

THE IN VITRO DIFFERENTIATION OF MAST CELLS

Cultures of Cells from Immunized Mouse Lymph Nodes and Thoracic Duct Lymph on Fibroblast Monolayers

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ABSTRACT

When cells from lymph nodes or thoracic duct of mice hyperimmunized with protein antigens are cultivated on embryo monolayers in the presence of the antigen, numerous clones of mast cells appear. The histochemical and ultrastructural characteristics of the cells permit their identification as mast cells and distinguish them from the phagocytic histiocytes that usually arise in abundance in similar cultures from unimmunized mouse cells or from immunized mouse cells cultured in the absence of the antigen. Only a few colonies of mast cells appeared in the latter cultures. The basis for the induction of mast cell differentiation is not known.

Mast cells are highly specialized inhabitants of vertebrate connective tissue. Their unique complement of heparin, alkaline proteolytic enzymes, and biogenic amines has morphological expression in characteristic granules. The ease with which fibroblasts may be maintained in dispersed cell cultures suggests the possibility that mast cells which keep such close company in vivo might also thrive in an in vitro system. Most mast cells in adult rodent connective tissues as well as those free in the peritoneal cavity are essentially resting cells; they have largely completed their synthetic processes and along the way ceased their mitotic activity, so that, if investigation of differentiation is the objective, mast cell precursors must be sought out and cultured under conditions compatible with differentiative processes.

Previously, the differentiation of mast cells has been described in cultures of mouse thymus cells grown on an embryonic fibroblast monolayer (7, 9). In this report we describe conditions for the differentiation of mast cells from cells of lymph node and thoracic duct lymph obtained from immunized mice.

METHODS

Monolayers

Embryonic fibroblast monolayers were prepared from several strains of mice (C₅₇B1/6, BALB/c and C₃H) as previously described (8). 1-cm-long embryos were optimal. Monolayers in the second or third passage were usually used; in some cases, monolayers up to the sixth passage were used. Although attention

to the details of preparing the monolayers produced reasonably consistent results, there was some variability in the capacity of different batches of monolayers at different times during their life span to support differentiation of mast cells. For this reason, most cultures were initiated on several different batches of monolayers.

Immunization

The standard primary course of immunization consisted of twice-weekly intraperitoneal injections of 0.2 ml of horse serum, calf serum, or 1 mg of ovalbumin in 0.2 ml of saline for 6 consecutive wk. A booster consisted of a single injection of 0.4 ml of serum or 2 mg of ovalbumin.

Mast Cell Precursors

Free cells were obtained from peripheral and mesenteric nodes of several mice by thorough mincing. The free cells were washed and plated on established embryonic monolayers at an initial concentration of $2-3 \times 10^7$ cells per Petri dish. Thoracic duct lymph was obtained¹ by the method of Reinhardt and Li (13) and cultured under identical conditions.

Media

Embryo fibroblast feeder layers were established in Waymouth's medium with 10% newborn calf serum and maintained in a 0.5% lactalbumin hydrolysate in Earle's salt solution containing 10% calf serum. Culture of lymph node and thoracic duct cells on monolayers was carried out in Dulbecco's modification of Eagle's medium,² supplemented with 20% horse serum. Culture medium was changed every 4 days.

Culture Technique

The lymphoid cells were plated on monolayers maintained in 60-mm-diameter plastic Petri dishes (Falcon Plastics, Los Angeles). The cultures were maintained at 37°C in 5% or 10% CO₂ in air. Subcultures were made by one of two procedures: (1) cultures were treated with trypsin and the resulting suspension plated on empty dishes or onto an already established monolayer, or (2) those cells free in suspension were transferred to a new monolayer.

Observation of Cultures

Living cultures were frequently observed and photographed. Smears of both suspended cells and

¹ We are indebted to Mrs. R. Caffrey Tyler for helping us with this procedure.

² The description of this medium previously reported in Table 2 of reference 8 is misprinted; L-threonine, 9.52 g, should have been included, and L-phenylalanine was used in the amount of 6.6, not 9.52 g.

cells dissociated with trypsin were fixed in methyl alcohol and stained for 5 min with 0.1% toluidine blue in 50% ethanol acidified to pH 3.5, washed, dehydrated in absolute alcohol, cleared in xylene, and mounted in permount. Smears were also stained with alcian blue-safranin (16), May-Gruenwald-Giemsa stain and a naphthol-diazonium coupling procedure for demonstrating acid phosphatase (2), and by the periodic acid-Schiff (PAS) reaction. For electron microscopy, mast cells adherent to the monolayer were washed with buffered saline and the intact monolayer gently peeled from the Petri dish and fixed for 4 hr in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 containing 0.1% alcian blue (3). The cells were washed in 0.18 M sucrose in 0.1 M cacodylate buffer, pH 7.4, and embedded in Epon 812 according to the procedure of Luft (12). Thin sections were stained with uranyl acetate and lead tartrate sequentially.

Evaluation of Mast Cell Growth

When the mast cells were largely in suspension, useful counts could be obtained. However, because many mast cells were present in colonies of varying sizes as well as diffusely over the monolayer, meaningful counts were not always possible. Neither trypsin nor EDTA proved satisfactory in obtaining quantitative release of mast cells adherent to the monolayer. At low concentrations of trypsin, many mast cells remained on the intact monolayer, while at concentrations of trypsin or EDTA high enough to disperse the monolayer, there was lysis of mast cells.

In some experiments, colonies or clones of mast cells on the monolayers were counted after fixing and staining intact dishes in Petri dishes. The colonies of mast cells, which stained metachromatically with toluidine blue, were readily counted under 10 magnification with a dissecting microscope.

RESULTS

Unlike cells from thymus, normal mouse lymph node cells gave rise to only small numbers of mast cells in culture. In preliminary experiments, however, it was noted that, when the lymphoid tissue used to prepare cells for culture was derived from mice immunized with horse serum, mast cells appeared in large numbers in the monolayers and in suspension.

Experiments were, therefore, designed to investigate the effect of immunization on the appearance of mast cells in culture. The results of experiments in which horse serum was the antigenic stimulus in C₅₇B1/6 and BALB/c mice are summarized in Tables I and II. Other strains of mice (SWR and Swiss white) were also immunized, but the high incidence of anaphylactic

TABLE I
Effect of Immunization with Horse Serum on the Differentiation of Mast Cells In Vitro

Donor strain	Immu- nized*	Number of cultures	Good mast cell growth	Good histio- cyte growth	Poor growth of both cell types
C ₅₇ B1/6	+	11	9	1‡	2
C ₅₇ B1/6	-	11	0	6	5
BALB/c	+	3	3	0	0

* The donor animals were immunized with horse serum as described in the text, and cultures were prepared 7-67 days after the last injection. In some experiments an antigen booster was administered 1-6 days before initiation of culture. Neither the booster nor the interval between the last immunization and time of culture significantly affected the subsequent growth of mast cells.

