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# Functional variations in liver tissue during the implantation process of metastatic tumour cells

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**Summary.** We have examined several properties of sinusoidal cells in the unaffected tissue of micrometastasis-containing livers. Tumour cells from either B16 melanoma (B16F10) or Lewis lung carcinoma (LLC) were injected intrasplenically in syngeneic mice and sacrified on the 7th day. Light and scanning electron microscopy (SEM) showed tumour cells in hepatic veins and sinusoids in close contact with endothelial walls and macrophages. Following quantitative analysis of SEM images from sinusoidal walls it was found that endothelial fenestrae from B16F10 or LLC-colonized livers were diffusely reduced both in size and density/$\mu m^2$ throughout the sinusoid wall, although especially affected zone 3 segments. Following the intrasplenic injection of 1 μm fluorescent latex particles 1 h prior to sacrifice of the mice a significant reduction of the latex particle uptake by sinusoidal cells was detected in B16F10-colonized livers (27% of controls) which was in contrast to the significant increase in LLC-colonized mice (180% of controls). Despite the focal character of the tumour cell implantation process, hepatic sinusoidal cells reacted diffusely to metastatic cells. However, over liver acini, endothelial cell changes were mainly expressed in zone 3 while phagocytic properties mainly varied in zone 1 and depending on the tumour type. Although the significance of these sinusoidal changes on metastatic development is unclear, data suggests that "soil" conditions in the liver are different before and after being metastasized by tumour cells.

**Key words:** Liver sinusoidal cells – Endothelial fenestrae – Hepatocyte succinate dehydrogenase activity – Kupffer cell phagocytosis – Liver metastasis

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## Introduction

The metastatic development of malignant cells depends on functional interactions between selected properties of tumour cells and host reactivity (Nicolson and Poste 1983; Weiss et al. 1988). It has been claimed that only those tumour cells able to escape the inhibitory effects of organs in which they lodge, or to adapt to the particular biological conditions of these organs, can succeed in establishing metastases (Horak et al. 1986). As a consequence, different patterns of metastatic efficiency and organ colonization have been reported both in clinical and experimental studies of cancer progression (Sugarbaker 1981; Weiss 1985).

All these observations closely connect with Paget's "seed-and-soil" hypothesis (1889), which proposed that, although haematogenously disseminated tumour cells are carried in all directions they only live and grow if they lodge in an organ that is congenial. At present there exists a number of studies on the possible mechanisms of tissue-specificity for tumour cells adhesion (Auerbach et al. 1987; Chung et al. 1988) and survival and growth (Horak et al. 1986; Nicolson and Dulski 1986). However, the majority of current views consider target organs as passive tissues with static properties. Moreover, when studying host tissue reaction, it is usually only the regional concentration of host defenses in the colonized organ which is taken into account (Williams et al. 1985).

This study focuses on the possible variations produced in the tissue structure and function of normal organs once they have been colonized by circulating tumour cells. Our study centers on the liver, a major organ for metastasis formation (Cady 1983). Specifically, we have examined the sinusoidal endothelial and Kupffer cell variation

concomitant with the initial colonization of this organ (implantation) by metastatic tumour cells.

We have detected a diffuse tissue reaction in the liver as a whole which contrasts with the focal character of early stages in the metastatic process. In addition, these phenomena represent a good example of organ response to metastatic tumour cells and it appears that the "soil" conditions offered by the liver to tumour cells, before and after metastasis are different.

## Materials and methods

Inbred C57Bl/6 mice, 6 weeks old, were purchased from Iffa Credo Laboratories (France). They were housed 5 animals to a cage and given food and water *ad libitum*.

Parental LLC and B16F10 tumour cells (Fidler 1973) were maintained in tissue culture in a medium made of DMEM (Sigma Chemicals Co., St. Louis, MO, USA) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma Chemical Co., St. Louis, MO, USA), to which 10 per cent fetal calf serum (Calbiochem-Behring Corp., La Jolla, CA, USA) was added. Cell cultures were maintained on plastic, and were incubated in a humidified atmosphere of 5 per cent $CO_2$ at 37° C and routinely monitored for mycoplasma infection. For passage, cells were detached with 0.1 per cent trypsin (Difco Labs., Detroit, MI, USA)-EDTA 2 mM (Merk, Darmstadt, FRG). Cells were transferred every 4 days and cultured for no more than 1 month.

