounts or tissue sections as described above.

Vasculature of solid tumours.—Rats were anaesthetized and approximately 10^5 W256 cells, suspended in 0·05 ml Tyrode's solution (pH 7·2), were injected beneath the visceral peritoneum of the fundus of the stomach. The abdomen was closed in layers with sutures and stainless steel clips. These rats were killed 5–10 days later and the vasculature was impregnated with India ink/gelatine suspension as described above. The stomach was removed from each rat and cut widely open along the greater and lesser curvatures; the anterior wall was washed, fixed, dehydrated, cleared and mounted *en face* on a slide for microscopic study of the tumour vasculature.

Investigation of effects of x-irradiation of the tumour and tumour bed.—Vascular changes in the diaphragm and parietal peritoneum induced by growth of tumour cells in the peritoneal cavity were studied in rats given a single dose of 1500 rad x-rays to the 2-cm wide section of the trunk containing the whole of the diaphragm, using the technique described previously for local thoracic irradiation of rats (van den Brenk *et al.*, 1973). These rats were injected i.p. with 2 $\times$ 10^7 W256 cells 2 h after irradiation and killed 4–5 days later, when the circulation was perfused with India ink/gelatine and *en face* mounts of the irradiated diaphragm and unirradiated parietal peritoneum of the lower abdomen were prepared for examination. In a further experiment rats were injected i.p. with 5 $\times$ 10^5 W256 cells and 4 or 5 days later the entire peritoneal cavity (including the diaphragm) was locally irradiated with 1000 rad. These rats were killed 2 days later and the vasculature of the diaphragm and parietal peritoneum was studied.

To study the effects of lethally irradiated (LI) W256 cells on the peritoneal vasculature, freshly harvested tumour ascites fluid held in a disposable syringe was exposed to a single dose of 10,000 rad *in vitro*—a dose of x-rays which prevents replicative growth of the tumour by reducing cell proliferative integrity of the population to $<10^{-7}$. A group of 3 rats were injected i.p. with $\sim10^8$ freshly prepared LI tumour cells daily for 4 days. The rats were anaesthetized 6 h after the last injection; the vasculature was washed out and perfused with India ink/gelatine and the diaphragm and peritoneal surfaces were prepared for examination as described above.

Tumour immunization—Rats were immunized against W256 tumour by i.p. injection with 10^6–10^7 LI W256 cells twice weekly for 3 or more weeks, as described previously (van den

Brenk *et al.*, 1971). Microvascular changes induced in the peritoneum of these rats by i.p. injection with 10^7 viable W256 cells were studied.

RESULTS

Peritoneal reactions to ascites tumour growth

Ascitic growth of W256 and Y-P388 tumours causes rapidly progressive anaemia in rats due to loss of blood into the peritoneal cavity; the ascites fluid of rats killed 5 days after i.p. injection with 10^7 tumour cells contained 3–5 g haemoglobin/100 ml. Examination of the ink-impregnated peritoneal vasculature showed that this loss of blood originated from the parietal and visceral peritoneal vasculature and was due to the exuberant growth of clusters of capillary sprouts produced by the microvasculature of the diaphragm, omentum and other peritoneal coverings; sprouts were not seen in normal rats which had not been inoculated with tumour cells (Figs. 1 and 2), nor on the diaphragmatic pleura in rats with ascites tumour (Fig. 2b), *i.e.* the reaction was localized to surfaces in contact with growing tumour cells. On the other hand, pleural angiogenesis, but not peritoneal angiogenesis, developed when W256 cells were grown in the pleural cavity by intrapleural injection of rats with the tumour or as the result of ulceration of the surface of the lung by tumour nodules which had grown after an i.v. injection of tumour cells. The sprouts originated not only from the subserous vascular layer but also from the adjacent underlying layer of parallel intermuscular capillaries. The affected microvasculature was dilated and had developed saccular swellings which rapidly enlarged and developed into clusters of protruding sprouts; those in which blood frequently remained after perfusion were freely permeable to red blood cells as well as to the India ink/gelatine suspension used for impregnating the vasculature, which filled and outlined the sprouts and also freely entered the peritoneal cavity. Individual sprouts remained more rounded in shape than

those seen in granulation tissue. Their plump shape (Figs. 1b, 2a and 2b) is attributed to the resistance of the covering mesothelium to their migration, which also appeared to inhibit clotting of blood on the peritoneal surfaces. The omentum was usually reddened with nodular thickenings which were covered with peritoneal sprouts (Fig. 3a). These nodules consisted of relatively avascular solid deposits of tumour lying deep to a densely vascularized surface layer; the tips of the interlacing sprouts of this surface were directed outwards towards the peritoneal cavity (Fig. 3b) and were more frequently covered with adhering blood clot than those of other peritoneal surfaces. The density of peritoneal sprouts and the total blood present in the ascitic fluid progressively increased with time after inoculation of the tumour cells.

