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INFILTRATION OF TUMOUR CELLS INTO CULTURES OF ISOLATED HEPATOCYTES

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SUMMARY

MB 6A lymphosarcoma and TA3 mammary carcinoma cells have previously been shown to infiltrate from liver blood vessels, where they had been arrested, into the liver parenchyma. The same tumour cells were presently added to cultures of isolated hepatocytes, 24 h after their isolation.

Both tumour cell types adhered to both dorsal and lateral surfaces of hepatocytes. The lymphosarcoma cells rapidly infiltrated between the hepatocytes. They first extended pointed pseudopods between the liver cells, and when the tumour cell body intruded, they deeply invaginated the liver cells at an interhepatocyte boundary. The MB 6A cells accumulated between and under the hepatocytes, and after 24 h virtually all cells were contained within the cultures.

TA3 cells also invaginated hepatocytes, not only at interhepatocyte boundaries, but all over the exposed surface. They did not extend pseudopods. The process was much slower than with MB 6A cells: After 24 h a few TA3 cells were completely encircled by hepatocytes. These observations indicate that the mechanism of infiltration is different for the 2 tumour cell types.

Part of the TA3 cells did not invaginate the hepatocytes. Several of these cells spread on the hepatocyte surface, attaining a flattened shape.

TA3 cells formed extensive tight junctions with the hepatocytes, sometimes sealing an intercellular lumen that resembled both a tumour acinus and a bile canaliculus. Also desmosomes were occasionally formed.

The hepatocyte cultures appear to be a suitable model for studying the mechanism of liver infiltration, not only of tumour cells, but also of leucocytes.

INTRODUCTION

Blood-borne tumour cells that have been arrested in the liver may infiltrate through the sinusoidal endothelium and into the liver parenchyma (Dingemans, 1973, 1974). Infiltration may be of decisive importance for the formation of metastases, since it enables the cells to escape from the blood stream, in which most tumour cells rapidly die (Roos & Dingemans, 1979). In certain circumstances normal leucocytes may also infiltrate into the liver (Dingemans, Roos, Van den Bergh-Weerman & Van de Pavert, 1978).

Infiltration does not seem to proceed similarly in different tissues. For instance, lymphosarcoma cells that rapidly and massively infiltrate into the liver, remain inside the blood vessels of the lungs (Dingemans, 1973). Tumour cells and also leucocytes, added to monolayers of endothelium, derived from arteries or umbilical cord veins, move through gaps between the endothelial cells (Kramer & Nicolson, 1979; Beesley

et al. 1979), whereas in liver sinusoids they move through openings in, rather than between, the endothelial cells (Roos, Dingemans, Van de Pavert & Van den Bergh-Weerman, 1977, 1978; Dingemans *et al.* 1978). Also subcutaneously transplanted leukemia cells move through temporary migration pores in, and not between, endothelial cells, when infiltrating into the tumour blood vessels (De Bruyn & Cho, 1979). These examples indicate that the extent and mode of infiltration are not only due to properties of the infiltrating cells but also of the infiltrated tissue. Thus, the mechanism should be studied separately in different tissues, and the appropriate normal cells should be employed in models *in vitro*.

Previously we have reported that the perfused mouse liver is a practicable model to study liver infiltration (Roos *et al.* 1977, 1978). The obvious advantage of this model is the preservation of the 3-dimensional structure of the liver. However, the disadvantages are also apparent: the limited duration of experiments, the low tumour cell–liver cell ratio and the relatively large volume of perfusion medium. In addition, the study of infiltration in the intact liver is complicated, because 2 interactions of the infiltrating cells have to be considered, firstly with endothelial cells and next with hepatocytes. Many of these problems would be avoided if infiltration could be studied in cultures of isolated hepatic sinusoidal endothelial cells and of isolated hepatocytes. We have not yet been able to culture the endothelial cells and, as far as we know, nobody has. Hepatocyte cultures, however, are widely used for the investigation of a variety of phenomena. At least in the first few days after isolation, the cultured hepatocytes resemble those in the intact liver (Wanson, Drochmans, Mosselmans & Ronveaux, 1977; Sirica *et al.* 1979). We have found that tumour cells infiltrate into such hepatocyte cultures. In the present report we describe the morphology of this phenomenon. The model allowed for a detailed analysis, resulting in the notion that lymphosarcoma and mammary carcinoma cells may have a different mechanism of infiltration. The hepatocyte cultures may be a useful model to study liver infiltration, not only by tumour cells, but also by leucocytes.

MATERIALS AND METHODS

Tumour cells

MB 6A murine ascites lymphosarcoma, TA3/Ha and TA3/St murine ascites mammary carcinoma cells were maintained as described previously (Roos *et al.* 1977, 1978).

Hepatocyte isolation

Male or female rats, strain R, 2–3 months old, were anaesthetized with Nembutal (100 mg/kg) and rinsed with ethanol. Within a laminar flow cabinet, livers were perfused *in situ* through a 16G cannula inserted into the portal vein and a needle (internal diameter: 2 mm) in the abdominal vena cava. Perfusion media were first Ca-free medium for approx. 5 min and then 0·2 % collagenase (Sigma, St Louis, Mo.) in Ca-containing medium for approx. 20 min at 37 °C. The latter medium was recirculated. The composition of the media was as described by Seglen (1973). The collagenase medium (approx. 300 ml) was bubbled with 95 % O_2 + 5 % CO_2 through a glass filter. Foaming was prevented by a drop of Antifoam A (Sigma, St Louis, Mo.). Perfusion pressure was approx. 20 cm H_2O (200 N m^{-2}) and the flow rate approx. 30 ml/min.

