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Vessel invasion by tumour cells

An ultrastructural study*

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Summary. The present electron microscopic investigation explored the mechanism through which the cells of an experimental fibrosarcoma implanted in the liver of syngeneic mice broke through the continuous (unperforated) endothelium of peri-tumour veins to enter the blood stream, as well as the immediate reaction of blood cells to the tumour breakthrough.

It was found that at their point of contact with the endothelial tube of the peri-tumour vein, the tumour cells caused the endothelial basement membrane to disappear and they entered the vein lumen either by inducing an opening of interendothelial junctions, or by causing intensive vacuolation and disintegration of individual endothelial cells – and thus producing gaps for their passage into the lumen. Both mechanisms of entry were sometimes observed in the same tumour.

At their point of breakthrough into the vein lumen, many neoplastic cells were immediately covered by a dense platelet aggregate or they were surrounded by numerous polymorph leucocytes (neutrophils more often than eosinophils) that stuck to their surfaces and sometimes caused a focal disappearance of the tumour cell plasma membrane at the site of polymorph-tumour cell contact. Occasionally polymorph lysosomal granules migrated to such contact areas, and the plasma membrane of the contacting polymorph disappeared as well. Finally, some polymorphs apparently plunged into the cytoplasm of vessel-invading tumour cells, while others were seemingly phagocytised by the latter.

Key words: Neoplasia – Metastasis – Vessel Invasion – Endothelium – Neutrophils – Eosinophils – Platelets

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Introduction

Several years ago we produced in this laboratory through chemical carcinogenesis a rapidly growing, locally very aggressive fibrosarcoma in Balb/c mice, that has since been maintained through periodic transplantation (once every 3 weeks) in syngeneic hosts for more than 8 years (Constantinides and Harkey 1985). We recently found that although this tumour has never been able to invade vessels and metastasize systemically when implanted in a subcutaneous or intramuscular site, it will extensively invade peri-tumour veins and widely metastasize into distant organs when implanted in the liver or the kidney (Constantinides et al. 1987). The purpose of the present study was to explore with the transmission electron microscope (1) the mechanism through which the tumour cells break through the continuous endothelium of the peri-tumour veins in the liver, and (2) the immediate reaction of blood cells at the point of tumour breakthrough. This objective is of particular interest in view of the fact that so far the great majority of electron microscopic investigations in this area have focussed on the in vitro interaction between neoplastic cells and endothelial monolayers or on the extravasation of tumour cells after their intravenous injection (i.e. on a late stage of the metastatic process), rather than on the in vivo invasion of vessels, the crucial first step of the metastatic process.

Materials and methods

A tiny fibrosarcoma specimen roughly 1 mm size was implanted into the liver of 10 adult male Balb/c mice following a procedure described previously (Constantinides et al. 1987). Twelve days after implantation, at a time when the tumour implants had become vascularised spheroid masses approximately 5–6 mm in diameter surrounded by a basket of thin walled veins, the animals were killed with nembutal and a roughly 1.5 mm thick slice through the whole implant and the surrounding liver pa-

renchyma was obtained from every mouse and fixed immediately in buffered formaline. Subsequently, 10 small blocks of tissue from the tumour-liver border zone, including tumour implant periphery, peri-tumour veins and adjacent liver parenchyma were cut out of each formaline-fixed slice, post-fixed in glutaraldehyde-osmic acid and processed for electron microscopic study. Semi-thin (1 μ thick) toluidine blue stained sections of all 100 blocks were first examined light microscopically and from the 30 of these blocks that showed evidence of imminent, suspect or actual vessel invasion by tumour cells, ultra-thin

