A Neoplastic Connective Tissue Mast Cell Capable of Continuous Growth In Tissue Culture*

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ABSTRACT

A neoplastic connective tissue mast cell from a dog mast cell sarcoma has been grown in tissue culture for 50 passages over a period of 2 years. The cells were grown as monolayer cultures in glass bottles, using Eagle's basal medium fortified with calf serum. The cultures were contaminated with an *Alkaligenes sp.* for 10 months but finally were sterilized bacteriologically by treatment with specific antiserum combined with antibiotics.

The cells grow in a fibroblastic pattern, and contain mitochondria, mast cell granules, and lipid granules or droplets. The mast cell granules stain basophilic with Giemsa's stain and metachromatically with azure A or toluidine blue. They also stain with Sudan black B and with periodic acid–Schiff stain. The interphase nuclei are vesicular, contain from 1 to 20 nucleoli, and frequently show bizarre outlines. Multinucleate cells are often seen, as are mitotic figures. Extracellular fibrous material occurs in all cultures and apparently originates from the cell surface. This material does not have the structure of connective tissue fibers and has not been identified.

The cells develop an increased number of metachromatic granules when grown in medium containing heparin and an increased number of sudanophilic granules when grown in medium containing stearic acid. Only small amounts of histamine were present in the tumor from which this cell line was derived and in the cells grown in tissue culture.

INTRODUCTION

The connective tissue mast cell has recently been reported to be involved in the metabolism of several compounds of biologic importance (1–9) and to be strikingly affected by certain drugs and hormones (10–12). Mast cells are difficult to ob-

tain in large numbers uncontaminated by other cell types and for this reason attempts were made to isolate a mast cell line capable of continuous growth in tissue culture in the hope that this would provide a continuous source of material for biochemical study.

Previously Paff *et al.* (13–15) have reported growth in tissue culture of mast cells from mast cell tumors, and Zitcer *et al.* (16, 17) have grown mast cells from lesions of urticaria pigmentosa. These investigators maintained the mast cells in culture for several months but eventually the cells died. In addition, Compton (18) and Riley (19) both mention growing mast cells in tissue culture. Transplantable mast cell tumors have recently been developed (20, 21). One of these has been

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adapted to growth as an ascites tumor (20), and is capable of growth as suspended cells in tissue culture (22). This report describes the isolation and some of the morphologic and physiologic properties of a cell line that was derived from a mast cell sarcoma of a dog. This cell line has been maintained in culture for 50 passages over a period of 2 years and appears to be capable of continuous growth.

Methods and Materials

Isolation of the Cell.—The cells were obtained from mast cell sarcomas of dogs.[1] Tumors were removed aseptically at surgery or autopsy. The tumors were minced with scissors, and the mince was treated with a solution of 0.25 per cent trypsin in buffered saline at room temperature with continuous stirring until a dense suspension of cells was obtained (23). The cells were sedimented by low speed centrifugation, washed with growth medium, and again sedimented in the centrifuge. The cells were resuspended in growth medium and aliquots were planted in milk dilution bottles or 1 liter Blake bottles. The cultures were incubated at 37°C.

The growth medium was Eagle's basal medium (24) fortified with 20 per cent calf serum. The medium was changed at intervals of 3 to 7 days depending on the rate of acid production by the cells. When the cells had multiplied sufficiently to cover the surface of the glass they were gently loosened by means of a rubber-covered glass rod. The suspension was transferred to a centrifuge tube and the cells were collected by centrifugation. The supernate was decanted and the cells were resuspended in fresh medium and dispersed by drawing the suspension into a pipette and discharging it rapidly several times. Aliquots of the suspension were then planted in new bottles containing additional fresh medium.

Preservation of Cells.—Cells were preserved by freezing in growth medium containing 10 per cent glycerol by the procedure of Swim, Haff, and Parker (25). The cells were frozen by placing the sealed tubes on a block of solid carbon dioxide within 10 minutes of suspending the cells in glycerol-containing medium.

Cytologic Studies.—Morphology of the living cells was studied by direct observation in T-flasks and by use of double coverslip preparations. The latter were prepared from coverslips on which the cells were grown by placing the coverslips in the culture bottles before

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[1] The tumors used in this study were provided by Dr. Peter H. Craig and Dr. Mark W. Allam of the School of Veterinary Medicine, University of Pennsylvania, Philadelphia, and Dr. David L. Coffin of the Margaret M. Caspary Center for Veterinary Research, New York. The histologic diagnoses were made by Dr. Craig or Dr. Coffin.

inoculation. Metachromasy in the living cell was demonstrated by exposing the cells to a solution of toluidine blue (National NU 8 or 13) in a concentration of 1:100,000 to 1:200,000 in Eagle's medium from which phenol red was omitted.

