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Changes of Histological and Proliferative Indices in the Walker Carcinoma with Tumour Size and Distance from Blood Vessel

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Abstract—*The spatial distribution of viable carcinoma cells, necrosis, vascular volume, connective tissue, growth fraction, labelling index, mitotic index and grain count per cell nucleus was determined on autoradiograms of the Walker carcinoma 256 for tumours of different size and also as a function of distance from blood vessel. From the edge to the centre of the tumours, the portion of carcinoma cells, vascular volume and portion of connective tissue decrease whereas growth fraction and labelling index, at large, remain constant. From day 8 to day 12 after transplantation the percentage of carcinoma cells decreases from 65 to 24% and that of necrosis increases from 9 to 49%, whereas the frequency of stroma elements and the proliferative indices remain constant. With increasing distance from blood vessel all the parameters investigated show pronounced changes. The numbers of viable and of proliferating carcinoma cells decrease exponentially with distance from blood vessel and reach the 50% levels at distances of 100.2 ± 1.3 μm and 83.6 ± 3.4 μm (± S.D.), respectively.*

INTRODUCTION

THE RESPONSE of tumours to therapeutic treatments depends not only on the intrinsic sensitivity of the tumour cells but also on a number of structural parameters. These parameters characterize the rather complex tissue architecture of solid tumours, which comprises the parenchymal tumour cells, in most studies considered as the principal target, as well as the stroma, made up of various tissue elements and providing the essential vascular framework. In addition, local formation of necrotic foci is known to occur in growing tumours, at the boundary areas of which highly intricate problems arise involving proliferation, starvation and death of cells. Within such regions there must be cells that are particularly resistant to treatment, and this is therefore a practical as well as a fundamental problem [1].

During the last two decades, there has been a search for structural regularities in animal tumours in which, however, a rather limited number of investigators have been involved. These studies showed the existence of two types of spatial dependencies of histological and proliferative indices: (a) changes occurring across a tumour cross section from the edge towards the centre and (b) changes occurring with increasing distance from blood vessel.

Regarding the tumour parenchyma at different positions across the tumour, Hermens and Barendsen [2] measured the ^{3}H-thymidine labelling index for a transplantable rhabdomyosarcoma and found a pronounced decrease towards the centre of the tumour. Rockwell *et al.* [3] showed for the KHJJ tumour of the mouse, as Aherne *et al.* [4] did for a BALB/c mouse sarcoma, that mitotic index, labelling index and growth fraction were higher at the periphery compared to the centre. Furthermore Hermens and Barendsen [5] and Rockwell *et al.* [3] reported a prolongation of the intermitotic time of the tumour cells near the centre, whereas Aherne *et al.* [4] as well as Nishioka in studies on sarcoma 180 of the mouse [6] did not find any difference.

As far as the existence of small structural units in the vicinity of blood vessels is concerned, already Thomlinson and Gray [7] found viable zones of tumour parenchyma limited in their spread by necrotizing beyond a critical distance from the capillary. Later,

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several authors (cf. [8, 9] and the references given there) showed qualitatively by ^{3}H-thymidine uptake that inside these viable tissue regions, zones of proliferation are preferentially located in close vicinity to blood vessels.

The only quantitative autoradiographic investigations published up to now are those by Tannock [10, 11]. He found that in regions near blood vessels, mitotic and labelling index as well as the growth fraction were approximately double the values in the zones bordering onto necrosis. No significant changes were observed in the cell cycle times, although a recent investigation [12] indicates that the mean cell cycle is prolonged in the cells adjacent to necrosis compared to those adjacent to capillaries.

In the present study, we investigated the structural regularities of an animal tumour using the Walker carcinoma 256 of the rat, that has most frequently been used for experiments for several decades. The changes of histological and proliferative indices were measured quantitatively over the entire area of tumours of different age as well as in relation to the distance from blood vessel.