‡ In one culture, good growth of both mast cells and histiocytes occurred.

TABLE II
Effect of Immunization and Exposure in Culture to Antigen on Mast Cell Growth

Immunization*	Antigen in culture	Time in culture	Mast cell colonies/ plate
Horse serum	Horse serum	Days	
		10	646
		15	755
		19	1545
		24	1238
Saline	Horse serum	10	22
		15	78
		19	62

* Immunized mice were given a standard primary course and a single booster (see text). 25×10^6 lymphoid cells were plated on each monolayer culture.

death prevented adequate study. Significant numbers of mast cells appeared only when the cultures were initiated with lymph node cells from immunized animals, and there was, in general, a reciprocal relationship between the growth of histiocytes and that of mast cells. In only one culture was there good growth of both mast cells and histiocytes. In other cultures, either mast cells or histiocytes grew in good number.

An attempt was made to investigate the influence of the presence of the antigen in the culture medium on the appearance of mast cells. Since horse serum was routinely incorporated in the culture medium, two other antigenic stimuli were employed in these experiments. In Table III are presented the results of an experiment in which lymph node cells from mice immunized with calf serum were used. The cells were cultured either in the presence or in the absence of calf serum in the culture medium. Ovalbumin was also tested, and the results of

TABLE III
Effect of Immunization and Exposure of Cells in Culture to Antigen

Exp.	1% calf serum in the cultures	Cells in culture	
		Mast cell	Histiocytes
A	+	+++	-
	-	+	+++
B	+	++	-
	-	±	+++

In experiment A the donor animals were given the standard primary course of immunization (see text) with calf serum and a booster after 50 days. Cultures were initiated 2 days after the booster injection. The animals in experiment B were given only a primary course and the cultures from these animals were initiated 50 days after the final injection.

TABLE IV
Effect of Immunization and Exposure of Cells in Culture to Antigens

Immu- nization of donor	Ovalbumin in culture	Number of cultures	Predominant cell type		Poor growth of both cell types
			Mast cells	Histio- cytes	
+	+	5	4	1	0
+	-	5	1	3	1
-	+	3	0	2	1
-	-	3	0	2	1

Donor mice were primarily immunized with ovalbumin and the culture was prepared 40-41 days after the final injection (see text). In two experiments, mice were given a booster after 40 or 131 days and cultures were initiated within 10 days after the final injection.

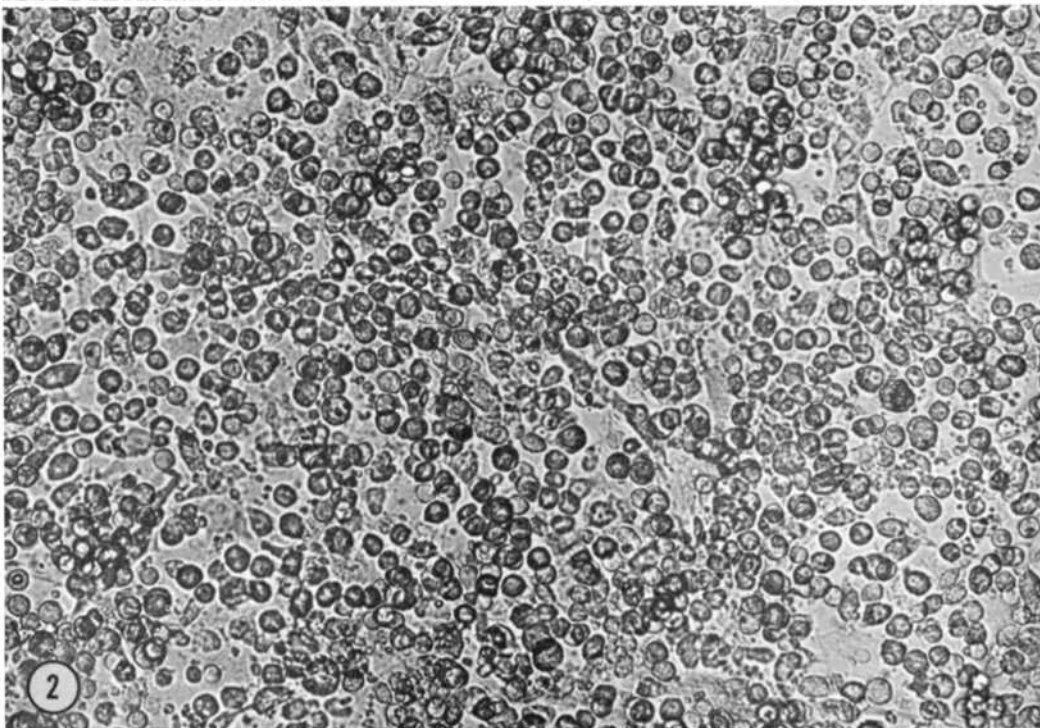
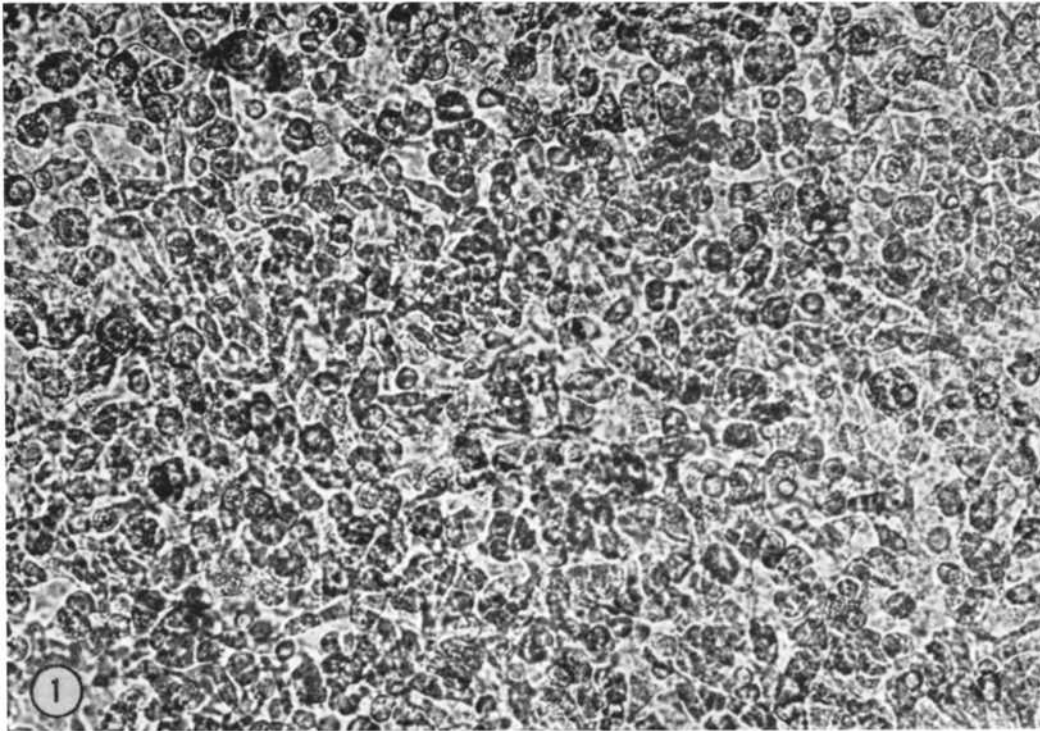