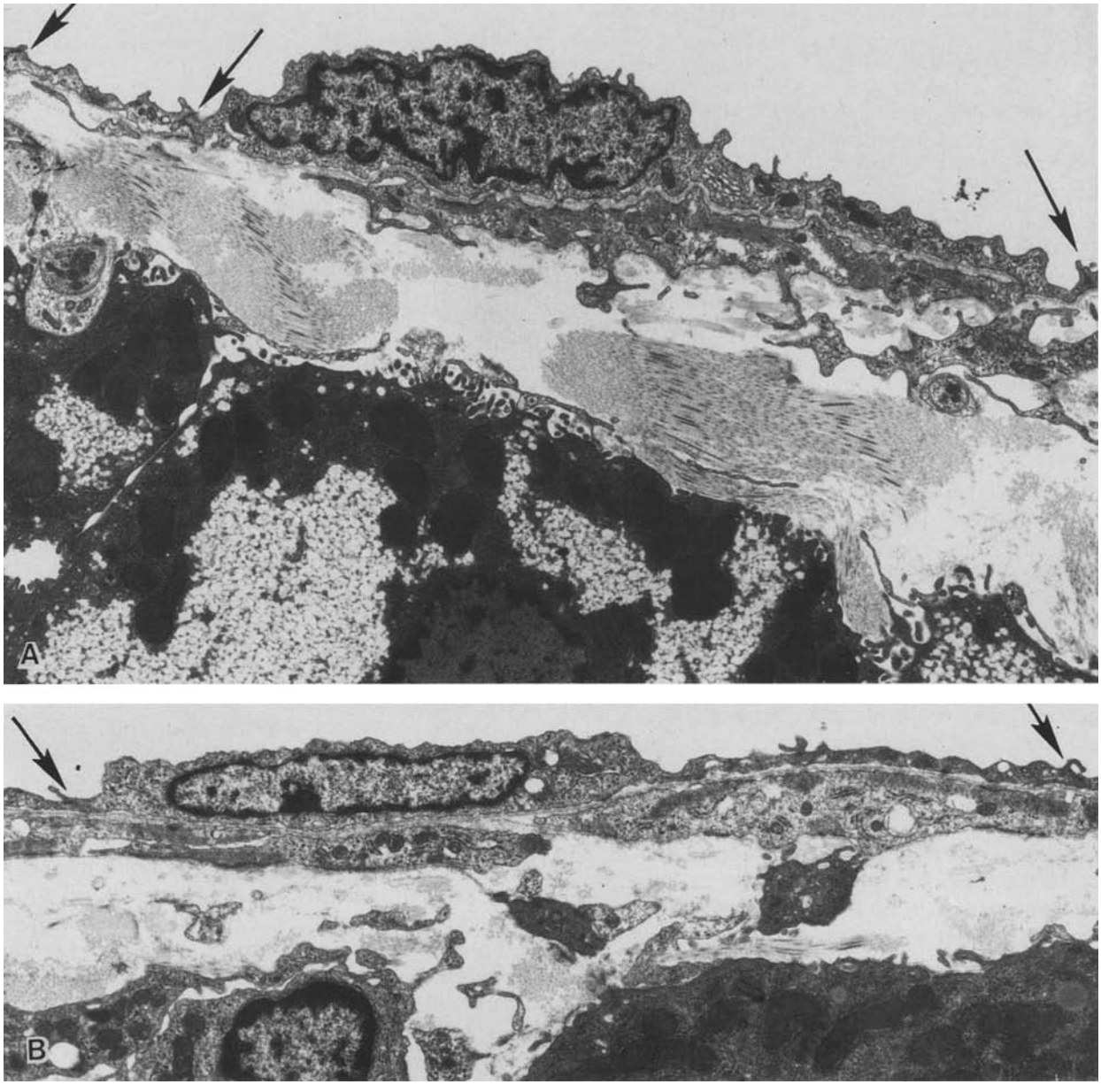


Fig. 1. A Typical wall of a peri-tumour vein that runs between the tumour implant and the surrounding liver parenchyma, as it always appears on the side of the vein that faces the hepatic parenchyma (the opposite from the one facing the tumour); the empty space above is the vein lumen. The wall consists of a thin and continuous (unperforated) endothelium with numerous closed interendothelial junctions (three of which are indicated by arrows), an underlying thin basement membrane, and a discontinuous layer of pericytes underneath the latter. A row of hepatocytes at the bottom of the print with microvilli, lucent (unstained) glycogen spaces, and a bile canaliculus at left faces the vein wall. The space between the vein wall and the hepatocytes contains some collagen bundles and a few remnants of extremely thin, perforated sinusoid walls adjacent to the microvillous hepatocyte surfaces. × 3000. **B** Another typical peri-tumour vein wall segment from the side of the vein that faces the hepatic parenchyma at the bottom of the print, with closed interendothelial junctions (*arrows*), basement membrane and pericytes. This picture shows that sometimes monocytes like the one on the left – but never polymorphs – patrolled the space between vein wall and hepatocytes. × 3000