After removal, the liver was disintegrated by gentle combing with a sterile fork. The cells were gently shaken in collagenase medium and filtered through nylon netting (pore diameter: 47 μm). Hepatocytes were purified by centrifugation at 60 *g* for 30 s, washing 4 times with 40 ml Earle's Balanced Salt Solution (EBSS) without Ca and Mg (Flow, Irvine, Scotland).

We mostly used rat hepatocytes because of the higher yield. Observations were similar, however, in cultures of murine hepatocytes, that were obtained in the same way, using a perfusion pressure of 5 cm H_2O (50 N m^{-2}) and a flow rate of approx. 3 ml/min.

Hepatocyte culture

Hepatocytes were cultured on gas-permeable membranes in 50-mm Petriperm dishes (Heraeus, Hanau, Germany) (5 ml). Subconfluent or confluent cultures were established by adding 3×10^6 or 5×10^6 cells, respectively, in a concentrated suspension to 5 ml of Dulbecco's modification of Eagle's medium (DMEM) (Flow, Irvine, Scotland), containing 10 % foetal calf serum (FCS) (GIBCO, Paisley, Scotland) and 20 mM HEPES. The cultures were incubated at 37 °C in 5 % CO_2–air. After 1 h, the cultures were rinsed twice with phosphate-buffered saline (PBS), to remove as many non-viable cells as possible, and incubated in fresh medium. We have also cultured cells on 60-mm Falcon plastic Petri dishes. Observations in these cultures were similar. Petriperm dishes were preferred since more hepatocytes survived and generally in better condition, especially in the confluent cultures.

Addition of tumour cells

Hepatocyte cultures were rinsed with PBS 24 h after plating, and incubated in DMEM + 20 mM HEPES. Tumour cells were washed in PBS, and 5×10^6 or 8×10^6 cells were added as a concentrated suspension to subconfluent and confluent cultures, respectively. The dishes were incubated at 37 °C in 5 % CO_2–air. After different intervals, up to 4 h, the cultures were washed twice with PBS and fixed with 2 % glutaraldehyde in 0·067 M cacodylate buffer, pH 7·2 + 1 % sucrose. To part of the cultures 3×10^6 tumour cells were added, they were washed with PBS after 4 h, fresh DMEM-HEPES or DMEM-HEPES + 10 % FCS was added and the cultures were fixed after an additional incubation period of 20 h.

In some experiments, tumour cells were added to freshly isolated hepatocytes in the complete medium before plating.

Electron microscopy

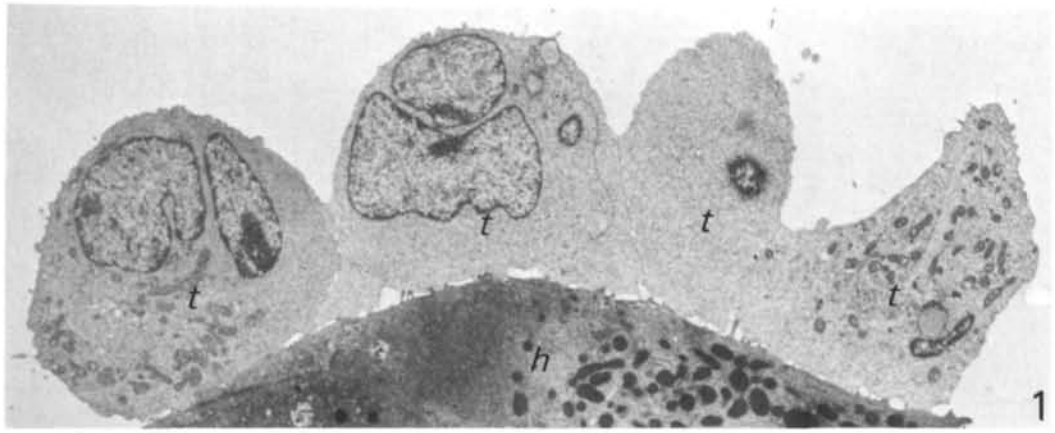
Cultures were postfixed with 1 % OsO_4, dehydrated and embedded *in situ*. Sections were cut either parallel or perpendicular to the substrate. Alternatively, for a quick assessment of the extent of infiltration, the cells were gently scraped off the dish after dehydration, pelleted and embedded. In plastic dishes cells were scraped off in propylene oxide. The cells were easily removed, since the plastic dissolved in the propylene oxide. Sections were cut with a diamond knife, stained with uranyl acetate and lead oxide, and observed in a Philips 301 electron microscope.

RESULTS

Hepatocyte cultures

Isolated hepatocytes adhered to the Petriperm dishes within 1 h. After 24 h the cells had spread, in the confluent cultures in part as a double layer. Where hepatocytes were in contact with each other, their membranes were straight and contiguous. Intercellular lumina, resembling bile canaliculi, were present and sealed by tight junctions (Figs. 2, 11).