In rats with 4-day-old W256 ascites tumour, which had been injected i.v. with India ink to label macrophages in tissues, the ascites fluid contained approximately 4×10^7 tumour cells/ml and 5×10^4 labelled macrophages/ml. Typical large macrophages distended with ink were also found in the diaphragmatic peritoneum of these rats, in close association with capillary sprouts.

Effect of local x-irradiation of the diaphragm

Pre-irradiation of the diaphragm with 1500 rad in rats significantly reduced the volume and slightly lowered the concentration of blood in tumour ascites fluid (Table). In these animals the irradiated diaphragm showed greatly reduced angiogenesis (Fig. 1c), whereas typical sprouts had grown out from the vasculature of the unirradiated parietal peritoneum of the lower abdomen. The diaphragmatic vessels were somewhat dilated and tortuous, and occasional buds and saccules were present along their length which were much smaller than normal sprouts and less permeable to the injected ink. These appearances were characteristic of the
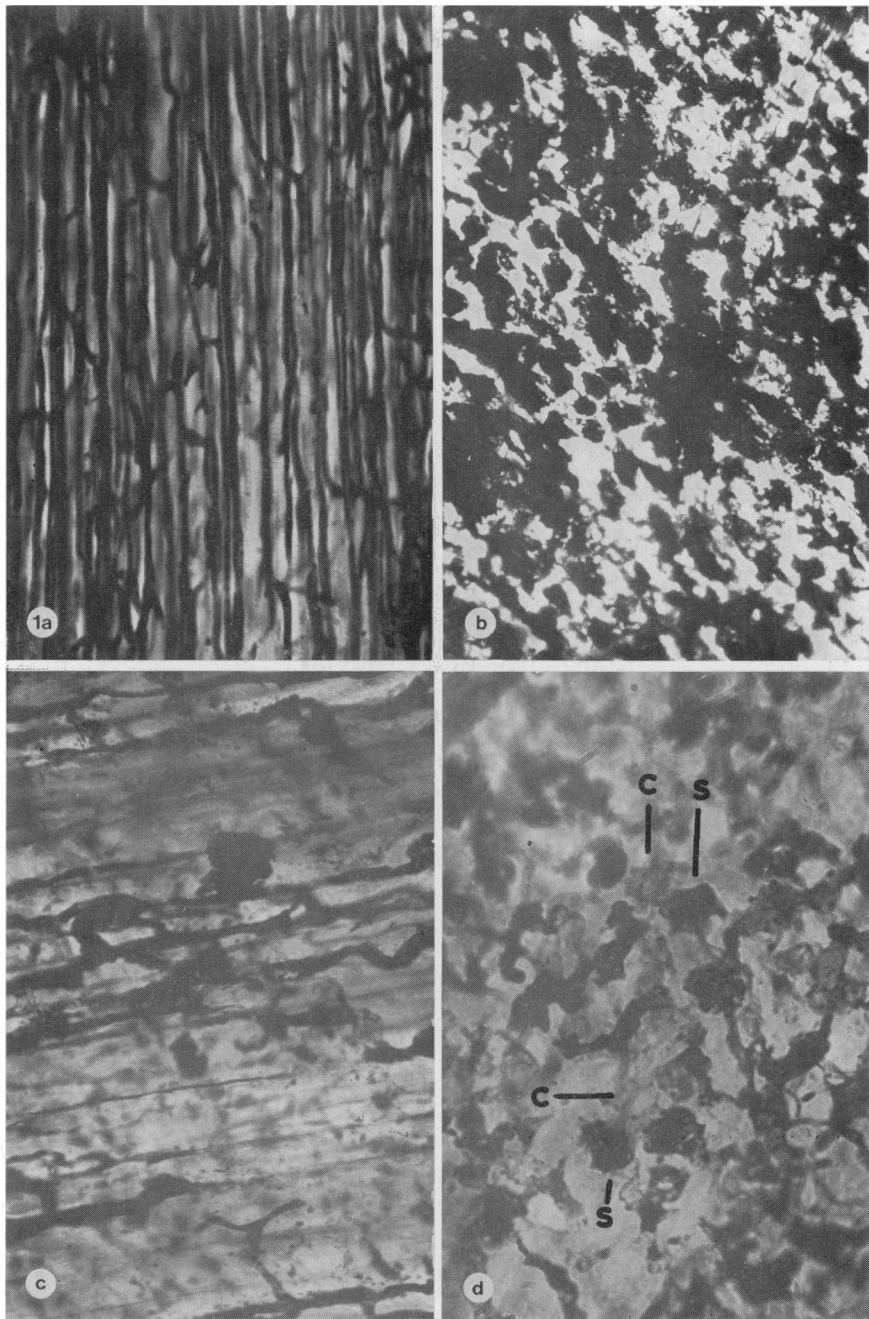
FIG. 1.—Preparations of diaphragm mounted *en face* of rats following impregnation of vasculature with India ink/gelatine suspension.
(a) Normal rat showing subperitoneal intramuscular capillaries (× 65).
(b) Rat with 4-day-old W256 ascites tumour showing dense clusters of capillary sprouts (× 65).
(c) As in (b) but in rats pre-irradiated with 1500 rad X-rays to diaphragm immediately before trans-plantation of tumour cells, showing marked reduction in formation of capillary sprouts (× 165).
(d) Rats injected daily for 4 days with approximately 10^8 lethally irradiated (LI) W256 cells, showing sprouts (S) closely associated with adhering LI cells (C) (× 165).
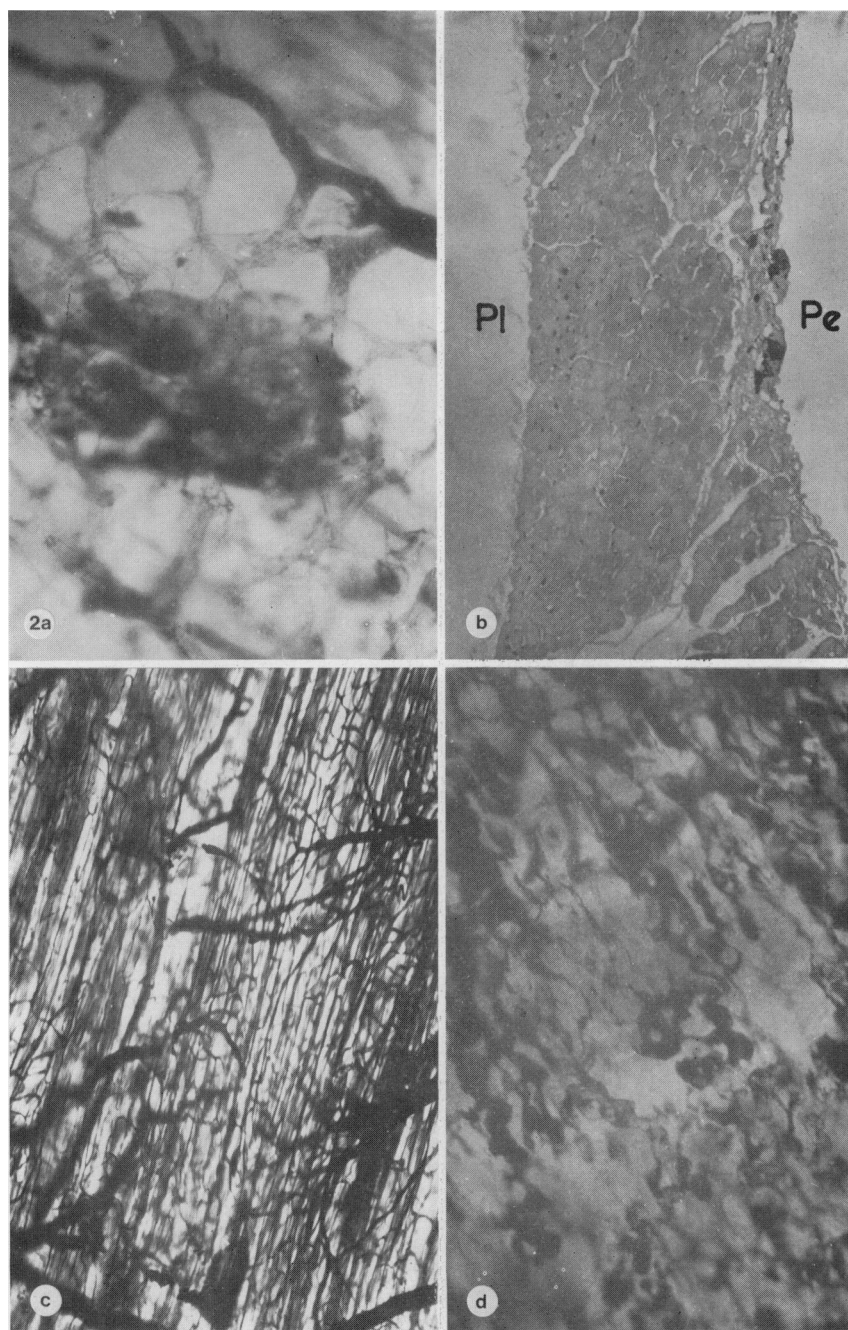
Fig. 2.—(a) Typical cluster of ink-filled sprouts formed by capillaries in parietal peritoneum of rat with 4-day-old W256 ascites tumour growth ($\times 265$).