Experimental hepatic metastases were produced according to the method of Kopper et al. (1982). Briefly, the anesthetized mouse (Nembutal, 50 mg/kg) was positioned on its right side and a small subcostal incision was made. The spleen was then exposed and a standard inoculum of $5 \times 10^5$ viable tumour cells in 0.1 ml of $Ca^{2+}$ and $Mg^{2+}$-free Hank's balanced salt solution (HBSS) was injected into the upper pole of the spleen with a 27 gauge needle. Both the abdominal wall musculature and the skin were then closed in one layer. Some control mice received the same innoculum without tumour cells.

Mice were killed under anesthaesia and their peritoneal cells were harvested for reinjection into syngeneic mice in 8 ml of HBSS, by peritoneal lavage. The cells were centrifuged at 200 g at 4° C and resuspended in $Ca^{2+}$ and $Mg^{2+}$-free HBSS at a concentration of $5 \times 10^6$ cells per ml (>90% were macrophages). 0.1 ml of this cell suspension was then injected intrasplenically to five mice as described above.

Liver samples from both control and hepatic metastasis-bearing mice were obtained on the seventh day post-injection. Liver samples from the peritoneal macrophage-injected mice were obtained 24 h postinjection. For liver tissue preparation for SEM, animals were anesthetized with ether and perfused via the aorta, first with tempered Ringer's solution containing 100 U/ml heparin and then with 2.5% glutaraldehyde in phosphate buffer at a flow rate of 0.75–1 ml/min. After organ fixation, small tissue blocks were excised and prepared for SEM as described previously (Vidal-Vanaclocha and Barberá-Guillem 1985).

The study of endothelial fenestrae was carried out in ten sinusoidal walls of 50 $\mu m^2$ selected at random in the proximal (zone 1) and distal (zone 3) areas of two different liver acini per animal. As five mouse groups were used for each tumour type, a total 50 sinusoidal fields per acinar region were studied. For the quantitative measurements of number, geometrical diameter, area of endothelial fenestrae (porosity), and sieve plate-forming fenestrae (percentage of clustered fenestrae, considering as sieve plates clusters with five or more fenestrae whose distance from each other was shorter than 50 nm), each of the selected sinusoids were studied at the same magnifications (×15000) and quantitative data of fenestrae were obtained using an interactive image analysis system (Kontron-Zeiss, W.G.).

Some liver were removed and frozen immediately in liquid nitrogen for succinate dehydrogenase histochemistry. For specific staining of the hepatic tissue an in situ histochemical reaction was produced on 10 μm cryostat sections as described previously (Barberá-Guillem et al. 1989a). Briefly, tissue slides were incubated at 37° C with 2–3 ml of a substratum solution made of sodium succinate (Merck, W.G.), nitrobluetetrazolium III (Sigma Chem. Co., St. Louis, MO) and phosphate buffered salts, and after an approximately 20 min incubation period, slides were rinsed in phosphate buffer, fixed and mounted. The morphometric estimation of hepatic tissue areas occupied by high and low SDH activity hepatocytes was carried out by using an integrated image analysis system (Southern Microcomp. Instruments, Inc., Atlanta, GA).

The phagocytic activity of Kupffer cells in normal and tumour-bearing animals was also evaluated on the seventh day post-injection of tumour cells. Ten mice per experimental group were injected intrasplenically with 1 μm in diameter fluorescent latex particles ($1.25 \times 10^8$ in 0.2 ml) one hour before animals were sacrificed and liver samples immediately frozen in liquid nitrogen. Then, an in situ histochemical reaction for the hepatocyte SDH activity was produced on cryostat sections, as described above and the number of fluorescent latex particles in the acinar zone 1 (high-SDH activity tissue) and zone 3 (low-SDH activity tissue) of the hepatic tissue were counted on twenty microscopic fields (area: 0.84 $mm^2$) per liver under fluorescence light.

The level of significance in the differences between mean values were computed with Student's *t*-test.