FIGURE 1. Living culture of histiocytes. The histiocytes are spread confluent over the embryo monolayer. Only a few mast cells, small, round, and smooth in appearance, are present. Thoracic duct cell culture from unimmunized mice, first passage, 29 days in culture. $\times 230$.

FIGURE 2 Living culture of mast cells. The mast cells are spread extensively over the monolayer, and many mast cells float above the monolayer. Thoracic duct cell culture from mice immunized with horse serum, first passage, 28 days in culture. $\times 230$.

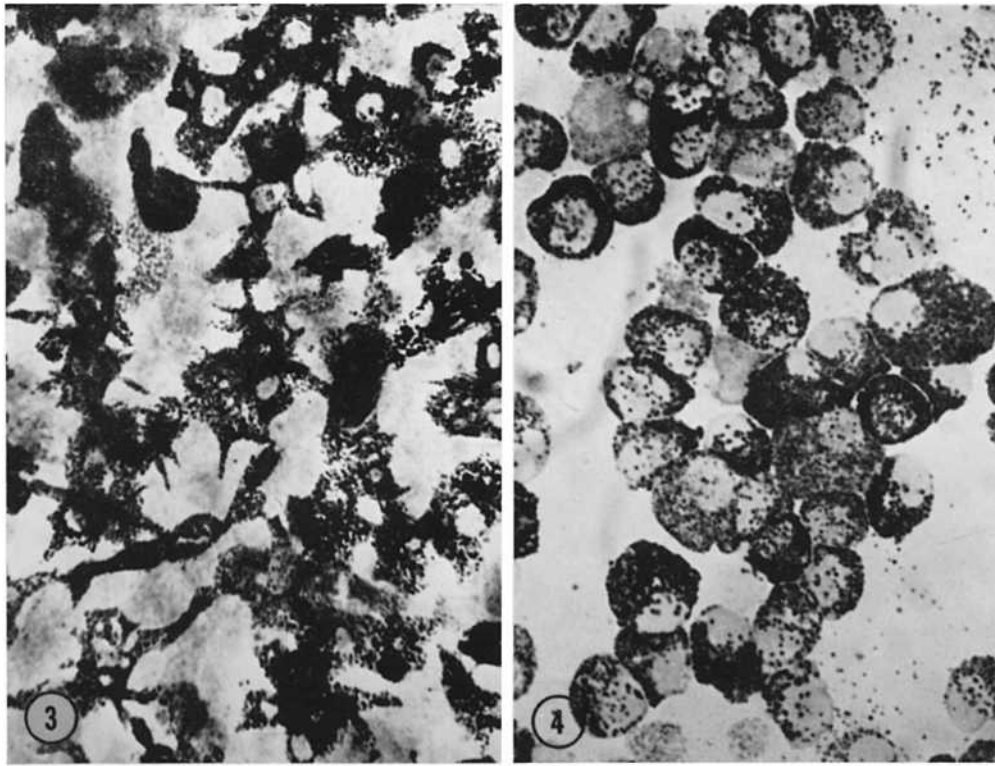


FIGURE 3 Mast cells on embryo monolayer. Cells grown from lymph node cells from mice immunized with horse serum have spread over the embryo cell monolayer. 40-day culture, first passage. Stained with toluidine blue. $\times 400$.

FIGURE 4 Mast cells in smear made of supernatant after 33 days in the second passage of a culture of thoracic duct cells from mice immunized with horse serum. Stained with toluidine blue. $\times 674$.

two experiments with this antigen are presented in Table IV. With both calf serum and ovalbumin, immunization and subsequent exposure of the cells to the antigen in culture were necessary for mast cell growth.

Cytologic Observations

In living cultures, mast cells and histiocytes could, with some experience, be readily differentiated. In suspension, mast cells were characteristically present as smooth-surfaced regular spheres. Histiocytes had irregular contours and contained a variety of vacuoles and granules. On the monolayers (Fig. 1), histiocytes were identified by their abundant cytoplasm filled with refractile droplets and vacuoles of engulfed material; mast cells tended to be smaller and

more discrete than histiocytes and were more refractile (Fig. 2). Mast cells were definitively identified by the metachromatic staining properties of their cytoplasmic granules (Figs. 3-5). The majority of mast cells in several cultures examined contained predominantly alcian blue-staining granules when the alcian blue-safranin procedure was applied. Safranin-staining granules were present in small numbers.

