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Ultrastructure of invasion in different tissue types by Lewis lung tumour variants

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Summary. Ultrastructural studies on the interactions of low and highly metastatic 3LL tumour lines with the basement membranes (BMs) of capillaries, veins, muscles, nerves and adipose tissue were performed by injecting tumour cells into the foot pad of mice. Haematogenous dissemination is the principle route of metastasis formation. Cells from the highly metastatic line were able to penetrate the blood vessels more efficiently than those from the low metastatic line. This difference was mainly due to a more pronounced diapedesis-like activity of the 3LL-HH cells, and partly to the altered intratumour vessel architecture in the highly metastatic tumour line. There was no difference between the two lines in the ultrastructure and frequency of invasion of nerves and adipose tissue BMs. However, in the highly metastatic line an extremely efficient penetration of muscle cell BM was observed. These results provide further evidence that the interaction of tumour cells with the BMs of different tissue types is one of the main determinants in local and distant dissemination.

Introduction

In the process of metastasis formation tumour cells interact with different extracellular matrix elements and host cells (Nicolson 1982; Nicolson and Poste 1983; Warren 1981). The most important interactions take place between tumour cells and basement membranes (BMs) and endothelial cells of the different tissues or organs during tumour invasion and progression (Liotta 1986). The BMs of different tissues are not of the same biochemical composition (Martinez-Hernandez and Amenta 1983; Thorgeirsson et al. 1985) and the cell surface components of their endothelial cells are also different (Auerbach et al. 1987; Nicolson 1982).

Hydrolytic enzymes, such as collagenase IV (Liotta et al. 1980), cathepsin B (Sloane and Honn 1984) and heparanase (Nakamura et al. 1983) have been shown to have a role in the penetration of tumour cells through BM. Moreover, an elevated activity of these enzymes has been observed in highly metastatic tumour lines compared to their low metastatic counterparts (Liotta et al. 1980; Nakamura et al. 1983; Sloane and Honn 1984; Tryggvason et al. 1987). In vitro investigations on the interactions between tumour and endothelial cells have revealed that the highly metastatic tumour cells have an increased adhesive capacity to the endothelial cells and that more tumour cells are able to invade the endothelial layer than in low metastatic tumour lines (Korach et al. 1986; Waller et al. 1986). All these observations have been made on isolated BM components and in endothelial cell cultures. The in vivo interactions between the tumour cells of different metastatic ability and the different BMs have been less well studied.

In the present ultrastructural investigation we studied the in vivo interactions of the low and highly metastatic lines of the Lewis lung carcinoma (3LL) (Pal et al. 1983, 1985) with the BMs of capillaries, veins, muscles, nerves and adipose tissue when tumour cells were injected into the foot pads.

Materials and methods

In our experiments inbred C57 B1/6 mice from our institute were used.

The low metastatic 3LL tumour line was maintained by serial intramuscular transplantations (2×10^5 cells, hind leg muscle). The highly metastatic 3LL-HH tumour line, formerly established by in vivo selection, was maintained by serial intrasplenic transplantations of tumour cells obtained from metastatic foci in the liver (Pal et al. 1983, 1985).

A single-cell suspension from the 3LL line was obtained from a 7-day-old primary tumour; the 3LL-HH line was taken from a 14-day-old liver metastasis. Tumour tissues were cut by crossed scalpels and filtrated through a four-fold gauze. After centrifugation (1000 rpm) and washing (TC-199) the viability of the cells was determined with trypan blue solution; it was 50–60% and 40–50% in the case of the 3LL and 3LL-HH cells, respectively.

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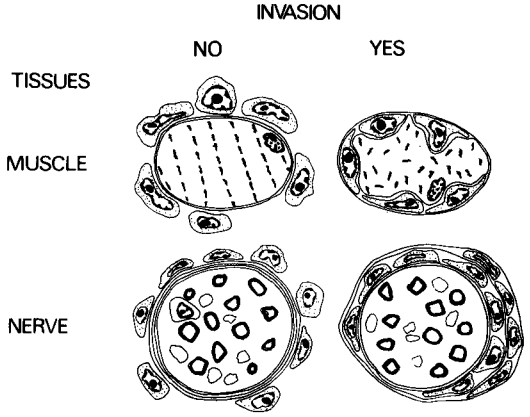


Fig. 1. Schematic representation of morphological criteria of invasion

For morphological studies from both tumour cell lines 10^6 viable cells were injected subcutaneously into the left hind foot pad of the animals. The tumours, together with the pad skin, were removed on the 5th, 7th, 8th or 9th day. The tissues were cut into pieces of 0.5×2 mm and were fixed for 2 h at 4° C in 2.5% glutaraldehyde (TAAB). After washing the tissue pieces were post-fixed in 2% OsO_4 (Merck), dehydrated in graded series of alcohol and embedded in Epon 815 resin (Polyscience). With this procedure we obtained 100 blocks from which 500 semi-thin and 100 ultrathin sections were cut. The photographs were taken by JEOL 100-B electron microscope. The semi-thin sections were stained with 0.5% toluidine blue (pH 8.5), while the ultrathin ones were contrasted with 2% alcoholic uranyl acetate and lead citrate.