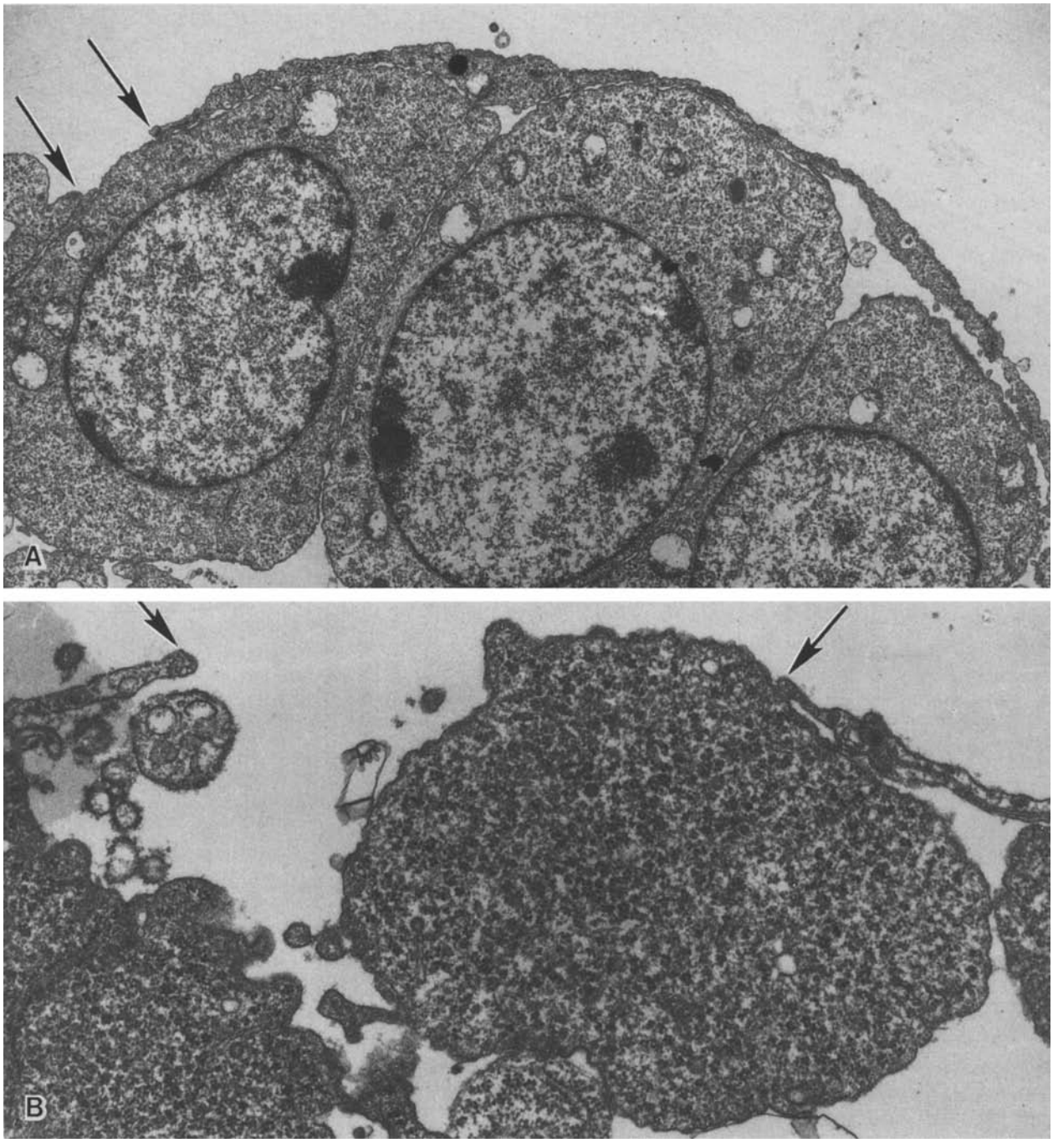


Fig. 2. A Wall segment of a peri-tumour vein from the side of the vein that faces the tumour implant (the opposite from the side that faces the hepatic parenchyma). Three tightly packed fibrosarcoma cells are pushing against the thin vein endothelium, stretching it and causing it to bulge into the lumen above. The endothelial basement membrane and the pericytes have disappeared at this area of tumour-endothelial contact and a small gap begins to develop in the endothelium at left (between the 2 arrows) seemingly the result of an opened interendothelial junction. There is no break in the endothelial plasma membranes at either edge of the gap. ×3000. **B** A more advanced stage in the opening of an interendothelial junction in the endothelium of a peri-tumour vein at the side of the vein that faces the tumour cells. It has produced a large gap in the endothelium (between the 2 *arrows*) through which a highly anaplastic fibrosarcoma cell brimming with polyribosomes is beginning to enter the vein lumen above. The gap has become as wide as the tumour cell directly underneath it. ×12000

sections of the areas of interest were further secured for transmission electron microscopic study of the vessel invasion process.

Results

As observed and documented in our earlier light microscopic study (Constantinides et al. 1987) most tumour cells were seen to invade the small thin-walled veins that had developed between the tumour implants and the surrounding liver parenchyma (the "peri-tumour" vessels) rather than the microvessels that had sprouted into the tumour implants themselves (the "intra-tumour" vessels). The present electron microscopic study showed that the wall of the preferentially invaded peritumour veins consisted of a continuous (unperforated) endothelium with numerous closed interendothelial junctions that was invested by a basement membrane and occasional pericytes, and their lumen, with a diameter of 100–200 μ was significantly larger than that of the intra-tumour microvessels or the normal liver sinusoids (Fig. 1).

At their point of close contact with the peritumour veins, the tumour cells caused the vein basement membrane to disappear, and they penetrated through the vein wall through two different mechanisms, which were often seen in the same animal: (a) by inducing an opening (unzipping) of the junctions between the endothelial cells of the veins (Fig. 2), and (b) by causing intensive vacuolation, necrosis and disintegration of individual endothelial cells and thus opening a gap for their passage into the lumen (Fig. 3). Sometimes tumour cells caused both endothelial vacuolation and opening of interendothelial junctions.

At their point of breakthrough into the vein lumen, the tumour cells often elicited the following two reactions of formed blood elements: (a) an aggregation of platelets on the surface of the tumour cell that was entering the lumen (Fig. 4), and (b) an adherence of many polymorph leucocytes (neutrophils more frequently than eosinophils) to the surfaces of the penetrating tumour cells (see Fig. 5). Sometimes at such sites of intimate contact between polymorphs and tumour cells lysosomal granules of the leukocytes migrated to the contact area (Fig. 6) and the plasma membrane of the tumour cell (or of both cells) disappeared (Fig. 7). Furthermore, what appeared to be free (extracellular) lysosomal granules were occasionally seen close to the polymorph-tumour cell contact areas. When a whole cluster of tumour cells broke into a vein lumen, polymorphs and platelets were attaching not only to its most superficial cells but often also infiltrated between the cells of its uppermost layers.

In two animals, several neutrophil polymorphs were embedded within the cytoplasm of vessel-invading tumour cells. Some of these neutrophils seemed to have penetrated actively into the tumour cell cytoplasm, since they were not enveloped by a phagosomal sac membrane (Fig. 8), while others appeared to have been swallowed up by the tumour cells since they were enclosed in phagosomal sacs.

Most above described ultrastructural changes associated with vein invasion by tumour cells were present in 8 of the 10 animals studied. Their frequency of incidence in those 8 mice is listed in Table 1.

Discussion

We found that our neoplastic cells crossed the walls of small veins to enter their lumen by disolving their basement membrane focally, opening their interendothelial junctions and destroying individual endothelial cells.

The focal dissolution of the veins' basement membrane prior to endothelial crossing by the fibrosarcoma cells was similar to the focal capillary basement membrane destruction by capillary-invading Erlich Ascites tumour cells described by Vlaeminck et al. 1972. It was most probably caused by collagenase type IV and other enzymes that are known to be secreted by many malignant cells (especially highly invasive lines) as seen in several recent in vitro studies of basement membrane and extracellular matrix degradation by chemical tumour action (Starkey et al. 1984; Thorgeirsson et al. 1985; Terranova et al. 1986; Liotta 1986).

The opening of interendothelial junctions by tumour cells has so far been documented ultrastructurally in in vitro studies of endothelium and tumour cultures, and in vivo studies of cancer cell penetration of lymphatic vessel walls as well as of extravasation from blood capillaries. The in vitro studies showed that several types of cancer cells can open interendothelial junctions in endothelial monolayers by inducing retraction (contraction) of adjacent endothelial cells (Kramer and Nicolson 1979; Almasio et al. 1983; and Vlodavsky et al. 1983). The investigations of lymphatic vessels revealed that certain tumour cells will extravasate from such vessels (after intravenous injection) by opening and slipping through their endothelial junctions, and that they can also enter lymphatics from a local foot pad injection site through the same process (Carr et al. 1976; Van der Velde and