The histiocytes, in contrast to mast cells, contained many vacuoles (Fig. 6), and were phagocytic, readily ingesting carbon particles added to the culture medium. Many of the histiocyte vacuoles stained with PAS and were positive for acid phosphatase. These bodies increased in number during the culture period. Similar vacuoles were not evident at any time in mast cells

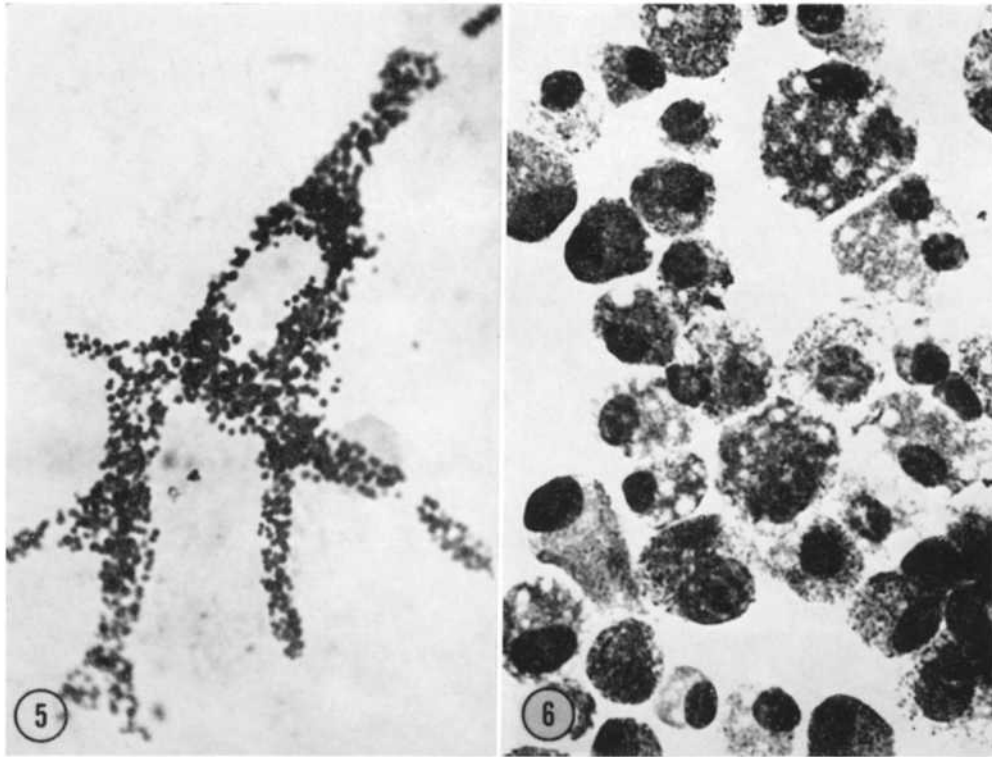


FIGURE 5 High magnification of a mast cell on the embryo monolayer from a culture of lymph node from mice immunized with horse serum. The mast cells in these cultures typically have large metachromatic granules. 40 days in culture. Stained with toluidine blue. $\times 1500$.

FIGURE 6 Histiocytes in smears of supernatant from a culture of lymph node cells from normal mice; after 26 days in culture. Numerous vacuoles are present in the cytoplasm of these histiocytes. Stained with May-Gruenwald-Giemsa. $\times 674$.

and, conversely, the inclusions in histiocytes were not metachromatic under the conditions used for staining mast cell granules.

Electron Microscopy

The mast cells were usually present in clusters. The surface of the cells commonly exhibited numerous villous projections which were long and sinuous (Fig. 7) and contained no cytoplasmic organelles. The mitochondria were not remarkable, with both ellipsoidal and circular cross-sections evident and moderate numbers of cristae present. A well developed Golgi system located close to the nucleus was frequently seen (Fig. 8), composed typically of several stacks of smooth-surfaced lamellae and numerous vacuoles. Centrioles were observed in the cell center. In cells with sparse granules, the rough-surfaced

endoplasmic reticulum, although never highly developed, was more extensive (Figs. 9 and 10) than in the cells which contained many granules (Fig. 11). The mast cell nuclei were generally ovoid with frequent indentations; the chromatin was aggregated along the nuclear membrane and nucleoli were frequently identifiable (Figs. 7 and 9).

The specific granules were characteristically surrounded by a closely applied membrane (Figs. 12 and 13). In immature-appearing cells, the granules consisted of a dense irregular mass surrounded by numerous small vesicles (Fig. 12). The proportions of the two components in granules varied considerably, so that some granules were comprised exclusively of the small vesicle component, while in the most mature-looking cells no vesicular element could be discerned in the