MATERIALS AND METHODS

Tumours

For transplantation, 0.05 ml of a tumour homogenate containing 2×10^6 cells was injected subcutaneously into the thigh of male BD1 rats weighing 230–260 g. As shown earlier [13], this tumour grows exponentially until day 9 after transplantation, later its growth rate decreases continuously. We investigated six tumours of two characteristic stages of growth, i.e., tumours in exponential growth and tumours in a stage of decelerated growth. In the first group, the animals were sacrificed 7 or 8 days after inoculation when the doubling time of the tumour volume derived from the slope of the exponential part of the growth curve amounted to 22.4 hr [13]. Tumours of the second group were 11 and 12 days old; at this age volume doubling time was about 100 hr [13].

Autoradiography

For single labelling, 0.2 μCi per gram body weight ^{3}H-methyl-thymidine (^{3}H-TdR; spec. act. 21 Ci/mM) were injected intraperitoneally on the 7th and 11th day post inoculation. Multiple labelling was performed by five injections of the same amount of ^{3}H-TdR repeated at 5 hr intervals. Since the length of S phase was determined to be 6.4–6.6 hr and the cell cycle to be 18 hr [13] virtually all of the proliferating cells are labelled by this procedure (cf. Steel [1], pp. 101–104). In the following, the percentage of cells labelled after repeated injections with ^{3}H-TdR is named 'growth fraction'. One hour after the last injection the animals were killed by cervical dislocation. Slices of 4–6 mm thickness were excised from the median region of each tumour together with parts of associated muscular and subcutaneous tissue. Prior to fixation in 8% formalin, the slices of the larger tumours (day 11 and 12) were bisected. The samples were embedded in paraffin and sectioned at 3 μm. Autoradiography was performed using the stripping film technique (Kodak AR10). Slides were exposed for 4 weeks at 4°C in sealed light-tight plastic slide boxes containing $CaCl_2$ for desiccant. After developing and drying the slides were stained with haematoxylin and eosin and inspected using a microscope with a projection unit (Telepromar, Leitz, Wetzlar, Germany). Since the background of the autoradiograms was determined to be about 0.8 grains per nucleus (60 μm^2), cells with 3 and more grains per nucleus were considered as being labelled.

Counting procedure

For determining the histological and proliferative indices, and their variations across the entire tumour, the area of each section was subdivided into subunits about 1 mm^2 in size and analyzed according to the method of Chalkley [14]. Per subunit 10–15 microscopic fields, 200 μm in size were projected consecutively onto a screen bearing 20 points distributed at random. The events coinciding with each point were recorded. Since one is in effect moving a random point through the tumour, the score for any tissue element gives a measure of its portion of the total volume.

For investigating the microenvironment of blood vessels, all the cells in the transit region between the inner wall of an intact vessel and the nearest necrotic region were analyzed. Vessels with diameters ranging from 10–25 μm were selected which have been cut almost transversely and which were surrounded by an undamaged layer of endothelial cells and labelled tumour cells indicating undisturbed circulation. Sixteen transit regions, 50 μm wide and 200–240 μm long, subdivided into 10 μm steps were investigated.

The histological elements were classified by their morphology as viable (i.e. morphologically intact) tumour cells, necroses, connective tissue, leucocytes (i.e. all infiltrating blood-borne cells), endothelial cells and vascular volume.

RESULTS

Spatial distribution of histological and proliferative indices in tumours of different age

Figure 1 shows the results obtained with a tumour at day 8 after transplantation. At that time, the tumours have the shape of half-ellipsoids, being about 6 mm wide and about 12 mm long. The central area of the section was divided into 42 subunits about 1 mm^2 in size, as shown on the lower part of Fig. 1. In the region indicated more than 12,000 cells were analyzed. It is obvious that the percentage of morphologically intact tumour cells decreases from 67.9% near the muscles to 61.5% on the side adjacent to the subcutis. In the centre of the tumour, the percentage of viable tumour cells shows a minimum. In the same region, the vascular volume and the portion of connective tissue reach minima, too, whereas the percentage of necroses is highest. In these tumours about 50 areas of confluent necrotic material per section are observed having a mean diameter of about 200 μm but scarcely exceeding 500 μm. They contain about 40% of the necroses scored, the others being scattered mainly as single events over the entire area but being more abundant in the centre. The numbers plotted in Fig. 1 (chart A) comprise a mean of 93.2% of the points scored. The remaining 6.8% are mainly indifferent events, leucocytes as well as a few endothelial cells.