Cells for fixation and staining were grown on coverslips as described above. Fixatives appropriate for the staining procedures were used. The metachromasy of fixed cells was demonstrated by staining with 0.1 per cent azure A (National NAz 12) in 30 per cent ethanol (26). Dehydration and clearing of these preparations were accomplished by immersing the coverslips sequentially in acetone, acetone-xylene 2:1, acetone-xylene 1:2, and xylene. The coverslips were immersed in the first three reagents for 3 to 5 seconds each and were kept in xylene for 5 minutes to several hours. Periodic acid-Schiff (PAS) staining was performed by the method of Lillie (27), as was the allochrome stain (28). Mallory's phosphomolybdic acid stain (29) and Masson's trichrome stain (30) were also employed. Lipid stains were made with Sudan black B by the method of Chiffelle and Putt (31). Silver impregnation was accomplished by the protargol technique modified for use with tissue culture material by Murray and Stout (32).

For electron microscopy the cells were grown on mica coverslips, fixed with buffered 1 per cent osmic acid, and air-dried. The surface casts used were prepared by the method of Coman and Anderson (33).

Histamine Assay.—Histamine was determined by the method of Lowry *et al.* (34).

Preparation of Antiserum.—Antiserum was prepared in a rabbit using as antigen a suspension of phenol-killed bacteria grown on trypticase-soy broth. A total of 6.5 ml. of vaccine was administered intravenously over a period of 5 weeks. One week after the last injection the animal was bled and sterile serum was prepared.

Materials.—The vitamins, amino acids, and glutamine of Eagle's medium were obtained from Microbiological Associates, Inc., Bethesda, Maryland. The calf serum was a commercial product or was prepared from sterile calf blood obtained locally. All sera were heated at 56°C. for 30 minutes prior to use. All other reagents were commercial products.

EXPERIMENTAL

Isolation of the Cell.—Attempts were made to grow cells in tissue culture from 5 mast cell sarcomas of dogs. All 5 tumors yielded cells which grew in a fibroblastic pattern and contained many granules or vacuoles. Cells from the 1st, 2nd, 3rd, and 5th tumors studied multiplied for 4 to 8 passages and then died. Cells from the 4th tumor have continued to multiply for 50 passages over a 2 year period. The details of the isolation of this cell are presented in Table I. The isolation of this cell line was complicated by contamination with both yeast and bacteria. The yeast was readily eliminated by use of the antibiotic eulicin, but the bacteria, which

TABLE I

Isolation of Neoplastic Mast Cell in Tissue Culture*

Cell source	Passage	Duration of passage	Medium‡ supplement	Comment
Thoracic nodes	1–8	12 wks.	None	Cells died after 8th passage.
Cervical nodes	1–11	10 wks.	None	
	12	4 wks.	None	Growth began in many foci 3 wks. after planting.
	13–16	4 wks.	None	
	17	10 days	Eulicin§ Polymyxin	Contaminated with yeast and Alkaligenes sp.
	18–31	6 mos.	Polymyxin Tetracycline Circulin Furadantine Chloramphenicol	Cultures contaminated with Alkaligenes sp. Antimicrobials added singly and in combination. All except furadantine appeared non-toxic. Cells ultimately died.
	23	17 days	Polymyxin	Cells replanted after being frozen 6 mos., still contaminated.
	24	17 days	Polymyxin	Contaminated with Alkaligenes sp.
	25–28	10 wks.	Polymyxin Antiserum‖	All cultures for Alkaligenes sp. negative after 4 wks.
	29–42	26 wks.	None	Cultures bacteriologically sterile.
	43	4 wks.	None	Growth began in many foci 3 wks. after planting.
	44–50	6 wks.	None	Cultures for bacteria and pleuropneumonia-like organisms negative.

* Tumor was obtained from a boxer dog in which it was present as metastases to cervical and thoracic nodes from a primary on the lip.

‡ The medium contained penicillin, 100 U/ml., dihydrostreptomycin, 50 μg./ml., and nystatin, 30 U/ml. added as a routine.

§ All antimicrobial agents were added in a concentration of 25 μg./ml. except furadantine which was added at 30 μg./ml.

‖ Antiserum was stored at −15°C. and was added to the medium in a concentration of 1 to 2 per cent immediately after medium changes were made.

were identified as an Alkaligenes sp.,[2] resisted treatment with several antibiotics[3] but were finally eliminated by use of both polymyxin B sulfate and an antiserum prepared against the specific organism. During

[2] We are indebted to Dr. Elwood L. Foltz of the Pepper Laboratory of the Hospital of the University of Pennsylvania, Philadelphia, for the identification of this organism.