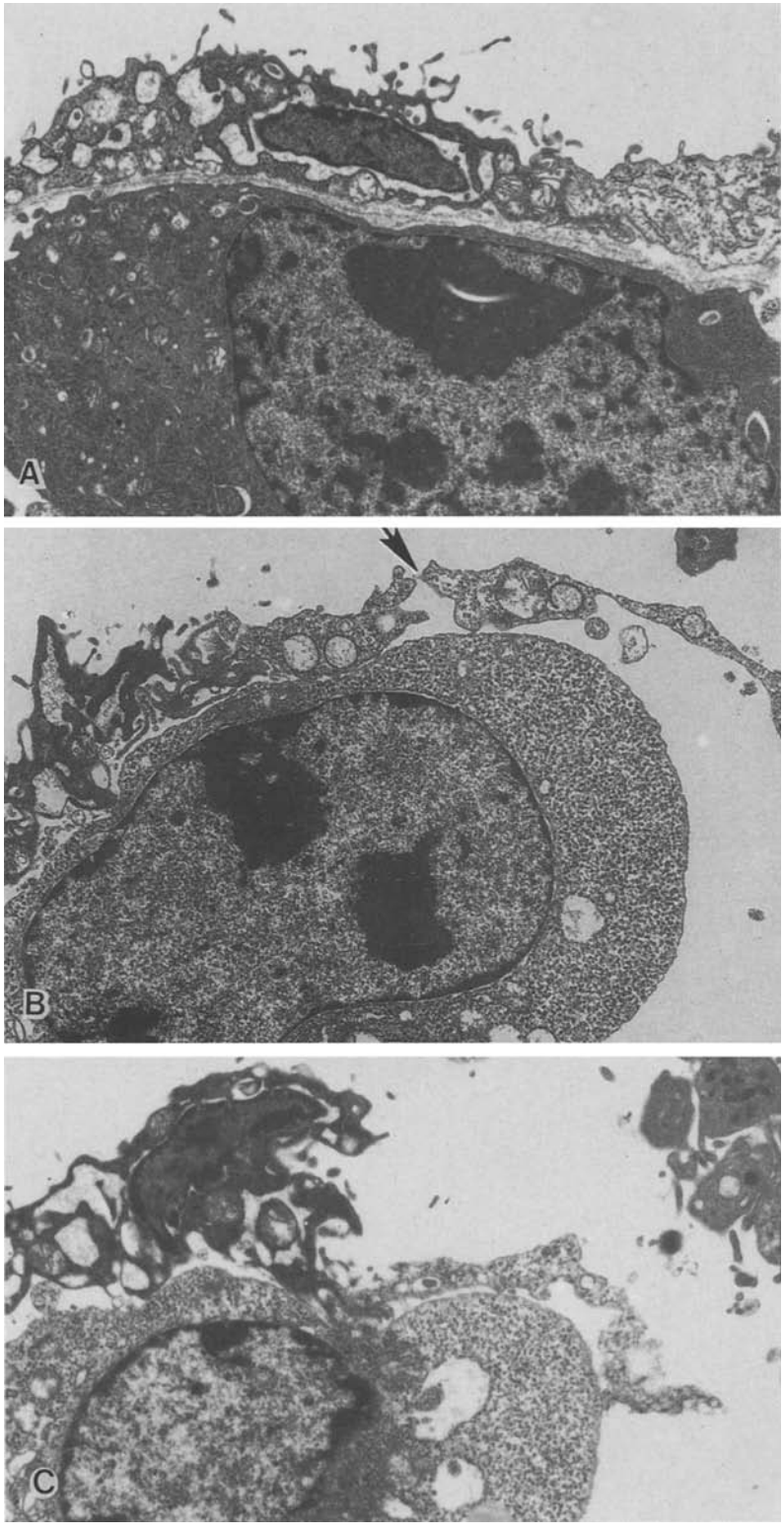


Fig. 3A–C. Destruction of individual peri-tumour vein endothelial cells (endotheliolysis) at sites of intimate contact between vein wall and tumour cells; the vein lumen is the upper space of the print in each case.
A Extreme swelling of endoplasmic reticulum sacs (including the perinuclear sac) in the left one of two endothelial cells overlying a huge fibrosarcoma cell; the junction between the two endothelial cells is still closed. × 3000
B Marked vacuolation of two endothelial cells and opening of the junction between them (at the arrow) over a large neoplastic cell. A platelet is approaching the injured endothelium. × 3000
C Extreme vacuolation and disintegration of an endothelial cell over a large tumour cell. A large gap has developed in the endothelial lining of the vein at right in association with the endothelial disintegration. × 3000

Carr 1977). The studies of extravasation from blood capillaries found that some cancer cells, after intravenous injection, emigrate from such vessels into the brain by pushing pseudopods and slipping through their endothelial junctions (Kawaguchi et al. 1982), while others, after subcutaneous inoculation, reach liver sinusoids and seemingly exit from them through pores or by causing retraction

