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EFFECT OF TUBULIN-BINDING AGENTS ON THE INFILTRATION OF TUMOUR CELLS INTO PRIMARY HEPATOCYTE CULTURES

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SUMMARY

The influence of tubulin-binding agents on the infiltration of murine MB6A lymphosarcoma and TA3 mammary carcinoma cells into primary rat hepatocyte cultures was studied. Colchicin, nocodazole and vinblastin reduced the number of infiltrating lymphosarcoma cells, probably by interfering with the adhesion of these cells to the exposed hepatocyte surface. However, the subsequent infiltration of cells that did adhere was not affected or even slightly stimulated. The reduced adhesion appears to be due to an effect on both the MB6A cells and the hepatocytes.

In contrast, adhesion of TA3 cells was not reduced and infiltration was markedly enhanced by these agents, due to an effect on the TA3 cells but probably not on the hepatocytes.

These observations support previously described morphological evidence for differences between the infiltration mechanisms of the two tumour cell types. It is concluded that the system within the hepatocytes involved in adhesiveness of the exposed surface to MB6A cells is distinct from that mediating other types of adhesion. The tendency of TA3 cells to invaginate hepatocytes may be due to disturbances in tubulin-dependent processes.

INTRODUCTION

Previously, we (Roos, Van de Pavert & Middelkoop, 1981) have described the infiltration of MB6A lymphosarcoma and TA3 mammary carcinoma cells into hepatocyte cultures as a model for infiltration into the intact liver during the formation of liver metastases (Roos, Dingemans, Van de Pavert & Van den Bergh Weerman, 1977, 1978). The MB6A cells adhered to the hepatocytes and intruded into the cultures at hepatocyte boundaries. They first extended a pointed pseudopod between the liver cells, and subsequently invaginated the hepatocytes deeply while migrating between them. The MB6A cells accumulated within the cultures, between and under the hepatocytes.

The TA3 cells also adhered to the hepatocytes and some of them also invaginated the liver cells. However, this invagination occurred not only at hepatocyte boundaries, but all over the exposed hepatocyte surface. The invaginating TA3 cells generally retained a rounded shape and did not extend pseudopods. Some of the TA3 cells that did not invaginate spread over the hepatocyte surface. We concluded that there were differences between the infiltration mechanisms of the two tumour cell types.

Mareel & De Brabander (1978*a*, *b*) have reported that tubulin-binding agents (TBA) like nocodazole inhibit invasion of tumour cells into cultured embryonic tissues. Such agents are also known to influence cellular activities like adhesion,

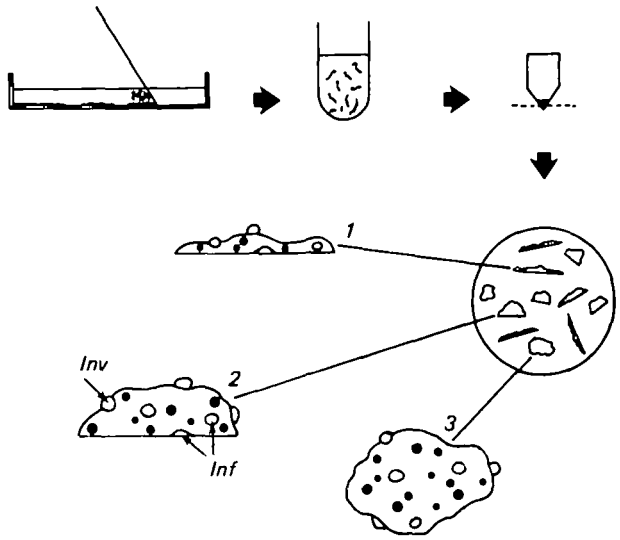

Fig. 1. Schematic illustration of the method used for quantification. Fixed cultures were scraped off the dish, fragmented and pelleted. In sections through these pellets multiple fragments can be seen, cut at different angles to the substrate: *1*, perpendicularly; *2*, obliquely; and *3*, parallel. Examples of tumour cells, designated infiltrated (*Inf*) or invaginating (*Inv*) are indicated. Black dots represent hepatocyte nuclei. For further details see text.

spreading and migration: adhesion and spreading of macrophages (Cheung, Cantarow & Sundharadas, 1978; Cheung & Terry, 1980), granulocytes (MacGregor, 1976) and fibroblasts (Ivanova, Margolis, Vasiliev & Gelfand, 1976) are reduced or retarded by colchicin and colcemid, and migration is enhanced in macrophages (Cheung *et al.* 1978; Cheung & Terry, 1980) and monocytes (Crispe, 1976), not influenced in granulocytes (Ramsey & Harris, 1972) and epithelial cells (Dipasquale, 1975; Dunlap & Donaldson, 1978), and inhibited in fibroblasts (Gail & Boone, 1971). Since adhesion, spreading and migration play a role in the interaction of tumour cells with cultured hepatocytes, we investigated the effects of TBA in this model system. These effects were different for MB6A as compared to TA3 cells, thus supporting the notion that these cells differ in certain aspects of their infiltration mechanisms.