[3] The circulin used was generously provided by Dr. Andrew J. Moriarty of the Upjohn Company, Kalamazoo, Michigan.

the period of bacterial contamination all the cells which had been carried continuously in culture were lost, and the cells now extant were derived from a single ampoule of cells which had been frozen for 6½ months.

At the present time the cells multiply slowly when compared to cells of strains HeLa or L. Quantitative data on growth rates based on cell counts have not been obtained because mechanical dispersion of cells scraped from glass is incomplete with many small clumps of cells remaining. Both trypsin and versene in buffered saline appear to be toxic. The usual inoculation procedure employed has been to replant a mature culture in 2 or 3 bottles of the same size as that from which the cells were harvested. Under these conditions 7 to 14 days are required for a culture to reach full growth. The cells grow equally well in Eagle's basal medium fortified with 10 or 20 per cent calf serum, and medium containing the lower concentration of calf serum is now employed. Eagle's basal medium fortified with 5 per cent calf serum did not support growth of the cells.

Morphology of the Living Cells.—Throughout 50 passages the cell has maintained the same morphology. Fig. 1 is a photograph of a 4 day culture in the 21st passage showing the fibroblastic growth pattern. Most of the cells attach to the glass individually, but with each passage several masses of cells about 0.5 mm. in diameter attach to the glass and individual cells migrate from the periphery. Fig. 2 shows the granules which are present in the living cells. As is discussed below, these appear to be both mast cell granules and lipid granules. Structures resembling mitochondria can be seen in living cells examined with the phase contrast microscope, but cannot be seen in this photograph. Fig. 2 also shows the pleomorphism which is characteristic of these cells.

Morphology of Nuclei.—The interphase nuclei are vesicular and contain from 1 to 20 nucleoli. Multinucleated cells are common. In many cells mitotic figures are present, and multinucleate cells have been observed in which the nuclei were in synchronous mitosis. Abnormalities of mitosis, usually multipolar mitotic figures, are frequently encountered. Many cells, particularly in older cultures, show bizarre nuclear configurations suggesting that budding or amitotic nuclear division occurs (Fig. 3). Small structures appearing to be micronuclei or nuclear buds are often seen, and in some cells nearly 100 of these have been observed.

Properties of Mast Cell Granules.—Detailed study of the mast cell granules has been carried out. Significant numbers of fibroblastic cells without

such granules have not been seen at any time. In addition to these granules, cells which have been dehydrated and cleared contain round, clear areas, larger than the mast cell granules, that appear to represent lipid granules which have been extracted by the solvents. The mast cell granules usually stain purple with Giemsa's stain although red granules are often seen (Fig. 4). However, all the mast cell granules in a given cell have the same color with this stain. These granules are usually round although elliptical forms occur. Their size frequently varies within a particular cell and between different cells. They are usually distributed around the nucleus or nuclei, and frequently a paranuclear clear area is present. They also appear in elongated cell processes, and in some cells accumulate near one end. These granules have been observed in cells undergoing mitosis. Mast cell granules appearing to be outside of cells are often present.

Mast cell granules are plentiful in most of the cells in young cultures, but in cultures about 9 days of age or older the granules of many of the cells appear to be smaller and to be present in decreased numbers. However, in all cultures, cells which contain the full complement of mast cell granules of the usual size are frequently observed. When cultures 9 days old or older are replanted into new bottles large mast cell granules reappear beginning on the 1st day and reach their greatest numbers in 3 to 6 days.

The mast cell granules stain metachromatically with azure A, and the metachromasy persists after dehydration with acetone and clearing with xylene. Prior to dehydration the granules are red and after dehydration vary in color from red to purple. The cytoplasm and nuclei are purple before dehydration, but after dehydration this metachromasy is much less pronounced or is absent.

Metachromatic granules can be observed in living cells incubated in medium containing toluidine blue. Staining of the granules begins within 5 to 10 minutes of exposure to the dye. The cytoplasm and nuclei remain colorless or become pale blue. The metachromatic granules lose their red color with time, and this has been observed to occur over a period of 10 minutes or less. Control preparations of mouse fibroblasts of strain L do not show metachromasy when incubated for up to 3 hours in medium containing toluidine blue.

The mast cell granules and cytoplasm, particularly that near the nucleus, are red when stained by the PAS technique. Cells stained without periodic acid oxidation occasionally have pink cytoplasm but the reaction is inconstant and the color much less intense than that obtained after periodic acid oxidation.