The cultures contained some non-viable cells and cell debris, that could not be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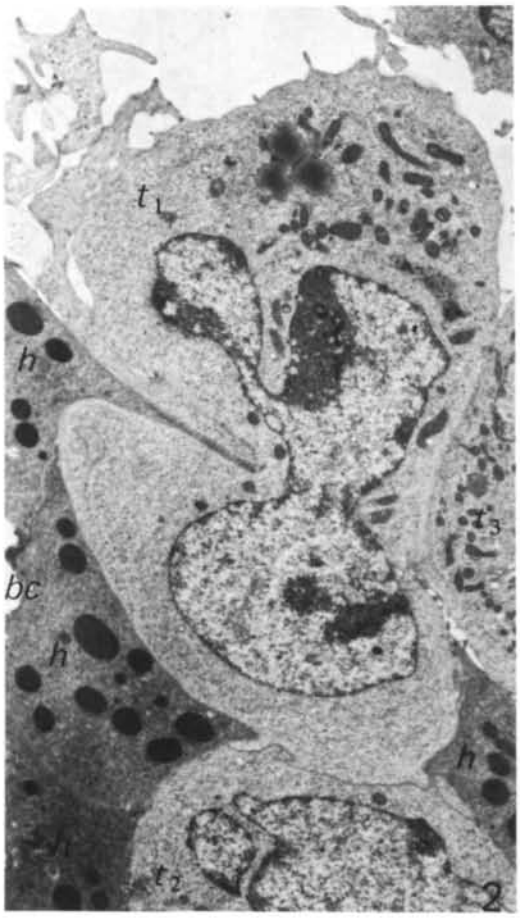
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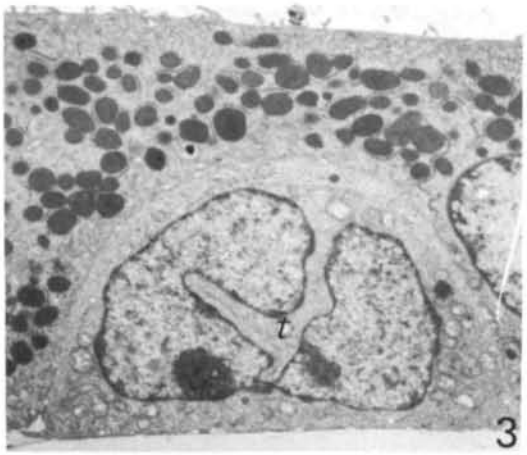
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removed, because they strongly adhered to the viable hepatocytes, which phagocytosed the debris and partly encircled the dead cells.

Adhesion of tumour cells to hepatocytes

When hepatocyte cultures were extensively washed more than 30 min after tumour cell addition, 30–60% of the MB 6A and TA3 cells remained attached to the hepatocytes. These ascites tumour cells did not attach to the substrate. In subconfluent cultures, the tumour cells adhered to both dorsal and lateral surfaces of hepatocytes. The cells that remained in suspension were not non-adhesive: they attached to hepatocytes, when the suspension was added to another hepatocyte culture. When the tumour cells were mixed with freshly isolated hepatocytes and the mixture plated and washed after 1 h, many TA3 cells remained attached to the hepatocytes, whereas MB 6A cells did not.

Lymphosarcoma cells

In the electron microscope, MB 6A cells were seen to be attached to hepatocytes at the tips of multiple protrusions or at smaller or larger patches of cell surface contiguous with the hepatocyte membrane (Fig. 1). The MB 6A cells rapidly infiltrated the cultures. Already after 30 min, MB 6A cells were seen located between hepatocytes (Fig. 2) or between hepatocytes and substrate (Fig. 3). The tumour cells moved between the hepatocytes from a position on top of the cultures (Fig. 2), and in subconfluent cultures also from a lateral position. Intrusion of the tumour cell body was sometimes seen to be preceded by the insertion of a pointed pseudopod between the liver cells (Fig. 5). Intrusion by the cell body led to deep invagination of the hepatocytes (Figs. 2–4) and sometimes even to invagination of the hepatocyte nucleus (Fig. 4). The cell surface of the deformed hepatocytes remained in close contact with the tumour cells (Fig. 2). Partly infiltrated cells were sometimes constricted (Fig. 2). Studying serial sections we found that all invaginating cells were located between hepatocytes. Also cells that infiltrated over the substrate always moved at hepatocyte boundaries, as was easily seen in sections parallel to the substrate. Observations in thin sections indicated that some MB 6A cells might be completely encircled by a single hepatocyte (Fig. 4), but studying serial sections this was never affirmed. After 24 h,

