THE MORPHOLOGY AND BEHAVIOR OF NEOPLASTIC MAST CELLS CULTIVATED IN VITRO*

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PLATES 10 AND 11

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Tumors composed of mast cells (1) have been shown to contain heparin in amounts that vary with the granular content of the neoplastic cells. This variation in the granules finds expression in the size, number, and tinctorial and optical properties of the cytoplasmic particulate matter (2). Cytological characteristics of this sort can be observed in great detail in tissue culture and furthermore the evolution and differentiation of the cell structures can be followed more advantageously under such conditions than in the original living tissues. Since the literature contains no comprehensive data relative to the morphology and behavior of mast cells in tissue cultures, this technique was therefore applied to two anaplastic mast cell tumors.

Technique

The tumor tissue fragments were obtained from two dogs. In every instance one-half of the fragment was fixed in Zenker formol or 10 per cent neutral formalin and sectioned and stained in hematoxylin and eosin, iron-hematoxylin, Mallory's aniline blue, and other stains; the other half was placed in several cubic centimeters of whole dog's blood under aseptic conditions for later cultivation *in vitro*. This latter procedure was followed because the tissue had to be transported across the city from the operating room to the tissue culture laboratory. Once there, the fragments were washed free of blood in Tyrode solution and then placed in dog serum for subdivision preparatory to planting in hanging drops and roller tubes.

The hanging drop cultures were prepared by mixing one drop of chicken blood plasma, two drops of normal dog serum, and one drop of chick embryo extract. Before clotting occurred, a fragment of tumor was added. In making roller tube cultures, the tubes were first lined with chicken blood plasma, ten to fifteen small tumor fragments were then set in the lining, and two drops of embryonic extract added. After clotting occurred, each tube received 2.5 cc. of normal dog serum and 0.5 cc. of chick embryo extract. All cultures were incubated at 38°C. and the drum which supported the roller tubes revolved ten times per hour. With both the hanging drop and roller tube cultures transplantation was done as seemed necessary because of beginning plasma liquefaction or to prevent deterioration of the cells.

Toluidine blue, a basic aniline dye, was used for staining the cells. This was done because "the only criterion by which a mast cell can be recognized is the presence in its protoplasm of certain granules which stain electively and metachromatically with basic aniline dyes" (3).

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The stain was prepared by adding to Tyrode solution enough grains of powdered toluidine blue to impart a sky-blue color to the solution. Hanging drop cultures were stained by freeing the paraffin-sealed coverslip and flooding the slip with the Tyrode-toluidine blue solution. No fixation was used since the prime object was to stain the granules metachromatically in the living cells. In the case of roller tubes the cultures were stained by adding the saline stain directly to the fluid medium or by replacing the fluid medium with the saline-toluidine blue solution. Either procedure gave a good result.

Some of the cultures were fixed in Zenker formol or 80 per cent alcohol and stained with Heidenhain's iron-hematoxylin.

In addition to direct microscopic examination of unstained and stained material, cinematographic continuous time-lapse photographs were made of individual cells as well as of cells in groups over periods varying from 6 to 12 hours.

OBSERVATIONS

Tissue culture of the two tumors utilized revealed differences in growth, morphology, and behavior of the neoplastic mast cells, and hence each growth will be described separately.

Tumor I.—A 9 year old male Boston terrier was brought to the hospital for surgical removal of a cutaneous nodule lying over the crest of the left ileum. This growth had been present for a year and after removal was identified as a mast cell tumor. Three weeks later the dog was returned to the hospital with twelve new skin nodules distributed over the trunk. No further surgery was attempted except to obtain biopsies for tissue culture purposes. The animal remained under observation for 4 weeks and became so cachectic that the owner requested its destruction. During this time the number of growths had increased to forty-five. These were subepidermal spherical nodules scattered over the surface of the trunk but not on the legs. In size the growths varied from 4 to 18 mm. in diameter and were elevated 2 to 4 mm. above the surrounding normal skin. The superficial epithelium was usually ulcerated and the nodules appeared greyish-tan on section. The left axilla was filled with an ulcerated mass measuring 14.5 cm. in diameter and 7.0 cm. in thickness. This large growth had developed within a period of 7 weeks. Autopsy showed small metastatic nodules up to 1.0 cm. in diameter in the liver, spleen, and lungs.

