Eur J Cancer Clin Oncol, Vol. 18, No. 2, pp. 155–165, 1982.
Printed in Great Britain.

0277-5379/82/020155-11$03.00/0
Pergamon Press Ltd.

Differences in Vascular Pattern Between the Spontaneous and the Transplanted C3H Mouse Mammary Carcinoma

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Abstract—*The transplanted C3H tumor differs from the spontaneous in its multi-focal origin and in its increasing loss of differentiation. Loss of differentiation is principally important in that even the partial branching into lobes, which occurs in the spontaneous tumour, is lost and that the parenchyma no longer consists of single-layered and multi-layered tubules, as in the spontaneous tumour, but of multi-layered only. Absence of branching removes the interlobular pressures, which in spontaneous tumours lead to infarction of the efferent vessels and sinus formation. Consequently, sinuses are absent from the transplanted tumour and the circulation is thereby improved. In contrast, since the vascular pattern of the spontaneous tumour is preserved and capillaries do not penetrate into the multi-layered tubules, although these have developed into solid cylinders, the diffusion pathway of oxygen is extended, resulting in necrosis at their cores. Accumulating necrotic fluid is at first removed by lymphatics, but subsequently invades the efferent vascular system. These changes are reflected in the pattern of tumour growth and in the radiation responses.*

INTRODUCTION

FOR THE experimentalist, syngeneic transplants have many advantages over spontaneous tumours and in consequence most recent work on the radiation responses of the C3H mouse mammary carcinoma has been performed with transplants. The clinician, however, is concerned both with primary and secondary tumours and needs to be aware of the likely differences in the radiation response of each. For this reason an investigation of the spontaneous C3H tumour [1] is now followed by a similar investigation of the transplant. McCredie *et al.* [2] have described the differences in the growth and morphology of the spontaneous tumour and its transplants up to the 900th generation, and there is almost complete agreement between their findings and those described here. The difference between their account and this is that theirs is a statistical analysis of growth, cell size, relative volume of tissues and relation of blood vessels to tumour nodules, while this is a descriptive account of the pattern of the vasculature and of its development in relation to the growth of the tumour as a whole.

C3H transplants differ from spontaneous tumours in their multifocal origin and in the changes that constitute 'progression' ("The development of a tumour by way of permanent irreversible qualitative changes in one or more of the characters of its cells". Foulds[3]). The multifocal origin tends to obscure the pattern of the vasculature, but is generally less important in its effect on the vascular pattern than the changes constituting progression. These changes operate to make the tumour more anaplastic and, though they do not affect the vascular pattern directly, they indirectly cause changes which must profoundly modify the responses to radiation and to hyperthermia.

MATERIALS AND METHODS

A spontaneous C3H tumour, 15–20 mm in diameter, after discarding necrotic portions, was finely minced in a minimum of sterile, isotonic saline. About 0.1 ml of the mince was implanted by trocar under the skin of the abdomen of male C3H/He mice about 5 mm to the left of the middle line. The tumours that developed were measured by callipers twice weekly along three dimensions. Three sets of 20 tumours each were prepared. The first two sets were used to provide material for the study of the vascular pattern and its development.

Accepted 29 September 1981.

The third set was used to compare the pattern of growth with the extent of necrosis.

The methods of tumour preparation were those used for spontaneous tumours[1]. Thin microtome sections stained in haematoxylin and eosin or Van Gieson were used for detailed histological examination. Cryostat sections, 200 μm thick, and sliced tumours mounted in resin were employed to give a 3-dimensional picture of the vasculature. Details of the methods involved have already been published[4]. In outline, in both the thick sections and the slices, the blood vessels are revealed by staining in benzidine and H_2O_2. This combination gives the erythrocytes a black colouration and is particularly valuable in indicating the state of constriction or dilation of vessels *post mortem*. In the thick sections, the blood vessels are traced onto transparent acetate sheeting using a Projectina microprojector. The tracings are then stacked vertically one above another on a light box and, when seen from above, provide a 3-dimensional picture of the vasculature. For mounting in resin, the tumours are first halved or sliced and, after staining, are passed through successive ethanols into ethyl acetate and finally into the special resin (Resin 8116 supplied by B.I.P. Chemicals Ltd., Warley, Worcs., U.K.). The specimens are then transferred to glass moulds and catalysed resin substituted for the original resin. The catalyst causes the resin to set in about 10 days and the resulting block, after polishing, shows in considerable detail the pattern of vasculature of the tumour in relation to that of the surrounding tissues, and also the pattern of necrosis.

RESULTS

The pattern of growth

The implanted tumour, like the spontaneous, has a warty appearance, but for an entirely different reason. In the spontaneous tumour the appearance results from incomplete branching, the tumour seeming to mimic the branching of normal gland, but the branches never separating from the trunk and remaining appressed to it and to each other. In the transplanted tumour, on the other hand, each wart is the product of a single fragment of the original mince. Initially, each fragment develops individually (Fig. 1), but later the products fuse and the line of demarcation provided by the connective tissue with which each was surrounded is lost. If the number of fragments from which the tumour is formed is small, the vascular patterns of each reveal the multifocal origin (Fig. 2). If the number is large, the multifocal origin is soon obscured (Fig. 3).