The growth fraction amounting to 94.5% near the muscles (Fig. 1, chart B) decreases slowly across the tumour and approaches 87.6% near the subcutis. In a similar way, the mitotic index changes from 5.5 to 3.3%. The number of grains per labelled carcinoma cell after multiple injections shows a pronounced local variation, dropping from 38 grains per nucleus to 10.5.

The data were also grouped in such a way as to yield the longitudinal variation of the parameters investigated. But most of the changes could not be proved to show clear trends. For example, in the horizontal groups of subunits from top to bottom of the area indicated on Fig. 1, the growth fraction amounted to 91.5, 90.4, 91.8, 92.1, 93.9, 91.4 and 94.4%, respectively.

Figures 2 and 3 show the local variation of viable and of labelled carcinoma cells as well as the distribution of necrosis determined for tumours in the stage of reduced growth rate. In these relatively large tumours, extended coherent regions of ischemia are observed as represented by the dotted areas in Figs. 2 and 3. They are located predominantly on the side of the subcutis and, to a lesser extent, on the

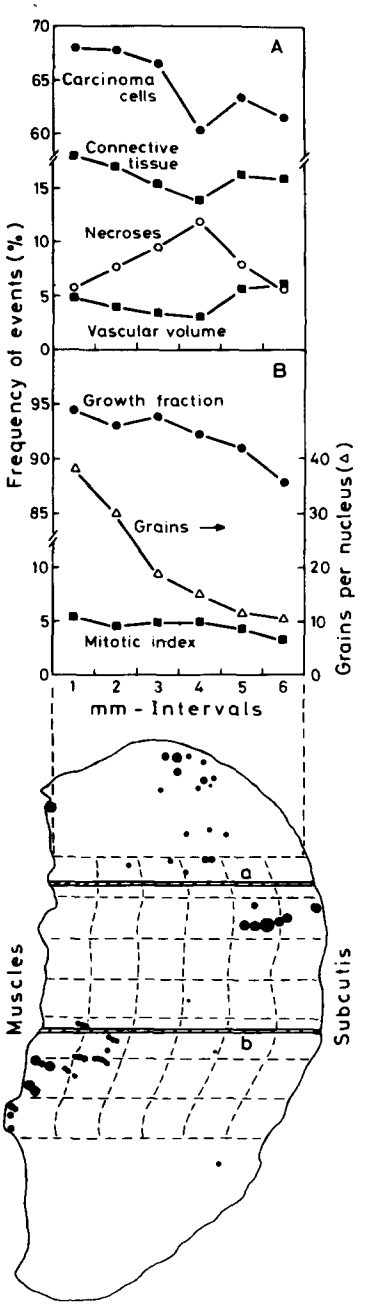

Fig. 1. Spatial distribution of histological and proliferative indices across a Walker carcinoma 256 at day 8 after transplantation. The dark areas represent necrotic regions.
A. Histological indices: carcinoma cells, connective tissue, necrosis and vascular volume.
B. Proliferative indices: growth fraction, mitotic index, grains = median number of grains per nucleus determined from autoradiograms by counting the grains of all carcinoma cells localized in the two shaded zones (a) and (b) across the tumour.

side of the muscles. In order to quantify these observations, more than 13,000 cells were scored in corresponding sections of either tumour. The portion of morphologically intact carcinoma cells amounts to about 60% on the side of the muscles; this portion decreases continuously, reaches figures much below 10% near the centre of the tumour and increases again over the last millimeters near the subcutis. The frequency of necroses shows an inverse dependence; it increases continuously over half the tumour width, reaches a broad maximum width 70–85%, and decreases again near the edge of the tumour.