The quantitative measurements of tumour invasion of the muscle fibres and peripheral nerves were determined in 5-, 7-, 9-day-old and in 9-day-old tumours respectively. The percentage of the cross-cut muscle fibres and nerves in which the signs of invasion could be observed was determined on semi-thin sections at a magnification of 400 × in the case of both tumour lines (Fig. 1).

Results

Invasion of both the lymphatic and blood vessels of the pad skin was observed in both tumour lines, although vascular invasion predominated. Invasion of the lymphatic capillaries took place by diapedesis between the endothelial cells in both tumour cell lines. Fragmentation of the endothelial cells was not seen during this process (Fig. 2). In contrast, in the invasion of the blood vessels considerable differences were observed between the two tumour lines.

In the low metastatic 3LL tumour we found that stromal vessels with a continuous BM predominated. However, some veins with elastic layers also occurred. In these vessels the tumour cells pushed the BM and the endothelial cells deeply into the lumen, thus separating them from the connective tissue (Fig. 3a). The tumour cells invaded the BM (Fig. 3d) and sometimes the endothelial cells with small pseudopodia. This was followed by the complete fragmentation of endothelial cells (Fig. 3a) and tumour cells entering the lumen (Fig. 3a, b). Occasionally fragmentation of the endothelial cells – which were pushed into the lumen – was observed before the tumour cells had invaded the BM (Fig. 3c). In veins with elastic layers this process was preceded by the penetration of the tumour cells through the natural holes of the elastic layer by a diapedesis-like movement (Fig. 3a).

In the highly metastatic tumour line, however, a different mode of invasion was observed. In the 3LL-HH tumour most of the stromal vessels had no BM. The cells that were in direct contact with the endothelial layer induced retraction and fragmentation of endothelial cells (Fig. 4) without pushing them into the lumen of the vessels. The holes caused by the fragmentation were penetrated by a diapedesis-like movement (Fig. 5a, b). This movement of the 3LL-HH cells was also observed through the wall of veins with elastic layers and BM.

Muscular invasion in both tumour lines was first manifest by the penetration of tumour cells in between the muscle cells which were separated from each other by oedematous connective tissue.

In the 3LL tumour the extent of invasion of muscle cells remained constant throughout the time of examination and occurred only in about 1.5% of fibres (Table 1). Electron microscopic investigations revealed tumour cells between the intact muscle fibers, not penetrating the BM (Figs. 6, 7).

In contrast, in the 3LL-HH tumour, between the 5th and 9th days invasion of the muscle cells increased markedly and at day 9, 25 times more muscle cell were invaded than in the 3LL tumour (Table 1). Under the electron microscope an apparent local solubilization of the BM of the muscle cells in direct contact with the tumour cells was seen (Fig. 8a, b). Through the holes produced tumour cells penetrated into the space between the muscle cells and the BM and spread on the surface of the latter (Figs. 8a, 9a). Usually plenty of tumour cells got underneath the BM of a single muscle cell, thus diminishing the connection between the muscle cells and their BM (Figs. 8a, 9a). In these "separated" muscle cells signs of degeneration were observed, with dilatation of the sarcoplasmic reticulum, disorganization of myofibrils and fragmentation of the plasma membrane (Fig. 9b). Occasionally the complete lysis of the muscle cells was seen, while the BM remained intact (Figs. 10, 11).

Table 1. Morphometric analysis of the muscle and nerve fibre invasion by 3LL tumour cells

Cell type	Day	Muscle fibres		Nerve fibres	
		Total number[a]	Incidence of invasion (%)	Total number[a]	Incidence of invasion (%)
3LL	5	921	1.5	ND	ND
3LL-HH		1057	5.2		
3LL	7	881	1.0	ND	ND
3LL-HH		987	15.8		
3LL	9	1741	1.6	323	67.4
3LL-HH		1171	43.0	287	87.0

3LL, Low metastatic Lewis lung tumour cells; 3LL-HH, highly metastatic Lewis lung tumour cells

[a] Total number of cross-sectioned muscle or nerve fibres studied in semi-thin sections