## Results

As shown in previous work (Barberá-Guillem et al. 1988a, 1988b), by day 7 post-intrasplenic injection of B16 melanoma or Lewis lung carcinoma cells no macroscopic growth had developed in the liver. The examination of the livers by light microscopy showed small micrometastatic foci composed of a few cells inside liver tissue which did not disturb the general structure of the tissue (Fig. 1). By SEM the hepatic microcirculation appeared sparsely infiltrated by tumour cells and, when present, the cells were found in isolation or in small groups of a few cells each within the medium and small caliber portal branches, where they were in close contact with liver endothelium and macrophages (Fig. 2).

Following the SDH histochemical reaction (Fig. 1), hepatic tissue from normal and tumour-bearing mice was highly stained around portal tracts (high SDH activity hepatocytes defining zone 1) and slightly around central veins (low SDH activity hepatocytes defining zone 3). After the

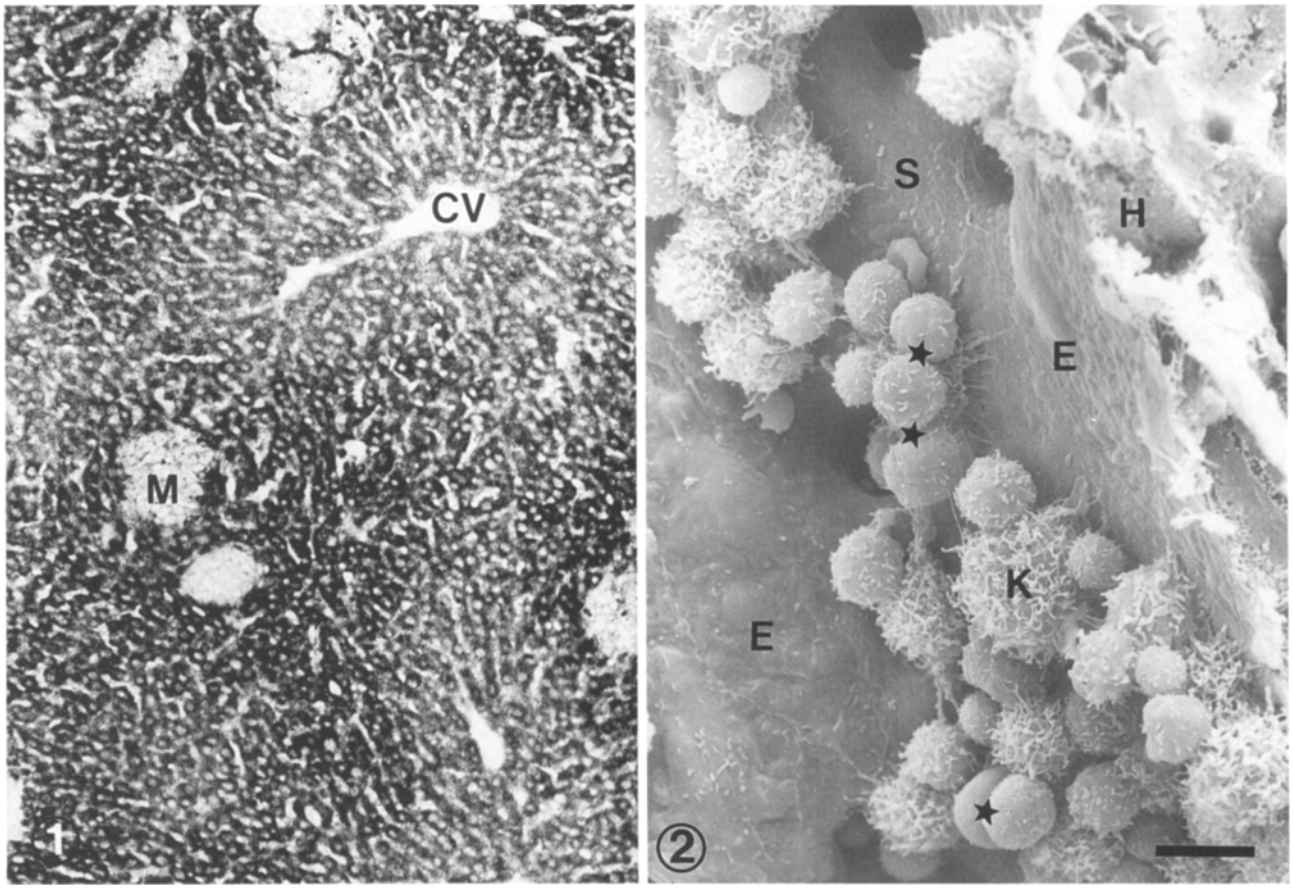



**Fig. 1.** Liver tissue section following a succinate dehydrogenase histochemical reaction obtained from an B16F10 melanoma intrasplenically injected mouse sacrified on the 7th day of the tumour evolution. Micrometastases (M) were identified as small unstained foci scattered throughout liver tissue. High SDH activity hepatocytes were defined as dark areas (zone 1) surrounding portal veins. Low SDH activity hepatocytes were observed as light areas (zone 3) surrounding central veins (CV) in which metastatic foci were never found. ×100