MATERIALS AND METHODS

Tumour cells

MB6A murine ascites lymphosarcoma and TA3/Ha murine ascites mammary carcinoma cells were maintained as described previously (Roos *et al.* 1977, 1978).

Hepatocyte isolation and culture

Rat hepatocytes were isolated as described previously (Roos *et al.* 1981), but the culture method was modified. The gas-permeable Petriperm dishes were treated for 15 min with 2 % newborn calf serum (NBCS, Gibco) in phosphate-buffered saline (PBS), and subsequently washed. The hepatocytes were seeded in Dulbecco's modified Eagle's medium (DMEM, Flow)

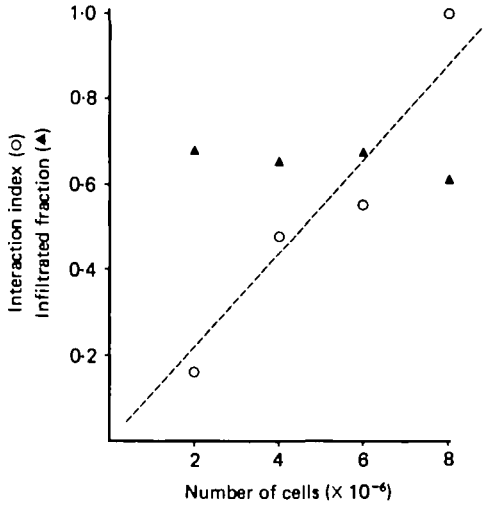

Fig. 2. Interaction index (○) and infiltrated fraction (▲) of MB6A cells in a representative 5-h experiment, in which different numbers of cells were added to replicate cultures.

supplemented with 20 mM-HEPES, 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Behring) and 20 ng/ml dexamethasone (Collaborative Research). The mixture of hormones was the same as that described by Marceau, Noel, Deschenes & Valet (1980), and the concentration was as recommended by Collaborative Research. Insulin and transferrin were dissolved in DMEM/HEPES at 50 μg/ml, filter-sterilized and diluted. Dexamethasone was dissolved at 0·2 mg/ml in ethanol and diluted 1:10000. After 1–2 h the cultures were washed and either this serum-free hormone-containing medium (H-cultures, to be used with TA3 cells) or DMEM/HEPES + 10% NBCS (S-cultures, to be used with MB6A cells) was added. The only reason for this difference was experimental convenience: adhesion of MB6A lymphosarcoma cells to hepatocytes was much reduced in H-cultures. This was not the case with TA3 cells. On the other hand, S-cultures were less convenient in the 24 h experiments usually performed with TA3 cells, since tubulin-binding agents (TBA) tended to detach hepatocytes upon prolonged exposure of S-, but not of H-cultures, as was also found by Galivan (1981). For both TA3 and MB6A cells there was no difference between the two types of cultures in the effects of TBA on interactions with hepatocytes.

Tubulin-binding agents

Nocodazole (a gift from Dr De Brabander, Janssen Pharmaceutics) and colchicin (Merck), dissolved in dimethylsulphoxide (DMSO) or ethanol at 5 mg/ml were diluted in DMEM/HEPES. Lumicolchicin was prepared by exposing a solution of 5 mg/ml colchicin in ethanol to ultraviolet light. The conversion was monitored spectrophotometrically at 267 and 350 nm (Wilson *et al.* 1974). In control experiments DMEM/HEPES containing 0·2% DMSO or ethanol was used. Vinblastin sulphate (Lily) was dissolved in PBS and diluted in DMEM/HEPES. In some experiments either hepatocyte cultures or tumour cells were pretreated with colchicin for 45 min at 37 °C in 5 ml DMEM/HEPES. They were washed free of colchicin immediately prior to the addition of the tumour cells to the hepatocyte cultures.