The cells appear to contain two different structures which stain with Sudan black B. The smaller of these stain gray to black, and have the same size and distribution as the mast cell granules. The larger structures stain intensely black and appear to be lipid granules (Fig. 5). Acetone extraction of stained cells removes the dye from both structures. If the cells are restained only the smaller structures take the dye, but, in addition, round, clear areas are present which apparently contained lipid granules which were removed by the solvent. Treatment of the stained cells with propylene glycol removes the dye from the lipid granules and restaining colors these structures as before.

Extracellular Fibrous Material.—The cultures contain extracellular fibrous material that stains red with Giemsa's stain (Fig. 6). This material has been present since the early passages. It increases in quantity throughout the growth period. It usually exists as fine, delicate strands, but coarse fibers and amorphous masses of material which stain like the fibers are present in older cultures. The fibers originate in close proximity to the cells. They pursue a random course in the culture, lying across, under, and alongside the cells, and form a network between the cells. Branching of the fibers has been observed frequently. With Giemsa's stain many of the cells have sharply defined, red staining bands coursing over the cell surface in one or more places, and these usually are continuous with a fiber extending beyond the cell boundaries.

Classical connective tissue stains were applied to the fibers. The fibers stained blue with Mallory's stain, green with the Masson trichrome stain using light green as the fiber stain, blue with the allochrome stain, and red with the PAS technique. Preparations stained with the protargol technique contained black fibers in areas of dense cell growth but fewer fibers appeared to stain with the silver as compared to Giemsa's stain.

The fibrous material was also studied by electron microscopy. An electron micrograph of a surface cast of a culture of the mast cell is shown in Fig. 7. The fibers originate from the surface of the cells and course from there to the surface of the coverslip. They are irregular in width, have poorly defined margins, and branch frequently. No

periodicity such as is characteristic of connecteiv tissue fibers is present (35). In addition to the fibers, very long, thin, cytoplasmic processes appear in the cultures. Many of these have terminal enlargements, some of which seem to contain granules (Fig. 8).

Physiologic Studies.—Attempts were made to enhance granule formation by supplementing the medium with various substances. On the assumption that inorganic sulfate might enhance mucopolysaccharide synthesis, sodium sulfate was added to growth medium in concentrations of 10 and 25 μmole/ml. and cells 13 and 21 days old were exposed to it for periods up to 5 days. No increase in granule formation could be detected by Giemsa's stain in these cells.

It has recently been demonstrated that mouse fibroblasts will develop metachromatic granules when exposed to heparin *in vivo* (36). For this reason cells from 10 day cultures were exposed to medium containing commercial heparin at concentrations of 0.5 and 1.0 mg./ml. The cells developed large numbers of granules which were red or purple when stained with Giemsa's stain, were metachromatic when stained with azure A, and were similar in appearance to those normally present. Figs. 9 and 10 are photographs of cells from a control culture and a culture exposed to heparin. The granules were present 1 day after exposure to heparin and increased in number for 3 days, at which time nearly all the cells contained many granules. Medium containing 1.0 mg./ml. of heparin appeared to be toxic to the cells if used for longer than 3 days. When cells with an increased number of granules induced by heparin treatment were grown in normal medium for an additional 5 days the number of granules in the cells appeared to diminish. These experiments with heparin were repeated using 3 day cultures of cells of the mouse fibroblast, strain L, and the same results were obtained.

It has been reported that mast cells may contain lipid granules and that these cells may ingest lipid material (1, 18). Because of these reports cultures 3 days old were exposed to medium containing stearic acid at a concentration of 5 and 10 mg. per cent in order to determine whether this would cause accumulation of lipid in cells of the present line. The stearic acid was added as an ethanolic solution, and the final concentration of ethanol was 0.8 per cent. Control cultures were grown in medium containing this concentration of ethanol without added stearic acid. Figs. 11 and 12 show that the presence of stearic acid in the medium markedly increased the quantity of lipid in the cells. Similar effects, although less pronounced, were obtained when cholesterol was added at a concentration of 5 mg. per cent. These experiments were repeated using 3 day cultures of cells of the mouse fibroblast, strain L, and similar results were obtained.

Histamine.—Sufficient tissue was obtained from 2 of the 5 tumors to permit a portion to be assayed for histamine. The tumor which yielded the stable cell line contained only 2.3 μg. of histamine per gm. of tissue while the other tumor contained 199 μg./gm. These 2 tumors were processed in a similar fashion in that both were frozen shortly after they were obtained and were stored at $-15°C$. for 24 hours before the assays were performed. Histamine assays have been carried out on several occasions on 0.1 gm. quantities of the mast cell grown in tissue culture. Material reacting as histamine was present in concentrations of less than 5 μg./gm. of wet cells.