**Fig. 2.** Scanning electron micrograph of a small-sized portal vein where can be seen tumour cells from Lewis lung carcinoma (★) closely connected to Kupffer cells (K) and endothelial cells (E). Hepatocytes (H), Sinusoidal lumen (S). ×1500, (*scale bar:* 10 μm)

measurement of the hepatic tissue areas occupied by hepatocytes with high and low SDH activity in liver from both normal and tumour-bearing mice, which were colonized by either B16F10 melanoma or LLC cells, we found no significant variations in the high-to-low SDH activity areas (Table 1).

In mice injected with HBSS, there were no differences with respect to the fenestration pattern of the normal liver (Table 2). In animals injected with HBSS-suspended peritoneal macrophages, the number of pores per surface unit was not different from the number in normal mice, and pore-clustering into sieve plates also followed the normal pattern. There was, however a slight increase in the size of the pores and, therefore, in the percentage of endothelial surface occupied by pores (porosity) (Table 2).

**Table 1.** Effect of the hepatic colonization by B16F10 melanoma or Lewis lung carcinoma tumour cells on the distribution of high and low succinate dehydrogenase activity (SDH) hepatocytes at the acinar level

| Experimental groups | Percentage hepatic tissue area[a] | |
|---|---|---|
| | High SDH activity hepatocytes | Low SDH activity hepatocytes |
| Control livers | 45.03 ± 0.52 | 50.84 ± 1.11 |
| B16 melanoma-colonized livers | 48.57 ± 2.23 | 47.22 ± 2.83 |
| LL carcinoma-colonized livers | 46.72 ± 4.49 | 49.36 ± 5.18 |

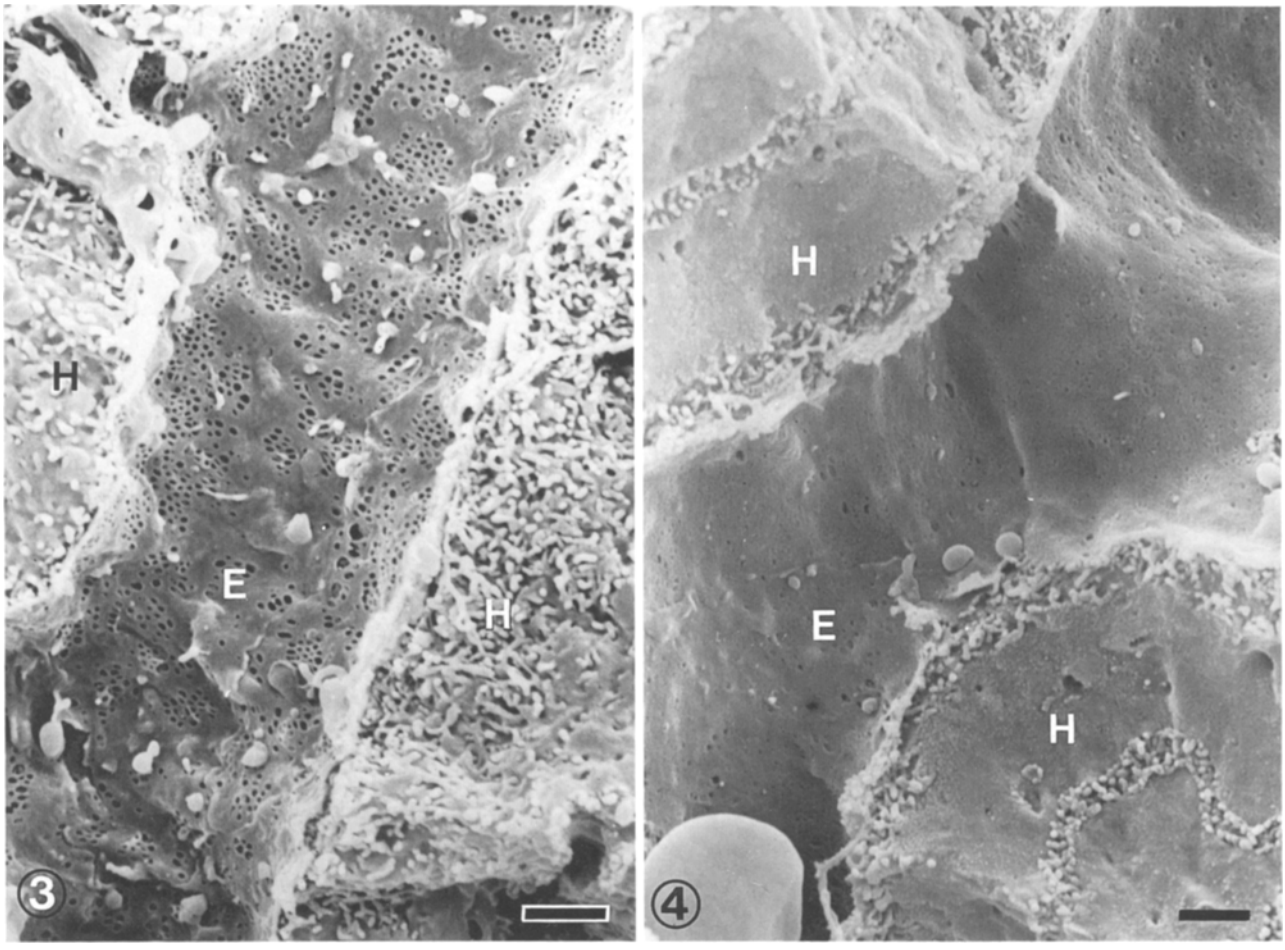
[a] Data are expressed as mean values ± SD. No significant differences were found for each tissue area between experimental groups ($p < 0.01$). The hepatic tissue area occupied by vascular structures (portal and central veins) represented 3 to 5% of the total area