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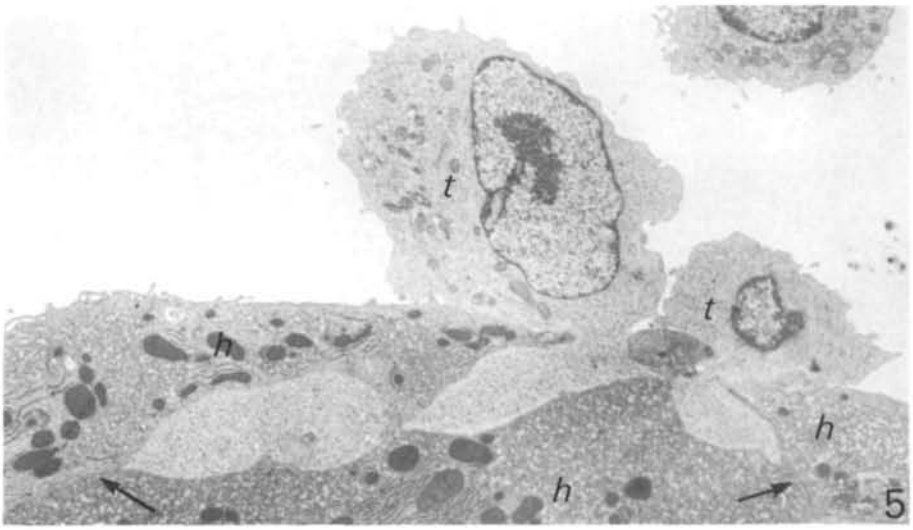
Figs. 1–4. Symbols: *bc*, bile canaliculus; *h*, hepatocyte; *t*, tumour cell. Cultures fixed 3 h after tumour cell addition.

Fig. 1. MB 6A lymphosarcoma cells adhering to hepatocytes. × 3300.

Fig. 2. Section, perpendicular to the substrate through a double layer of hepatocytes. Substrate is to the lower left. One MB 6A cell (t_1) is infiltrating between hepatocytes. It has been preceded by t_2. Another cell (t_3) is still located on the dorsal surface of the hepatocytes. × 4950.

Fig. 3. MB 6A cell located under a hepatocyte. × 4200. (Cells scraped off a plastic dish; substrate is visible as a thin line.)

Fig. 4. MB 6A cell apparently encircled by a single hepatocyte. Note invagination of hepatocyte nucleus (upper left). × 5600.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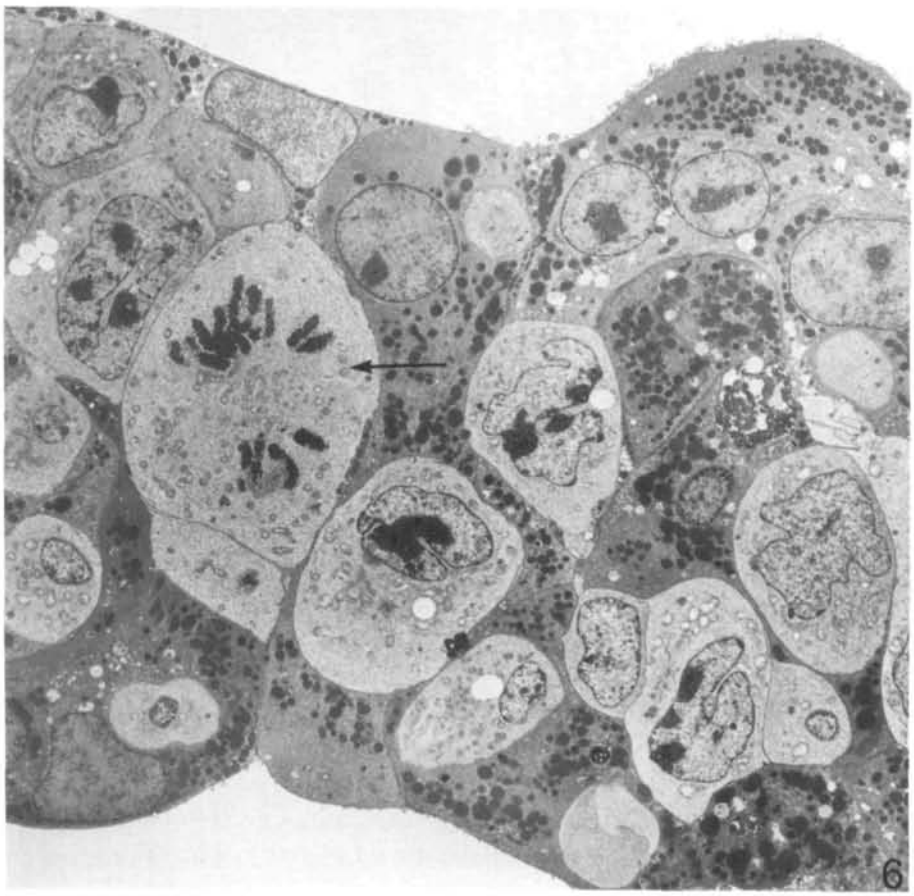
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virtually all MB 6A cells were located between or under hepatocytes and very few were still attached to the exposed surface (Fig. 6).