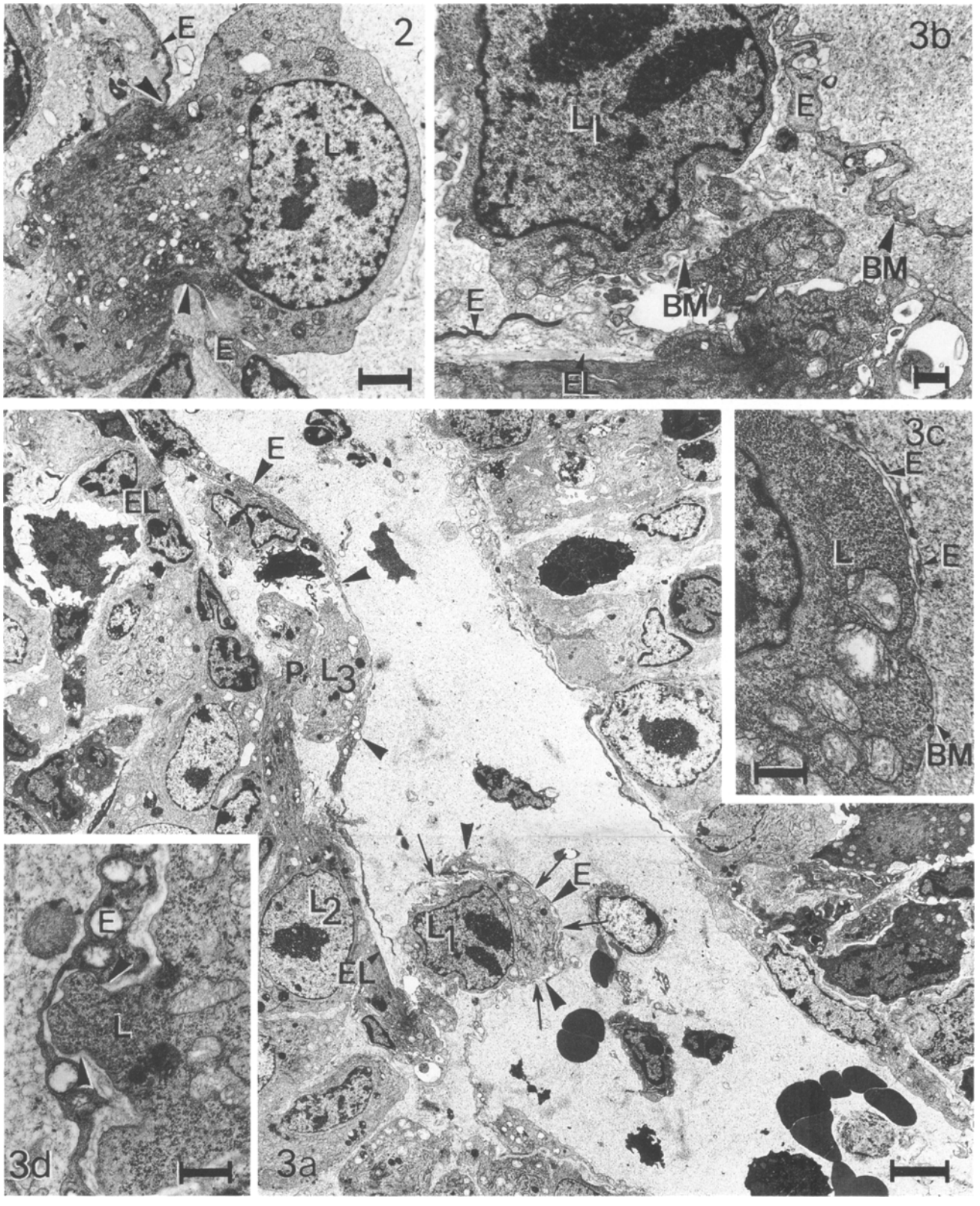
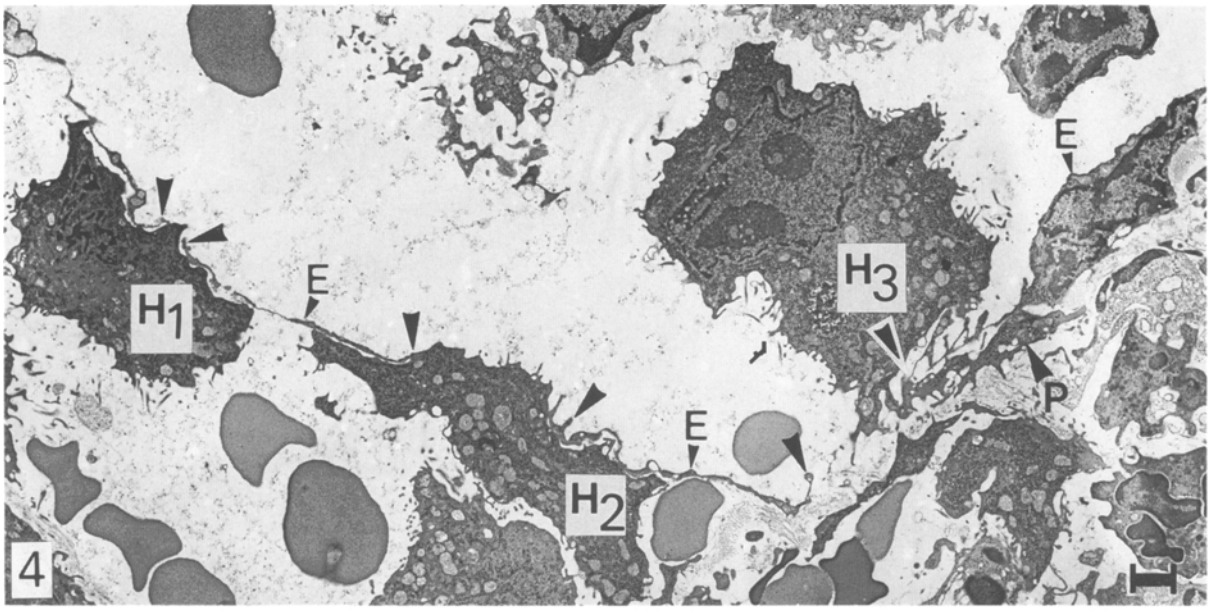
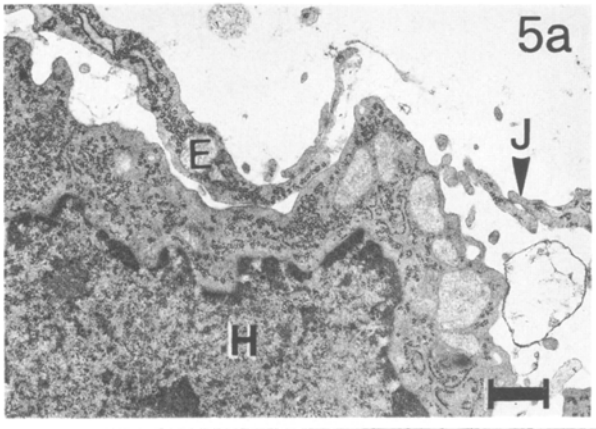
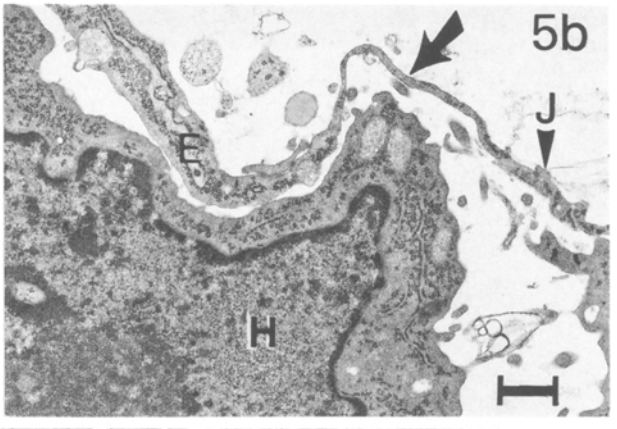