DISCUSSION

This mast cell isolated from a dog mast cell sarcoma appears to be capable of continuous growth in tissue culture in that it has undergone 50 passages over a period of 2 years. The tumor from which this cell was derived contained other cells in addition to mast cells. Coverslip preparations were first examined in the 4th passage and only mast cells were present at that time. Both Paff et al. (13) and Zitcer et al. (16) have previously reported that only mast cells appeared in their cultures. In the early passages cytologic study of this mast cell was limited to staining with Giemsa's stain, observation of living cells, and supravital staining with toluidine blue. Within the limits of these studies the cell now has the same cytologic characteristics that were observed soon after it was placed in culture.

The prolonged contamination of the cultures with the *Alkaligenes sp.* had no apparent lasting effect on the cells. The use of antiserum in addition to antibiotics was effective in destroying the bacterial contaminant after antimicrobial agents alone had failed. Presumably both complement and specific antibody were present in the antiserum although this was not determined because of insufficient material.

The cell can be identified as a mast cell because it retains many of the morphologic characteristics of normal mast cells obtained directly from animals (1). Thus it contains granules which are usually basophilic when stained with Giemsa's stain, and are metachromatic when stained with toluidine blue or azure A. In addition, the granules are PAS-positive (37) and stain with Sudan black B (38). Grossfeld (39) has reported that metachromasy appears in living cells other than mast cells when they are exposed to solutions of toluidine blue. In living cells of the present line it is difficult

not to ascribe significance to the metachromasy observed since the reaction occurred immediately, was limited to the granules, and the controls were entirely negative.

The granules of normal mast cells in tissue sections have been reported to be usually PAS-negative (18, 40), although Jorpes *et al.* (37) found the granules of mast cells in the subcutaneous tissue of young rats to be PAS-positive. They suggest that the PAS reaction in mast cell granules is due to the presence of heparin monosulfate since this compound stains by this technique while heparin does not. If it may be assumed that the PAS staining of the granules is due to the presence of mucopolysaccharide it is interesting that Grossfeld *et al.* (41) have reported that chondroitin sulfate produced by fibroblasts in tissue culture is not fully sulfated. Possibly this mast cell produces heparin which is not fully sulfated and this may account for the positive PAS reaction of the granules.

Cells stained with Sudan black B contain gray to black-staining structures which are believed to be mast cell granules, and large, black-staining structures interpreted as lipid granules. It is possible that some of the smaller structures are lipid droplets, although most of these appear to resist extraction with acetone. The experiments with solvent extraction indicate that the mast cell granules contain lipid, and that this lipid is of different nature from that present in the lipid granules or droplets. It has been reported that acetone does not extract Sudan black B from mast cell granules (38). Our results thus differ from those found with mast cells obtained directly from animals in that acetone does extract the dye from the mast cell granules although these granules are insoluble in this solvent.

The experiments in which the present strain of cells were grown in medium containing added stearic acid or cholesterol demonstrate that mast cells *in vitro* are able to accumulate lipid in their cytoplasm as has been reported for mast cells *in vivo* (1, 18). However, the significance of this is uncertain because the presence of lipid in the cytoplasm appears to be a general finding in cells grown in tissue culture (42). The accumulation of sudanophilic granules in cells grown in medium containing added stearic acid may also be a general property of cells in culture since the present mast cell line, the L cell, and the cells from human aorta studied by Rutstein *et al.* (43) all show this reaction, and the presence of soap in the medium also enhances the formation of lipid granules in tissue culture cells (44).

Exposure to medium containing heparin resulted in the formation of an increased number of metachromatic granules in the mast cell and in the appearance of metachromatic granules in the mouse fibroblast, strain L. The rate of accumulation of these granules and their general appearance is similar in the two cell lines. Thus by analogy it would seem likely that the granules formed in mast cells exposed to heparin arise directly from the added heparin, although it is possible that the heparin in the medium was degraded by the cells and thereby provided substrate for the synthesis of new heparin.

The extracellular fibrous material which appears to originate from the surface of the cells does not have the structural characteristics of connective tissue fibers although it has the tinctorial properties reported for these fibers. The location of the material on the cell surface and the positive PAS reaction suggest that it is mucoprotein that is secreted by the cell. The formation of strands of this material may be related to movement of the cells. Further study will be necessary to establish its identity. The long cytoplasmic processes seen in the electron micrographs may be the same structures as have been described by Bloom *et al.* (45) in mast cells obtained directly from a mastocytoma.