(b) Transverse section of diaphragm showing sprouts in peritoneal surface (Pe) but not in pleural surface (Pl) of rat with ascites tumour ($\times 65$).

(c) Diaphragm (en face) of W256 tumour-immunized rat 4 days after intra-peritoneal injection of 10^7 W256 cells, showing complete absence of angiogenesis ($\times 26$).

(d) Vasculature of diaphragm of rat given 1000 rad X-rays to the whole abdomen 4 days after inoculation with W256 cells and killed 2 days later; showing retraction of capillary sprouts ($\times 65$).
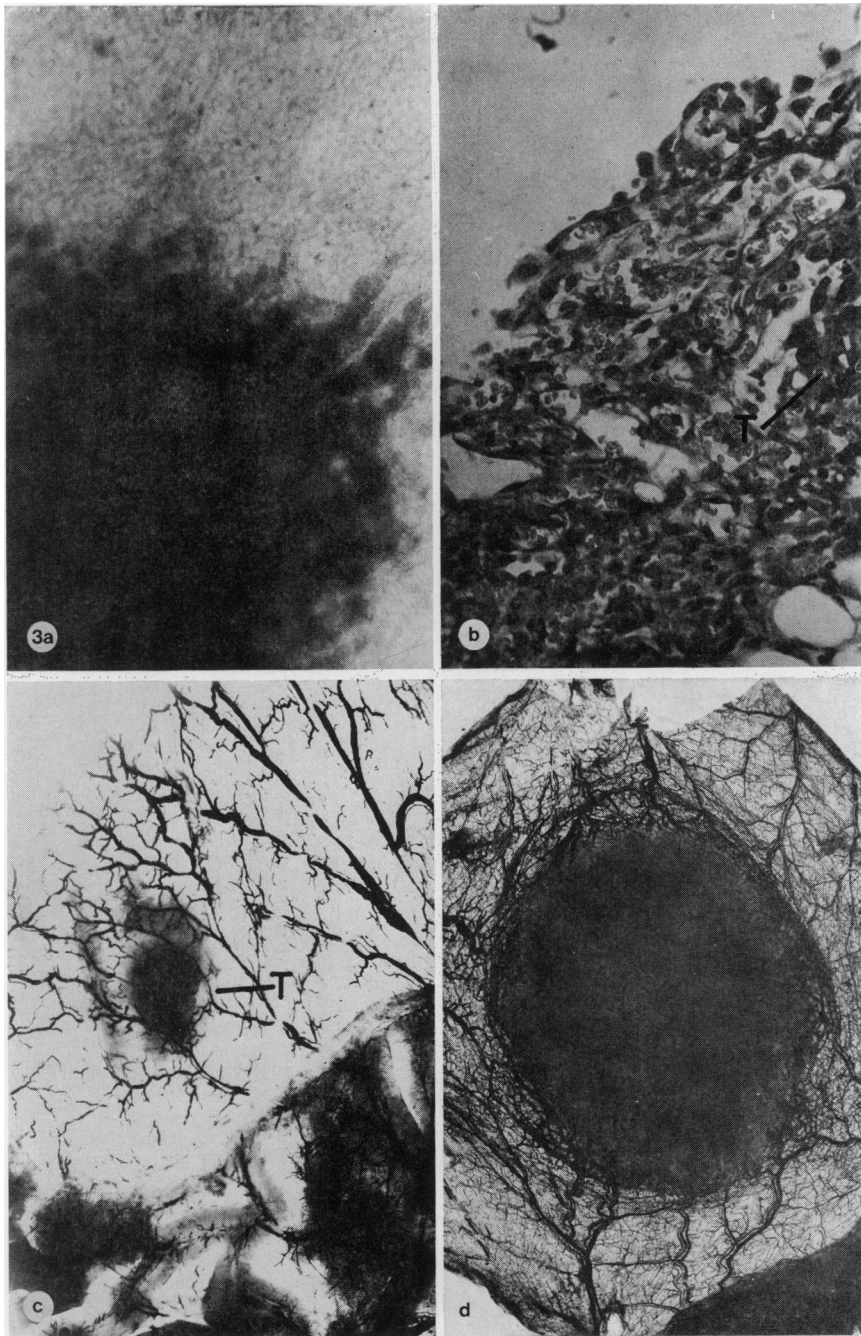
FIG. 3.—(a) Edge of omentum of rat with W256 ascites tumour (*en face*), showing profuse ink-filled
capillary sprouts covering the surface (× 65).
(b) Capillary sprouts (as in (a)) seen in histological section of omentum in rat with ascites tumour
growth which covered solid tumour (T) growing in the omentum (haematoxylin–eosin stain;
× 165).
(c) *En face* preparation showing vasculature of fundus of stomach and solid tumour of ink-impreg-
nated rat, 2 days after subserous injection of stomach with 10^5 Y-P388 tumour cells.   The tumour
(T) is already deeply stained with blood extruded from sprouts (approximately × 4·5).
(d) Same preparation as in (c) of an 8-day-old Y-P388 tumour, showing peripheral neovascularization
and accumulation of pools of free blood within the " devascularized " central regions of the tumour
(approximately × 4·5).