To be able to employ the cultures as an experimental model, it would be desirable to express the above observations in some quantitative parameter. Examination of serial sections or perpendicular sections through *in situ* embedded cultures is rather laborious when a large number of tumour cells has to be examined quickly. We therefore chose to use sections through pellets of subconfluent cultures that had been scraped off the dish. The culture fragments, that remained separated in the pellets, were sectioned in random directions. The substrate side was recognizable as an extended straight side of the fragment, except of course when the fragment was cut more or less parallel to the substrate. We enumerated the cells that were completely

Table 1. *Percentage of encircled tumour cells*

Interval after tumour cell addition, h	MB 6A	TA3/Ha	TA3/St
3	57 ± 9	3 ± 3	n.d.
24	95 ± 3	11 ± 5	12 ± 6
24 (premixed)	90 ± 9	10 ± 5	11 ± 4

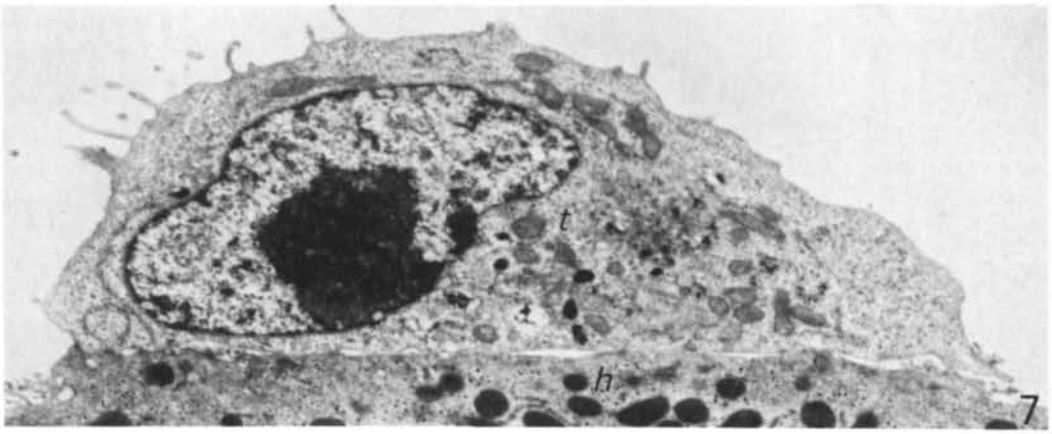
The percentage of tumour cells in contact with hepatocytes that was completely encircled by hepatocytes or located between hepatocyte and substrate. This percentage was determined in random sections through pelleted cultures. Shown are averages of 3–4 experiments and standard deviation. Premixed: tumour cells were mixed with isolated hepatocytes before plating. n.d. not done.

encircled within the section by hepatocytes or contained between hepatocytes and substrate, as a percentage of the total number of cells in contact with hepatocytes. This percentage was 57% after 3 h and 95% after 24 h (Table 1). The percentage was quite variable after time intervals shorter than 3 h.

When MB 6A cells were added to freshly isolated cells, before plating, and the resulting cultures were fixed after 4 h, many cells were observed between and under the hepatocytes, but no MB 6A cells were observed to be attached to the exposed surface, except at interhepatocyte boundaries. After 24 h, these cultures were indistinguishable from 48-h cultures, to which the tumour cells had been added 24 h after plating (see Table 1).

Fig. 5. Two MB 6A cells (*t*) extending pointed pseudopods between hepatocytes (*h*). Arrows indicate boundary between hepatocytes (3 h after tumour cell addition). × 4700.

Fig. 6. Hepatocyte culture, 24 h after addition of MB 6A cells, sectioned parallel to the substrate. Arrow, MB 6A cell in mitosis. × 1800.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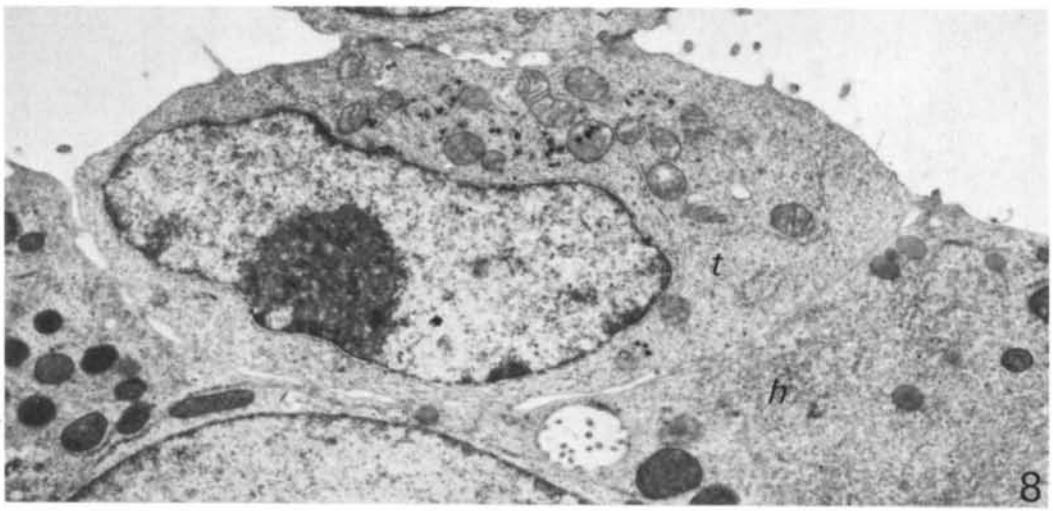
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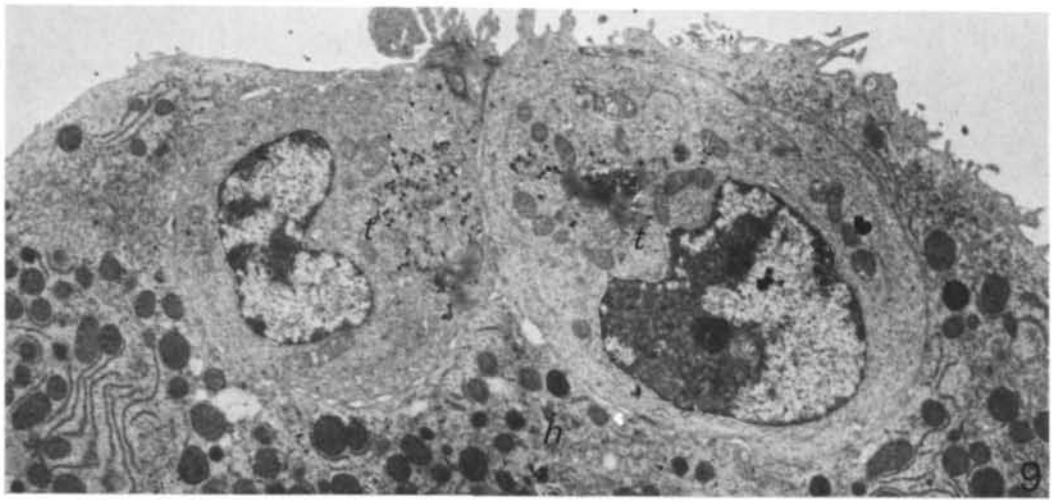
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Mammary carcinoma cells