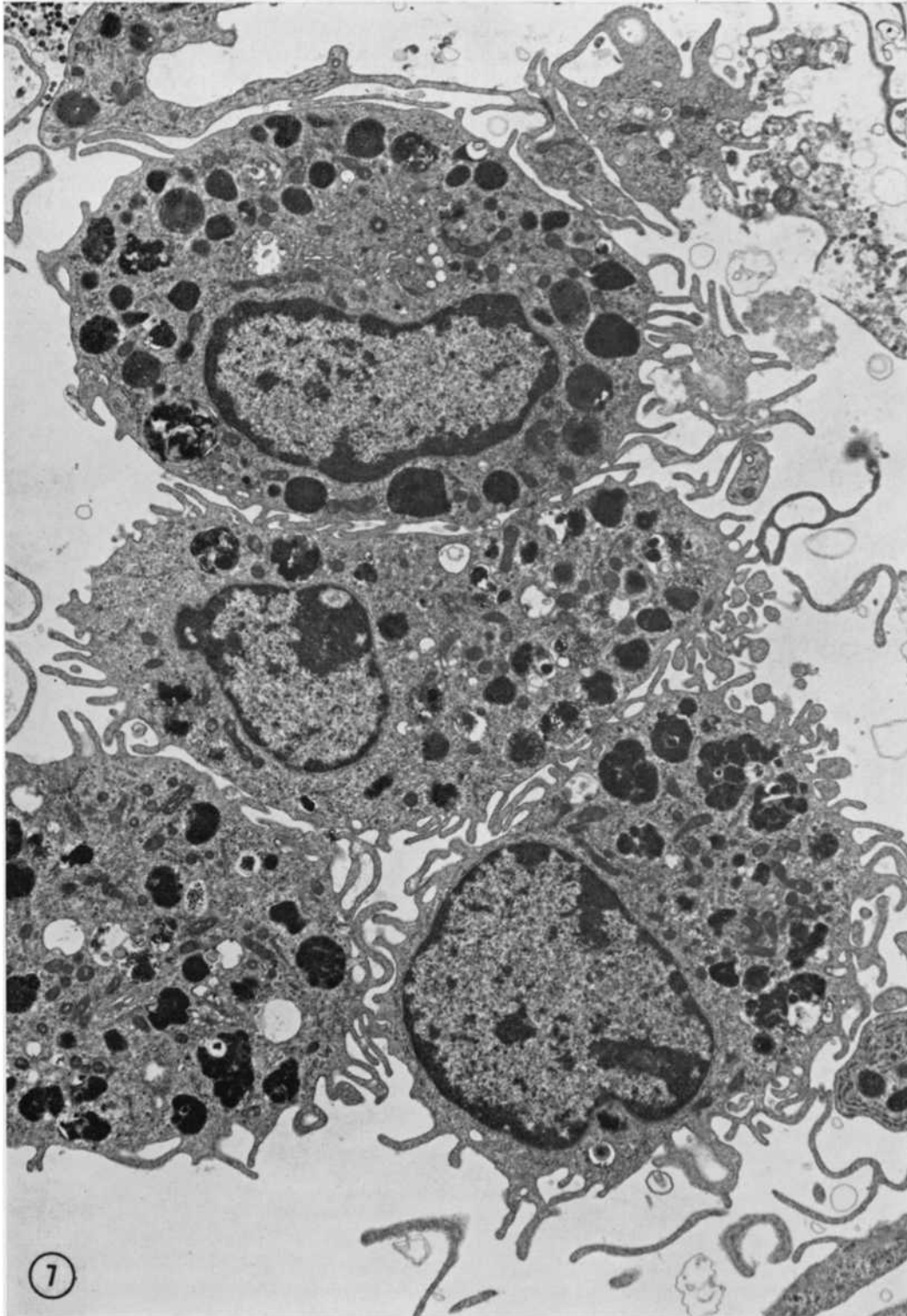


FIGURE 7 Electron micrograph of four mast cells from a 28-day-old culture. The numerous villi are well demonstrated. A variety of granules are evident in the several cells. A well developed cell center with centriole and Golgi structure is present in the uppermost mast cell. Only scattered segments of rough endoplasmic reticulum are present in these cells. The nuclei with the peripherally clumped chromatin are typical of mast cells fixed with glutaraldehyde. $\times 9,000$.

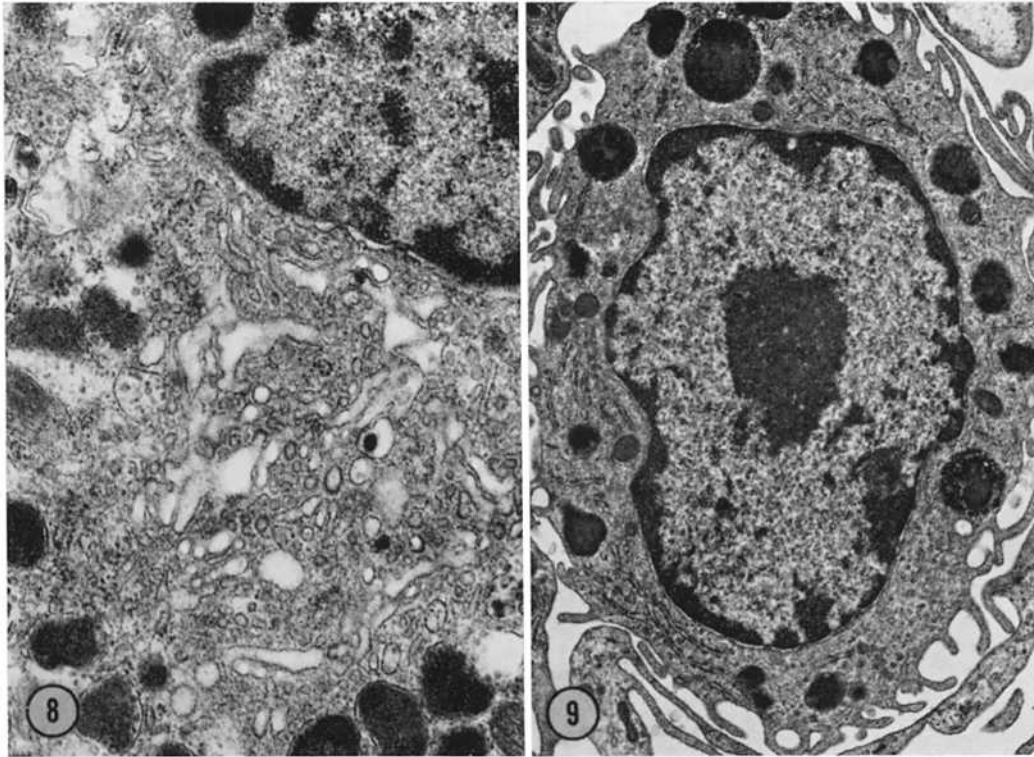


FIGURE 8 Electron micrograph showing the cell center of a mast cell from a 10-day-old culture. Numerous Golgi vacuoles and less frequent saccules are present. In some of the vacuoles, small dark granules are evident. Similar granules have been suggested to represent granule precursors in the normal maturation of rat mast cells. $\times 26,500$.

FIGURE 9 Electron micrograph of a mast cell from a 28-day-old culture. This cell has relatively few granules with varying proportions of dense matrix and peripheral vesicles. The rough-surfaced endoplasmic reticulum is moderately well developed in this cell. A large nucleolus such as is seen in this micrograph is not uncommon in tissue culture mast cells. $\times 10,000$.

homogeneously dense granules (Fig. 13). In general, the mast cells in culture resembled immature mouse mast cells present in embryonic connective tissue (5).

Differentiation of Cells on Monolayers

MAST CELLS: Very few mast cells were present in the initial inocula from mouse lymph nodes, and those that could be identified had the appearance of mature cells packed with normalized, strongly metachromatic granules. The early sequence of events in cultures in which mast cells ultimately differentiated was identical to that exhibited by lymphoid cells from unimmunized animals on monolayers (8). The number of lymphocytes decreased during the first few days;

then there was a distinct increase in large lymphoid cells both on the monolayer, where they formed small colonies, and free in suspension. Significant numbers of identifiable mast cells in small aggregates of two to ten appeared in clones of immature lymphoid cells in the most favorable cultures between 4 and 5 days. The number of cells in a colony then increased over the next few days to 15-30. With the increase in the size of the mast cell colonies, the number of granules per cell also increased. At about 8 days, mast cells appeared in suspension in the media. In active cultures, the mast cell colonies dispersed (Fig. 14) and extensive distribution of mast cells over the monolayer was established by 20-30 days (Fig. 15). Subsequently, mast cells decreased in number,