Very little histamine could be detected in the tumor from which the present cell line was derived or in the cells in culture. Cass *et al.* (46) reported that one of several mast cell tumors contained very little histamine and explained this as due to improper handling of the excised tumor. This would not seem to apply here since both tumors which were assayed for histamine were handled similarly and yet one was found to contain large quantities of this substance. The absence of histamine in the present cell line may be an indication of dedifferentiation of the cell occurring when it became malignant in the host.

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EXPLANATION OF PLATES

PLATE 167

FIG. 1. Unstained 4 day living culture in the 21st passage. × 330.

FIG. 2. Unstained 20 day living culture from the 42nd passage, examined with the phase contrast microscope. × 1,300.

FIG. 3. Cell from 3 day culture in the 41st passage, stained with Giemsa's stain and examined with the phase contrast microscope. × 5,300.

FIG. 4. Cells from 2 day culture in the 41st passage stained with Giemsa's stain. × 3,300.
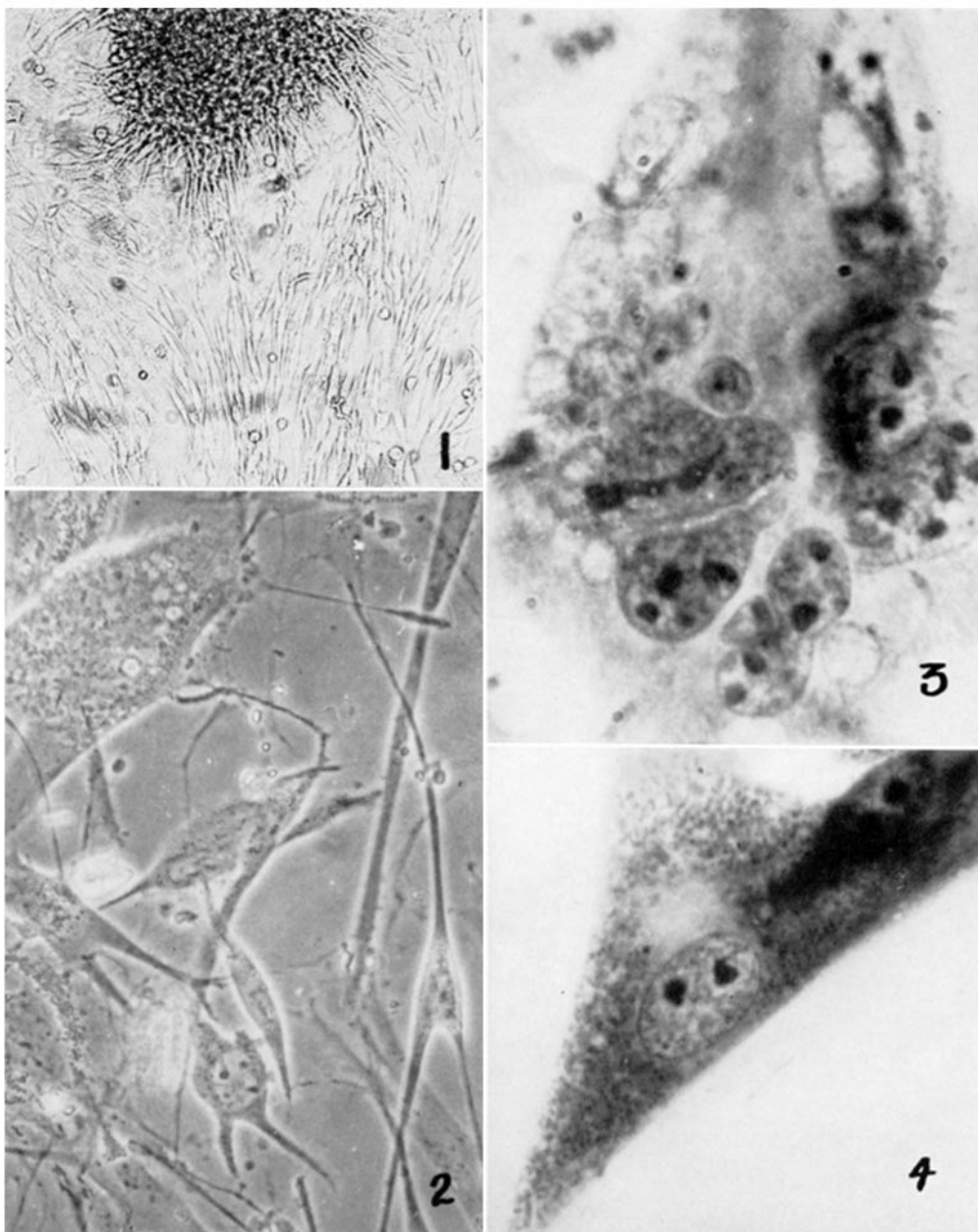
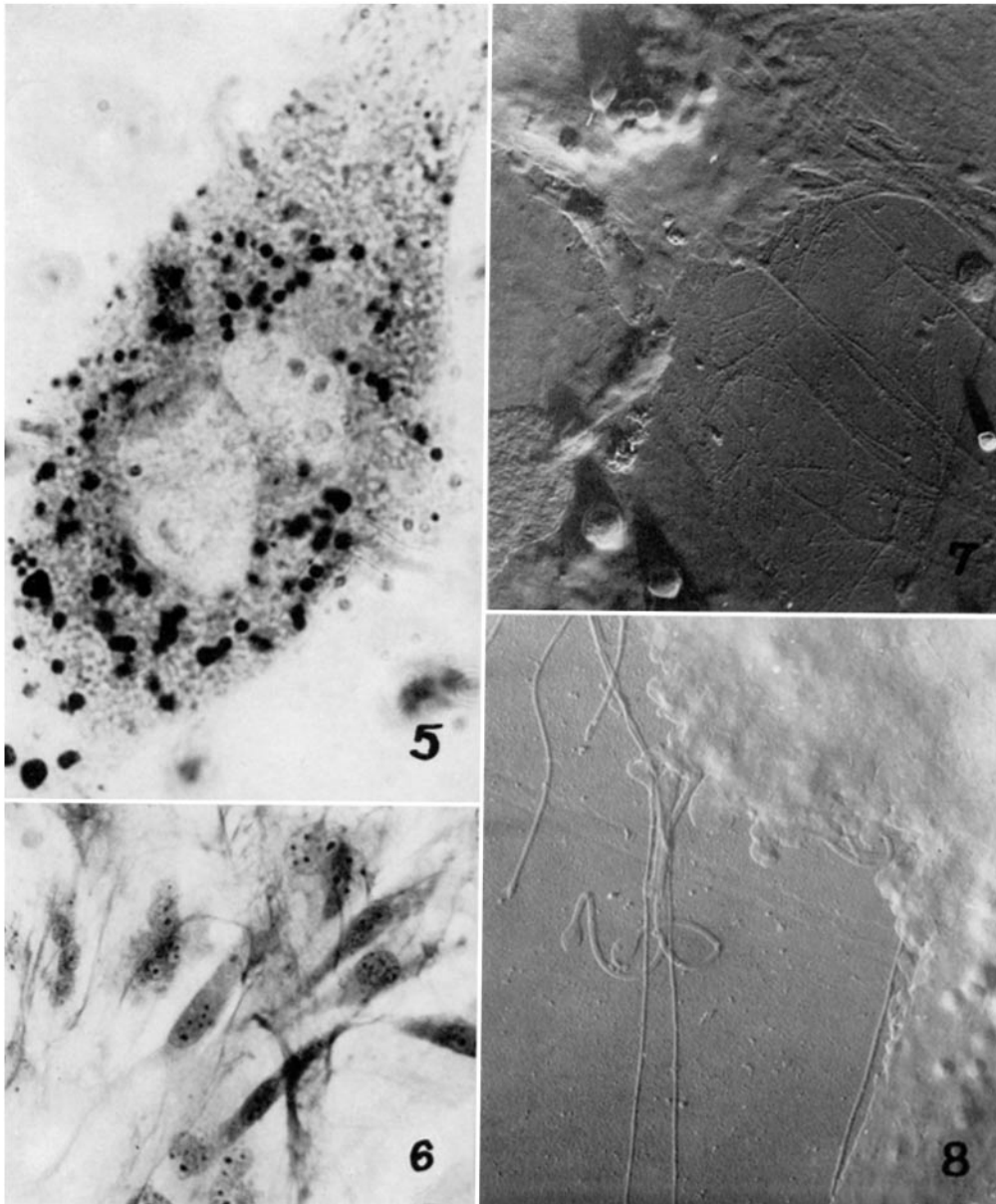(Williams *et al.*: Mast cell capable of growth in tissue culture)

FIG. 5. Cell from 9 day culture in the 36th passage stained with Sudan black B and examined with the phase contrast microscope. × 5,300.