The labelling index of carcinoma cells (Fig. 2, chart B) does not deviate significantly from the mean value of 39.6%, although showing some scatter in regions with only a few viable cells present. In a similar way, the growth fraction (Fig. 3) is rather constant with a mean of 88.3% in spite of the large variation

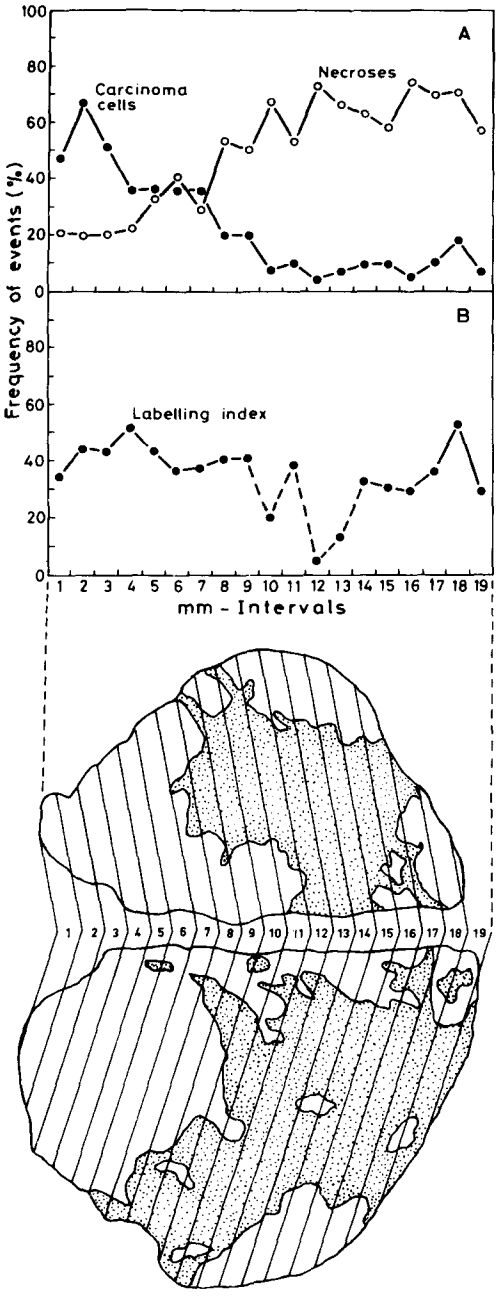

Fig. 2. Spatial distribution of viable carcinoma cells, necrosis and labelling index of carcinoma cells across a Walker carcinoma 256 determined at day 11 after transplantation after single labelling. Dotted areas represent necrotic regions.