The tumor nodules consisted of dense collections of closely packed mast cells which had invaded the corium and subcutaneaus tissues. Occasional neutrophils, eosinophils, lymphocytes, histiocytes, and plasma cells were scattered among the neoplastic cells. A capsule and trabeculae were absent and there was a sparse fibrous stroma.

The neoplastic mast cells were round, oval, or polyhedral and the round or oval nucleus was either centrally or eccentrically located in the cytoplasm. The nuclear structure was somewhat vesicular and one to three acidophilic nucleoli were present. There was considerable variation in cellular dimensions and the nuclei of the larger cells were usually hyperchromatic, with large nucleoli and often a bizarre shape. Nuclear hyperchromatism and hypertrophic nucleoli occurred not uncommonly in smaller cells as well. Cells of great size containing from two to eight nuclei were not unusual and there were many abnormal mitotic figures. Sections stained with toluidine blue revealed a relatively few reddish-purple, fine, delicate metachromatic granules. In the

occasional mature mast cell present in the same section, the granules were coarser and usually so numerous as to obscure the nucleus.

Imprints of the tumor treated with Wright's stain more clearly demonstrated the granules and the quantitative variations that existed in the different cells. The granules had a fine delicate appearance that contrasted with the coarser granules seen in imprints of normal mast cells.

Cultivation in Vitro.—Tumor I grew very slowly in tissue culture and the lag phase was greatly prolonged; no growth was observed in hanging drop or roller tube culture until 8 to 10 days or more had elapsed. During this phase no cells of any kind wandered out into the medium. When migration and growth were finally discernible the type of growth in the tubes differed from that in hanging drops.

In the roller tubes the first indication of growth activity was the outward advance of a few contiguous cells at one or two points along the circumference of the fragment. The growth had an appearance not unlike epithelium, the cells extending out in a single layered sheet. The shape of the cells varied, but on the whole they were polygonal or rounded. No long spike-like protoplasmic processes were present (Fig. 1).

The first cells to grow out contained only one nucleus, but with extension of the sheet of tissue some with two and three nuclei appeared. Fig. 1 shows such a sheet as it appeared after 16 days' incubation of a roller tube. The cell shapes and number of nuclei are faithfully represented but the low magnification did not permit accurate representation of cytoplasmic structures.

The most striking feature of the cells was the appearance of the cytoplasmic granules; even in the unstained condition their uniform size and great number were evident. When stained with toluidine blue they assumed the violet color of metachromasia and contrasted sharply with the faint blue of the nuclei. Every cell of the growth showed these same distinctive cytoplasmic structures. Only mast cells had grown out so that these sheets of tissue represented pure cultures, despite the fact that the original tumor fragment contained connective tissue and other cellular elements removed from the skin and tela subcutanea.

Hanging drop cultures made from the same material presented a quite different picture. The cells extended out in strands and a great range of shapes was encountered, varying from the spindle form to elements with many processes (Figs. 2 to 4). Some of these last were eight to ten times as long as the cell body and exceedingly thin; when cytoplasmic granules were present in them these lay in single file. Other processes were more dendritic in appearance, tapering rapidly from a broad base to a point from which a thin extension, often of considerable length, arose (*e.g.* Fig. 5).

The cells in the hanging drop cultures held granules varying considerably in size, though in any given cell all had nearly uniform dimensions. Only oc-

casionally one could perceive within a single cell granules which varied from barely visible to coarse (Fig. 6). When stained with toluidine blue the granules gave the violet color characteristic of metachromasia. With the technique used, not all of them were stained at the same time, those in the peripheral cells of the colony usually staining first. To stain those close to the original fragment exposure to the dye for several hours was sometimes necessary and by this time the color of the cells which had stained first had faded. Sometimes not all of the granules in a single cell could be stained. Some of the granules in one part of a cell would remain free of dye, while granules of similar appearance in adjacent areas of the same cell were deep violet (Fig. 3). In a few elements none of the granules could be stained despite the fact that morphologically they were identical with adjacent cells which showed all granules metachromatically stained.