TA3 cells adhered to hepatocytes at smaller or larger patches of contiguous cell membrane (Figs. 7, 8). Part of the TA3 cells invaginated hepatocytes more or less deeply (Figs. 8–10), whereas other cells did not (Fig. 7). The latter cells spread to different degrees on the hepatocyte surface, attaining a more or less flattened shape (Fig. 7). Invaginating cells were mostly rounded, but some cells were ellipsoid in shape, particularly those that did not invaginate deeply (Fig. 8). Extension of pseudopods by TA3 cells was not observed.

Studying serial sections, we found that in contrast to MB 6A cells, invaginating TA3 cells were located all over the exposed surface and not necessarily at an inter-hepatocyte boundary. Like MB 6A cells, TA3 cells were never completely encircled by a single hepatocyte.

Using the same quantification method as for MB 6A cells, we found 3% encircled TA3 cells after 3 h, and approx. 12% after 24 h (Table 1). It should be noted that the percentages for different tumour cell types cannot be readily compared even when the cells were added to replicate hepatocyte cultures, because the percentages depend to a certain extent on the average diameter and the deformability of the tumour cells.

Adherent TA3 cells formed junctions with the hepatocytes. Junction formation was seen already after 1 h, and some cells had formed extensive junctional complexes after 3 h (Fig. 10). These junctions were similar to those sealing bile canaliculi (Fig. 11) and thus presumably tight junctions. Using freeze-fracturing, it was recently established that they are indeed tight junctions (C. A. Feltkamp *et al.*, unpublished). In addition, some desmosomes were formed (Fig. 12) and occasionally an intercellular lumen, sealed by tight junctions, surrounded by smooth tumour cell membrane and villous hepatocyte surface, thus partly resembling a mammary tumour acinus and partly a bile canaliculus (Fig. 13).

In Fig. 14, a photomicrograph of a 1-μm-thick section is shown, cut parallel to and approx. 3 μm above the substrate, of a confluent culture, 10–12 μm thick, 24 h after addition of TA3 cells. The TA3 cells that have invaginated to such depth are readily identified. Studying serial parallel sections, we found that some of the TA3 cells were contained within the cultures, but that most of the TA3 cells were not completely encircled.

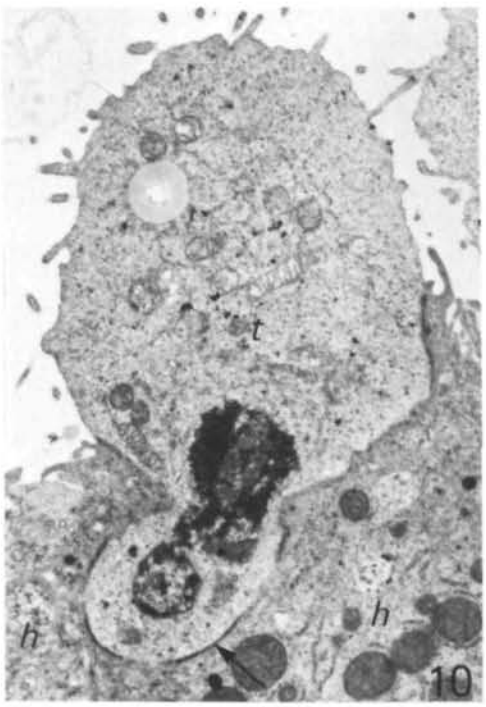
When TA3 cells were mixed with freshly isolated hepatocytes before plating, instead of adding them to 24-h cultures, and fixed 3 h later, TA3 cells were observed on the exposed surface, but they did not invaginate the still-rounded hepatocytes.