TABLE.—*Effect of Local Pre-irradiation of the Diaphragm with a Single Dose of* 1500 *rad X-rays on the Production Of Tumour Ascites Fluid in Rats* 4 *Days after Intraperitoneal Injection of* 3×10^6 *W256 Tumour Cells*

		Ascites fluid	
Treatment (No. of rats)	Volume (ml)	Total tumour cell population ($\times 10^8$)	Haemoglobin concentration (g/100 ml)
Unirradiated (5)	$1 \cdot 7 \pm 0 \cdot 3$	$1 \cdot 24 \pm 0 \cdot 26$	$1 \cdot 5 \pm 0 \cdot 26$
Irradiated (6)	$0 \cdot 9 \pm 0 \cdot 1$	$1 \cdot 29 \pm 0 \cdot 14$	$1 \cdot 2 \pm 0 \cdot 25$

* Rats were injected with tumour cells 5–10 min after irradiation.

effects of irradiation on angiogenesis in growing granulation tissue (van den Brenk, 1959); in those studies it was shown that a dose of approximately 1500 rad was required to reduce the mean rate of growth of sprouts by 50%. An attempt was made to irradiate the whole peritoneal cavity of 6 rats with 1500 rad immediately before transplantation to inhibit uniformly angiogenesis induced by ascites tumour cells, but only one rat survived 4 days after inoculation of 10^7 W256 cells; the remainder died from peritonitis caused by radiation damage to the intestine. Less than 0·1 ml of milky ascites fluid was recovered from the one survivor; this fluid contained $>10^8$ tumour cells/ml but was virtually free from blood. Local irradiation of the abdomen of rats with 1000 rad given 4–5 days after i.p. injection with ascites tumour cells, and when several ml of heavily blood-stained ascites tumour had formed in the peritoneum, markedly inhibited further growth of sprouts, caused retraction of sprouts which had formed before irradiation (Fig. 2d) and resulted in vascular patterns similar to those observed in the pre-irradiated diaphragm (see Fig. 1c). Also the ascites tumour volume and the concentration of blood in the ascites fluid were slightly reduced by this dose of x-rays.

Effect of LI tumour cells on the peritoneal vasculature

Daily i.p. injection of rats with 2 ml of neat LI (radiation sterilized) ascites fluid caused vascular sprouts to develop in the peritoneum of the diaphragm and other regions of the abdomen. These changes were similar to those induced by viable proliferating tumour cells, but angiogenesis was much less advanced; the sprouts were smaller and fewer in number and had mostly formed in close relationship with LI cells where these had adhered to the peritoneum (Fig. 1d). After 4 days the peritoneum contained several ml of tumour-cell-laden and blood-stained reddish-brown fluid in which a high proportion of the tumour cells were enlarged and had the characteristics of " radiation-giant " cells; mitosis was absent but a majority of the LI cells excluded the dye, nigrosin. Intraperitoneal injection of rats with saline, plasma, blood or with heat-killed W256 cells did not induce peritoneal angiogenesis. The reduction of angiogenesis induced by LI cells was reflected by a reduction in the blood which leaked from the vasculature into the peritoneum.

Tumour-immune rats

In rats immunized with repeated i.p. injections of LI W256 cells given for an overall period of 3 weeks or more, 10^7 or more i.p.-injected viable W256 cells failed to grow or produce ascites tumour fluid. The peritoneal surfaces showed no signs of neovascularization; the vessels appeared normal in arrangement and calibre and sprouts were absent (Fig. 2c).