Within many of the proliferating mast cells an area free from granules was present adjacent to the nucleus (Fig. 4). In the unstained cell close examination was necessary to distinguish this area from the nucleus and only in stained preparations was it quite clearly cytoplasmic.

Cinematographic studies were made on the hanging drop preparations. By time-lapse exposure technique it was possible to demonstrate unmistakable ameboid movement. Some mast cells which had grown out from the original fragment could be seen to return toward the fragment or to move among stationary neighboring cells whereas other cells failed to retract their protoplasmic processes or change shape appreciably over an observation period of 6 hours. Division was seen to occur only by amitosis; no mitotic figures were observed. Preliminary to division it was noted that the protoplasmic processes were retracted, the cell became rounded, and the granules more refractile, with result that the cytoplasm transmitted light less freely. Using these criteria one could predict division. Amitosis followed with the formation of two daughter cells of equal size, which soon moved away from each other and again extended their protoplasmic processes into the surrounding medium.

Tumor II.—Since this is the same tumor described in the preceding paper (2), a mastocytoma of immature cell type, the history and gross description of the tumor need not be repeated. Microscopically it consisted of dense collections of mast cells which were scattered below the pars papillaris of the corium and infiltrated the subcutaneous tissues. There was an irregular fibrous connective tissue stroma which in some regions showed trabecular formations. Some areas were heavily fibrosed and in others the connective tissue proliferation was minimal. Haphazardly distributed among the neoplastic cells were occasional histiocytes, neutrophils, plasma cells, eosinophils, and lymphocytes.

In general the tumor cells were similar to those of Tumor I. Cellular pleomorphism was as prominent but mitotic figures were fewer and of the normal type. Staining with toluidine blue indicated that the metachromatic granules were somewhat more plentiful and that they resisted decolorization more strongly. The granules had the same fine delicate structure as those of

the preceding tumor and hence contrasted with the coarser granules of normal mast cells and of the tumor mast cells first reported by Bloom (1).

In imprint preparations, the granules were essentially similar in number and structure to those in Tumor I.

Cultivation in Vitro.—2 days after planting pieces of Tumor II in roller tubes an excellent outgrowth of cells appeared from the entire circumference of some of the original fragments. The cells did not grow out as contiguous elements having the appearance of an epithelium but as spindle-shaped cells. Under low magnification the cultures therefore looked much like cultures of fragments from the ventricle of the 8 day chick embryo heart. Under higher power, however, the spindle-shaped cells were seen to contain the characteristic cytoplasmic granules which stained purple with toluidine blue. The granules were especially numerous in the cell body surrounding the nucleus, though occasionally a granule-free area lay adjacent to the latter.

The hanging drop cultures also showed differences between the growing cells of this tumor and those of Tumor I. As in the roller tube, the lag phase was far shorter and the growth more rapid. Proteolytic activity was considerably greater as evidenced by the fact that the fibrin clot was more rapidly liquefied, an occurrence necessitating more frequent transplantation of the growing tissue to a fresh clot. Occasional mitotic figures were observed, a fact confirmed in sections stained with iron-hematoxylin. Finally, in Tumor II more cells were present which showed a mixture of granules ranging in size from bare visibility to coarse (Fig. 6). Cells filled with coarse granules alone were less numerous than in cultures from Tumor I.

DISCUSSION

From the standpoint of the phenomena of tissue growth in culture, the most peculiar and perhaps significant observation made in our cultures of the mast cell tumor is the fact that only mast cells grew. Several previous observations and conclusions lead to an interesting line of thought in this regard. Heparin has been shown by Fischer (4, 5), Goerner (6), and Zakrzewski (7–10) to inhibit cell growth. Jorpes (11) after reviewing the work of a series of investigators, has concluded that the granular substance of the normal tissue mast cells is heparin. A paper published herewith (2) has shown that a mast cell tumor may have as much as fifty times the heparin content of normal tissues, and has provided reason to suppose that the heparin content varies with the number and size of the metachromatic granules within the cells. If the cultivated cells were producing heparin *in vitro* it seems possible that this material acted to prevent growth of the other cells in the tumor fragment.