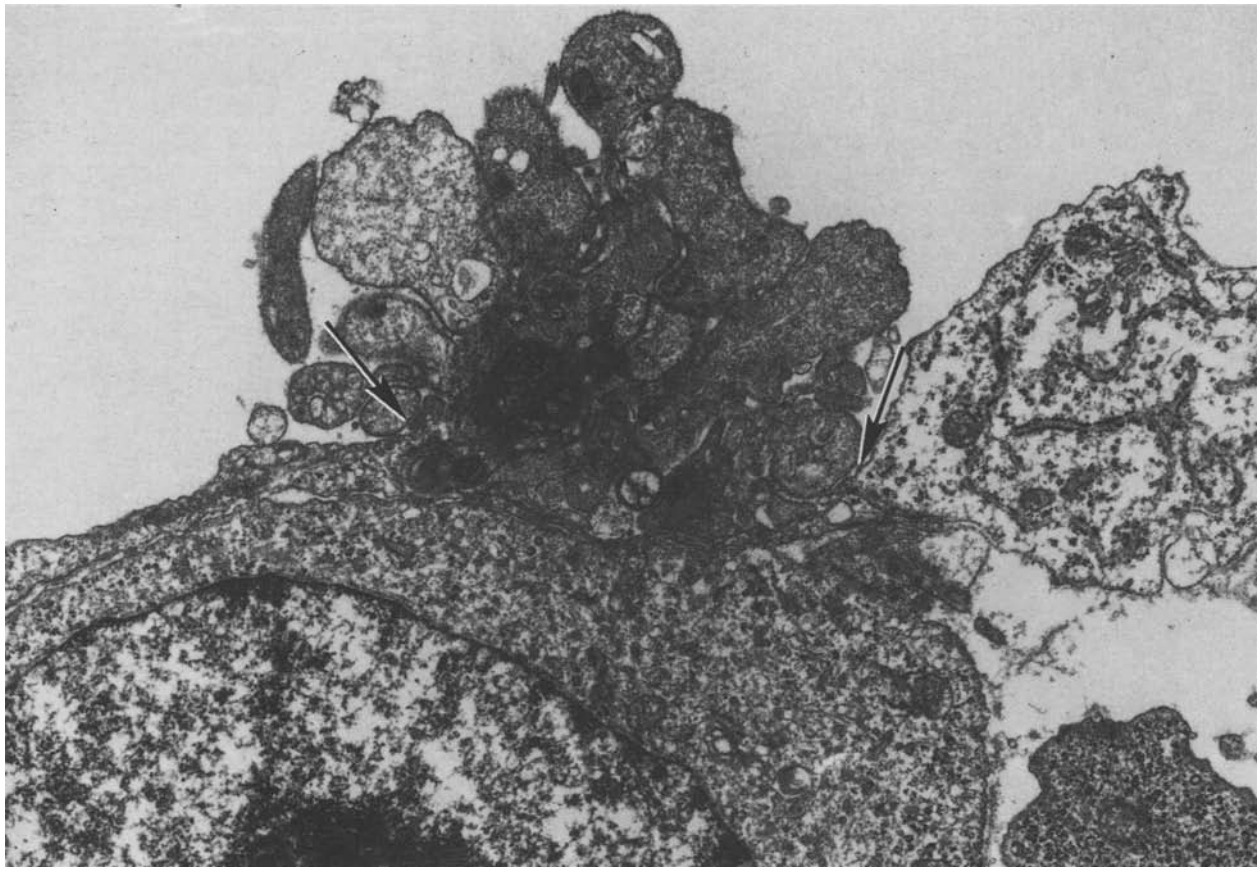
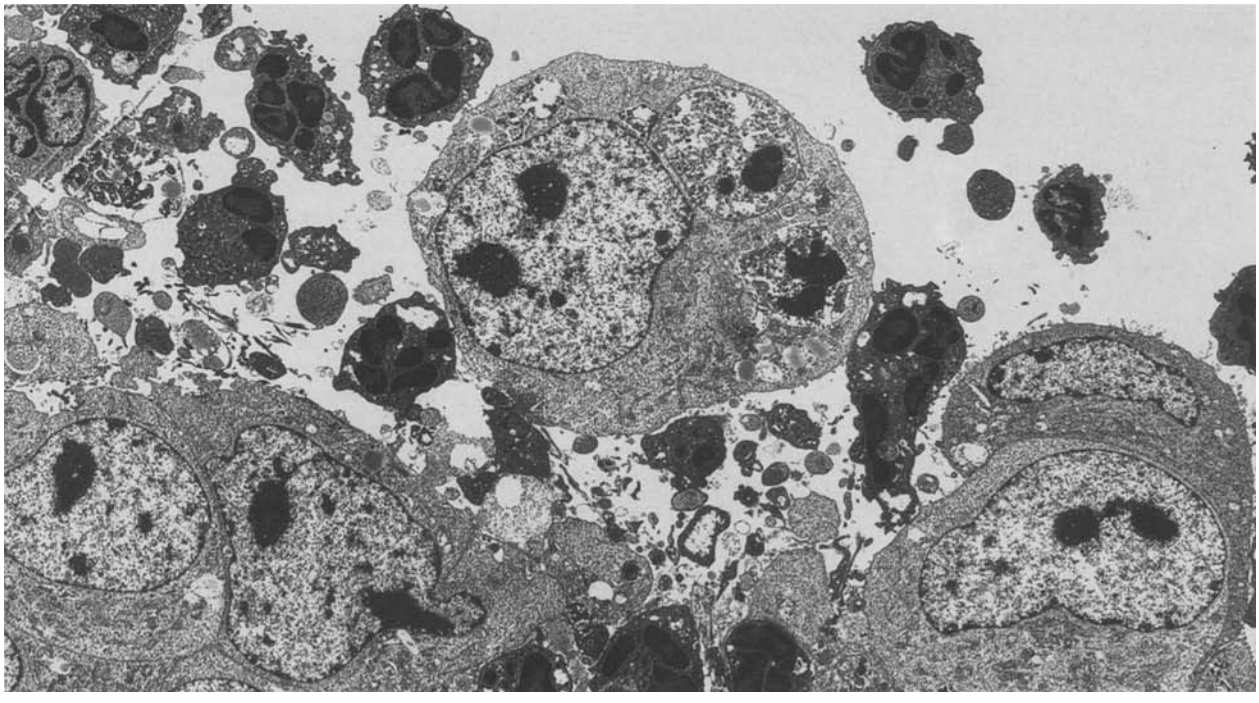


Fig. 4. A large fibrosarcoma cell (in the lower half of the print) faces a widely opened interednothelial junction (the gap between the *arrows*) in the wall of a peri-tumour vein whose lumen is the empty space above. A compact platelet aggregate has formed on the part of the neoplastic cell exposed to the lumen and has plugged the gap produced by the opened interendothelial junction. $\times 7000$