**Table 2.** Assessment of the fenestration pattern in endothelial cells of the proximal (acinar zone 1) and distal (acinal zone 3) segements of liver sinusoids from normal, HBSS-injected and peritoneal macrophage injected mice[a]

| Fenestration parameters | Normal livers | | HBSS-injected mouse livers | | Peritoneal macrophage injected mouse livers | |
|---|---|---|---|---|---|---|
| | Zone 1 | Zone 3 | Zone 1 | Zone 3 | Zone 1 | Zone 3 |
| No. Fenestrae/$\mu m^2$ of endothelium | 8.8±0.5* | 15.4±0.4 | 10.4±1.4* | 17.3±0.5 | 10.9±0.2* | 15.4±0.6 |
| Diameter (nm) | 75.0±0.2* | 85.8±0.3 | 73.9±0.3* | 82.9±0.3 | 95.5±0.4** | 96.5±0.4** |
| Porosity[b] | 3.9±0.4* | 8.9±0.4 | 4.5±0.4* | 9.3±0.1 | 7.8±0.1** | 11.2±0.8 |
| Sieve plate-forming fenestrae[c] | 10.9±1.3* | 25.8±1.4 | 7.3±1.5* | 22.6±2.5 | 12.9±0.4* | 22.6±1.9 |

* $p < 0.01$, zone 1 versus zone 3
** $p < 0.01$, normal livers versus HBSS-injected mouse livers or peritoneal macrophage-injected mouse livers

[a] Data are expressed as mean values ± SEM
[b] As percentage of the total endothelial surface
[c] As percentage of the total number of fenestrae




**Figs. 3, 4.** Scanning electron micrographs showing endothelial cells (E) lining sinusoidal walls in normal (*Fig. 3*) and B16 melanoma colonized livers (*Fig. 4*). Endothelial fenestrae from normal sinusoids distributed on the surface of endothelial cells forming sieve plates. In tumor cell-infiltered livers, sinusoidal lining endothelial cells showed significant changes in the fenestration pattern, especially in distal segments (zone 3 sinusoids), where number, diameter and clustering of fenestrae highly reduced with respect to controls. Hepatocytes (H). ×4400, (*scale bar:* 1 μm)