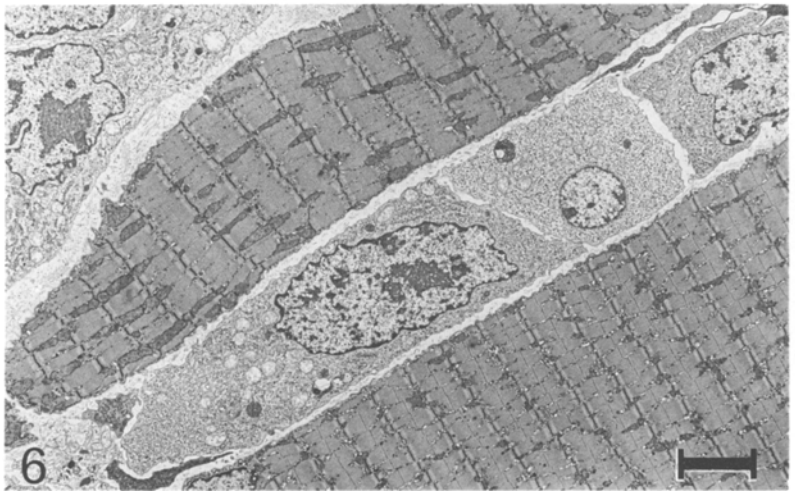
Fig. 2. 3LL tumour cell (*L*) apparently entering a lymph-vessel lumen (*arrow heads*) while the endothelium (*E*) is intact. ×4500; *bar* = 2 μm