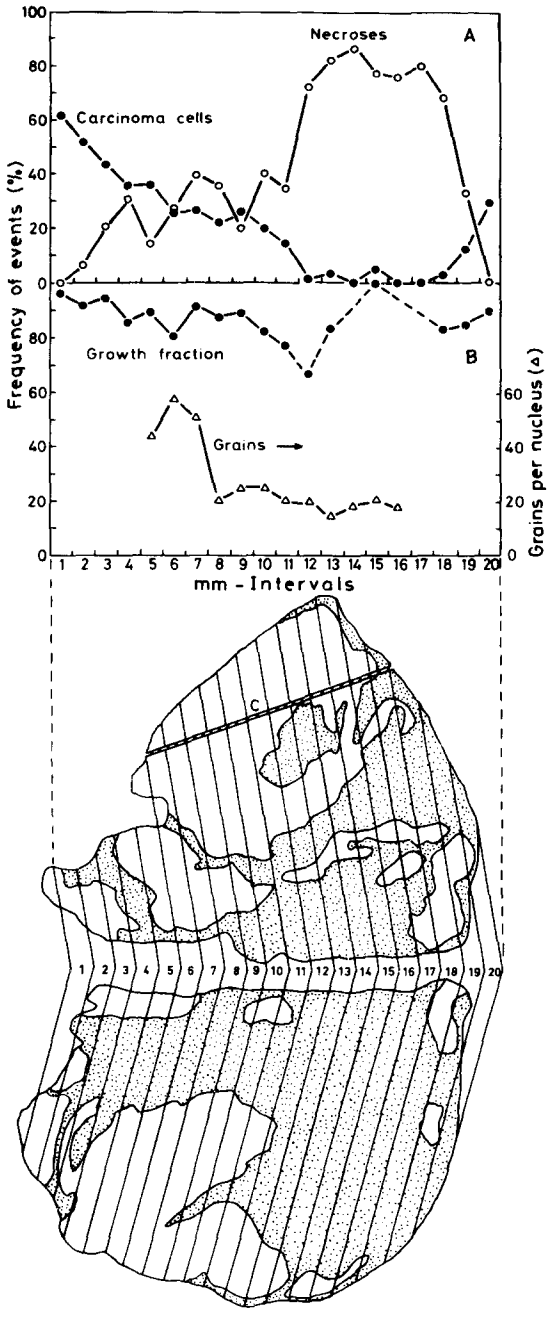

Fig. 3. Spatial distribution of viable carcinoma cells, necrosis and growth fraction of carcinoma cells across a Walker carcinoma 256 determined at day 12 after transplantation after multiple labelling. The median number of grains per nucleus was determined from autoradiograms by counting the grains of all carcinoma cells localized in the shaded zone (c) across the only larger part of the tumour without necrotic regions.

in the number of viable carcinoma cells observed.

The number of grains per nucleus (Fig. 3, chart B) was counted for all carcinoma cells within an area, 50 μm wide, across the only larger part of the tumour without necrotic regions. From left to right the grain count decreases by a factor of 3, i.e., in a similar was as has been observed for the small tumours (Fig. 1).

Age dependence

Figure 4 shows the variations of histological and proliferative parameters in tumours of

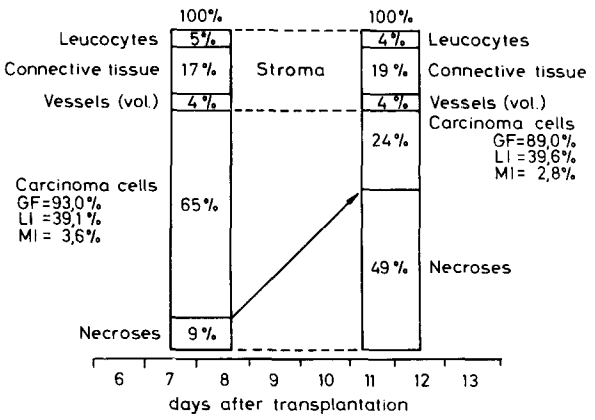

Fig. 4. Changes of histological and proliferative indices of the Walker carcinoma 256 as a function of time after transplantation.

different age. The data plotted are based on the analysis of a total of more than 48,000 cells scored in six different tumours. The stroma elements constituting 26 and 27%, respectively, do not show any change with age.

The same result is obtained for leucocytes, connective tissue and the vascular volume. In contrast, during the time interval investigated, the viable carcinoma cells decrease from 65% to 24%, whereas the necroses increase from 9 to 49%.

In spite of these dramatic changes observed in the number of viable carcinoma cells their proliferative indices remain unchanged. That means, the labelling index, the growth fraction as well as the mitotic index are the same for tumours in exponential growth as well as for those in the stage of decelerated growth. This demonstrates that most carcinoma cells that appear morphologically intact also proliferate. If resting cells are present in this tumour, their amount is well below 10%.