**Table 3.** Assessment of the fenestration pattern in endothelial cells of the proximal (acinar zone 1) and distal (acinar zone 3) segments of liver sinusoids colonized by tumour cells from B16F10 melanoma or Lewis lung carcinoma[a]

| Fenestration variables | B16F10 melanoma colonized livers | | LL carcinoma colonized livers | |
|---|---|---|---|---|
| | Zone 1 | Zone 3 | Zone 1 | Zone 3 |
| No. Fenestrae/$\mu m^2$ of endothelium | 8.0±0.5 | 8.8±0.6* | 8.2±0.7* | 11.0±0.8** |
| Diameter (nm) | 63.3±0.2** | 68.1±0.2** | 69.4±0.2[4,5] | 73.5±0.2** |
| Porosity[b] | 2.5±0.3** | 3.2±0.1** | 3.1±0.1 | 4.6±0.8** |
| Sieve plate-forming fenestrae[c] | 2.6±0.7** | 4.9±0.1** | 5.8±0.6** | 9.4±2.5** |

* $p<0.01$, zone 1 versus zone 3
** $p<0.01$, tumour cell colonized livers versus HBSS-injected mouse livers (Table 2)

[a] Data are expressed as mean values±SEM
[b] As percentage of the total endothelial surface
[c] As percentage of the total number of fenestrae

**Table 4.** Evaluation of the reticuloendothelial cell function in liver sinusoids colonized by malignant cells from B16F10 melanoma or Lewis lung carcinoma

| Experimental groups | Phagocytosis of latex particles[a] | | |
|---|---|---|---|
| | Total uptake | Partial uptake in acinar zone 1[b] | Partial uptake in acinar zone 3[b] |
| Control livers | 86±12 (1.0) | 87.2% | 12.8% |
| B16 melanoma-colonized livers | 24± 5* (0.2) | 86.3% | 13.7% |
| LL carcinoma-colonized livers | 155± 8* (1.8) | 80.0% | 20.0% |

* $p<0.01$, control versus tumour-colonized livers

[a] Data express the mean number of latex particles±S.D. per microscopic field (area: 0.84 $mm^2$). In parenthesis: relative values with respect to control livers
[b] As percentage of the total uptake

In contrast, the fenestration pattern of endothelial cells in the sinusoidal walls of micrometastatis-bearing livers did show a clear change with respect to controls (Figs. 3 and 4; Table 3). Density of fenestrae was clearly lower in the sinusoidal endothelial in liver colonized by tumour cells, especially in the zone 3 sinusoidal segments. The diameter of fenestrae was homogeneously smaller throughout the sinusoidal path. Consequently, porosity values were clearly reduced in sinusoidal walls from tumour-bearing mice throughout the sinusoidal length, although these values were even smaller in zone 3 sinusoidal segments. Moreover, the number of clustered fenestrae, (i.e., those forming sieve plates) was also much lower throughout the sinusoidal wall of livers from tumour-bearing mice.

Quantitative values of the phagocytic efficiency of sinusoidal cells in normal and colonized livers are presented in Table 4. As can be seen, different reticuloendothelial activity occurred in livers depending on the kind of tumour cells colonizing hepatic sinusoids. In liver tissue from B16 melanoma-injected mice, uptake of circulating latex beads was clearly depressed with respect to controls. Liver tissue from Lewis lung carcinoma-bearing mice revealed a phagocytic activity even higher than that in control mice. Despite these changes, the distribution of latex particle uptake in zones 1 and 3 or the hepatic acinus was not drastically altered, although in the case of LLC-colonized livers phagocytosis in zone 3 had increased slightly.

## Discussion

In the present study we have demonstrated that early in the metastatic process a generalized sinusoidal cell response takes place in liver tissue which coincides with the implantation of small foci of tumour cells, and that the main features of this reaction are a reduction in the number of endothelial fenestrae throughout the sinusoidal wall and an alteration of the phagocytic efficiency of reticuloendothelial cells.