Growth rate

The earliest stages of growth were difficult to detect by palpation in the living host. This was partly because of their flatness and partly because they originate from more than one fragment of the implant. Further complications were caused by some fragments starting to grow later than others, or by spontaneous regression after an early start. In the male recipients used, there was a delay of about 18 days before the presence of a tumour could be inferred with certainty. Foulds [3] describes a similar delay in the male BR mouse and states that prior implantation of pellets of diethyl stilboestrol reduces the delay and causes the tumour to grow as well in the male mouse as in the female. After the twentieth day, growth proceeds regularly and follows the pattern described by McCredie *et al.* [2] for their first generation transplants, i.e., Gompertzian with a growth/retardation ratio of about 10.

Figure 4 compares the growth rates of 14 tumours, all derived from a single source. From the graph and from the *post mortem* appearance of the tumours, at least four factors appear to control the rates of growth: (a) variation in the delay of fragments starting to grow is indicated both by the graph and by the *post mortem* appearance. In the graph, if the curves are extrapolated backwards they strike the abscissa at different points, and in the resin blocks, a small fragment growing actively is often associated with a larger body made up of fused fragments, each considerably larger than the small one. The distance between the small fragment and the large fused mass is too great for it to have been derived from them and, unless its growth rate is much less than theirs, it seems probable that it originated independently and started growth later; (b) variation in the number of fragments making up a tumour was clearly seen in a few small tumours in which the individual fragments could still be clearly recognised *post mortem*; (c) variation in the growth rates of individual fragments is hard to distinguish from (a), but seems to follow from the variation in the slope of the curves; (d) distortion of the recorded volumes by accumulation or loss of necrotic fluid, the evidence for which is given later. At least in the early stages of growth, spontaneous abortion of fragments may constitute a fifth variable, but though the impression gained during tumour measurement that this was occurring was strong, no certain evidence was obtained.

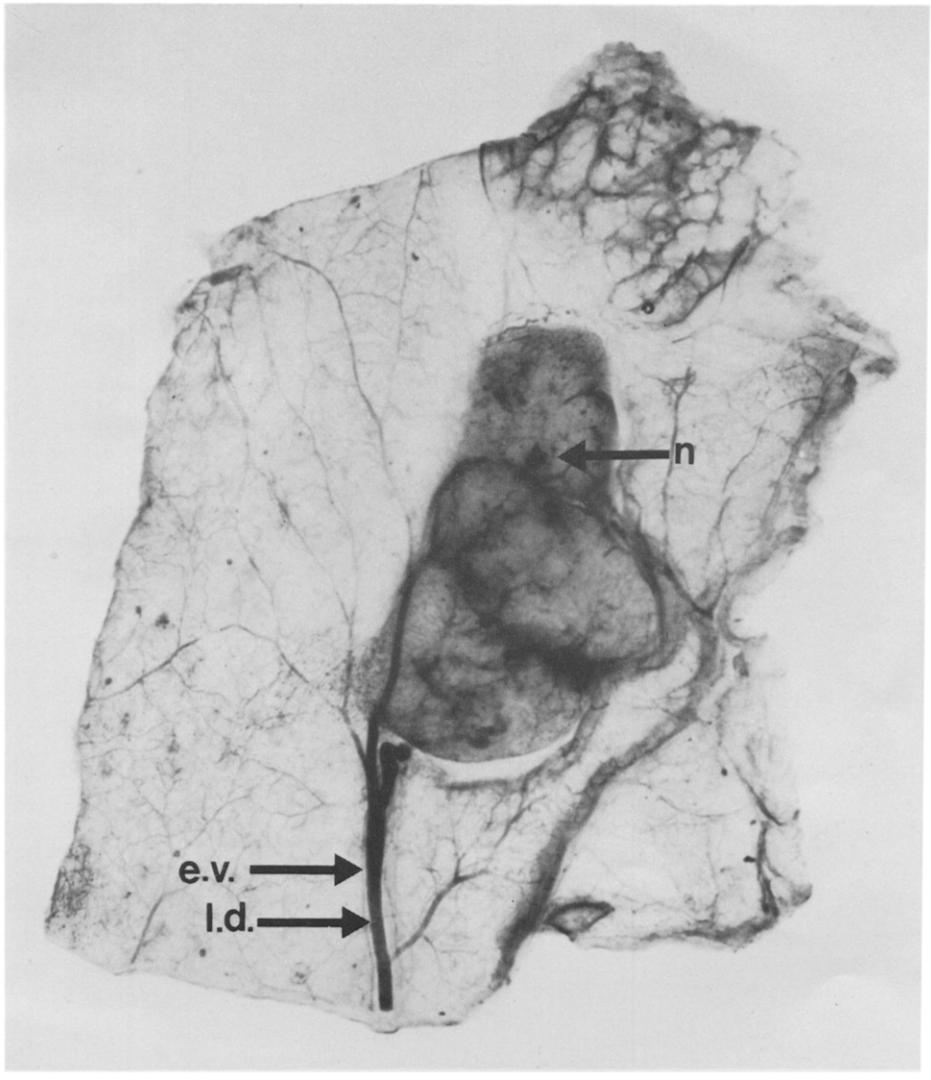

Fig. 1. *Abdominal wall and inner half of an implanted C3H tumour,* 12 mm *in length, stained in benzidine and cleared and mounted in resins. The tumour is made up of at least three and possibly four fragments which have only partially fused. Some necrosis is visible, but the efferent vascular system has not yet been captured. At this stage, removal of necrotic material is by the lymphatic, which projects on either side of the drainage vessel. Necrotic areas, n; lymph duct, l.d; efferent vessel, e.v.*