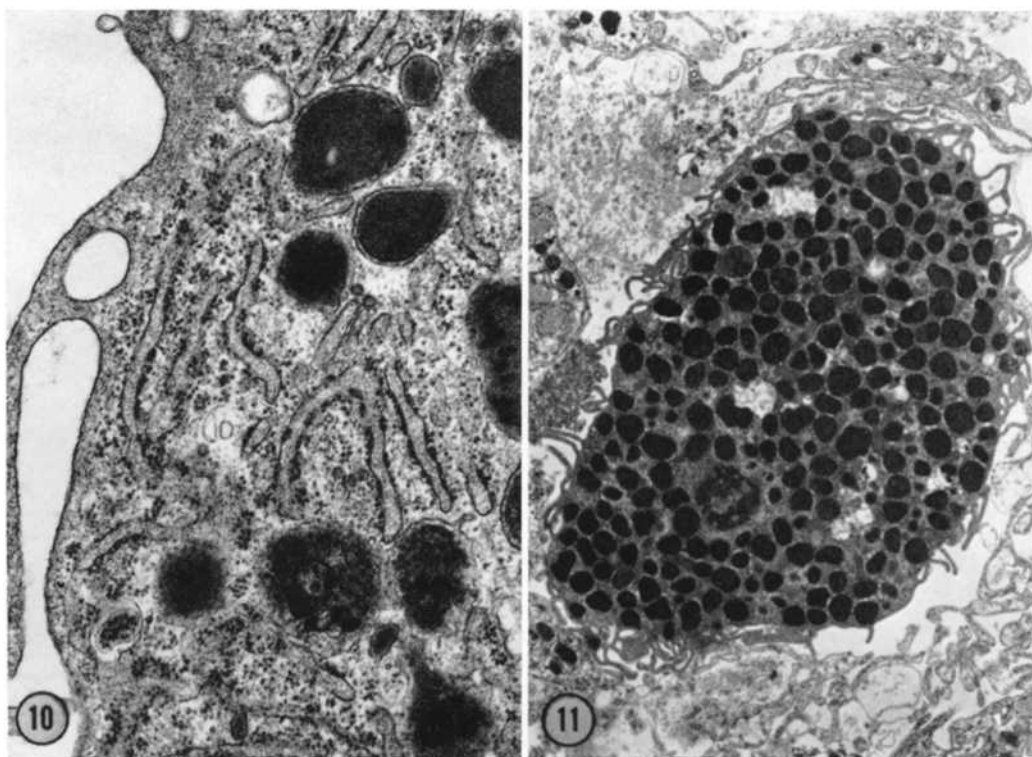


FIGURE 10 Electron micrograph of peripheral cytoplasm of a mast cell from a 10-day-old culture. The villus is seen to be free of organelles including ribosomes. Ribosomes, both free and attached to endoplasmic reticulum, are present in the cytoplasm proper. A well preserved perigranular membrane is evident around several of the homogeneously dense granules. $\times 27,000$.

FIGURE 11 Electron micrograph of a mast cell with many homogeneous granules. This cell from a 10-day culture looks more like a mature mouse tissue mast cell than most cells in tissue culture and may well represent a cell carried over from the lymph node. The section includes only a small portion of the nucleus. $\times 5,000$.

and subculture did not lead to further proliferation.

In cultures prepared from unimmunized mice a few colonies of mast cells occasionally appeared. These were limited in extent as well as number and were, in time, commonly overgrown by histiocytes. The granules in the mast cells from unimmunized animals (Fig. 16) tended to be smaller than those in cells from immunized animals (Fig. 17). There were also many more granules per cell in the cells from unimmunized animals. None of the large clones of immature mast cells prevalent in the cultures initiated from immunized animals was present in the cultures derived from unstimulated lymphoid cells.

HISTIOCYTES: In cultures of cells from un-

immunized animals, histiocytes appeared as a distinguishable cell type in small colonies between 4 and 6 days. There followed a progressive increase in colony size leading ultimately to confluency by 20–30 days. During this time, the histiocytes exhibited a progressive increase in their content of vacuoles, many of which were strongly PAS positive. When the cells were derived from immunized animals, histiocytic colonies appeared at the same time and increased to about 50 cells per colony during the 2nd wk. Proliferation then apparently ceased. Their attachment to the monolayer weakened, so that they tended to come loose; large inclusions appeared in the histiocytes, and disintegration ensued.

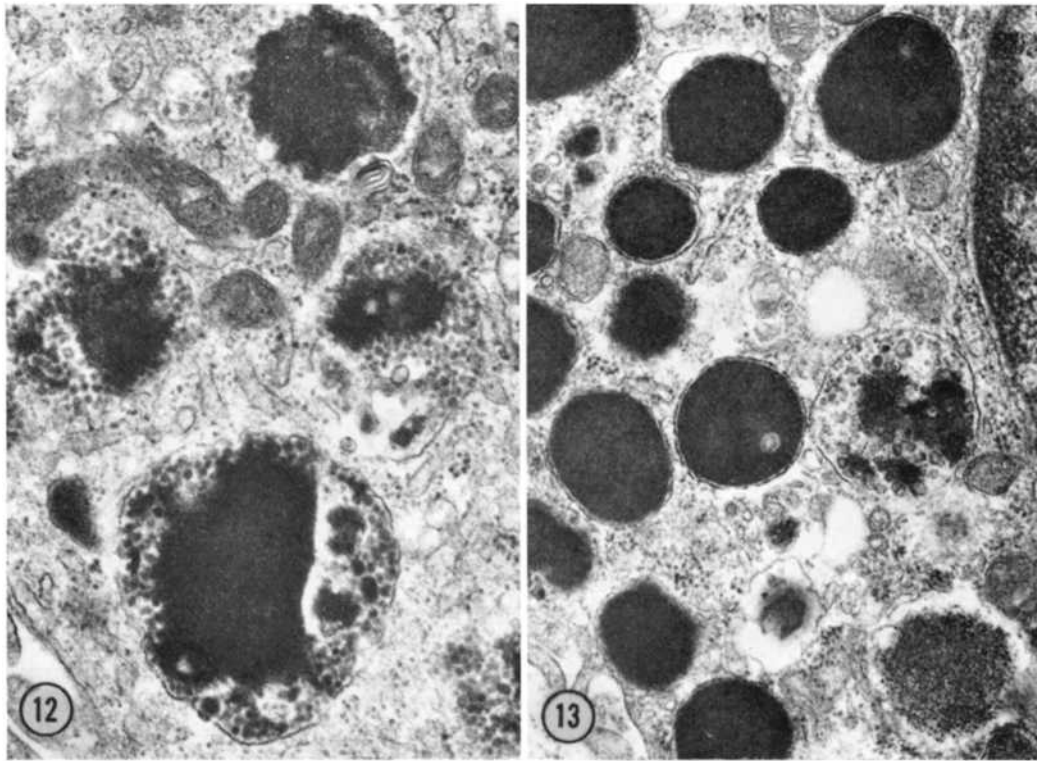


FIGURE 12 Electron micrograph of several granules from a mast cell 28 days in culture. The two components of the granules, small vesicles and dense matrix, are well illustrated in this micrograph. The perigranule membrane can be traced almost completely around the circumference of the largest granule. $\times 35,500$.