Addition of tumour cells

After 24 h the cultures were washed and DMEM/HEPES containing drugs or solvent was added. Then 8×10^6 MB6A or 4×10^6 TA3/Ha cells were added in the short experiments (up to 5 h) and 4×10^6 MB6A or 2×10^6 TA3/Ha cells were added in the case of 24 h incubations. The cultures were put in an incubator at 37 °C in 5% CO_2/air.

Electron microscopy

The cultures were fixed and processed for transmission electron microscopy as described previously (Roos *et al.* 1981). Some cultures were used for scanning electron microscopy: after dehydration these were critical-point-dried from CO_2, sputtered with Au/Pd in a Polaron SEM-coating unit and observed in a Cambridge Mark II scanning electron microscope at the Laboratory for Electron Microscopy of the University of Amsterdam.

Quantification of transmission electron microscopic observations

Cultures were scraped off the dish and pelleted. In sections through the pellets multiple culture fragments were seen, cut at different angles to the substrate (Fig. 1). The substrate side was easily recognized as the extended straight side of the fragments. The chance to encounter

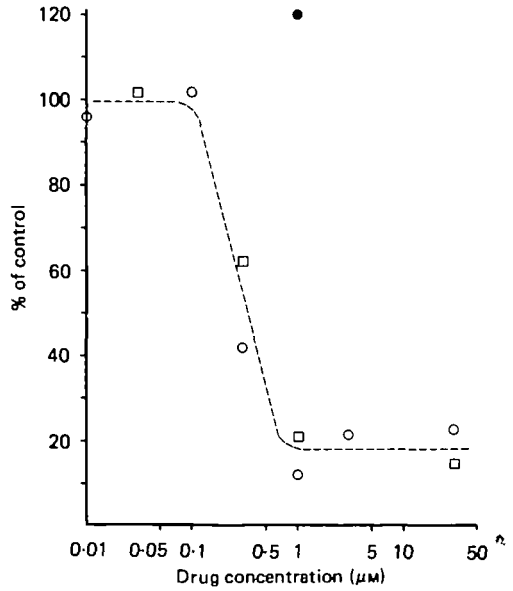

Fig. 3. Interaction index of MB6A cells as a function of drug concentration in a representative 5-h experiment. (○) colchicin; (□) nocodazole; (●) lumicolchicin.

completely encircled tumour cells within such sections increases when more cells have infiltrated and when partially infiltrated cells have invaginated the hepatocytes to greater depth. Thus the number of encircled cells per hepatocyte was used as a measure of the extent of infiltration. This parameter is determined by: (1) the total number of interacting cells per hepatocyte, and (2) the fraction of such cells that are completely encircled. Using hepatocyte nuclei to measure conveniently the number of hepatocytes, we thus employed two parameters.
(1) The *interaction index:* interacting tumour cells per hepatocyte nucleus;
(2) the *infiltrated fraction:* the fraction of interacting cells that was completely encircled.
Fig. 2. shows that the two parameters are independent within a relevant range of MB6A–tumour cell interaction. In this experiment the number of interacting cells was reduced by adding less MB6A cells. The interaction index was proportional to the number of added cells, but the fraction that had infiltrated during 5 h was similar in all cases.

In the case of TA3 cells, where complete encirclement was rare after short-term interaction, we used an additional parameter: (3) The *invaginating fraction:* the fraction of interacting

cells that were either partially or completely encircled. At least 30 % of the tumour cell circumference was surrounded in the case of partial encirclement.

In all experiments at least 150 hepatocyte nuclei were counted in each of the duplicate cultures. Data presented are the average of duplicates.

RESULTS

Effects of TBA on infiltration by MB6A lymphosarcoma cells

The interaction index of MB6A cells varied between 5-h experiments, performed on different occasions, in the range from 0·3 to 3·0. However, there was little variation between control values in duplicate cultures. The effects of the drugs were similar in all experiments and also in those with extremely high or low interaction in the controls. The results of one particular experiment are given in Table 1. Table 2 gives the averaged percentages of control values from three experiments. In the individual experiments, nocodazole and colchicin reduced the interaction index consistently to 10–30 % of control values. The infiltrated fraction, on the other hand, was nearly always somewhat increased. The reduced interaction index was still observed after 24 h. Vinblastin gave similar results as colchicin (not shown). Lumicolchicin had no effect. The concentration dependence for nocodazole and colchicin is given in Fig. 3; the effect is not seen at 0·1 μM and is close to maximal at 1 μM.

