Vascular Participation in Tumor Slice Metabolism.* (18725)

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The metabolism of tumor tissues has been the subject of numerous investigations, particularly in regard to oxygen consumption(1). In an effort to more closely correlate metabolic activity with the actual cancer cell content of a tumor mass Chalkley introduced a point pattern method of measurement(2). While good correlation is found between the per cent of epithelial cells and certain enzyme activities by such technics, this does not exclude the possibility that stromal elements are also participating in the respiration in some constant relationship with the tumor cells. More recently we have introduced the use of tetrazolium chloride for the measurement of the metabolism of tissue slices(3,4). This procedure has the advantage that the red insoluble formazan, formed as the result of the dehydrogenase activity of the tissues, is deposited at the site of reaction. Frozen section examination of the tissues after incubation of the tissue slice in tetrazolium chloride will then allow visualization of the cells involved (5-7). Advantage was taken of this phenomenon to study the participation of stromal and cancer cells of tumors as contrasted with such activity of normal tissues. Such activity could also be characterized by the use of selected enzyme inhibitors.

Our findings would indicate that in spontaneous breast carcinoma, arising in C3H and CFW mice and in a transplantable mammary carcinoma grown in C57 black mice, no formazan was deposited in the tumor cells, themselves; but rather was limited to the endothelial cells of the fine capillary network within the tumor and also to some extent in the adjacent connective tissue stroma. Similar findings were also noted in methyl cholanthrene induced epidermoid carcinoma of the skin of mice. This reaction was not inhibited by fluoride, malonate or azide.

Materials and methods. The tumors included in this study were spontaneous mammary carcinomas arising in female mice of the C3H and CFW strains, as well as transplantable mammary carcinoma implanted subcutaneously in CFW or C57 black mice. The control tissues included kidney, liver, adrenal, lung, normal mammary gland and skin. The animals were sacrificed by rapid crushing of the cervical cord and the tissues to be studied were immediately dissected out and sectioned into slices approximately 1 mm in thickness. In the case of the tumors, care was taken to employ only those areas which showed no gross evidence of necrosis. Five determinations were usually run on each tissue, and the determinations were run in duplicate except in the case of the adrenal where the small size of the organ made this impossible. Representative slices were placed in the incubation mixtures, each of which contained 3 ml of a 1% aqueous solution of 2,3,5-triphenyltetrazolium chloride (*TTC*) buffered with phosphate buffers to pH 7.2. The different mixtures had in addition one ml of (a) 0.9% sodium chloride, (b) 10^{-3} M sodium fluoride, (c) 10^{-3} M neutralized malonic acid, (d) 10^{-3} M sodium azide and (e) 2×10^{-4} M neutralized succinic acid. The tubes were placed in a rack in an incubator, maintained at 37°C

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and subjected to continuous gentle agitation for a period of one hour. At the end of this time they were removed, the solutions decanted and the tissues fixed in 10% formalin. If kept in the dark, the fixed tissues will be suitable for quantitative analysis or microscopic examination for at least a week. After fixation, the tissues were sectioned for histological examination on the freezing microtome and then the remaining tissue and the unsectioned duplicates were subjected to acetone extraction of the reduced TTC, the concentration of which is determined in μg per mg of acetone dried tissue according to the technic previously described(3,4). The sections for histological examination were studied both with and without counterstaining with polychrome methylene blue. If the sections are mounted in glycerine and kept in the dark they may be considered as semipermanent. However, it was our practice to examine such sections as soon as they were cut. In many instances the histological examination was made within a few hours of the time the animal was sacrificed.

Results. This report is primarily concerned with the observations on the tumor tissues. The findings on the other tissues will be discussed only in so far as they provide a control for pertinent observations made on the tumors.

The spontaneous mammary tumors arising in the C3H and the CFW strains and the transplantable mammary carcinoma behaved in a similar fashion in regard to the quantitative determination and the histochemical appearance. For this reason no differentiation between them will be made in the following discussion of the results.

After incubation the tumor slices in each of the different solutions were seen to be diffusely red in color. Frozen section examination revealed that the tumor cells, themselves, were free of any intracellular formazan deposition. In contrast, the vascular structure of the tumor was sharply outlined by the deposition of elongated crystals of reduced TTC within the cytoplasm of the endothelial cells comprising the capillaries of the tumor (Fig. 1). In counterstained sections the intimate relationship of the capillaries to the individual tumor cells is clearly demonstrated and corresponds to the descriptions of Algire and Chalkley of tumor vasculature(8). Most of the intrinsic vessels consist of naked endothelial tubes closely applied to the tumor cells in a fashion which is suggestive of the type of arrangement obtaining in endocrine glands. However, there is no indication that the vascular channels are discontinuous or sinusoidal in character. Perivascular fibroblasts noted around larger vessels also show intracellular deposits of reduced TTC. In addition a moderate amount of extracellular amorphous formazan deposition was scattered throughout the section. This latter finding is most marked in the tissues incubated in the presence of succinate. The qualitative histochemical picture was found to be essentially the same in the tissues which had been incubated in each of the different solutions, *viz.*, the formazan deposition was confined almost exclusively to the endothelial cells of the tumor capillaries, perivascular fibroblasts and stromal macrophages. No evidence of inhibition of such staining was observed to occur in the presence of the fluoride, malonate or azide, a finding confirmed by quantitative determinations of the μg of TTC reduced per mg of acetone dried tissue.