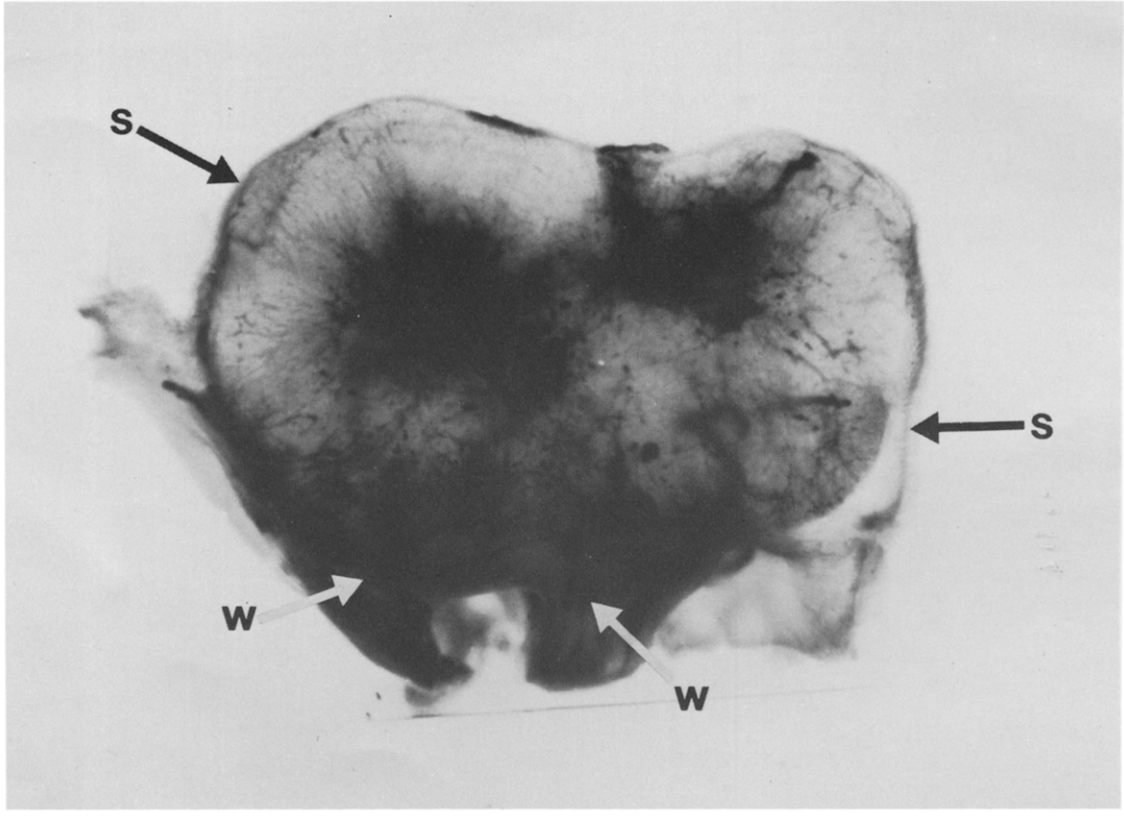

Fig. 2. Half of a C3H tumour, 19 × 11 × 11 mm, cut vertically, stained in benzidine and cleared and mounted in resin. The tumour has grown from 3 fragments, the largest occupying its left side and the two smaller the right. Very little necrosis is present, so that the pattern of the efferent vessels is exceptionally clear. Skin, s; abdominal wall, w.