Spatial distribution of histological and proliferative indices around blood vessels

Figures 5 and 6 show a detailed analysis of the spatial distribution of histological elements and labelled cells in the transit region between intact vessels and the nearest necrotic regions. The data represent mean values of 4 transit regions in tumours at day 8 (Fig. 5) and 12 transit regions in tumours at day 12 after transplantation (Fig. 6). Closest to the inner wall of a blood vessel mainly endothelial cells are found; these are surrounded by connective tissue as becomes especially obvious

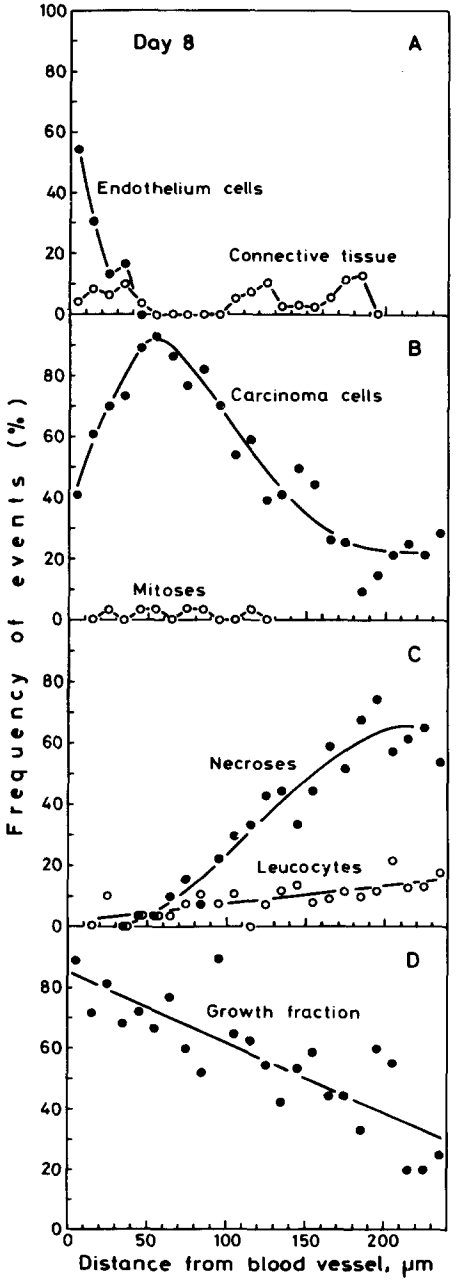

Fig. 5. Changes of histological and proliferative indices as a function of distance from blood vessel determined for the Walker carcinoma 256 at day 8 after transplantation.

from Fig. 6 (chart A). With increasing distance from blood vessel, the fraction of carcinoma cells increases continuously reaching a maximum of more than 90% at a distance of 50–70 μm. In the region where the carcinoma cells decrease again, an increase in necrotic cells is observed. Mitotic carcinoma cells are observed only in the viable parts whereas leucocytes are found predominantly in the necrotic region.

The percentage of labelled carcinoma cells decreases with increasing distance from blood vessel. In parallel, the amount of ^{3}H-thymidine incorporated per cell changes correspondingly as becomes obvious from the number of grains per nucleus (Fig. 5, chart D) which decreases from about 30 in the vicinity of a vessel to less than 10 in the necrotic region. In the latter region, the data points show a rather large scatter due to the few carcinoma cells being present.