FIGURE 13 Electron micrograph of granules from a mast cell 10 days in culture. Most of the granules evident exhibit morphology considered to represent the final stage of granule maturation, with homogeneously dense matrix and a closely applied perigranule membrane. A granule composed almost entirely of small vesicles is seen close to the nucleus. Several mitochondria are present in this micrograph. $\times 27,000$.

MAST CELL DIFFERENTIATION FROM THORACIC DUCT LYMPH CELLS: Cells obtained from thoracic duct were plated on monolayers. (No mast cells were present in thoracic duct lymph.) In cultures from both immunized and unimmunized animals, large lymphoid cells appeared between the 3rd and 6th day of culture. These cells in suspension were subcultured and gave rise to cultures of mast cells and histiocytes according to their derivation from immunized or unimmunized mice, respectively. The sequence of developments closely paralleled that described for lymph node cells.

DISCUSSION

The results indicate that immature mast cells will differentiate and replicate in cultures pre-

pared from suspensions of lymphoid cells under appropriate conditions. A sequence resembling that in embryonic connective tissue (4) is followed in the cultures; namely, that mast cells appear to arise from an ungranulated precursor, then during a predominantly replicative period the cells begin the synthesis of specific granules and finally attain a differentiated nonreplicative stage.

In rat embryos, the mast cells differentiate from mesenchymal cells at about 12 days (4). Nothing is known as yet of the *in vivo* precursor of mast cells in adult rodents. Although most mast cells in the adult rat have apparently either lost the capacity for mitosis entirely or else have an exceedingly long intermitotic period, prolifera-

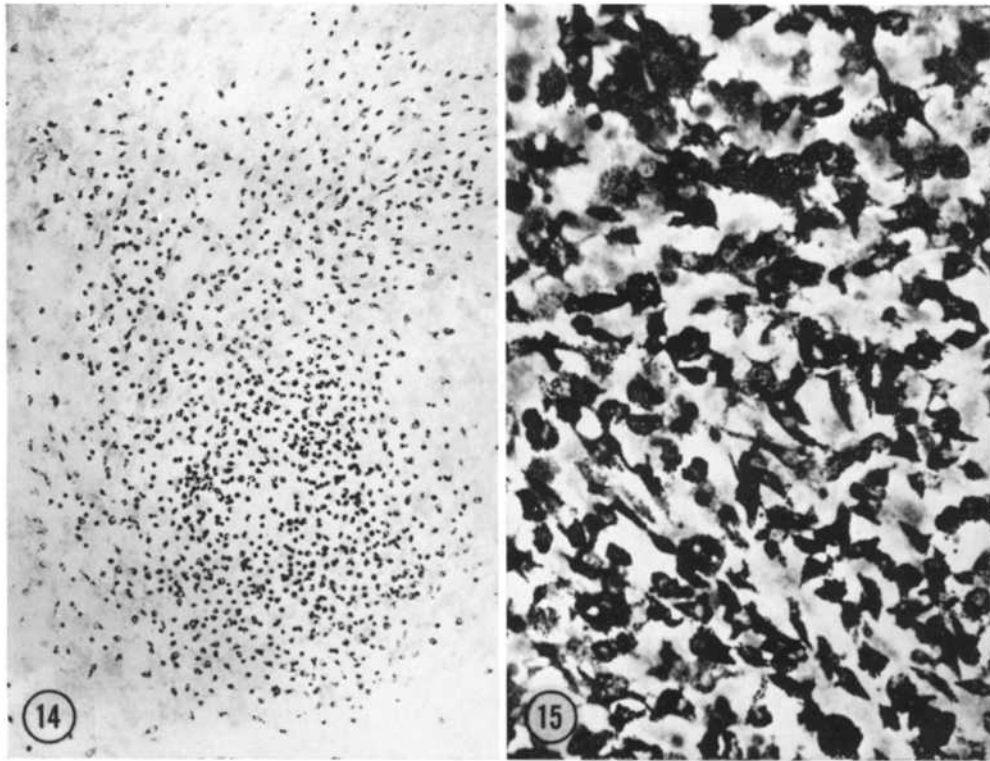


FIGURE 14 A clone of mast cells on embryo monolayer. The lymph node cells seeded on the monolayer were derived from mice immunized with ovalbumin. Stained with toluidine blue. $\times 30$.

FIGURE 15 Mast cells on embryo monolayer. Cells grown from lymph node from mice immunized with horse serum have spread over the monolayer. 40-day culture. Stained with toluidine blue. $\times 208$.

tion of mast cells can be induced by a variety of agents (1).

The derivation of the mast cells in the cultures cannot be assigned with absolute certainty. A number of considerations incline us to the opinion that the mast cells derive from precursors, lacking specific granules, present in the population of cells obtained from the lymph nodes or thoracic duct lymph.

Immature mast cells, characterized by small numbers of granules and large nuclei with prominent nucleoli, are not evident in the cells obtained from lymph node, and the observed sequence of maturation militates against the small numbers of mature mast cells giving rise to the colonies of immature mast cells. Further evidence against homoplastic differentiation is provided by the cultures initiated with thoracic duct cells among which no mast cells whatever were found.

The possibility that the mast cells do not derive

from any cells in the lymph node or thoracic duct lymph, but rather from the monolayer may also be raised. Definitive studies with chromosomal markers such as *T6* or irreversible inhibition of monolayer proliferation by X-irradiation have not yet been performed in the system described in this paper. In the case of differentiation of thymus cells to mast cells, X-irradiation of the monolayer, however, was not found to interfere with mast cell differentiation (Ginsburg, unpublished results). Against the likelihood that the fibroblast monolayers give rise to mast cells is the fact that, in cultures of monolayers to which no lymphoid cells are added, mast cells do not differentiate. Further, the fibroblasts of the monolayer are a homogeneous population of large flat cells, and transitions to colonies of round cells such as those in which immature mast cells appear have not been observed; finally, mast cells appear in good number on fibroblast