Fig. 3. a Elastic lamina-containing vein in the 3LL tumour. Tumour cells (L_1, L_3) are pressing the endothelium (*E*) into the lumen (*arrowheads*). In front of the L_1 tumour cell the endothelium is fragmented (*arrows*). Another tumour cell (L_2) penetrates the elastic lamina (*El*) with a cytoplasmic process (*P*). ×2000; *bar* = 5 μm. **b** Part of Fig. 3a at higher magnification. The tumour cell (L_1) has almost entirely crossed the basement membrane (*BM*). *El*, Lamina elastica; *E*, endothelium. ×6000; *bar* = 1 μm. **c** Intact BM in front of the tumour cell (*L*) entering into the lumen, while the endothelium (*E*) has already been fragmented. ×9000; *bar* = 1 μm. **d** Crossing of the BM (*arrowheads*) by a 3LL tumour cell (*L*). The endothelium (*E*) is still intact. ×9000; *bar* = 1 μm









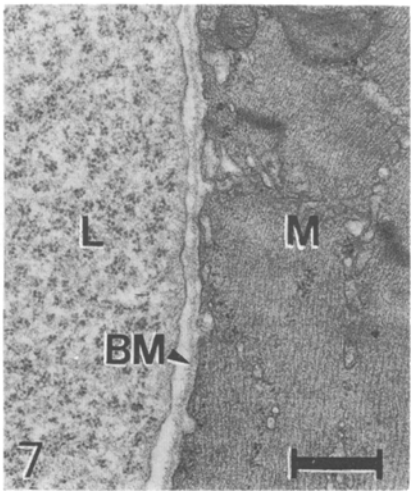


Fig. 4. Vessel without BM in the 3LL-HH tumour. Tumour cells (H_1, H_2, H_3) at different stages of intravasation (*arrowheads*). H_3 cell still has a small cytoplasmic process (*P*) in the connective tissue. *E*, Endothelium. ×2700; *bar* = 2 μm

Fig. 5a. 3LL-HH tumour cell (*H*) penetrating the endothelial lining (*E*) lacking BM by a cytoplasmic process. *J*, intercellular junction. ×8000; *bar* = 1 μm. **b** Cellular interactions shown in Fig. 5a at a different section. Note the absence of cellular junction in front of the tumour cell process (*arrow*). ×8000; *bar* = 1 μm

Fig. 6. 3LL tumour cells among muscle cells. There is no degeneration observable in the latter. ×2000; *bar* = 5 μm

Fig. 7. Intact basement membrane (*BM*) of the muscle cell (*M*) in the presence of a 3LL tumour cell (*L*). ×12000; bar = 1 μm