Figs. 7–9. Symbols: *h*, hepatocytes; *t*, tumour cell. Cultures fixed 3 h after tumour cell addition.

Fig. 7. A TA3/Ha cell that has spread on a hepatocyte surface. × 8100.

Fig. 8. A TA3/Ha cell that has spread on and slightly indented a hepatocyte. × 6250.

Fig. 9. TA3/Ha cells deeply invaginating a hepatocyte. × 6250.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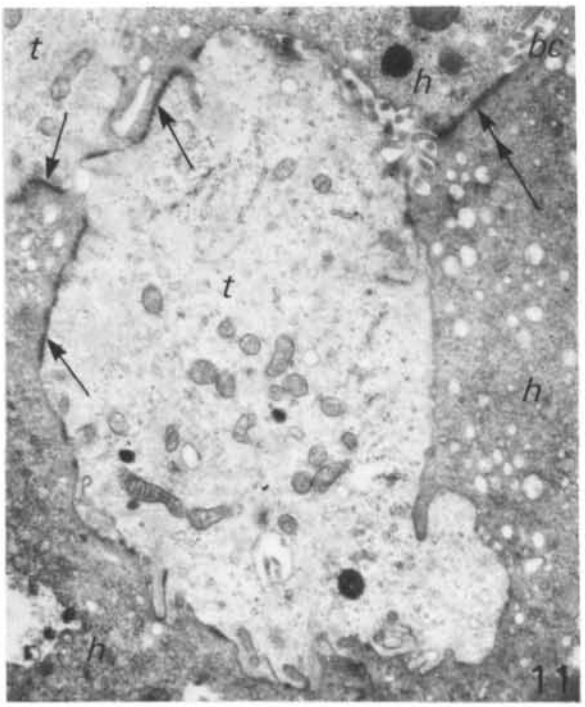
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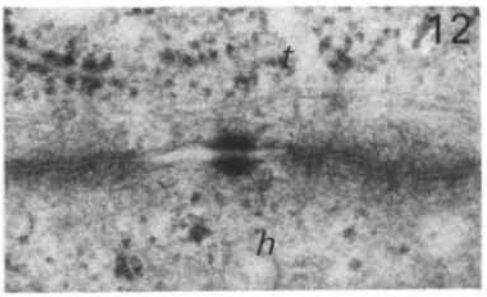
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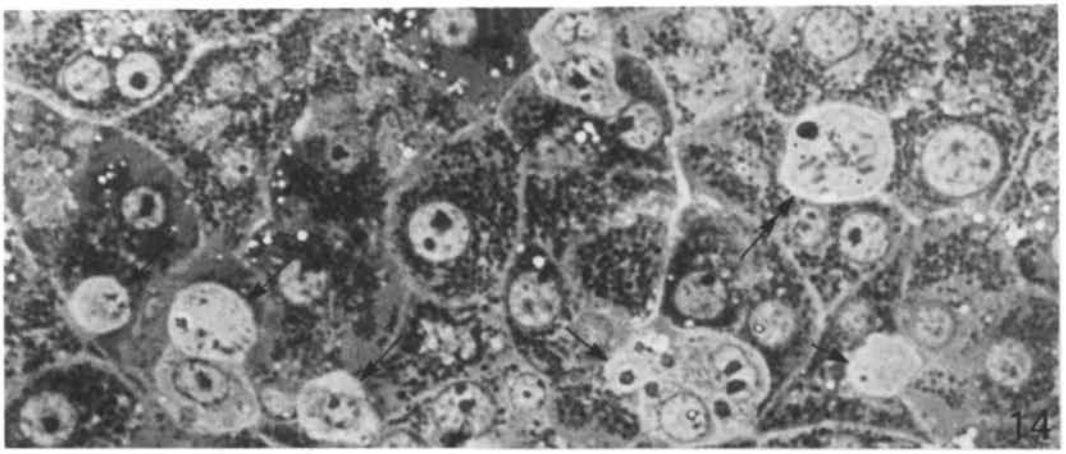
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After 24 h, the percentage of encircled cells was similar to that in 48-h cultures, to which the tumour cells had been added 24 h after plating (Table 1).

DISCUSSION

We have observed that 2 tumour cell types, MB 6A lymphosarcoma and TA3 mammary carcinoma, infiltrate into cultures of isolated hepatocytes. The 2 cell types have previously been shown to infiltrate the liver parenchyma when added to the perfusion medium of a perfused mouse liver, or injected into the portal system *in vivo* (Roos *et al.* 1977, 1978). In the intact liver, velocity of infiltration was quite different: up to 40% of MB 6A in contrast to less than 1% of TA3 cells were completely extravascular after 3 h. This corresponds with the presently reported large difference in the velocity of infiltration in hepatocyte cultures, as indicated by the percentage of completely encircled cells in sections through pelleted cultures (57 *vs.* 3%) after the same interval.