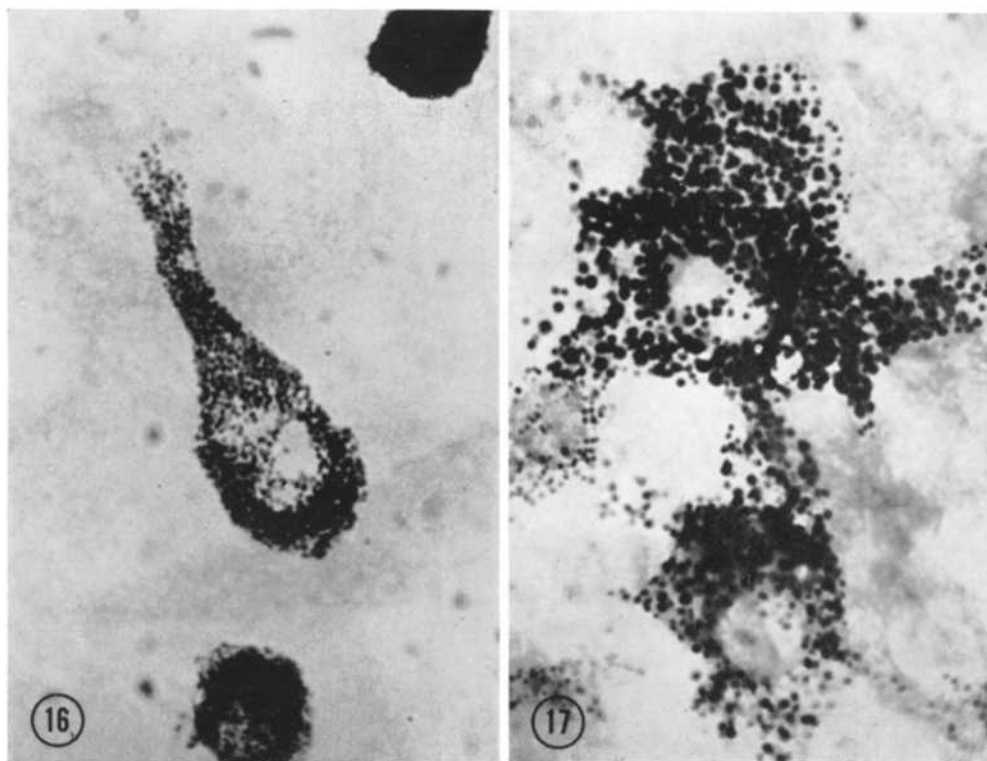


FIGURE 16 Mast cell in culture of lymph nodes from normal mice. The mast cells in these cultures have smaller granules than those in culture from immunized mice. 22 days in culture. Stained with toluidine blue. $\times 1500$.

FIGURE 17 Mast cell from a culture of lymph nodes from mice immunized with horse serum. The mast cells in these cultures have large metachromatic granules. 40 days in culture. Stained with toluidine blue. $\times 1500$.

monolayers that have been serially plated as many as six times, a procedure likely to exclude small numbers of specific mast cell precursors.

The number of mast cell precursors is difficult and hazardous to estimate on the basis of the tissue culture observations. If it is assumed that each colony in a tissue culture derives from a single lymphoid precursor with a cloning efficiency of 50%, mast cell precursors can be estimated, from the results recorded in Table II, to occur with a frequency of one in 10,000 lymphoid cells. If the cloning efficiency is, in fact, less than 50%, the number of precursors might be considerably greater than the one in 10,000 estimate. Contrariwise, if secondary clones are established from precursors that arise in primary clones, the estimate of the number of precursors would be too high.

Although strong evidence is lacking as to the

identity of the precursor of the mast cell, the potential of thoracic duct cells to differentiate to mast cells leads us to favor the small lymphocyte, since the larger cells in thoracic duct rapidly disappear *in vitro* (11). Further, the large lymphoid cells which precede the appearance of mast cells in culture resemble the cells which derive from small lymphocytes following exposure to a variety of blastogenic stimuli (10, 14). Identical reasoning leads to the same tentative conclusions for the identity of the histiocyte precursor in the cultures. In contrast, Volkman and Gowans (17) have provided substantial evidence that inflammatory histiocytes arise from monocytes derived from bone marrow. It may be that macrophages of differing types do not have the same precursors.

Mast cells as connective tissue cells having synthetic capabilities quite different from those

of fibroblasts present an opportunity to study the control of differentiation of a second connective tissue cell in vitro. The observation that antigenic stimulation of the donor animals and subsequent exposure to the antigen in culture are necessary for the establishment of mast cell cultures is surprising. Although increases in mast cell numbers, most likely on a proliferative basis, have been described following the administration of a diversity of local and systemic agents in vivo, only moderate proliferation of mast cells has been described in response to immunization (6, 15). In general terms, antigen may be considered to act either directly to stimulate mast cell proliferation and/or differentiation, or indirectly to inhibit histiocytes and thus permit

mast cell growth. Further experiments are required to determine the actual mechanism of antigenic stimulation.

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REFERENCES

1. BENDITT, E. P., and D. LAGUNOFF. 1964. *Progr. Allergy*. **8**:196.
2. BARKA, T., and P. J. ANDERSON. 1962. *J. Histochem. Cytochem.* **10**:741.
3. COMBS, J. W. 1966. *J. Cell Biol.* **31**:563.
4. COMBS, J. W., D. LAGUNOFF, and E. P. BENDITT. 1965. *J. Cell Biol.* **25**:577.
5. COMBS, J. W. 1966. *J. Cell Biol.* **31**:23A (Abstr.).
6. COTTIER, H., N. ODARTCHENKO, G. KEISER, H. HESS, and D. STONER. 1965. *Ann. N. Y. Acad. Sci.* **113**:612.
7. GINSBURG, H. 1963. *Ann. N. Y. Acad. Sci.* **103**:20.
8. GINSBURG, H. 1965. Methodologic Approaches to the Study of Leukemias. V. Defendi, editor. *Wistar Inst. Symp. Monograph* No. **4**:21.
9. GINSBURG, H., and L. SACHS. 1963. *J. Nat. Cancer Inst.* **31**:1.
10. GINSBURG, H., and L. SACHS. 1965. *J. Cellular Comp. Physiol.* **66**:199.
11. GOWANS, J. L. 1962. *Ann. N. Y. Acad. Sci.* **99**:432.
12. LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9**:409.
13. REINHARDT, W. O., and C. H. LI. 1945. *Proc. Soc. Exptl. Biol. Med.* **58**:321.
14. ROBBINS, J. H. 1964. *Science*. **146**:1648.
15. ROBERTS, A. 1966. *Am. J. Pathol.* **49**:889.
16. SPICER, S. S. 1960. *J. Histochem. Cytochem.* **8**:18.
17. VOLKMAN, A., and J. L. GOWANS. 1965. *Brit. J. Exptl. Pathol.* **46**:50.