The metachromatic cytoplasmic granules are the most characteristic structures found within proliferating mast cells. Since their granules may be concerned in the elaboration of heparin, the steps involved in their formation and dissolution become important. Our observations in cultured cells closely

accord with those of Oliver, Bloom, and Mangieri as reported in the associated paper on the heparin content and the cytoplasmic particles of tumor mast cells (2). These investigators correlated the heparin content of the tumors examined with the occurrence in the cells of granules differing in size and number and with differing tinctorial and optical properties. Their finding suggests an elaboration of pre-heparin, finely particulate matter into the coarse metachromatic granules of the mature heparin-containing cells. As yet we have not observed a complete cycle of granule formation within a single living cell, but the composite picture seen in the developing cells of the tissue culture lends itself to such an interpretation. Following staining with toluidine blue we were unable to detect within the nucleus either metachromatic granules (12) or a diffuse metachromasia (2, 12), findings which were the basis of Downey's conclusion that the granules are derived from the nucleus. In the cytoplasm, however, a sequence in granule formation is strongly suggested by the presence of fine and coarse granules and a corresponding variation in tinctorial reaction from a complete absence of metachromasia in the fine granules to the development of its intensely reddish-purple color in the coarse ones. The optical differences in the granules demonstrated by phase microscopy, noted in the living cells of the tumors (2), were also seen in the tissue cultures (Fig. 7).

Many observers believe that division in normal mast cells is solely by amitosis (13, 14). This was true in the cultures of one of the tumors we have studied, but division by mitosis was observed as well in the culture of the second tumors, an occurrence which may have been due to the relatively more anaplastic state of the neoplastic cells.

SUMMARY AND CONCLUSIONS

Fragments from two mast cell tumors of the dog have been cultured *in vitro*. Studies on the living and on fixed and stained preparations revealed the following:

Only mast cells grew out from the original tumor fragments though these contained other types of cells. They grew in some of the roller tube cultures in a sheet resembling an epithelium but in hanging drop cultures they lay separate and were irregularly spindle or star-shaped with long protoplasmic processes.

The cytoplasmic granules of the proliferating mast cells varied in size, number, and tinctorial properties. In most of the cells they stained metachromatically, in occasional cells some of the granules only could be stained, and in a few none could be stained.