Table 1. *Effects of tubulin-binding agents on the interaction of MB6A cells with hepatocytes in a representative 5-h experiment*

Treatment	Interaction index	Infiltrated fraction
Control (0·2 % DMSO)	0·89 (100 %)	0·32 (100 %)
Nocodazole, 3 μM	0·30 (34 %)	0·56 (175 %)
Nocodazole, 30 μM	0·21 (24 %)	0·48 (150 %)
Colchicin, 3 μM	0·28 (32 %)	0·44 (138 %)
Colchicin, 30 μM	0·25 (28 %)	0·49 (153 %)
Lumicolchicin, 3 μM	1·04 (117 %)	0·30 (93 %)

For explanation of parameters see Materials and Methods.

Table 2. *Effects of tubulin-binding agents on the interaction of MB6A cells and hepatocytes*

Treatment	Interaction index (%)	Infiltrated fraction (%)
Controls (0·2 % DMSO)	100	100
Nocodazole, 3 μM	43 ± 13*	161 ± 25
Nocodazole, 30 μM	29 ± 15	122 ± 34
Colchicin, 3 μM	22 ± 11	149 ± 46
Colchicin, 30 μM	22 ± 7	137 ± 17

Averaged percentages of control values of three experiments.
* Standard deviation

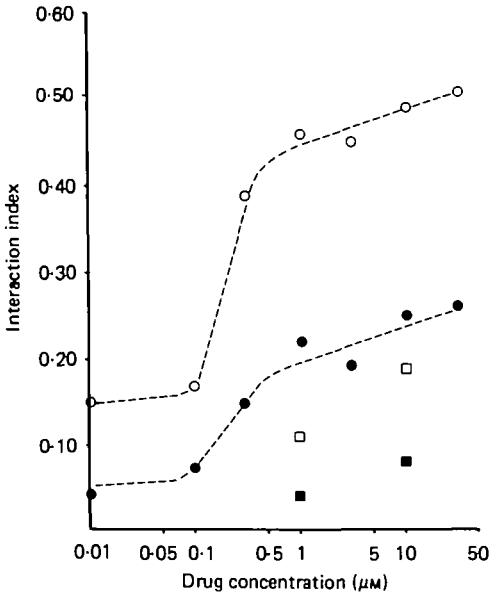

Fig. 4. Infiltrated fraction (●, ■) and invaginating fraction (□, ○) of TA3 cells as a function of drug concentration in a representative 24-h experiment. (○, ●) colchicin; (□, ■) lumicolchicin.

Table 3. *Effect of pretreatment with colchicin on the subsequent interaction of MB6A cells with hepatocytes*

Pretreated cell	Colchicin concn (μM)	Duration of experiment (h)	Interaction index* (%)
Hepatocyte	30	2	9
Hepatocyte	30	5	29
Hepatocyte	0·3	5	35
MB6A	30	2	25
MB6A	30	5	19

* Percentage of control values, average of two experiments.

Pretreatment of the cells with colchicin yielded results given in Table 3. The interaction index was not only reduced following treatment of MB6A cells, but also after pretreatment of hepatocytes. The latter effect is probably not due to uptake by MB6A cells of hepatocyte-released colchicin, since it was also seen after pretreatment with a suboptimal concentration (0·3 μM).

Table 4 presents the results of an experiment in which MB6A cells were allowed to attach for 1 h, and non-adherent cells were subsequently removed before addition of nocodazole. In both control and drug-treated cultures many MB6A cells detached in the subsequent 4 h, showing that binding to hepatocytes is reversible. However,

Table 4. *Effect of nocodazole on attached MB6A cells**

Treatment	Interaction index	Infiltrated fraction
Control	0·21 (100%)	0·83 (100%)
Nocodazole, 30 μM	0·08 (38%)	0·90 (108%)

* The MB6A cells were allowed to attach for 1 h, then non-adhering cells were removed. Nocodazole or 0·2 % DMSO (control) was added and the cultures were fixed after another 4 h.

detachment was more extensive in the presence of nocodazole, resulting in a lower interaction index.

TBA treatment affected the morphology of MB6A cells (Figs. 5, 6). Non-treated cells were mostly spherical and few had a uropod, whereas treated cells were rather irregular in shape; they were often elongated and had one or more globular or uropod-like extensions. In contrast, hepatocyte morphology was influenced very little, except that the cells were somewhat more rounded.