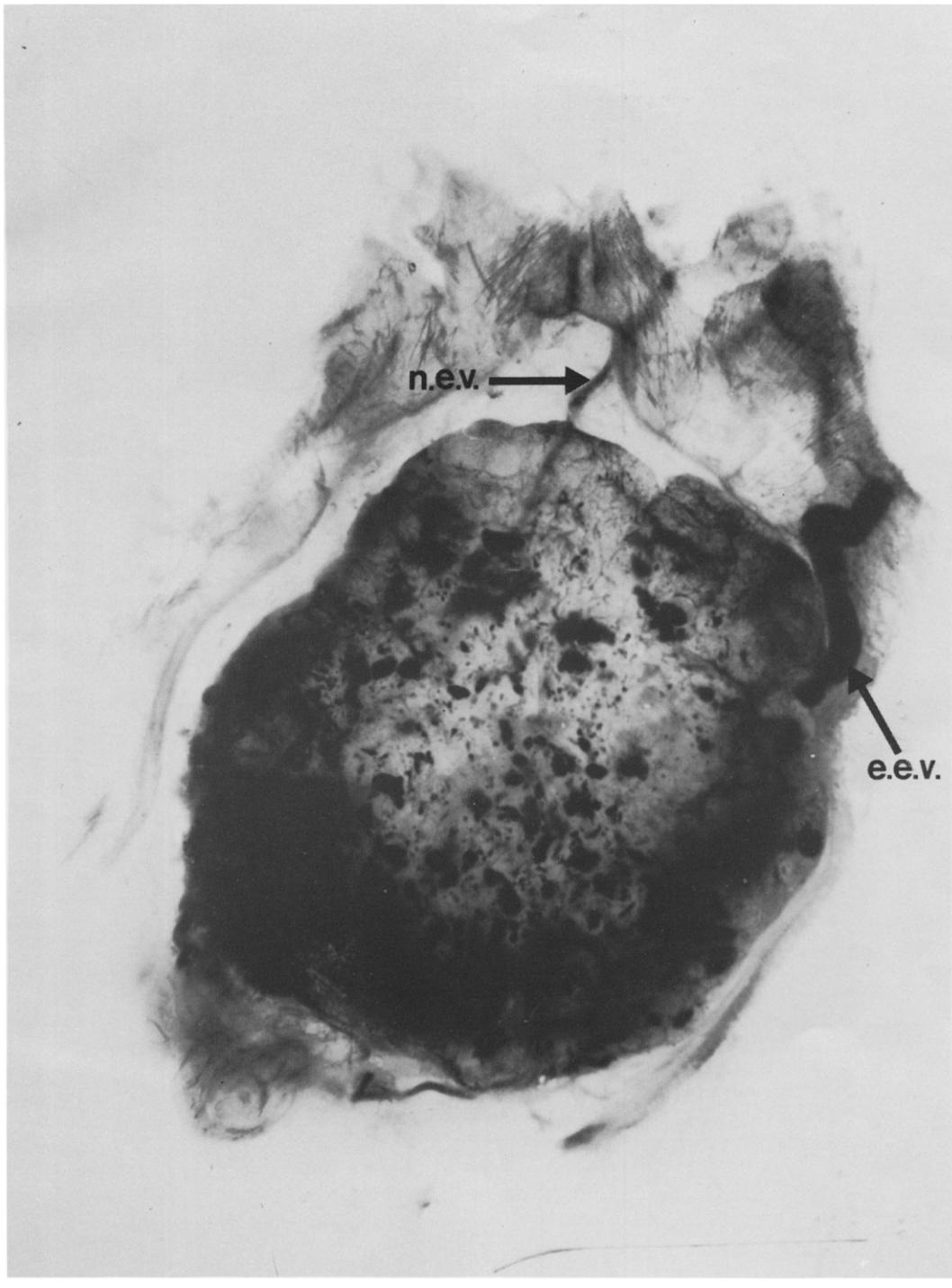

Fig. 3. Outer half of a 22 mm *diameter transplanted C3H tumour, stained in benzidine and cleared and mounted in resin. The tumour is composed of at least* 8 *fragments which have fused. The small, dark spots are the necrotic centres of the tubules which are starting to fuse. On the right, they have fused and taken over the vascular drainage system. The efferent vessel (e.e.v.) is greatly enlarged by addition of necrotic fluid in contrast to its normal sized partner (n.e.f.).*

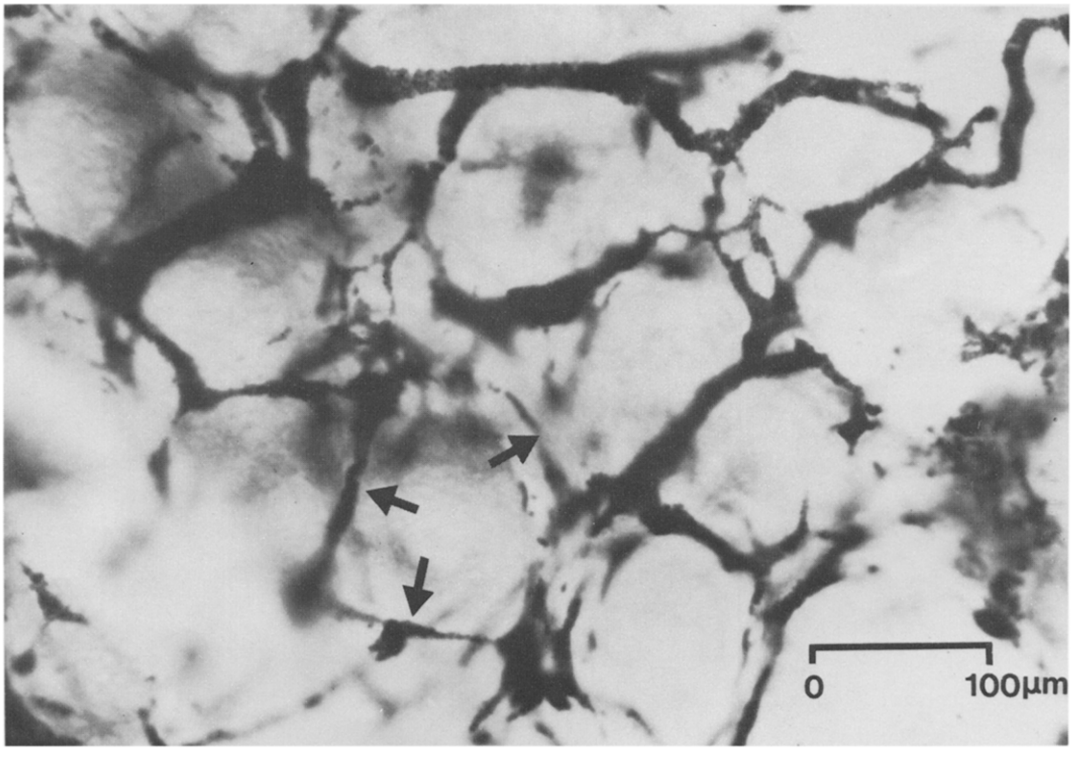

Fig. 6. Section, 200 µm thick, stained with benzidine and H_2O_2, of a C3H transplanted 8 mm-diameter tumour. A group of multi-layered tubules has been cut almost transversely. The blood vessels, which are characteristically irregular in diameter, form an imcomplete sheath round the tubules but do not enter them. There is no necrosis. Erythrocytes are just visible in some vessels.