We would like to stress that the observed sinusoidal reaction occurred when tumour cells were present in liver tissue simply as a small proportion of intravascular isolated cells, and as small-sized metastatic foci not yet disturbing hepatic structures – as demonstrated histologically and by the unchanged proportions of acinar zone 1 and 3 de-

fined by hepatocyte SDH activity. Therefore, a diffuse cell-to-cell action of metastatic cells on sinusoidal cells can ruled out, as can be haemodynamic effects secondary to this incipient colonization by tumour cells. We may also discard the possibility of influence caused by injection or manipulation of the portal tract since the effects were studied 7 days after the experiment was carried out. Moreover, no signs of alteration were found in the control animals subjected to identical surgical trauma caused by the intrasplenic injection of the same volume of saline solution, nor in control animals injected with the same volume of HBSS containing peritoneal macrophages suspended in the same concentration as used in the case of tumour cell injections.

Concerning the variations observed in the endothelial cells, we wish to stress the diffuse nature of these changes, as shown by the reduction of the fenestration pattern throughout the sinusoidal wall. Moreover, there was a zonal repercussion in this new expression of the endothelial surface since the porosity reduction was significantly higher in the zone 3 segments of the sinusoidal wall, despite the fact that the implantation of micrometastases occurred only in zone 1 of the hepatic acinus, as shown previously (Barbera-Guillem et al. 1989b).

Interactions between tumour cells and endothelial walls have been widely recognized and considered to be crucial step in the metastatic process (Nicolson and Poste 1983; Weiss et al. 1988). However, it is to be expected that such interactions occur only at implantation sites (Nicolson and Poste 1983). In a previous study, we showed that the implantation of B16 melanoma and Lewis lung carcinoma metastases developed only in zone 1, a finding that is apparently related with the type of endothelial cell present in the colonized sinusoidal segment (Barbera-Guillem et al. 1989b). Therefore, the diffuse reaction of the hepatic endothelium that we have detected can only be associated with these focal phenomena if it is assumed that intracellular signals can be generated in those endothelial cells interacting with metastatic cells, and that these signals subsequently spread from the point of tumour-endothelial cell contact to the whole of the sinusoidal endothelium. Although it is not possible to pinpoint the causes of the diffuse endothelial cell reaction, a possibility would be that unknown substances derived from tumour cells or host cells of the immune system could act on the endothelial cells, as has been observed in other studies (Campbell 1977; Starkey et al. 1984).

The variation of phagocytic properties detected in the livers of tumour-injected mice suggest the possible involvement of Kupffer cells in the hepatic colonization process of tumour cells. It is worth noting that phagocytic changes depend on the tumour type: while in B16 melanoma-colonized livers uptake of latex particles was much lower, in Lewis lung carcinoma-colonized livers this activity was significantly enhanced with respect to controls. It should also be pointed out that phagocytosis mainly occurred in the high-SDH activity area of liver acini without variation of the zone 1-to-zone 3 latex uptake rate. The alteration in the phagocytic potential of sinusoidal cells produced during the early phase of hepatic colonization could be associated with the set of functional properties expressed by macrophages against tumour cells or with the antigen-presenting role of these macrophages. It has long been known that tumour cells are capable of subverting functional activities of antitumour host cells. In fact, together with changes affecting cytotoxic effects of T lymphocytes and natural killer cells produced with increasing tumour burden (Lala et al. 1985; Barbera-Guillem et al. 1988a), phagocytic and chaemotactic functions of reticuloendothelial cells have also been reported to vary significantly depending on the tumour type and the stage of evolution of the tumour (Salky et al. 1967; Williams et al. 1985; Cianciolo and Snyderman 1986).

Finally, none of these changes can be associated with variations in hepatic zonal activity because the distribution percentage of hepatocytes with high and low SDH activity was never found to be altered. Therefore, without discarding the possibility of other functional changes in hepatocytes, it seems that tumour cell-induced changes in the endothelial cell fenestrae and phagocytosis activity are limited to these cell types and, despite the functional interrelation between hepatocytes and sinusoidal cells, the metastasis-related sinusoidal changes do not apparently affect the metabolic definition of the hepatic acinar zones, at least insofar as hepatocyte SDH activity is concerned.

Two suggestions arise from these results. The generalized reaction of the hepatic sinusoidal structure could be induced early in the metastatic process as a result of the implantation in the liver of a small number of scattered tumour foci. Furthermore, this liver tissue reaction appears to vary according to the colonizing tumour cells type. Our present knowledge of the metastatic process causes us to conclude that this reaction of the colonized tissue may affect the evolution of the metastasis itself since it seems clear that "soil" properties of colonized organs are different before and after being invaded by metastatic cells.

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