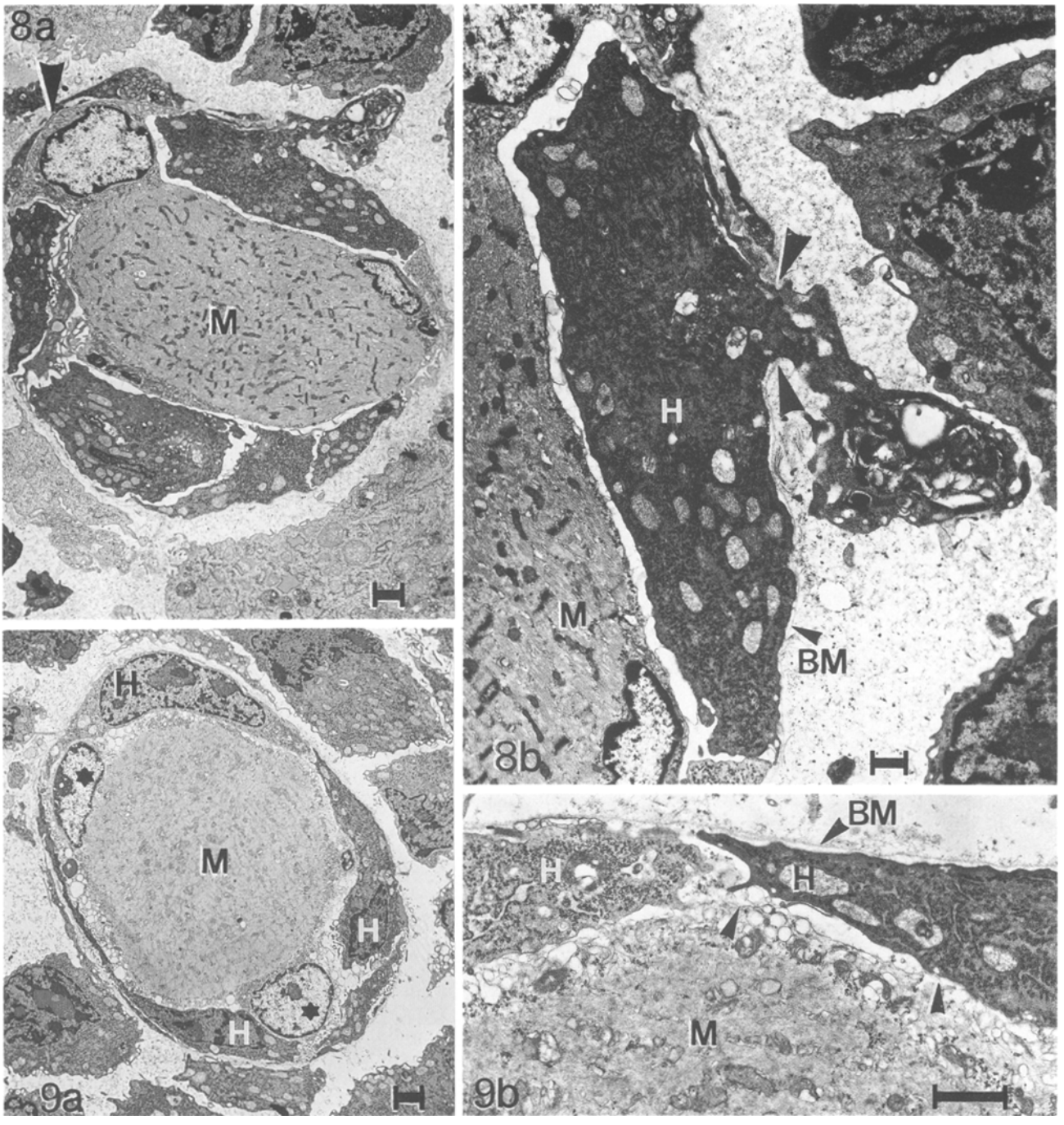


Fig. 8a. 3LL-HH cells have crossed the BM but have not invaded into the muscle cell (*M*) which is still in contact with the BM over a small surface area (*arrowhead*). ×2500; *bar* = 2 μm. **b** Part of Fig. 8a at higher magnification. Diapedesis of a 3LL-HH cell (*H*) through a micro-discontinuity (*arrowheads*) of the basement membrane (*BM*). *M*, Muscle cell. ×5700; *bar* = 1 μm

Fig. 9. a Invaded muscle cells, surrounded by 3LL-HH cells (*H*) showing karyolysis (*asterisks*) and dilatation of the sarcoplasmic reticulum. ×2500; *bar* = 2 μm. **b** Part of Fig. 9a at a higher magnification. Note the fragmentation of the muscle cell (*M*) plasma membrane (*arrowheads*) and the disorganization of the myofilaments. *H*, 3LL-HH cell. ×12000; *bar* = 1 μm

The invasion of the nerves took place in a similar way in both tumour lines. During the interval studied (5–9 days) the tumour cells were usually able to penetrate the multiple layers of the perineurial endothelial-like cells and their BM (Fig. 12). Tumour cells were rarely seen in the endoneurium, and the BM of the Schwann cells was never invaded. Morphometric analysis performed on day 9 showed no significant difference between the two tumour lines in the frequency of invasion of the peripheral nerves (Table 1).

The invasion of the adipose tissue was similar to that of the nerve fibres. Both tumour lines were able to penetrate the BM of the adipocytes. The cells then spread on the inner surface of the BM, coming into