Effects of TBA on infiltration by TA3 mammary carcinoma cells

The interaction index of TA3 cells was influenced very little by TBA. In individual experiments both increases and decreases were found, but on average there was little difference (Tables 5, 6).

The infiltrated fraction of TA3 cells varied between 0·03 and 0·19 in separate 24-h experiments with different batches of TA3 cells. It was, however, reproducible in duplicate cultures. The infiltrated fraction was increased by colchicin and nocodazole (Tables 5, 6). Similar results were obtained with vinblastin. Lumicolchicin had no effect. It was striking that in all experiments TBA increased the infiltrated fraction to a constant value of approximately 0·28, no matter how high or low the control value was.

In the case of TA3 cells, we used an additional parameter, the invaginating fraction, i.e. the fraction of TA3 cells that was partly or completely encircled. Although intended to be employed primarily in short experiments, it appeared to be useful also in the 24-h experiments. In Tables 5 and 6 it can be seen that the invaginating fraction was markedly increased by TBA, often to approximately 0·70, independent of the variable control values (0·15–0·42). The concentration dependence is shown in Fig. 4. This was often difficult to establish, using the infiltrated fraction as the only parameter, especially when control values were high. However, when the invaginating fraction was measured as well, unequivocal results were always obtained. The effect was absent at 0·1 μM-colchicin, increased sharply between 0·1 and 1 μM, and more gently above 1 μM.

Pretreatment experiments indicated that enhanced infiltration was due to an effect on TA3 cells and probably not on hepatocytes. The results of a representative 2 h experiment are given in Table 7.