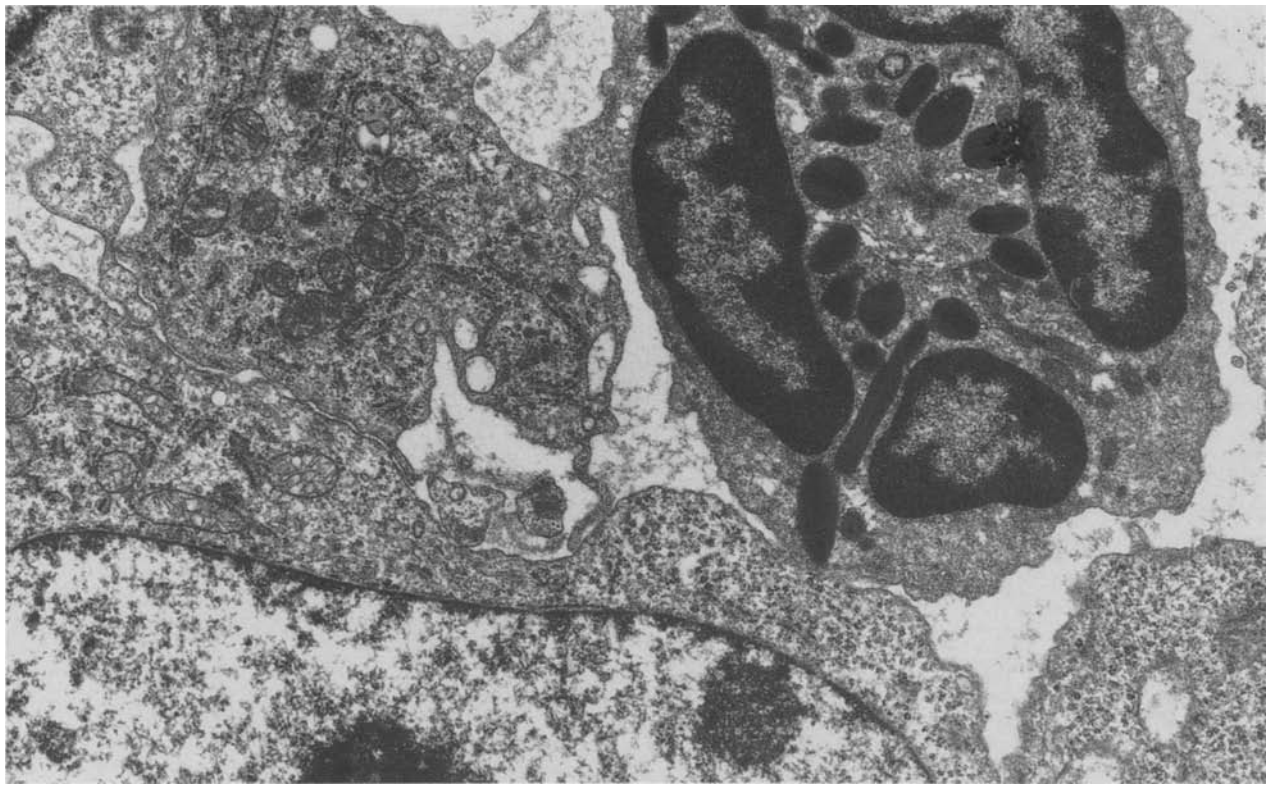


Fig. 6. An eosinophil polymorph leukocyte in the upper right has established contact with a tiny area of the surface of a large fibrosarcoma cell below it. A typical torpedo-shaped lysosome of the eosinophil has migrated to the site of contact between the two cells, where the plasma membrane of both cells is becoming fuzzy. × 7000

of their endothelial cells (Azarelli et al. 1985). The present study provides electron microscopic evidence that some neoplastic cells can also enter blood vessels by opening their endothelial junctions.

The process through which some fibrosarcoma cells open the endothelial junctions of the peri-tumour veins without endotheliolysis at their point of contact with the endothelium is unknown at present. It might involve secretion by the tumour cells of substances that dissolve the glue-like binding material between adjacent endothelial cells, or the secretion of vaso-active substances similar to inflammation mediators (such as amines, peptides and prostaglandins) that cause contraction of endothelial cells by stimulating their actomyosin filaments and thus produce interendothelial gaps (see overview in Constantinides 1984). Further research is needed to identify the junction-opening agent emanating from the cancer cells in this study, but whatever its nature may prove to be, it evindently requires contact or close proximity between tumour cells and endothelial junctions for its action.

The destruction of individual endothelial cells (endotheliolysis) leading to gaps in the peri-tumour vein walls that permitted entry into the vascular lumen of several other fibrosarcoma cells was evidently the result of some tumour cell action on the endothelium since it always occurred in the wall side facing (and closely contacted by) the tumour cells – never in the other side that was facing the normal hepatic parenchyma. It was most probably due to the production of an endotheliotoxic factor secreted by some of the neoplastic cells and

Fig. 5. A cluster of fibrosarcoma cells has broken into the lumen of a peri-tumour vein through a large gap due to endothelial destruction in its wall below the lower margin of the print. Numerous small polymorph leukocytes surround and begin to attach to the topmost neoplastic cell of the pack while others infiltrate deeper into the tumour cell cluster at the bottom of the print. In addition, to polymorphs have penetrated into the cytoplasm of the topmost fibrosarcoma cell (see higher magnification of the penetrated polymorphs in Fig. 8A. × 1000