FIG. 6. Fourteen day culture in the 42nd passage stained with Giemsa's stain. × 1,300.

FIG. 7. Electron micrograph of a surface cast of cells from an 18 day culture in the 38th passage, showing the extracellular fibrous material. × 5,000.

FIG. 8. Electron micrograph of a surface cast of cells from an 18 day culture in the 38th passage, showing the cytoplasmic processes. The rounded projections on the cell surface probably represent the mast cell granules. × 5,000.

(Williams *et al.*: Mast cell capable of growth in tissue culture)

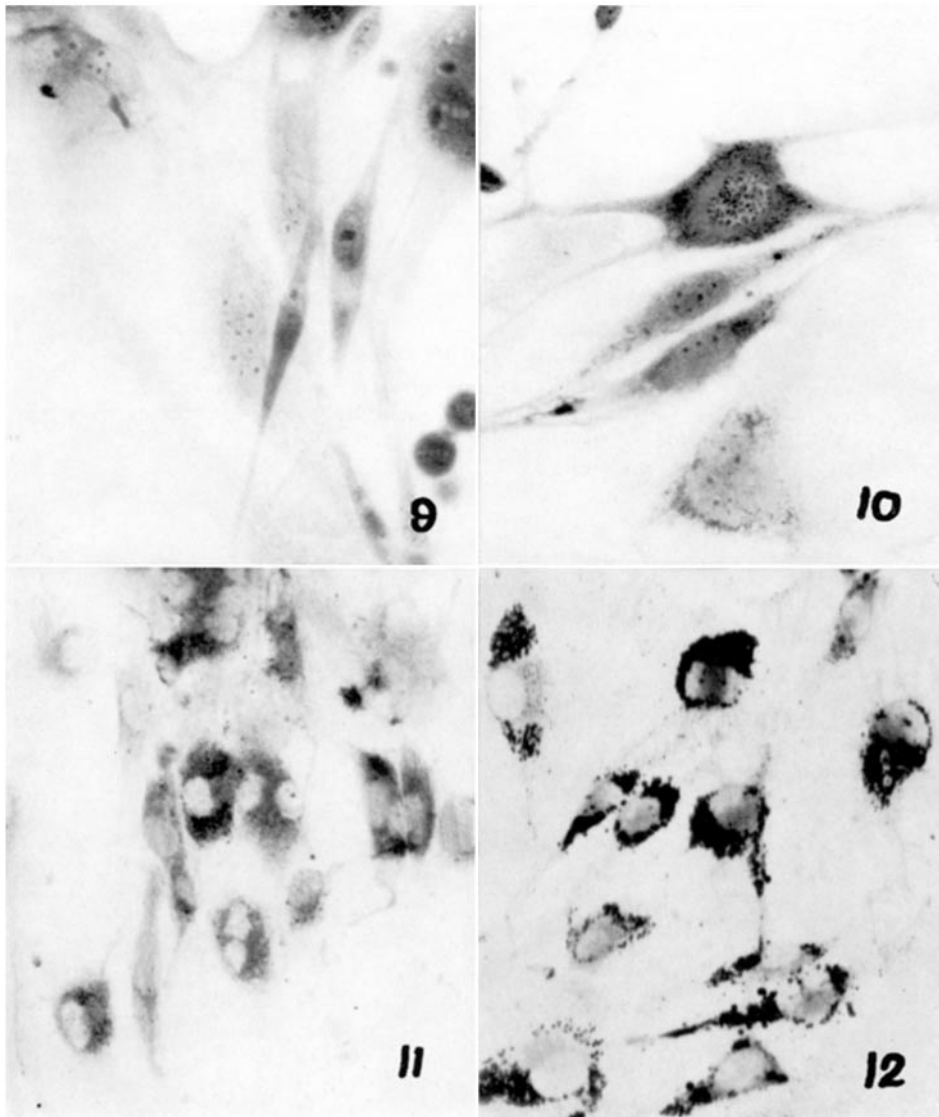FIG. 9. Cells from a 12 day culture from the 42nd passage grown on normal medium. × 1,300.

FIG. 10. Culture comparable to that shown in FIG. 9 but exposed for 3 days to medium containing heparin at a concentration of 1.0 mg. per ml. Giemsa's stain. × 1,300.

FIG. 11. Six day culture from the 36th passage grown on normal medium. Sudan black B stain. × 1,300.

FIG. 12. Culture comparable to that shown in FIG. 11, but exposed for 3 days to medium containing stearic acid at a concentration of 10 mg. per cent. Sudan black B stain. × 1,300.

(Williams *et al.*: Mast cell capable of growth in tissue culture)