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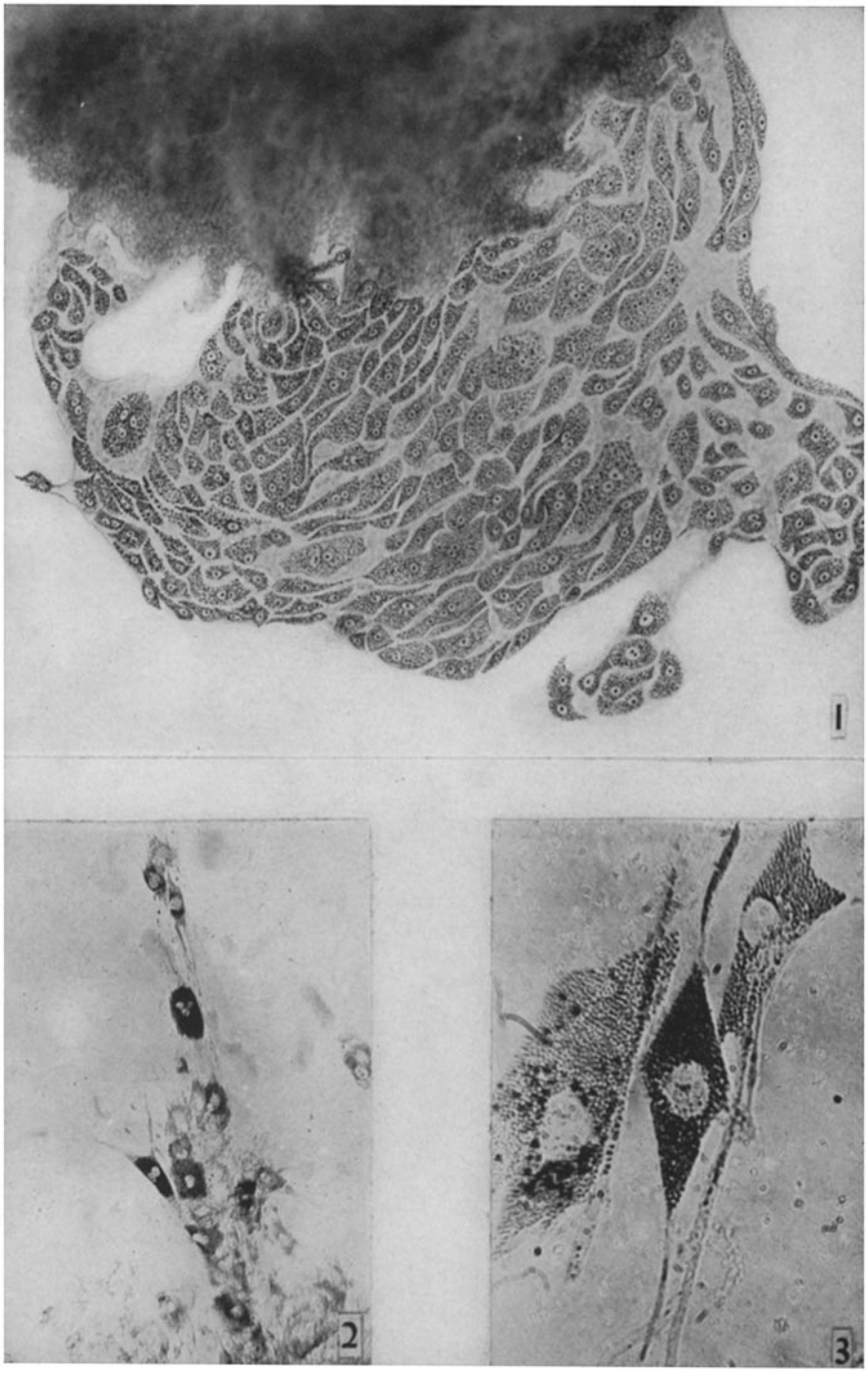
EXPLANATION OF PLATES

PLATE 10

FIG. 1. Tumor I. Sheet of mast cells resembling epithelium. Stained with toluidine blue after 16 days' cultivation in roller tube. Drawing, × 100.

FIG. 2. Tumor I. Group of mast cells cultured for 63 days in a hanging drop. In some of the cells the metachromatic granules are especially numerous about the nucleus. Toluidine blue. × 100.

FIG. 3. Tumor I. Three mast cells from a fragment grown for 55 days in a hanging drop culture. All of the granules in the central cell have taken the stain deeply whereas those of the cell on the left differ widely in this respect. Toluidine blue. × 430.



(Paff *et al.*: Neoplastic mast cells cultivated *in vitro*)