In order to obtain depth, the section was photographed with a Leitz Orthoplan NP 1 16/0.40 objective and subsequently enlarged. The limits of one tubule are indicated by three hollow arrows.

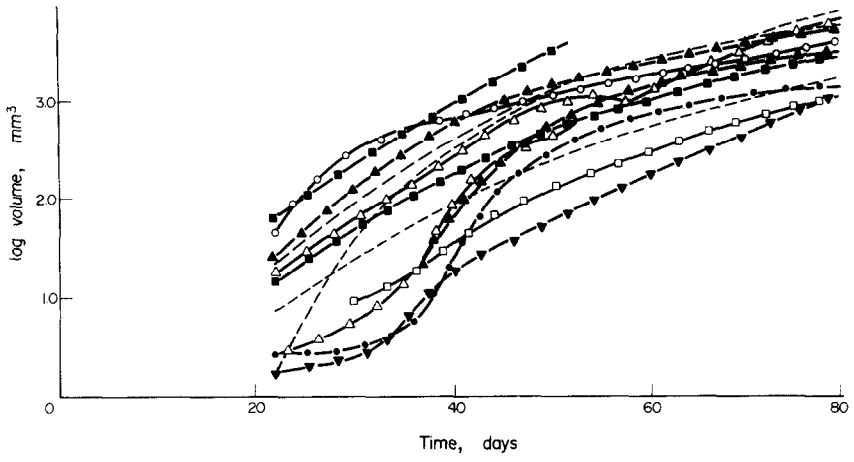

Fig. 4. The growth of 14 tumours derived from implants from a single source into male mice. Volumes were not plotted until the tumours exceeded 3 mm in depth. Measurements of the greatest diameter before this stage indicate an initial latent period characteristic of implantation into male hosts. Early growth rate is affected both by the delay in starting growth and by the number of tumour fragments. After 30 days, the slope of the curves is a true indication of the growth rate.

Histology

The histological structure of the spontaneous C3H tumour was described and figured in [1] and will only be summarised here. In that description, the effect of transformation of normal mammary gland was interpreted as occurring in two steps. These are illustrated in Fig. 5. In the first step, A–B, the alveolus is lost, but the duct remains with a wall consisting of a single layer of cells only. In some ducts the lumen remains patent, in others it closes. In most cases the duct is cylindrical, but cystic outgrowths also occur. Bresciani[5] has shown that in the initial transformation, a change occurs in the pattern of mitotic activity. In the normal, resting, post-pubertal gland, mitosis occurs only in a single zone, indicated by the arrow in Fig. 5, and serves for replacement of lost duct cells. In the transformed gland, mitosis may occur at any point in the duct, thereby causing extension and tumour growth.

The single-layered tubule is characteristic of early growth in the spontaneous tumour. The second step in transformation is its replacement by the multi-layered duct, (Fig. 5, B–C). It is possible that the change is brought about by a reorientation of the plane of cell division, but

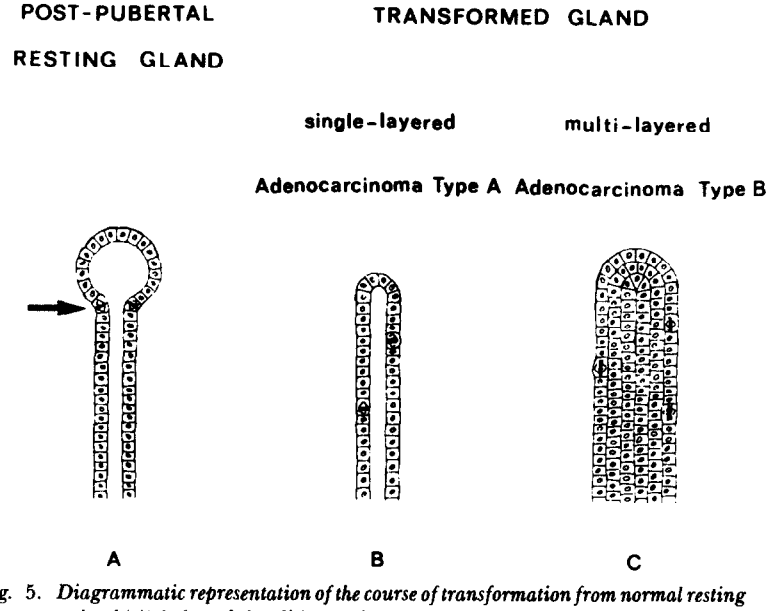

Fig. 5. Diagrammatic representation of the course of transformation from normal resting mammary gland (A) by loss of alveoli (B) to the multi-layered solid duct (C), characteristic of the later stages of spontaneous tumours and almost universal in transplanted tumours.

migration of the products of division cannot be excluded as an explanation. The change from the single-layered to the multi-layered tubule is regarded as part of the process of progression. Single-layered tubules are very rare in transplanted tumours. Only 2 examples were seen in 40 F_1 transplants from primaries in 5 different animals. In each case, the single-layered tubules formed less than 5% of the tumour volume.