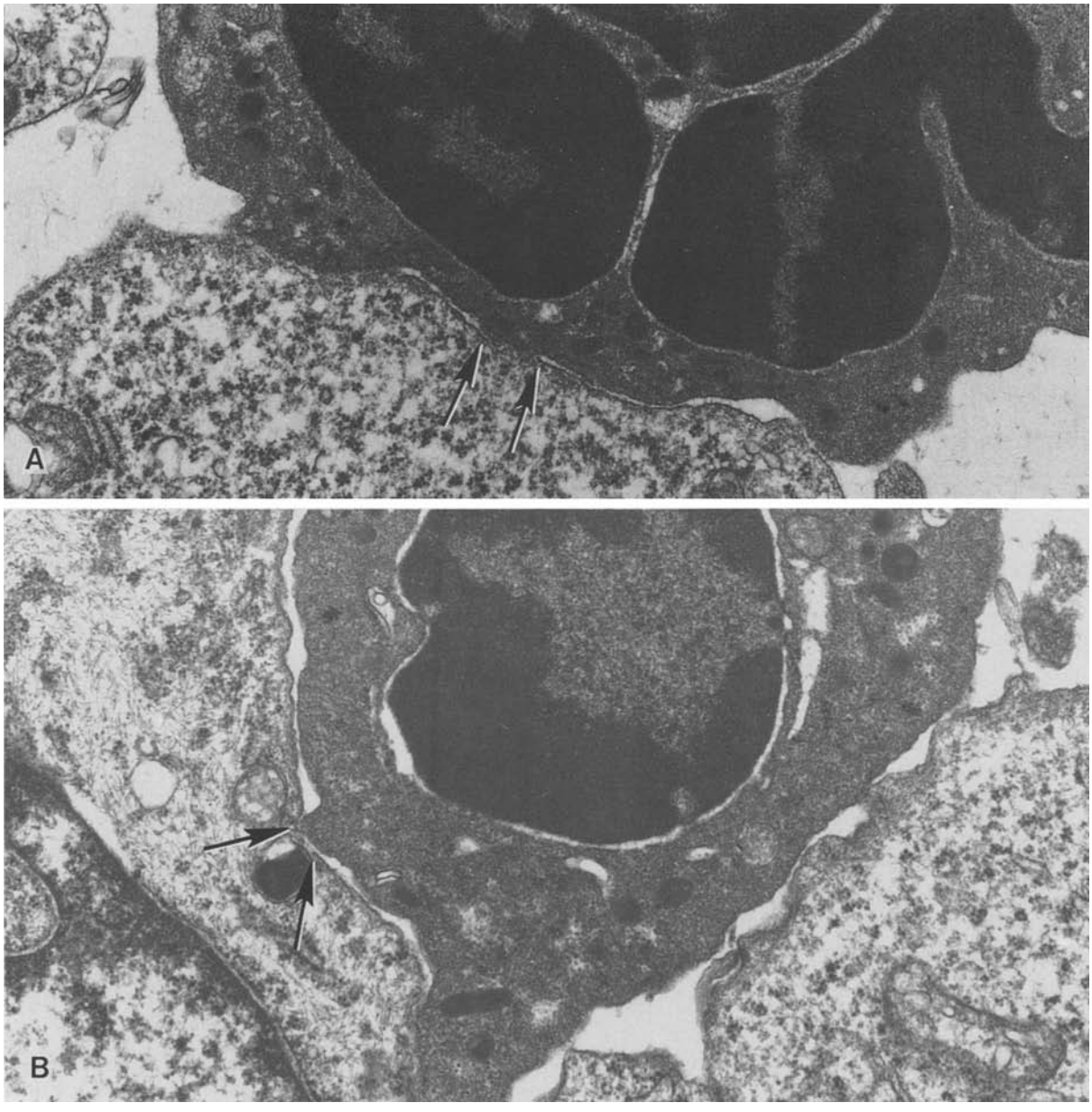


Fig. 7. A A neutrophil polymorph has established contact with the process of a fibrosarcoma cell below it. At one site of the contact area (between the two arrows) the plasma membrane of both cells has disappeared leading to fusion of the two cytoplasms. ×12000. **B** A neutrophil polymorph (with only part of one of its nuclear lobes visible in this field) has contacted two fibrosarcoma cells beneath it. At one point of the polymorph's contact with the left one of the neoplastic cells (between the two *arrows*) the plasma membrane of the fibrosarcoma has been interrupted. ×12000

acting at a very short distance from its production site.

Cytotoxic factors produced by certain cancer cells and capable of destroying normal host cells have been postulated for a long time on the basis of indirect evidence in the light microscopic era (Rössle 1949), and ultrastructural evidence in more recent years further supports their existence, as e.g. in the case of normal hepatocyte and striated muscle fiber destruction by tumour cell processes that plunge into their cytoplasm (Babai and Tremblay 1972; Babai 1976). Electron microscopic evidence of endotheliolytic action by cancer cells has, however, so far been reported only in extravasation of such cells from blood capillaries in one human lung biopsy (Fonk-Cussac et al. 1969) and in experiments with intravenous tumour cell injections in chick embryos (Vlaeminck et al. 1972). The re-