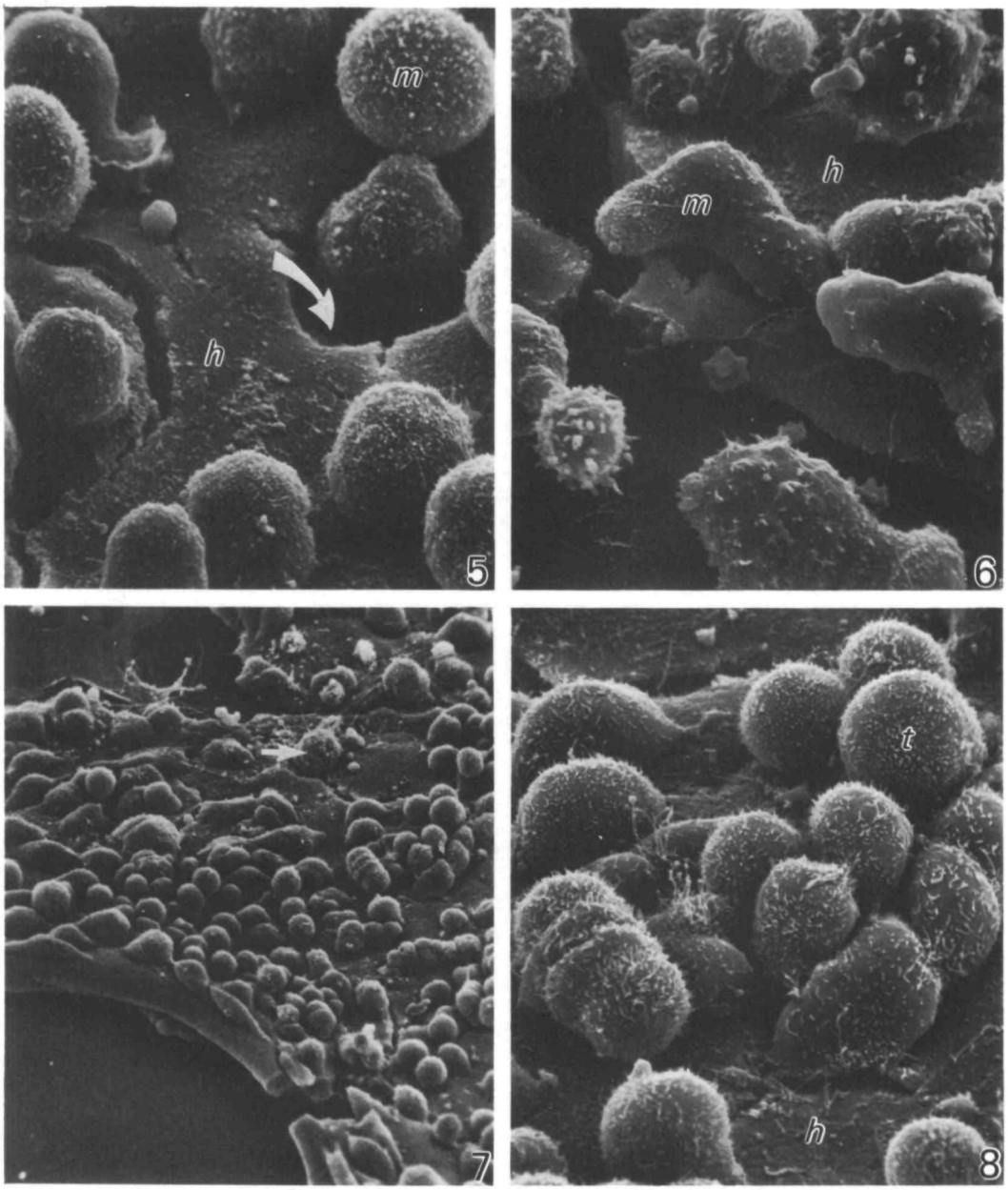

Fig. 5. MB6A lymphosarcoma cells (*m*) on a hepatocyte (*h*) culture. One of the MB6A cells is infiltrating between hepatocytes (arrow). The opening has widened due to shrinkage during critical-point drying. × 2000.

Fig. 6. MB6A cells (*m*) on a hepatocyte culture (*h*) with 30 μM-nocodazole in the medium. Note irregular shape of MB6A cells (compare with Fig. 5). × 2000.

Fig. 7. Hepatocyte culture, 24 h after addition of TA3/Ha mammary carcinoma cells. Arrow, dead hepatocyte. × 500.

Fig. 8. As Fig. 7. *h*, hepatocyte; *t*, TA3 cell. × 2000.

Table 5. *Effects of tubulin-binding agents on the interaction between TA3 cells and hepatocytes in a representative 24-h experiment*

Treatment	Interaction index	Infiltrated fraction	Invaginating fraction
Control	0·70	0·10	0·26
Nocodazole, 30 μM	1·09	0·24	0·72
Colchicin, 30 μM	0·68	0·25	0·71
Vinblastin, 30 μM	0·99	0·26	0·68
Lumicolchicin, 30 μM	1·14	0·08	0·19

Table 6. *Effects of tubulin-binding agents on the interaction between TA3 cells and hepatocytes: averages of six experiments*

Treatment	Interaction index	Infiltrated fraction	Invaginating fraction
Control	1·15 ± 0·36*	0·11 ± 0·07	0·28 ± 0·10
Colchicin, 30 μM	0·96 ± 0·31	0·27 ± 0·02	0·62 ± 0·10
Nocodazole, 30 μM†	n.d.‡	0·29 ± 0·04	0·77 ± 0·07

* Standard deviation.
† Three experiments.
‡ Not determined.

Table 7. *Effect of (pre)treatment with colchicin on the interaction between TA3 cells and hepatocytes in a representative experiment*

Treatment	Invaginating fraction
Control	0·38
Colchicin, 30 μM*	0·73
Pretreated hepatocytes†	
Control‡	0·16
Colchicin-treated	0·26
Pretreated TA3 cells†	
Control‡	0·06
Colchicin-treated	0·70

* Colchicin present during interaction.
† For 45 min, 30 μM; washed prior to experiment.
‡ Pretreated with 0·2 % DMSO.

The morphology of the interaction between TA3 cells and hepatocytes is shown in Figs. 7–12. In contrast to MB6A cells, the shape of TA3 cells was not affected by TBA. In TBA-treated cultures deeply invaginating cells were readily apparent (Figs. 9–10). It should be noted that in sections used for quantification such cells will often appear completely encircled like the ones in Fig. 7. Both control and treated