The vascular pattern

In both the spontaneous and the transplanted tumours, the arrangement of blood vessels is intimately related to the tubular structure. Each tubule, single-layered or multi-layered, is invested by a thin sheath of connective tissue, in which lies a dense network of capillaries unevenly spaced and varying widely in their diameter (Fig. 6). Though the multi-layered tubules may reach a diameter of 750 μm, capillaries never penetrate into them, remaining always part of the investing sheath. The pattern of the afferent vessels supplying these capillaries is identical with that of the spontaneous tumour. Within about 1 mm of entering the tumour, the supplying artery loses its muscular coat and is reduced to an endothelial lining heavily invested in collagen. About another 1 mm further on, this vessel gives rise to a large number of small branches, which at first invest it closely, but later leave it in bundles to penetrate between the tubules and, finally, after further branching, to connect with the very much coarser capillaries described above. The system has been described as horse-tail branching, the vertebrae of the horse's tail resembling the main supply vessel, and the hairs, the minute vessels to which it gives rise. In the implanted tumour, each fragment receives its arterial supply in this way and the supply route is therefore relatively shorter than in the spontaneous tumour, in which only three afferent branches are found.

The pattern of the afferent system of individual fragments of transplanted tumour resembles that of the spontaneous tumour. A main central drainage vessel, by means of branches perpendicular to it, collects blood from the capillary bed between the tubules (Fig. 7D). Because the transplanted tumour does not branch, the distal collecting vessels are not carried outwards, so that the characteristic circumferential vessels of the spontaneous tumour are absent. A still more important effect of the lack of branching is that interlobular pressures do not arise. In the spontaneous tumour, these led to the constriction both of the main drainage vessel at its base and

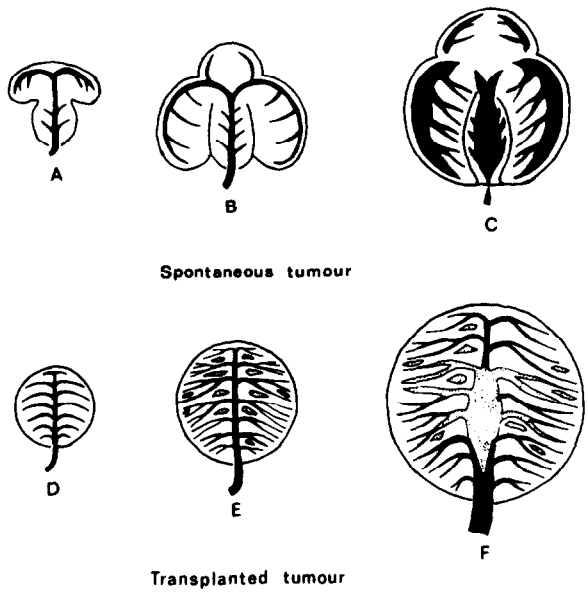

Fig. 7. Diagrammatic comparison of the origin and fate of the efferent vascular system of spontaneous and transplanted tumours. In the early stages (A and D), similar systems start to differ through the retention of branching into lobes in the spontaneous tumour. Further branching of the spontaneous tumour (B) intensifies the difference, while in the transplanted tumour, because of the thickening of multi-layered tubules, necrosis (dotted areas) appears. At a still later stage (C), the pressures generated between the lobes of the spontaneous tumour break down the efferent vessels into sinuses, which become dilated in consequence to the interruption to their normal system of drainage. In contrast, in the transplanted tumour (F), consequent on the fusion of necrotic areas and their eruption into the efferent vessels, a superficially similar accumulation of liquid occurs, but of blood and necrotic material, and not of blood alone.

of the circumferential vessels at their bases. The resulting infarction caused the dilatation of the vessels concerned and their transformation into sinuses (Fig. 7C). Without such interlobular pressures, the transplanted tumour lacks sinuses and its circulation is consequently improved.

Of the 60 transplanted tumours studied, in only three were single fragments found possessing the vascular pattern of the spontaneous tumour with dilated blood sinuses. In two, the fragments had developed too far to ascertain whether or not the characteristic branching of the spontaneous tumour was the cause, but in the third, lateral branches could clearly be seen compressing the base of the fragment and causing infarction.

Necrosis

In spontaneous tumours, necrosis is first seen at the centre of multi-layered tubules which have attained a diameter of at least 60 μm. The mean value from 25 measurements of the distance from the edge of the necrotic zone to the nearest blood vessel was 62 μm,

with a minimum of 30 and a maximum of 110 μm. These figures were taken to indicate that necrosis occurs where the pO_2 resulting from the diffusion of the gas from a capillary vessel has fallen below a critical value, and that the pO_2 must depend both on the length of the diffusion pathway and on the degree of saturation of the haemoglobin in the capillary. In spontaneous tumours, necrosis is not seen in single-layered tubules since the distance between their centres and the nearest blood vessel is always less than the critical value. In transplanted tumours, single-layered tubules are almost invariably absent and the multi-layered thicken at an earlier stage. Hence, necrosis which arises in precisely the same way in transplanted as in spontaneous tumours occurs at more loci and earlier. Initially, necrosis appears as scattered islands, but later the islands become confluent and eventually expand into the vascular drainage system (Fig. 3). In vessels thus invaded, the endothelial lining near the point of invasion often appears partially digested, even though an intact capillary lies adjacent to it, while at a greater distance from the point of invasion the endothelial lining remains intact.