Growth of solid W256 and Y-P388 tumours —vascular changes

Actively-growing primary and secondary solid W256 and Y-P388 tumours are

blood-red in colour. This colour is largely due to the presence of free blood in the tumour which cannot be removed by perfusion of its vasculature; the haemoglobin content of the tumour was found to be proportional to its wet weight (van den Brenk *et al.*, 1974*a*). At an early stage of its growth the tumour induces intense angiogenesis in the vasculature of the surrounding tumour bed which leaks blood into the tumour (Fig. 3c). Formation of capillary sprouts continues with further growth of tumour but remains largely confined to a narrow peripheral actively-growing rim of the tumour; within this outer reactive zone extravasated blood persists (Fig. 3d) but the tumour becomes relatively avascular because the newly-formed vessels fail to remodel and establish a circulation which meets the metabolic and structural demands of the progressively and rapidly proliferating tumour cell population. Thus, capillaries formed by the newly-formed sprouts degenerate and form the ragged remnants seen in the more central but viable parts of the tumour (Fig. 3d) as described previously (van den Brenk *et al.*, 1972). Although these appearances suggest that the newly-formed capillaries are being actively destroyed by growing tumour they appear to remain closed, since no significant leakage of the ink/gelatine suspension was observed. This indicates that the free blood in the tumour largely arises from angiogenesis at the periphery of the tumour and not from destruction of tumour blood vessels in the more central and necrotic regions of the tumour. We have previously demonstrated that when W256 and Y-P388 tumours invade the smooth muscle coat of the stomach, little neovascularization is observed and nutrition of the tumour largely depends on the capacity of the capillary networks which supply the muscle to subserve tumour growth— mature capillary networks already in existence which are similarly being obliterated as the tumour invades the musculature. It follows that the peripheral neovascularization induced by solid growth of rapidly growing tumours such as the W256 and Y-P388, despite its intensity, largely fails to result in an effective tumour circulation, and that central tumour necrosis develops. We have previously drawn attention to the possibility that the copious amounts of free blood present in such tumours in regions where the capillary circulation is absent or defective may contribute to the oxygenation and associated radio-sensitivity of tumour cells, and possibly affect their viability (van den Brenk *et al.*, 1972). The patent vasculature of the central regions of actively growing solid W256 and Y-P388 tumours was found to consist mainly of larger pre-formed vessels (arterioles and veins) of the normal tissue which had been spared during its invasion and replacement by the neoplastic tissue, or localized "tongues" of granulation tissue which were tumour-free and had grown into degenerating and necrotic regions of the tumour (van den Brenk, 1971).

DISCUSSION

Survival and progressive growth of spontaneous and transplanted tumours depend on the provision of a new blood supply by the tumour bed which subserves the metabolic requirements for growth of the tumour. With very few exceptions, such as very slowly growing and benign neoplastic conditions which can be accommodated by the pre-existing vasculature and stroma, and elicit little more than their remodelling, neovascularization accompanies tumour growth (Ludford and Barlow, 1944). In less rapidly growing but invasive tumours, labelling studies have shown that angiogenesis occurs at a rate closely related to the rate of growth of the particular tumour (Tannock, 1970). Similarly, it has been shown that in such tumours the rate of growth of fibrous stroma of the tumour bed is directly proportional to that of the tumour

(Brada, 1965). In very rapidly growing, undifferentiated solid tumours such as the W256 and Y-P388 in the rat, the implanted tumour cells rapidly induced intense angiogenesis, but the profuse formation of sprouts and new capillaries in the periphery of the tumour largely failed to develop into a vasculature which subserved the requirements of progressive growth, structure, form and metabolism of the neoplasm. As the tumour continues to grow and enlarge, these newly-formed capillaries rapidly degenerate when their distance from the growing edge increases; thus, necrosis develops in the inner portions of the tumour while angiogenesis continues to be induced apace at its invasive growing periphery (Figs. 3c and 3d), in a manner analogous to stimulation of angiogenesis in the repair of tissue injury. Indeed, it is difficult to distinguish any qualitative difference between the neovascularization of tissue repair and that of neoplasia. In both instances induction of capillary endothelial sprouting is the primary morphological event, and each is associated with leakage of blood from the growing sprouts into the affected tissues.

Neovascularization in non-neoplastic conditions such as growth of granulation tissue primarily depends on the capacity of capillary endothelium to react to a stimulus of yet unknown nature, and to develop migratory blind endothelial protrusions (sprouts); these subsequently anastomose and canalize and establish the microvasculature of granulation tissue which subserves tissue repair. This regeneration of blood vessels in granulation tissue can be observed as a dynamic process *in vivo* in a preparation such as the Sandison-Clark ear chamber in the rabbit (see Florey, 1970), which shows that blood plasma and corpuscles from the supply capillary arcades progressively enter the sprouts and are extruded from their tips. Within each sprout the erythrocytes are in a state of relative stasis, often held together in rouleau formation before being extruded by an ill-understood mechanism referred to as diapedesis. Electron microscopy has shown that this extraordinarily high degree of permeability of sprout endothelium to the cellular and non-cellular components of blood is accounted for by the development of spaces within the endothelial cells, which arise without initial communication with the lumen of the supply vessel (Clark and Clark, 1939), and are probably produced by a secretory activity of endothelial cells—a function which these cells appear well-equipped to perform on structural grounds (Cliff, 1963, 1965). The blood, which is being continuously extruded, accumulates, clots and forms a substrate in which the growing sprouts become embedded; in the clotted plasma a scaffold of interlacing network of fibrin forms which provides surfaces for the attachment and migration of newly-formed sprouts and thereby facilitates their forward growth. It is significant that during endothelial blastogenesis the sprouts become fibrinolytically inactive (Cliff, 1963; Pick and Cater, 1971); during ageing and maturation of the sprouts and establishment of the circulation of blood, an elaboration of endoplasmic reticulum and Golgi apparatus occurs, plasminogen activator enzyme is synthesized and secreted, and fibrinolytic activity of the endothelium is restored. This sequence of changes in permeability and enzyme activities of the vascular regenerate are adapted to subserve polarized forward growth of sprouts, followed by demolition of the fibrin scaffold they have formed by the " mature " endothelium of the newly differentiated supply capillaries. This demolition phase is supported by an influx of monocytes; these form macrophages which remove effete erythrocytes and insoluble matter of the clot and subsequently develop into the perivascular fixed tissue histiocytes of repair tissue (Ebert and Florey, 1939). The possibility that the extruded erythrocytes exert a specific function in angiogenesis does not seem to have been given sufficient consideration. It has been