The mechanism of infiltration of the 2 tumour cell types in the intact liver appeared to be the same: after traversing the endothelium, tumour cell protrusions in contact with hepatocytes, enlarged and became globular pseudopods that deeply invaginated the hepatocytes. These pseudopods enlarged further, until finally the whole cell was extravascular. In the cultures, whole tumour cells rather than pseudopods invaginated hepatocytes. This difference is probably due to the presence in the liver of endothelial cells, separating the tumour cell body and the hepatocyte. Therefore, protrusions extended through the endothelial cells, rather than the tumour cell body, made contact with the hepatocytes. Indeed, the pseudopods largely disappeared as soon as the tumour cells were completely extravascular.

Because many interactions were seen within one section, the hepatocyte cultures allowed for an extensive analysis of serial sections, which was not feasible in the intact liver. This analysis revealed that, in contrast to what had appeared to be the case in the intact liver, the mechanisms of infiltration of the 2 tumour cell types may be quite different. MB 6A lymphosarcoma cells extended pointed pseudopods between

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Fig. 10. TA3/Ha cell invaginating a hepatocyte, 3 h after tumour cell addition. Note junctions between tumour cell and hepatocyte (arrow). × 8100.

Figs. 11–14. Cultures fixed 24 h after tumour cell addition.

Fig. 11. TA3/St cell infiltrated between hepatocytes. Note junctions with hepatocytes (arrows) and similarity with junctions between hepatocytes (double arrow), sealing a bile canaliculus (*bc*). Section parallel to the substrate. × 11 000.

Fig. 12. Desmosome between a TA3/St cell and a hepatocyte. × 85 000.

Fig. 13. An intercellular lumen formed between a TA3/St cell and a hepatocyte, resembling both a tumour acinus and a bile canaliculus. × 37 500.

Fig. 14. Photomicrograph of a 1-μm-thick section through a confluent culture, 10–12 μm thick, cut parallel to and 3 μm above the substrate. TA3/St cells had been added to the confluent culture 24 h before. Tumour cells that have invaginated to this depth are readily identified (arrows). Note tumour cell in mitosis (double arrow). × 800.

hepatocytes, in contrast to TA3 mammary carcinoma cells. Why these pseudopods were not seen in the intact liver is not clear. Preceded by the pseudopods, MB 6A cell bodies were displaced to a position between hepatocytes, invaginating these cells only at hepatocyte boundaries. It seems likely that active movement of the MB 6A cells along the interhepatocyte surfaces is the cause of this type of infiltration. TA3 cells, on the other hand, invaginated all over the exposed surface, and the depth of invagination still increased when most TA3 cells had formed tight junctions with the hepatocytes and thus were presumably immobilized. It does not seem likely that infiltrating TA3 cells actively moved, especially since they remained rounded and did not exhibit any pseudopodial activity.

Part of the TA3 cells flattened on the hepatocyte surface, thus increasing the area of cell surface adhering to the hepatocyte. Infiltrating cells remained rounded; for some reason they may not have been able to attain a flattened shape. If so, the only way to increase the adherent surface area was for the hepatocyte to encircle the TA3 cell in a process not unlike their encirclement of non-viable hepatocytes. If this notion is correct, it would mean that TA3 cell infiltration is due to the activity of the hepatocytes rather than of the TA3 cells themselves.

It has been repeatedly reported that leukaemia cells, granulocytes and immunologically stimulated T-lymphocytes move into cell cultures (Haemmerli, Felix & Sträuli, 1977; Chang, Celis, Eisen & Solomon, 1979). The cultured cells were mostly fibroblasts, but also mesonephros and different types of tumour cells. Our observations on lymphosarcoma cells were different in a number of aspects. First, the MB 6A cells not only moved under the monolayer from a lateral position, but also between the cells from a dorsal position. In this respect, our observations resembled more those of tumour cells and leucocytes in endothelial cell monolayers (Kramer & Nicholson, 1979; Beesley *et al.* 1979). Secondly, the MB 6A cells did not attain an extensively flattened shape under the monolayers without affecting the shape of the cultured cells; they remained more or less rounded and deeply invaginated the hepatocytes. Thirdly, in contrast to the leukaemia cells and lymphocytes that moved in and out of the cultures at high speed, MB 6A lymphosarcoma cells tended to accumulate inside the cultures, suggesting that they were arrested in their interhepatocyte location. Part of these differences may be ascribed to our use of hepatocytes rather than fibroblasts. Indeed, a small number of lymphocytes, present in TA3/St cell populations, infiltrated the hepatocyte cultures in a manner comparable to lymphosarcoma cells, with conspicuous pointed pseudopods extended between the hepatocytes. Experiments with different types of leucocytes are in progress and will be reported separately.

We have not used hepatocyte cultures beyond 48 h after their isolation, because these cells exhibited a number of morphological changes like extensive flattening, development of stress fibres and extension of lamellipodia, that made them more and more dissimilar to intact liver hepatocytes. Concomitantly they became much less adhesive for tumour cells. It is known that cultured hepatocytes rapidly attain foetal characteristics (Sirica *et al.* 1979). Leung & Babai (1979) studied interactions of tumour cells with foetal hepatocytes. They noted some underlapping of the hepatocytes, but not the extensive invagination we observed. This might partly be due to their use of

different tumour cell types, but might equally well be due to their hepatocytes being foetal, rather than adult hepatocytes.

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