The explanation of this apparent anomaly is 'heterolysis', a term coined by Thomlinson (personal communication) to describe death and destruction caused by agents produced at a distance, as opposed to autolysis, the process of death and destruction consequent on the action of agents produced locally. In scattered islands of necrosis, death follows from autolysis. Once the blood vessels are invaded, the destruction of their endothelial lining is by heterolysis.

In tumours of less than 10 mm diameter, the lymph played an important role in removing necrotic material. This is clearly seen in Fig. 1, where a swollen lymphatic projects on either side of a swollen vein. In another tumour of the same size, a blisterlike structure enclosed it in part, narrowing as it left the tumour to about the same bore as that of the vessel in Fig. 1. Swollen lymphatics were not seen draining larger tumours and it seems likely that initially the fluid from the necrotic islands forces a channel to the exterior of the tumour and so is drained by the lymphatics, but with the enlargement of the tumour the channels find their way to the nearest efferent vessel and these then carry out the drainage of the liquid (Fig. 3).

Accumulation of necrotic fluid within a tumour has a marked effect on the tumour's apparent growth rate. While the fluid accumulates, the true growth rate is overestimated, but once the vascular system is invaded and a drainage system established, the growth rate is at first underestimated while excess fluid is being lost, and then reaches a steady value when equilibrium between growth and cell loss is established. This is the explanation of the two sigmoid curves in Fig. 4. No marked accumulation of necrotic fluid was present in the tumours at the time of death, but both showed well-marked drainage channels with no central reservoir of necrotic material to supply them.

Relation between the degree of necrosis and tumour volume and growth rate

Transplanted C3H tumours are mosaics made up by the fusion of many fragments; the products of each fragment might be regarded as units, each having its own volume, growth rate and degree of necrosis, and all sharing an almost identical environment. It had been hoped, using these units, to study in a single tumour the relation between (a) degree of necrosis and (b) volume and growth rate. The uncertainty, however, in ascertaining when each fragment started growth precluded this approach and consequently the comparison between degree of necrosis, volume and growth rate had to be made between individual tumours. The growth rates of tumours in 15 syngeneic mice were measured during a period of 80 days.

After the death of the mice, each tumour was halved and mounted in resin. The pairs of resin blocks were scattered on an X-ray viewer and then arranged in the order of the extent of necrosis. The degree of dilatation of the efferent vascular vessels and of the lymphatics formed a valuable guide in deciding the order. The process was repeated three times at three weekly intervals and the orders adjudged on the three different occasions never varied by more than two places for any tumour. The result is set out in Fig. 8, where the 15 tumours are arranged in order of volume, the largest on the left and the smallest on the right. In the lower histogram, the height of the column represents the position of the tumour in the order adjudged, the tallest column representing the most necrotic tumour. In the lower histogram, the heights of the columns are proportional to the rates of growth recorded after a steady rate had been achieved. For most tumours, this period extended from the 30th to the 80th day, but in some cases growth between the 50th and 80th day had to be substituted. The unit used is the reciprocal of the doubling time (in days). The lower histogram shows

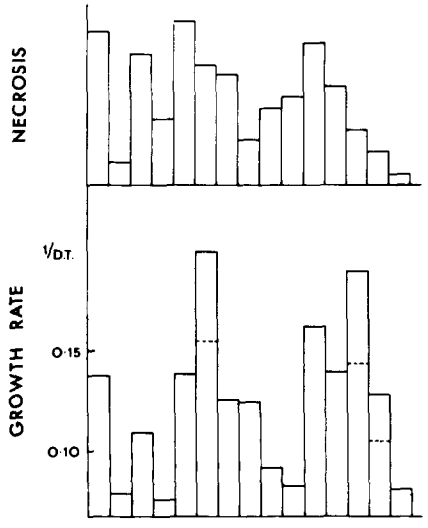

Fig. 8. A comparison of the volume, growth rate and extent of necrosis of 15 tumours derived from implants from a single source. The tumours are arranged in order of volume, the largest on the left, the smallest on the right. Growth rates for all tumours had been measured before the death of the hosts and are expressed as the reciprocal of the volume doubling time in days after a steady state had been reached, in most cases from day 50 to day 80. The degree of necrosis was estimated by arranging the cleared tumours in order according to the amount of necrotic material visible in them and the degree of distention of their drainage vessels.

There is no correlation between (a) either growth rate or extent of necrosis and (b) tumour volume. A loose correlation exists between growth rate and extent of necrosis, and this becomes more precise if allowance is made in the sixth, the thirteenth and the fourteenth tumours for the amount of liquid contained, which leads to a deceptively high estimate of the volume in the living animal.

scarcely any correlation between volume and growth rate, and the upper shows a slight tendency for larger tumours to be more necrotic than smaller. The two histograms together indicate a greater, but only partial, correlation between growth rate and degree of necrosis. The partial correlation becomes closer when allowance is made for the fact that the growth rates of the sixth, thirteenth and fourteenth tumours have been exaggerated by an exceptional volume of liquid within them. The area above the dotted lines represents the fluid enclosed, so that for true growth, comparison should be made between the degree of necrosis and the growth rate indicated by the dotted lines. Because of the need for corrections such as this and of the vagueness of the term 'degree of necrosis', a detailed mathematical analysis seemed inappropriate. The investigation showed that in general, fast-growing tumours are more necrotic than slow-growing ones, but the relation is not precise and, in view of the uncertainties in assessing rate of growth, an exact correlation would not be expected.