found that *in vitro*, under tissue culture conditions, erythrocytes, washed free of plasma, release a factor into the medium which exhibits " survival activity " by sustaining the viability of normal and neoplastic cells *in vitro*, and that their action in this respect is comparable to that of blood plasma (van den Brenk, unpublished). All cells, *in vivo* and *in vitro*, must be nurtured to remain viable, grow and replicate. It is therefore conceivable that during angiogenesis lengthening endothelial sprouts obtain from extruded erythrocytes the nurture previously provided by the blood circulating through their vessels of origin (the arcades of supply capillaries). That leakage of blood by capillary sprouts is an inherent characteristic of angiogenesis is shown by the accumulation of free blood produced by growth of granulation tissue in the Selye (granuloma) pouch in the rat; in this preparation the amount of blood is proportional to the weight of granulation tissue in the wall of the pouch and can be used to quantify angiogenesis in unirradiated and irradiated tissues (van den Brenk *et al.*, 1974*b*).

Mechanisms concerned with loss of blood from sprouts in wound healing are cogent to similar bleeding which attends angiogenesis induced by tumour cells. It is generally agreed that the rate of endothelial cell proliferation in the tumour bed, which begins early in tumour growth, must be closely coordinated with that of the tumour cells in order to subserve tumour metabolism (Thomlinson, 1973). Since the relentless demand on angiogenesis by rapidly growing tumours exceeds in duration that of non-neoplastic conditions, it is not surprising that angiogenesis fails to keep up with growth of tumour and leads to tumour necrosis. Another theory of tumour necrosis postulates that it is due not to a lack of capillary sprouting but to the failure by feeder arterioles to maintain an adequate flow of blood in the tumour vasculature (Thomlinson, 1973). No evidence has been obtained that progressive bleeding

from the microvasculature into a tumour occurs in central regions of the tumour or results from central necrosis; indeed, the escape of blood commences and continues at the periphery of a growing tumour where the sprouts grow and the tumour cells are viable and actively proliferating. It is noteworthy that macrophages are similarly concentrated in this peripheral region of angiogenesis and active tumour growth. The current interest in tumour immunity has concentrated attention on the participation of macrophages in local immune reactions, but their role in demolition associated with angiogenesis points to a likelihood that their function in tumours is similarly concerned with demolition and the remodelling of the tumour bed (stroma). Most of the erythrocytes which leak from growing sprouts in solid tumours rapidly lyse and stain the tumour tissue red, whereas in liquid tumours the erythrocytes are better preserved by the escape of blood from the sprouts growing in the lining of the body cavity which offers insufficient resistance to its flow, so that it accumulates, admixed with tumour cells. During the exponential phase of growth of Walker ascites tumour in the peritoneum over the first 4 days after tumour transplantation, the ascites fluid volume increased rapidly, but the concentration of blood in the fluid, measured as haemoglobin, remained relatively constant (1–2 g Hb/100 ml ascitic fluid), and the size and density of capillary sprouts on the peritoneal surfaces increased. Thereafter, as the rate of tumour cell proliferation decreased, the tumour cell concentration decreased, but the volume of ascites fluid increased and the concentration of blood also usually continued to increase. This strongly suggests that the blood present in ascites tumour fluid is primarily produced by angiogenesis, induced in the presence of living tumour cells. Pre-irradiation of the vasculature in experimental animals and in man inhibits the regeneration of endothelium and other tissues involved in the repair process, and these effects

are dose-dependent (van den Brenk, 1956, 1959; van den Brenk *et al.*, 1972). Similarly, pre-irradiation of the peritoneum inhibited angiogenesis induced by ascites tumour and reduced the concentration of blood in the tumour ascites fluid. It is noteworthy that x-rays cause vascular reactions in which permeability and fragility of the microvasculature increase and other (haematological) changes may be induced which are also conducive to haemorrhage. Nevertheless, two well-documented, important and beneficial effects of x-ray therapy in cancer are those of rapidly reducing local " haemorrhage " associated with tumour growth and of decreasing the vascularity of a tumour; these effects have been used to advantage in the control of bleeding from ulcerated tumours and to reduce potential bleeding at operation—particularly from the growing periphery of the tumour—by prescribing radiation as a pre-operative measure. Pre-irradiation of the whole abdomen of rats with a single dose of 1500 rad was similarly found to be effective in almost completely inhibiting bleeding into tumour ascites fluid in rats killed 3–4 days after inoculation.