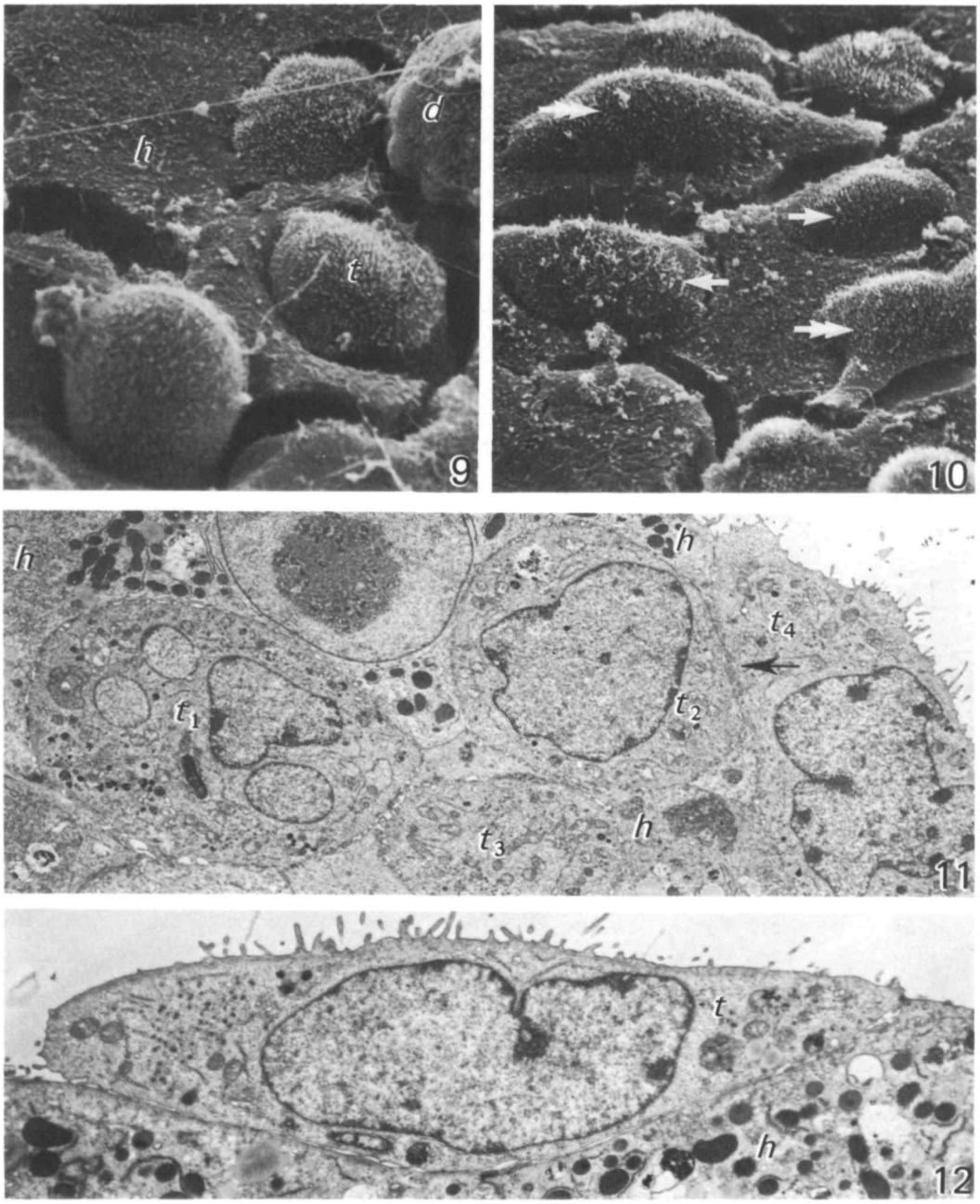

Fig. 9. Interaction of TA3/Ha cells (*t*) and hepatocytes (*h*) in the presence of 30 μM-nocodazole. Note deep invagination of hepatocytes by TA3 cells. *d*, dead hepatocyte. × 2000.

Fig. 10. As Fig. 9. Note invaginating cells (arrows) and cells that have flattened on the hepatocyte surface (double arrows). × 1600.

Fig. 11. Section through a hepatocyte culture (*h*), 24 h after addition of TA3/Ha cells (t_1–t_4). Colchicin (30 μM) was present in the medium. Note completely encircled cells (t_1–t_3), the thin rim of hepatocyte cytoplasm around t_3 (arrow), and the TA3 cell that has flattened on the hepatocyte surface (t_4). × 8300.

Fig. 12. TA3 cell that has flattened on a hepatocyte, in the presence of 30 μM-vinblastin. × 14200.

cultures contained TA3 cells that had spread over the hepatocyte surface (Figs. 10, 12). Cells of which the diameter parallel to the hepatocyte surface was more than twice the perpendicular diameter constituted approximately 10% of the interacting TA3 cells. This percentage was not influenced by TBA.

DISCUSSION

MB6A murine lymphosarcoma and TA3 mammary carcinoma cells infiltrate into primary rat hepatocyte cultures (Roos *et al.* 1981). This phenomenon has morphological characteristics in common with the infiltration of the same tumour cells in the intact mouse liver (Roos *et al.* 1977, 1978). The hepatocyte cultures may thus serve as a model to study the underlying mechanisms.