DISCUSSION

Progression in the C3H tumour

McCredie *et al.* [2], working with spontaneous tumours and first and 900th generation transplants, reported that taking the three classes of tumour in that order, there were: (a) an increase of initial growth rate; (b) an increase in the rate of retardation of growth; (c) a decrease of tumour differentiation; (d) an increase of necrosis; and (e) a decrease in the relative volume of cancer cells within the tumour. These findings, together with those reported here, show that all the changes from normal to malignant tissue do not occur at the original transformation. Through many cell generations a loss of differentiation occurs, manifesting itself at the cell level in a reduction of cell size and in increasing growth rate, and at the level of the whole tumour, in the loss of the branching habit and of the arrangement of cells into single-layered ducts.

It is these last two changes which are the indirect cause of the other principal modifications. The initial incomplete branching, which occurs in the spontaneous tumour and occasionally persists in the transplants, leads to the formation of blood sinuses, one of the most conspicuous characters in those tumours. The complete loss of branching in the transplanted tumour means the sinuses do not form and must thereby increase the efficiency of the circulation.

Like the loss of branching, the substitution of multi-layered for single-layered tubules is indicative of lack of differentiation. The ability of the normal gland to organise its cells into alveoli and patent ducts is progressively lost, first by failure to form alveoli and then by failure of the cells to arrange themselves in single layers around an open duct and, instead, to proliferate solid cylinders of tissue. The effect of this is to extend the diffusion pathway of oxygen and thereby increase the relative proportion of hypoxic and anoxic cells in the tumour. This, in turn, increases the extent of necrosis and leads eventually to the invasion of the efferent vascular system by fluid of necrotic origin. The tumour is thus characterised by the accumulation of this fluid and by the distention of its efferent blood vessels.

The vascular pattern in relation to radiotherapy and hyperthermia

The radiation responses of the C3H tumour have been exceptionally fully explored. Hawkes *et al.* [6] investigated the response of the spontaneous tumour and its first generation transplant to single and fractionated doses of

14 MeV electrons. They found that a dose split into two halves separated by 24 hr was more effective than a single dose, but only if the dose was in excess of 3000 rad for spontaneous tumours and 2500 rad for transplanted. From this and other evidence they concluded that the advantage obtained from fractionation resulted from increased oxygenation after the initial dose. They also concluded that the response to low doses came from the predominantly oxygenated cell compartment, whereas at higher doses it came from the predominantly hypoxic population. Following on from this, they deduced that the proportion of hypoxic cells in the transplanted tumour exceeded that of the spontaneous. They even went so far as to suggest that differences in the pattern of vasculature of the two tumour types might be responsible for the difference.

The work of Hawkes *et al.* [6] was followed by a long and detailed series of investigations by Fowler and his colleagues [7], using transplanted tumours only. They also demonstrated the benefit of fractionation, but found that the relationship between the number of fractions, their timing and the distribution of total dose among them was exceedingly complicated. The structures described here provide an anatomical background against which the experimental results can be visualised. From the structure of the tumour, the hypoxic fraction is believed to lie in the central portion of the multi-layered tubules. A first radiation dose would sterilize the outer, oxic portion of the tubule, but leave unaffected the inner, hypoxic area. The oxygen supply to this area would then be improved, since it would be brought nearer the surrounding sheath of capillaries. A delicate balance would obtain between the need to extend the time between fractions for the full advantage of reoxygenation to be reached and the need to reduce it to minimize the effect of repopulation. A similar, but reversed, situation is described by Hirst and Denekamp [8], who worked with the KHH mouse carcinoma, a corded tumour with central vascularisation and peripheral necrosis. Using thymidine labelling, they were able both to follow the migration of cells from a proliferative zone adjacent to the blood vessels across the cord to the necrotic zone at the periphery and to represent their results in quantitative form.

A further characteristic of the tumour in its radiation response consistent with the anatomical background is the early marked loss of volume following irradiation. Denekamp [9] quotes a loss of 11% in the first 24 hr after irradiation by 1–2 krad and considers this not to be a direct result of radiation, but to follow from the unmasking of the normal rate of cell loss when cell proliferation is halted by a radiation-induced mitotic delay.

The significance of the vascular pattern of the spontaneous tumour in hyperthermia was discussed in [1]. In the transplanted tumour, in view of the higher proportion of necrotic tissue, hyperthermia may be more effective.

Acknowledgements—I am grateful to Mr. P. H. Warren, who bred the mice and carried out the transplants, to Mr. F. Paice, the photographer, to Dr. A. Michalowski and Dr. T. Wheldon, who read the manuscript and gave much useful criticism, and to Dr. R. H. Thomlinson of the Regional Radiotherapy Centre, Mount Vernon Hospital, for his constant help and encouragement.

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