In contrast to the intracellular reduction of TTC by tumor capillaries and stroma such activity was minimal or absent in the vessels and stroma of normal mammary parenchyma, adrenal cortex and medulla, kidney, liver, skin and lymph node.

It was noted, however, that in the region of the precapillary sphincters there were perivascular cells which reduced the TTC. Some formazan deposition may also occur in inflammatory foci of the slices incubated in the TTC with saline or succinate. However, when these tissue slices were incubated in the presence of fluoride, malonate or azide no TTC reduction by the vessels or stroma was observed.

Discussion. The observation that the endothelial and stromal cells of these tumors actively reduce TTC is indicative of a unique stromal parenchymal relationship which has

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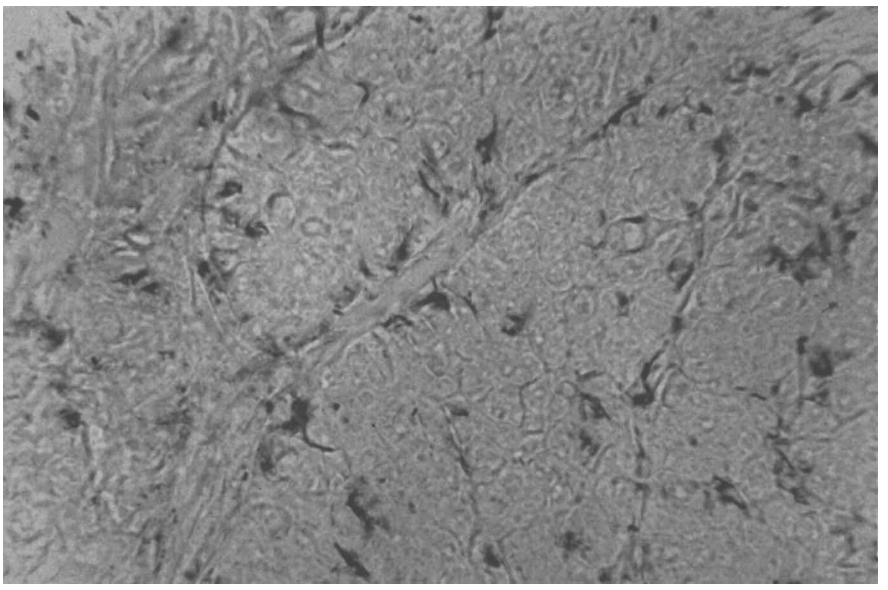

FIG. 1. Spontaneous breast carcinoma; C3H mouse, after incubation in TTC for 1 hr. 440×.

not been observed thus far in a wide variety of normal tissues. It should also be mentioned at this time that a similar although not as clearly defined phenomenon occurs in human cancer tissues; indication of the general participation of this type of activity in malignant tumors. Details of these studies will be reported shortly.

As connective tissue and stromal vessels usually show minimal oxygen consumption and dehydrogenase activity it has been tacitly assumed in the past that the larger part of such activity by a tissue slice represented the metabolic activity of the parenchymatous elements. While this is apparently true for normal tissues, the present observation would place serious limitations on such an assumption in regard to cancer tissues.

In consideration of an altered enzyme activity in tumor vessels, it should be noted that a marked diminution in alkaline phosphatase has been shown to occur in these vessels(9). Manheimer and Seligman point out that while ordinary histological methods cannot differentiate young vessels in malignant tumors from those in neighboring tissues, alkaline phosphatase determinations suggest a physiological difference. They call attention to the destructive action of bacterial polysaccharides on animal and human tumor vasculature as contrasted to the minimal effect on the vessels of normal tissues.

On the basis of these observations and our own, it would appear that the tumor cells and their associated stroma constitute a unique biochemical and biological entity. Further evaluation of this assumption appears worthy of more intensive investigation.

Summary. Tissue slices from mammary carcinoma and diverse normal tissues of mice of different strains were incubated in TTC solutions with and without fluoride, malonate, azide and succinate. Frozen section examination after such treatment revealed formazan deposition in the vasculature and stroma of the tumor but none in the tumor cells, themselves. This manifestation of dehydrogenase activity in the stromal vessels was not inhibited by fluoride, malonate or azide; a finding confirmed by quantitative determinations of μg of TTC reduced per mg of acetone dried tumor tissue. No such stromal dehydrogenase activity was found in slices of normal mammary parenchyma, adrenal cortex or medulla, kidney, liver, skin or lymph node. These findings are discussed in terms of a unique function of tumor capillaries.

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