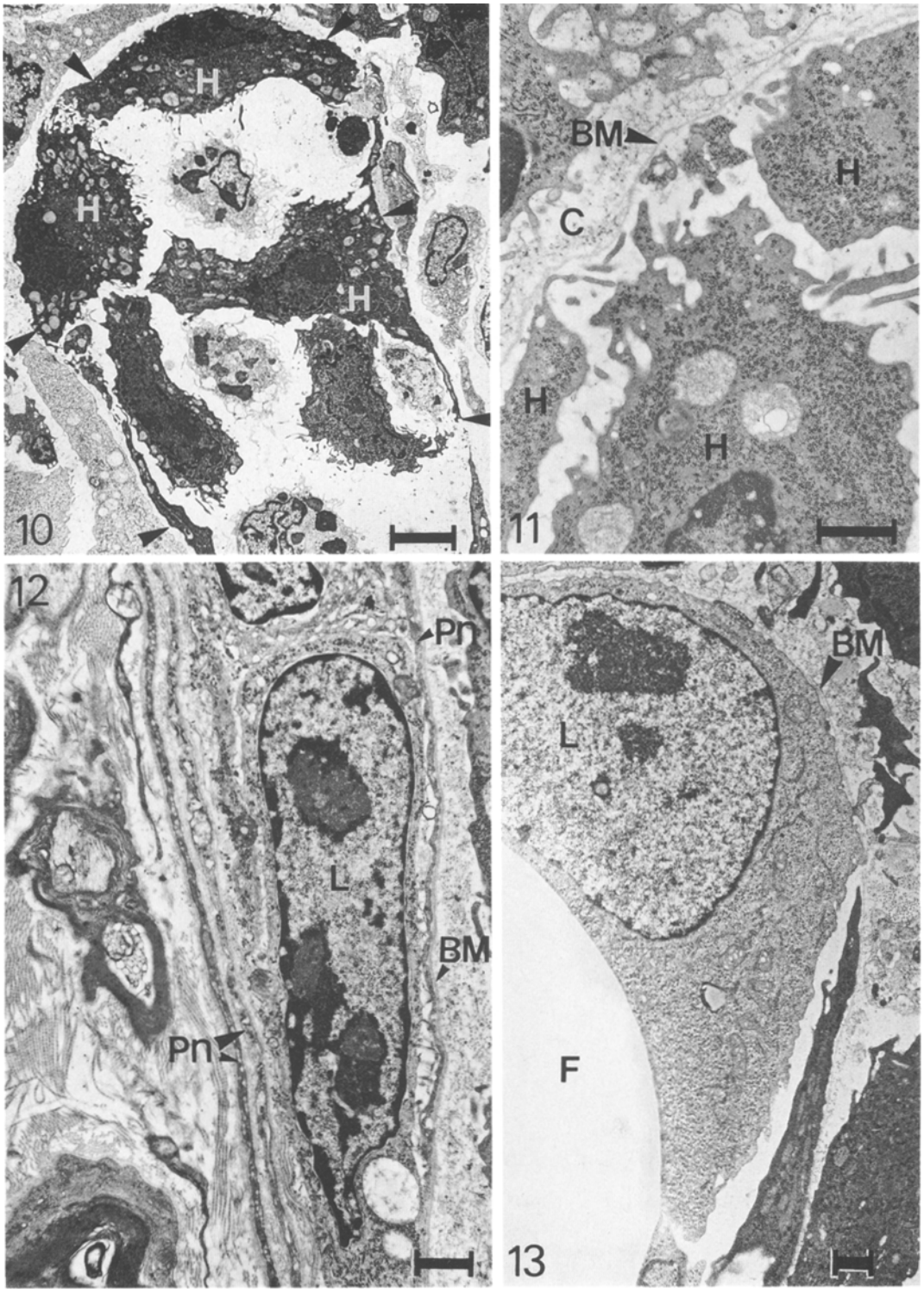


Fig. 10. Complete lysis of the muscle cells in the last phase of invasion. 3LL-HH tumour cells (*H*) adhering firmly to the BM (*arrowheads*). ×2000, *bar* = 5 μm

Fig. 11. Intact BM after the complete lysis of the muscle cells. *C*, Collagen; H, 3LL-HH cells. ×12000, *bar* = 5 μm

Fig. 12. Invasion of perineurial cells and their basement membrane (*BM*) by a 3LL tumour cell (*L*). ×4500; *bar* = 2 μm

Fig. 13. Invasion of an adipocyte (*F*) and its basement membrane (*BM*) by a 3LL tumour cell (*L*). ×5300; *bar* = 1 μm

contact with the plasma membrane of the adipocytes (Fig. 13). Degeneration of the adipocytes was not observed during the experimental period. Morphometrically, no difference was seen between the two tumour lines in the frequency of invasion of the adipocytes (data not shown).

Discussion

After having been injected into the foot pad Lewis lung tumour cells were able to invade both lymphatic and blood vessels. However, vascular invasion occurred more frequently; the haematogeneous route can therefore be considered the main mode of metastasis formation in both 3LL tumour lines. Invasion of the lymphatic vessels took place by a diapedesis-like process as was described for the Rd/3 anaplastic tumour (Carr et al. 1976), 13762 breast adenocarcinoma (Carr and Carr 1983) and BSp73ASML pancreas adenocarcinoma (Paku et al. 1986).