The studies of Folkman *et al.* (1971) of a *tumour angiogenesis factor* (TAF) in tumour cells has recently aroused much interest; they have devised experimental techniques which have clearly confirmed previous observations by Algire and Chalkley (1945) which indicated that both neoplastic and injured normal tissues produce diffusible factors which induce neovascularization, and that certain tumours such as the Walker tumour are particularly rich in TAF. Recent studies by Ausprunk and Folkman (1976) have shown that endothelial migration preceded endothelial mitogenesis in angiogenesis induced by V2 carcinoma cells in the rabbit, and that the [3H] thymidine-labelling index of the endothelium did not increase till 3 days after implantation of cells to the cornea. A much more rapid and vigorous onset of angiogenesis was observed by Brown (1976) in the parietal peritoneum of mice after i.p. injection of Erhlich tumour cells; the endothelial cell [3H]thymidine-labelling index increased sixteen-fold within 6 h after inoculation, and a similar early onset of cell proliferation occurred in the supporting (fibroblastic) tissues. These various observations do not indicate, however, that the new growth of stroma induced by a neoplastic stimulus differs intrinsically in nature from that induced by wounding or other non-neoplastic conditions such as the " take " of a normal tissue graft. Tumours vary considerably in the development and structure of their stroma which are affected by their origin, histology, rate of growth and location. In general, the vascular index (density) of a particular tissue, whether normal or neoplastic, is primarily determined by its metabolic activity; in respect of growth and regression the microvasculature is highly labile and the vascular index of a tissue adjusts to local changes in metabolic requirements which may be produced by changes in its growth, form and function (Virchow, 1858). Rates of neovascularization of tumours are similarly related to rates of tumour growth. Consequently, rapidly growing transplantable ascites tumours in the rat and mouse induce marked angiogenesis which is essentially normal in nature. In the ascitic form of tumour growth, sprouts develop and blood is extruded in the usual way, accumulating in the peritoneal cavity as a result of the lack of anatomical tissue barriers to the growing sprouts which would allow the extruded blood to clot and " anchor " the sprouts and thereby restrict the loss of blood. However, marked losses of blood are produced by angiogenesis in large solid tumours in animals which cause progressive anaemia. The causative role of tumour angiogenesis in anaemias associated with malignant diseases in man warrants further consideration for similar reasons. Somewhat unusual descriptions have been given of changes in blood vessels seen in tumours which have clearly been chosen to differ from conven-

tional concepts of their structure and form in normal tissues, and imply that a transformation of the stroma itself can arise in tumours which simulates that of the neoplastic cells. Thus, newly-formed vessels in tumours have been termed " small and simple " and are said to " often consist of irregular channels lined by endothelium only or by naked tumour cells " and to be prone to " 'suicidal' occlusion " (Willis, 1950). It could be argued that such structural aberrations are more apparent than real and that these appearances are perfectly consistent with angiogenesis proceeding in the unstable cellular environment of a tumour, which interferes with growth, differentiation, remodelling and function of the neovasculature.

Our observations that LI tumour cells induce angiogenesis indicate that induction of angiogenesis does not appear to depend entirely on active proliferation of the tumour cell, but on some other metabolic activity (possibly secretion of TAF). Thus, we were unable to induce peritoneal angiogenesis with heat-killed (non-metabolizing) tumour cells. It is interesting that significant angiogenesis was not induced in the peritoneum of tumour-immunized rats by intact tumour cells. This may have been due to a rapid cytolysis of the tumour cells in sensitized rats and the inadequate production of TAF. We have observed a similar local failure of tumour angiogenesis in immunized rats challenged with intact W256 or Yoshida tumour cells when the latter were injected into the anterior wall of the stomach, but failed to grow into solid tumour. This seems to indicate that angiogenesis fails when tumour cells rapidly lose their capacity to liberate TAF during immuno-destruction, and that the absence of peritoneal angiogenesis in immunized rats was not simply due to the sequestration of ascites tumour cells at the site of implantation by the omentum or mesenteries which prevented their peritoneal spread and reactions with surface capillaries.

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