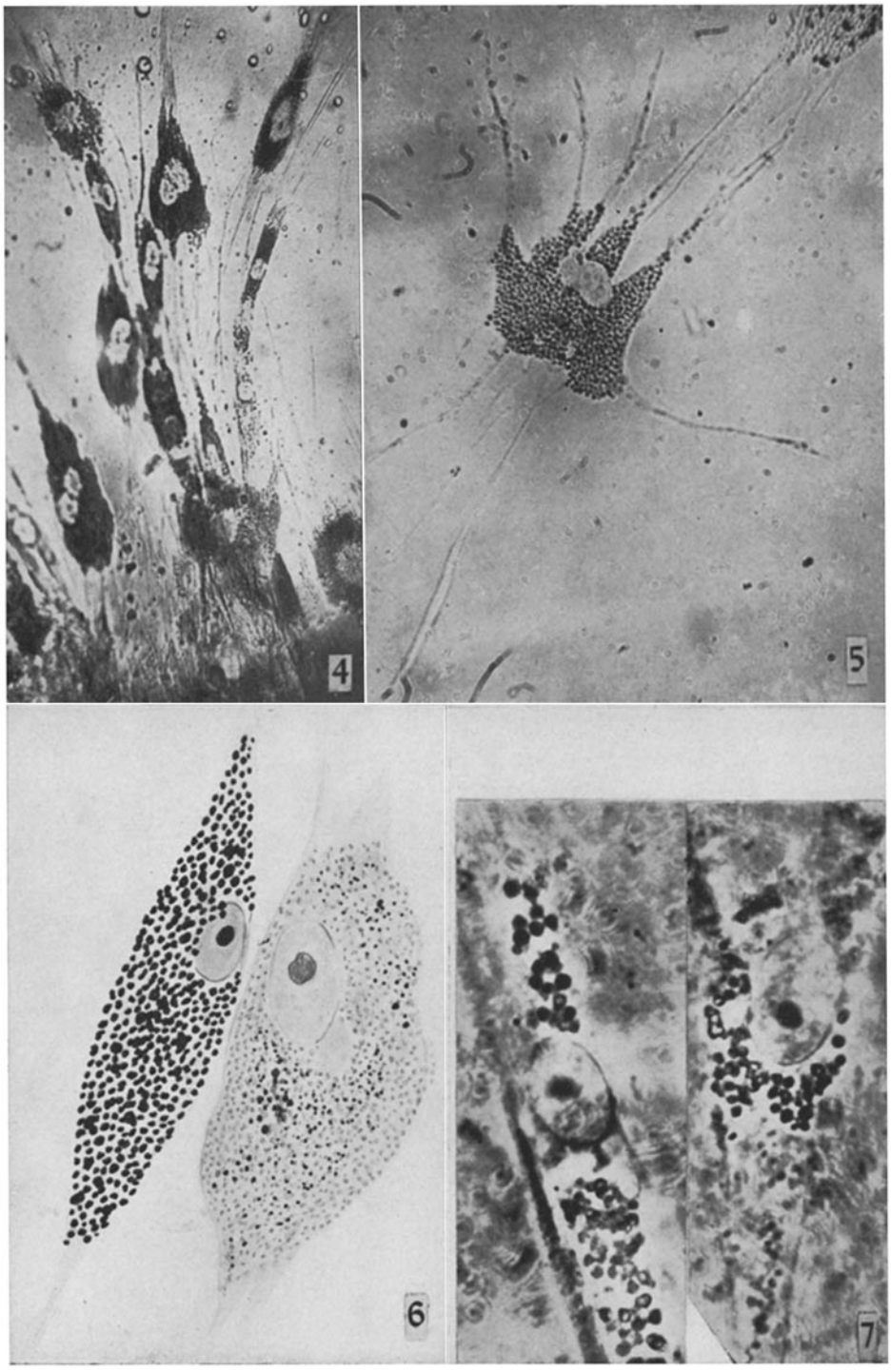
PLATE 11

FIG. 4. Tumor I. Mast cells grown 66 days in a hanging drop culture. Note cell processes, distribution of metachromatic granules, and the cytoplasmic area free from them near the nucleus. Toluidine blue. × 200.

FIG. 5. Tumor II. Mast cell from a hanging drop culture 45 days old. Note the size and distribution of granules and the long tenuous processes. Toluidine blue. × 430.

FIG. 6. Tumor II. Two cells from a 41 day old culture stained with Heidenhain's iron-hematoxylin. Note coarse granules in cell at left and fine granules in cell at right. Drawing, × 970.

FIG. 7. Tumor III. A photograph taken with the phase microscope of two living spindle-shaped mast cells containing coarse granules, from a tissue culture. Some of the granules appear homogeneous, whereas others have a shell of dense material surrounding a clearer central portion. Hanging drop, 11 days old. × 970.



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