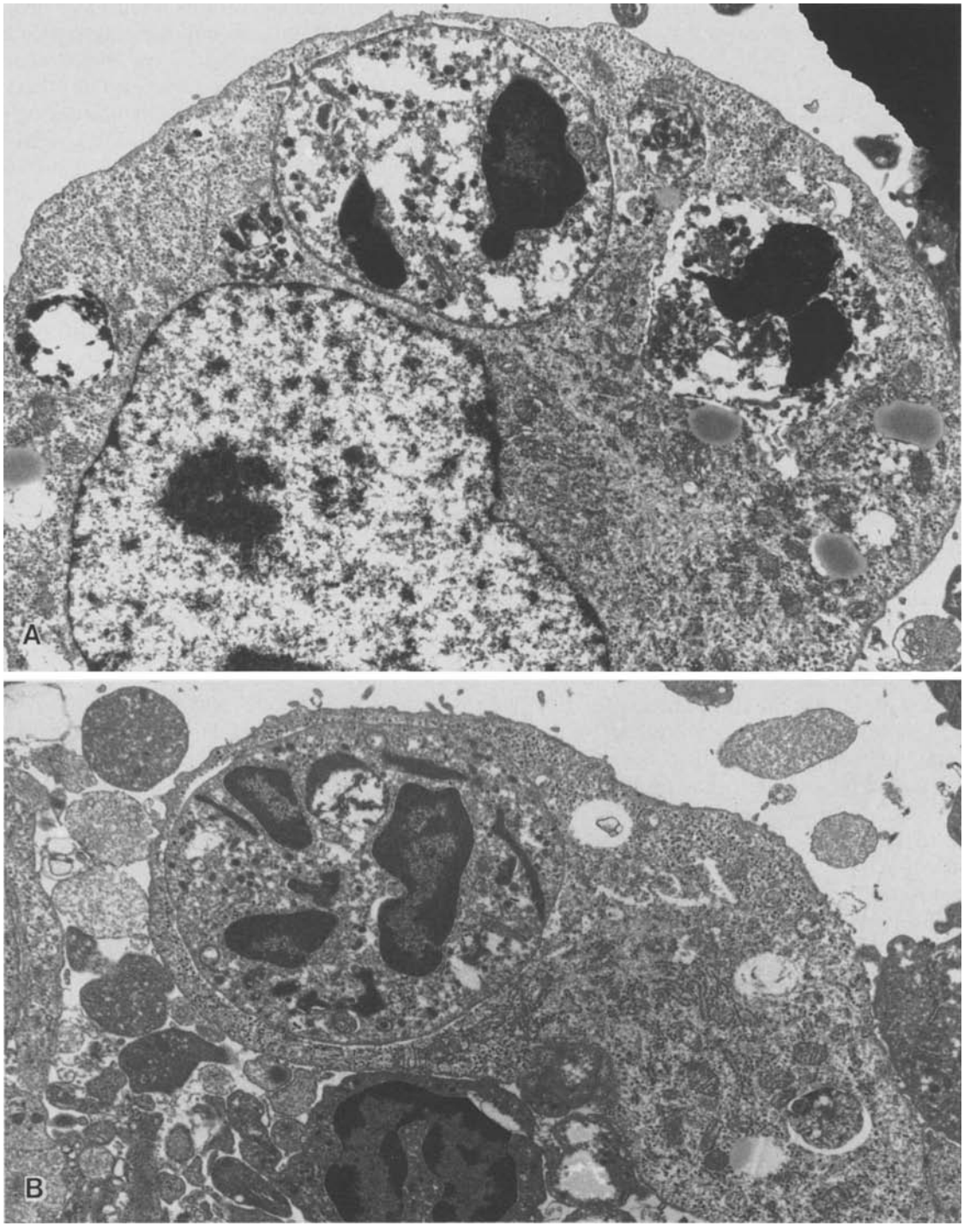


Fig. 8. A A higher magnification of the fibrosarcoma cell of Fig. 5 with the two neutrophils embedded in its cytoplasm, from another section. It clearly shows the absence of a phagosomal sac around the two polymorphs that have penetrated into the tumour cell, and the intracytoplasmic disintegration of the polymorph on the right. ×3000. **B** Another neutrophil embedded without an enveloping phagosomal sac in the cytoplasm of a fibrosarcoma cell that has just broken into the lumen of a peri-tumour vein. The tumour cell is partly surrounded by platelets and polymorphs. ×3000

Table 1. Incidence of various ultrastructural changes associated with vein invasion by fibrosarcoma cells observed in the 8 mice exhibiting such invasion

Specific ultrastructural changes associated with vein wall penetration by fibrosarcoma cells	Incidence
Focal basement membrane dissolution	7/8
Opened interendothelial junctions without endotheliolysis	5/8
Endotheliolysis at the point of contact between tumour cells and endothelial tube	7/8
Platelet aggregation on vessel-invading neoplastic cells	6/8
Polymorph attachment to vessel-invading neoplastic cells	8/8
Plasma membrane breaks of neoplastic cells at their point of contact with polymorphs	3/8
Active penetration of neoplastic cells by polymorphs	1/8
Phagocytosis of polymorphs by neoplastic cells	1/8

sults of the present study show that malignant tumour cells can destroy endothelial cells of blood vessels not only from the lumen side but also from outside. It is thus evident that the future identification of the endotheliotoxic factor(s) produced by certain malignant tumour cell could help develop neutralisers (anti-endotheliotoxins) that might inhibit or delay vessel invasion and thus facilitate the eradication of the primary tumour before any metastasis develops.

The fact that in the same tumour implant some fibrosarcoma cells opened interendothelial junctions without destroying endothelial cells while others induced endotheliolysis is evidently an expression of great phenotypic diversity among the neoplastic cells and is in line with recent insights into the marked functional heterogeneity and continuous emergence of new clones within both primary and secondary tumours (Nicolson and Poste 1983).

At their point of breakthrough into the vein lumen some – but by no means all – of the fibrosarcoma cells induced an aggregation of platelets mainly on the part of their surface that protruded into the lumen. Some experimental tumour types are known to cause platelet aggregation around their emboli that circulate in the blood stream or when they are added in vitro to platelet-rich plasma, while other types do not display any platelet aggregating activity (Gasic et al. 1973; Steiner 1982). Among the tumour species with the ability to aggregate platelets some seem to metastasize more vigorously because of this ability, as evidenced by the anti-metastasis effect of aggregation inhibitors or thrombocytopenia on such neoplasms, while the metastatic performance of others does not appear to be helped by their platelet aggregating property (Zbytniewski et al. 1981; Gordon et al. 1982; Ambrus et al. 1982a; Ambrus et al. 1982b; Willmott et al. 1983; Honn et al. 1985). The mechanism through which certain neoplasms cause platelet aggregation has been found to be complex, involving several processes such as the release of sialolipoprotein-rich plasma membrane microvesicles, phospholipid, ADP and prostaglandins and the activation of thrombin by the neoplastic cells (Lerner et al. 1983; Tohgo et al. 1984; Grignani et al. 1986), and it seems to be different in different tumour types (Esumi et al. 1987).

The immediate and vigorous reaction of neutrophil and eosinophil polymorph leukocytes to the vessel-invading fibrosarcoma cells at their point of penetration through the vein wall was unexpected, because so far all in vivo and in vitro studies of blood cell defense against tumour cell invasion and expansion – apart from humoral immune responses – have focussed exclusively on the role of mononuclear cells (cytotoxic T lymphocytes, "natural killer" cells and macrophages) in such defense (see review of Nicolson and Poste 1983). Yet there is no doubt that, at least in the case of some experimental tumours like our chemically induced fibrosarcoma, neutrophils and eosinophils represent an acute and immediate response of white blood cells to blood vessel invasion by neoplastic cells. At the point of tumour cell breakthrough into the vessel lumen many small polymorphs attached to each huge neoplastic cell and attacked it in two ways. The first and more frequent way consisted of the production of breaks in the tumour cell plasma membrane at the sites of polymorph-tumour cell contact through a process that, probably involved the release of polymorph lysosomal enzymes or other metabolites; it may be noted here that eosinophil granules, like the neutrophil ones, have been found to contain lysosomal enzymes (see review by Zucker-Franklin 1974). Sometimes the breaks occurred in both the polymorph and the neoplastic cell plasma membranes at their point of mutual contact, just as has been documented to occur at the sites of lymhocyte-tumour cell contact in other situations (Schönfelder and Wildführ 1977). The second and more rare attack mechanism consisted of the seemingly active plunge of polymorphs into the cytoplasm of tumour cells, as evidenced by the absence of an enveloping phagosomal membrane around

the neutrophils embedded within the neoplastic cells. A similar penetration of lymphocytes into the cytoplasm of various cells in vitro has been described as "emperipolesis", even though in some cases the embedded lymphocytes were shown to be phagocytically engulfed (Ioachim 1965).

In contrast to the active penetration of certain tumour cells by neutrophils, the seemingly true phagocytosis of some neutrophils by fibrosarcoma cells in one of our animals agrees with the known ability of certain tumours to phagocytise small particles (Rosner and Golomb 1982), parts of cells (Kerr and Searle 1972) or whole cells (Goldenberg et al. 1969; Falini et al. 1980; Ahmed 1981).

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