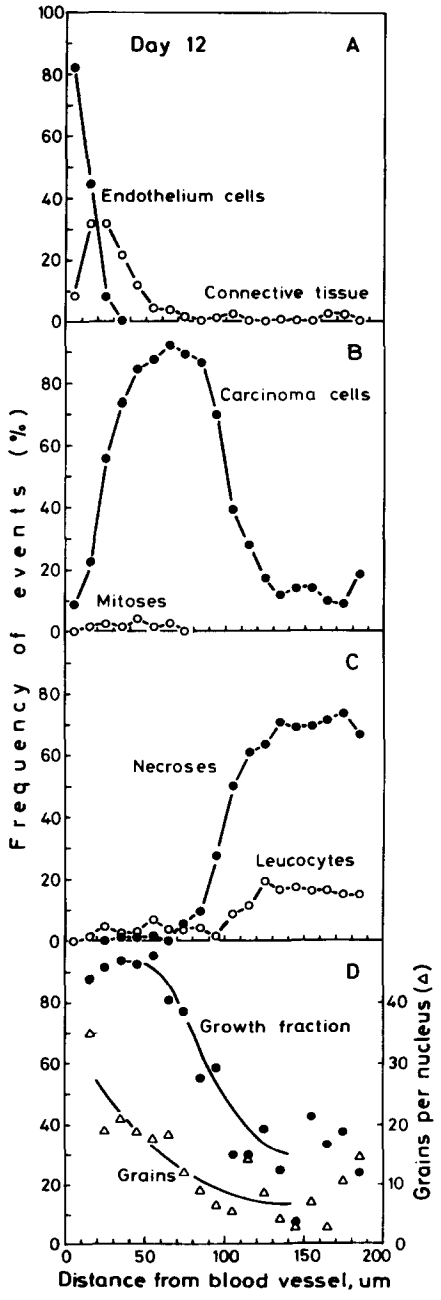

Fig. 6. Changes of histological and proliferative indices as a function of distance from blood vessel determined for the Walker carcinoma 256 at day 12 after transplantation.

The changes observed within the transit regions from blood vessel to necrosis seem to occur more generally in the smaller tumours compared to the larger ones. Although this has been found for the spatial distribution of carcinoma cells, necrosis, leucocytes and the growth fraction, we believe that the distribution in the tumours is influenced by artefacts mainly by overlapping of the rather close network of capillaries present in these tumours. The average distance of capillaries seems to be substantially shorter than 200 μm. So the chance is rather high that above or below the plane of the section analyzed there may have been vessels that influence the distribution of cells in the scored plane although the vessels have not been visible on the slide. This view is supported by the observation that the percentage of connective tissue shows some small maxima at larger distances from blood vessels: So we suppose that the "true" structure of viable tissue layers in relation to blood vessels can only be obtained from the analysis of the older and larger tumours. Figure 6 shows that at a distance of 100 μm from the inner wall of the nearest blood vessel the percentage of carcinoma cells as well as of necroses reach their half-maximum values. This may be regarded as an estimate of the thickness of the viable tissue layer.

DISCUSSION

It has been shown on Fig. 1, that in the centre of the 8 days old tumours the vascular volume, the portion of connective tissue and of viable carcinoma cells show minima whereas the number of necroses reaches a maximum. These findings indicate that the Walker carcinoma is supplied mainly from the side of the muscles and also, but to a lesser extent, from the side of the subcutis. Even these relatively small tumours show a reduced vascularisation in the centre giving rise to a higher frequency of necrotic cells and, concomitantly, to a small reduction of viable cells. But these small tumours, although possessing a rather close network of capillaries, still develop coagulative necrotic foci at some of its largest "meshes". These correlations

become even clearer from the older tumours where the supply and clearance of the central part is so insufficient that the majority of cells present become necrotic, and being not removed form large and confluent regions of ischemia. Only a few cords of viable carcinoma cells are found here around intact, mainly sinusoidal capillaries.

This picture, at a first glance, seems to be distorted by having found that the relative vascular volume does not change with age of the tumours (Fig. 4). But the vascular volume per se is an inadequate measure for the condition of blood supply in neoplastic tissue (cf. [16, 17]). Blood vessels tend to dilate during tumour growth, and inside large tumours central vessels are widened and frequently of sinusoidal type, thus leading to a reduction in surface per vascular volume. Furthermore, blood vessels mainly in central parts of larger tumours show vascular stasis although appearing morphologically intact [18].

In a preceding publication [13] we reported on the kinetic parameters of the parenchyma cells averaged over the entire Walker tumour. Within 12 days after transplantation, about 40% of the volume are occupied by necroses whereas the growth fraction of the non-necrotic compartment remains constant amounting to 96, 93 and 93% at day 5, 7 and 11, respectively. The more exact data plotted on Fig. 4 that are based on the analysis of more than 48,000 cells agree well with these earlier findings supporting the conclusion that the slowing-down of growth rate of the Walker carcinoma is primarily caused by an increased cell loss but not by a prolongation of cell cycle times or by the appearance of a substantial portion of non-proliferating cells.