Both 3LL tumour lines studied contained large stromal veins with elastic layer and capillaries with BM, but in the highly metastatic variant (3LL-HH) more stromal veins and capillaries lacking BM were present. A considerable difference was found between the two Lewis lung tumour lines regarding their invasiveness of these blood vessels. (Table 2 summarizes the invasive characteristics of the two tumour lines.) The low metastatic 3LL cells were not able to penetrate the endothelium of the subcutaneous blood vessels by a diapedesis-like movement as was observed in the lymphatic vessels. In contrast, they penetrated the elastic layer of the large veins by such a diapedesis-like movement, and they were also able (like breast cancer cells) to invade both the BM and the endothelial lining by pseudopodia (Song et al. 1986). The fragmentation of the endothelial cells was possibly due to mechanical pressure from the tumour cells (Kitinya et al. 1988). This theory is supported by the observation that endothelial cells separated from the tumour cells by an intact BM were also fragmented.

A proportion of the highly metastatic 3LL-HH cells were able to penetrate the endothelium of large veins with an elastic layer and BM transcellularly (by diapedesis), as well as the endothelium of capillaries without BM. The same type of penetration has been seen in liver sinusoids (by 3LL) (Timar et al. 1983) and also in the case of leaukaemic and melanoma cells (De Bruyn and Cho 1979, 1982). The retraction and fragmentation of the endothelial cells could be caused by a factor produced by the tumour cells (De Bruyn and Cho 1979, 1982; Constantinides et al. 1989).

The diapedesis-like movement of the highly metastatic tumour cells through blood vessel walls and the special neovasculature of the tumour may provide a better opportunity for these cells to enter the circulation than in the low metastatic 3LL variant.

In studying the invasion of other tissue types we demonstrated that the cells of both 3LL tumour lines were able to penetrate the BM of perineurial cells and adipocytes. However, at day 9 the highly metastatic 3LL-HH tumour line was 25 times more effective in the penetration of the BM of the muscle cells than the low metastatic 3LL line.

Table 2. Differences in the invasion of different vessel and tissue types between the low and highly metastatic Lewis lung tumour cell lines

Vessel and tissue type	Cell type	
	3LL	3LL-HH
Lymphatic vessels	By a diapedesis-like process between endothelial cells	
Blood vessels		
Elastic lamina	By diapedesis through the natural holes	
Basement membrane	Pushing into the lumen, fragmentation by pseudopods	By a diapedesis-like process through endothelial cells
Endothelium		
Muscle	Laying between the muscle fibres	Penetration of the BM, disorganization of the cell
Nerve	Penetration of multiple layer of perineurial cells and their BM	
Adipose tissue	Penetration of the BM of the adipocytes	

BM, Basement membrane

Before invasion of the muscle cell BM we did not observe significant distortion of the muscle cells, and the BM remained intact even after the complete dissolution of the muscle cells. Based on these observations, we do not think that the local dissolution of the BM is a consequence of the decreased BM synthesis of the muscle cells, as suggested by Gabbert et al. (1987) for sarcoma and carcinoma cell lines. In our experiments, we believe that the local dissolution of the BM was probably produced by hydrolytic enzymes. A similar type of invasion of the muscle cells was described in different tumour lines by Babai (1976). The highly metastatic 3LL-HH cells spread on the inner surface of the BM but did not enter the muscle cell itself, as described by Galasko and Muckle (1974) in the case of the VX2 carcinoma. This observation indicates an increased affinity of the 3LL-HH cells to the BM lamina rara. Their high affinity, which was also observed in in vitro studies (Lapis et al. 1986), may have an important role in their more efficient extravasation compared with the low metastatic variant. The degeneration and lysis of the muscle cells was due to the fact that they became separated from their BM by the interposed tumour cells, and thus lost their oxygen and nutritional supply.

There may be a cellular subpopulation in the highly metastatic 3LL-HH tumour which is able to produce specific lytic enzymes in an increased amount or with an increased activity as seen in other systems (Liotta et al. 1980; Nakamura et al. 1983; Thorgeirsson et al. 1985). These enzymes would be able to degrade all BMs of the studied tissues.

Our data provide further support to the theory that the interaction of tumour cells with the BMs of a wide range of tissue types is crucial in the invasion (Dingemans 1988). Relying on the definite, although poorly characterized differences in the composition of BMs of the different tissues (epithelia, endothelium, muscles, nerves and adipose tissue) we support the view that the selective recognition and degradation of BMs could be partially responsible for organ-specific metastasis of tumour cells (Nicolson 1988).

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