In this paper we have shown that the extent of infiltration is influenced by the tubulin-binding agents (TBA) nocodazole, colchicin and vinblastin. The fact that these structurally dissimilar agents all had the same effect, in contrast to lumicolchicin, which does not bind tubulin, strongly suggests that infiltration is influenced by tubulin-dependent processes. Which processes these are and how tubulin is involved are not known. The concentration dependence was quite similar to that observed in many other TBA-influenced phenomena, e.g. secretion (Eichhorn & Peterkofsky, 1979), segregation of transport protein from phagocytic membrane (Ukena & Berlin, 1972), turnover of phosphatidylinositol (Schellenberg & Gillespie, 1977) and hormone-induced changes in cyclic AMP levels (Rudolph & Malawista, 1980). This concentration dependence is compatible with breakdown of cytoplasmic microtubules (Ukena & Berlin, 1972) being the cause of the effects of TBA. However, since the above phenomena are primarily membrane processes and since there is increasing evidence for the existence of tubulin in membranes (Berlin, Caron & Oliver, 1979), it is also possible that some of the effects of TBA are actually due to their interference with the unknown functions of this membrane-bound tubulin.

Infiltration by the MB6A lymphosarcoma cells was diminished due to a reduction in the number of interacting cells. We presume that this is caused by reduced adhesion to the exposed hepatocyte surface. TBA-treated MB6A cells had a very irregular shape, suggesting that TBA interfered with proper control of cell shape, as was also found for several other cell types (De Brabander *et al.* 1977). Treated cells may therefore be less able to flatten against the hepatocytes, resulting in a smaller adherent surface area, decreased strength of adhesion and therefore easier detachment. The observation that nocodazole enhances detachment supports this interpretation.

The infiltrated fraction of MB6A cells was unchanged, or even slightly increased. This indicates that the infiltration between hepatocytes by the few cells that did adhere was not affected, or even accelerated. If it is assumed that this infiltration is dependent upon active locomotion of MB6A cells, this finding is in agreement with enhanced locomotion of macrophages (Cheung *et al.* 1978; Cheung & Terry, 1980), monocytes (Crispe, 1976) and granulocytes (Ramsey & Harris, 1972) by TBA.

We have recently obtained evidence that two different hepatocyte surface molecules are involved in the interaction with MB6A cells (O. P. Middelkoop & E. Roos,

unpublished data). We found that Fab fragments prepared from antisera against sinusoidal surface-enriched, and against contiguous surface-enriched, liver plasma membranes reduced the interaction index and the infiltrated fraction, respectively. We concluded that one of the putative molecules mediates adhesion to the exposed surface, and the other adhesion to the contiguous surface, and that the latter is thus involved in the infiltration process proper. Our present findings indicate that adhesion via the exposed molecule is inhibited by TBA treatment of hepatocytes, whereas the adhesion to the contiguous surface is not. This is the more remarkable since other hepatocyte adhesion phenomena are not influenced by TBA; neither mutual adhesion between hepatocytes nor their adhesion and spreading on a substrate is interfered with, as reported by De Brabander *et al.* (1978) and confirmed by us. Also adhesion to TA3 cells was not affected. In this context, it is interesting to note that adhesion of MB6A cells to the exposed hepatocyte surface was reduced in cultures that had been established in a medium containing insulin and dexamethasone (see Materials and Methods). Preliminary experiments indicate that this reduction is due to insulin. Dexamethasone actually appears to increase adhesion. Also here, the infiltrated fraction of MB6A cells and the interaction with TA3 cells were not influenced. These observations indicate that the system within hepatocytes mediating adhesion of the exposed surface to MB6A cells is clearly distinct from that involved in other types of adhesion. The study of these different systems may eventually explain why MB6A cells invaginate hepatocytes only after contact with the contiguous surface, whereas TA3 cells may invaginate the exposed surface also (Roos *et al.* 1981).

The number of TA3 cells that invaginated hepatocytes was markedly increased by TBA. Pretreatment experiments indicated that this was solely due to an effect on the TA3 cells. This indicates that tubulin-dependent processes exert some control over properties of TA3 cells that cause them to invaginate hepatocytes. The constant level seen after TBA treatment was apparently the extent of invagination in the complete absence of such control. The variation in control values may then be due to differences in the extent of tubulin-dependent control. The question as to why such a disparity exists between batches of tumour cells obtained from different mice is being investigated.

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