The variation of histological and proliferative indices with increasing distance from blood vessel may be compared with the findings of Tannock [10, 11]. He investigated two transplantable mammary tumours of the mouse, a BICR/SA1 adenocarcinoma [10], which exhibits a corded tissue architecture throughout the tumour, and a C3H tumour [11], which forms distinct cylindrical cords of viable parenchyma in certain regions. In the two tumours, the viable tissue layers around capillaries had thicknesses in the range of 60–120 μm with a mean of about 90 μm, and of 100 ± 20 μm, respectively. Our data recorded for 10 μm intervals make possible a rather exact determination of the tissue layer dimensions. In Fig. 7, the percentage of morphologically intact and of proliferating carcinoma cells are plotted in a half-log scale as a function of distance from blood vessel. The regression lines drawn show that the frequency of carcinoma cells ($r = -0.9960$) and of proliferating carcinoma cells ($r = -0.9794$) decrease exponentially with increasing distance. When using the 50% levels to characterize the thickness of the layers, we calculated from the data of Fig. 7 a distance of 100.2 ± 1.3 μm ($\pm$S.D.) for the thickness of the viable layer and a

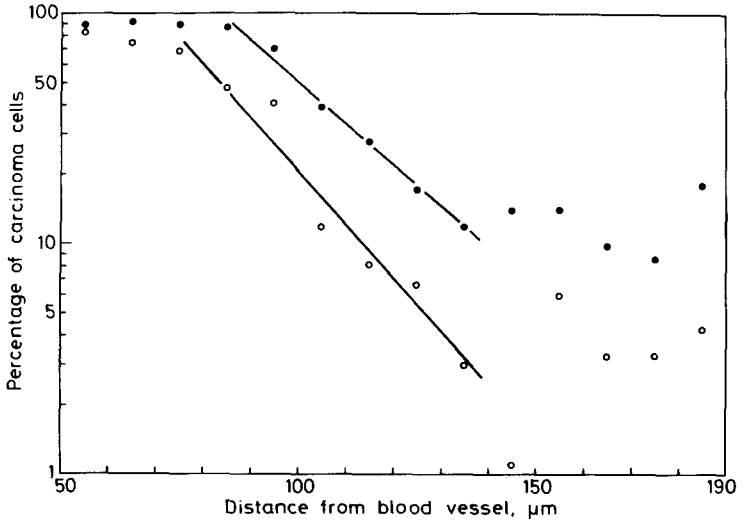

Fig. 7. Distribution of viable and proliferating carcinoma cells around blood vessels determined for the Walker carcinoma 256 *at* day 12 *after transplantation.* ● *Frequency of morphologically intact carcinoma cells (data from Fig.* 6, *chart B).* ○ *Frequency of proliferating carcinoma cells determined by multiplying the percentage of carcinoma cells (Fig.* 6. *chart B) with their corresponding growth fraction (Fig.* 6, *chart D).*

distance of 83.6 ± 3.4 μm for the thickness of the proliferating region. The absolute distances *in situ* are somewhat larger since in paraffin-embedded samples shrinkage can never be avoided.

"The interdependence of parenchyma and stroma probably lies at the heart of what determines the overall growth rate of tumours" (Steel [1] p. 163). In addition, there are some indications that cells in the border regions adjacent to necrosis may have a radioresistance 10 times higher (or even more) than that of fast proliferating cells [9]. If these highly resistant cells are capable to regain their mitotic activity under certain conditions such as after inactivation of proliferating tumour zones, then the optimum therapeutic dosage depends on very complex conditions. This indicates the need for further investigations into the interdependence of parenchyma and stroma in the growth of tumours and their influence on proliferation kinetics and response to therapeutic treatments.

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