AGGREGATION AND TRANSFORMATION OF RAT LYMPHOCYTES ON RAT EMBRYO MONOLAYERS

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ABSTRACT

Lymphocytes from Sprague-Dawley rats have been cultured on monolayers of embryo-derived fibroblasts from the same outbred strain. Under these conditions the lymphocytes form aggregates, and transformation of small lymphocytes to lymphoid blast cells occurs within these aggregates. Transformation is characterized cytologically by enlargement of the nucleus, dispersion of nuclear chromatin, and the appearance of a prominent nucleolus. The principal cytoplasmic changes are an increase in cytoplasmic volume, a marked increase in number of ribosomes, and a clustering of ribosomes. These changes parallel those seen in the transformation of lymphocytes caused by a variety of treatments. One apparent difference is the paucity of lysosomes and lipid inclusions in the lymphocytes that transform on the monolayer.

INTRODUCTION

When lymphocytes from lymph nodes or the thoracic duct of the rat are plated on xenogeneic embryo monolayers, large lymphoid cells arise through the transformation of small lymphocytes, and lysis of the cell monolayer follows (1–4). When lymphoid cells from immunized mice are plated on either syngeneic or allogeneic monolayers, differentiation to mast cells occurs, while cells from untreated mice yield cultures of histiocytes (5). A different sequence is observed when suspensions of Sprague-Dawley rat lymphocytes are plated on fibroblast monolayers prepared from Sprague-Dawley embryos.

In such cultures, lymphocytes form multiple aggregates on the surface of the monolayer, and lymphoid blast cells appear within the aggregates. The blast cells undergo mitosis; the aggregates increase in size, and cells are seeded into the culture medium. It is the purpose of this paper to describe the aggregation and transformation of rat small lymphocytes on embryonic monolayers.

METHODS

Embryonic fibroblast monolayers were prepared from Sprague-Dawley rats as previously described (1, 2). The monolayers were initiated by plating from 5×10^4 to 2×10^5 cells per 60-mm Falcon plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.) in 4 ml of medium consisting of 0.5% lactalbumine hydrolyzate in Earl's saline supplemented with 10% calf serum. These plates were kept for periods ranging from 1 to 2 wk before use and had a final cell population of $1.5–2.5 \times 10^6$ fibroblasts.

Suspensions of lymph node and thoracic duct cells were prepared as described previously (2, 5). Usually 3×10^7 cells in 4 ml of Dulbecco's medium plus horse serum were added to a single monolayer Petri dish (1).

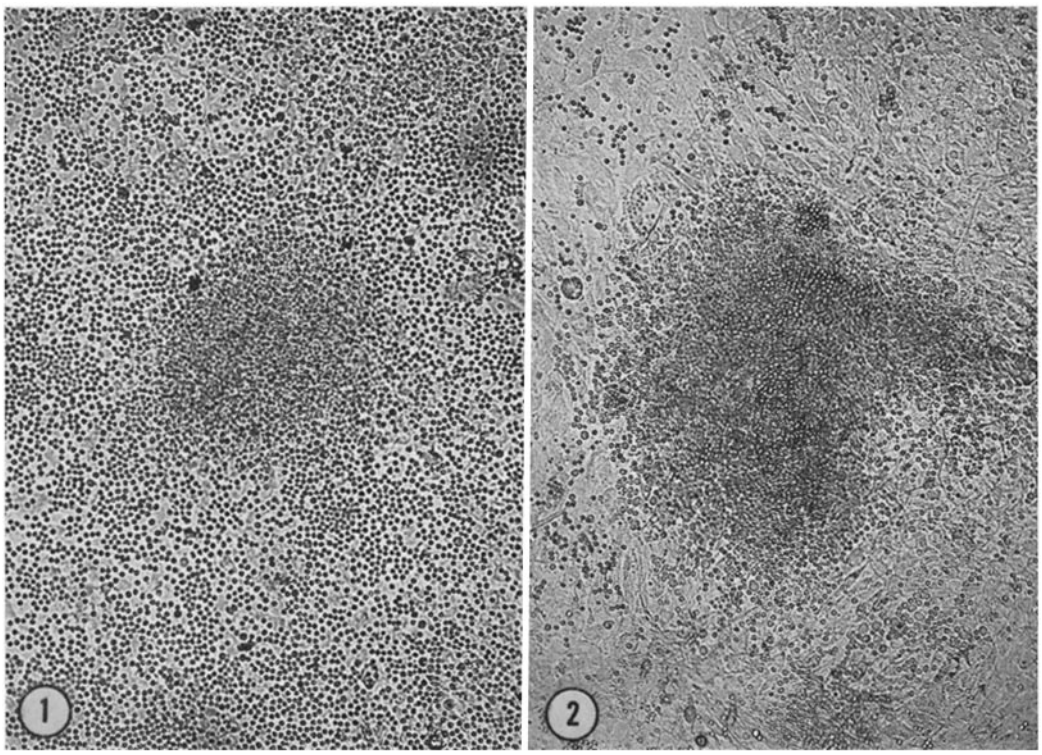


FIGURE 1 2 day old culture of lymph node cells of Sprague-Dawley rats on Sprague-Dawley monolayer. Many small lymphocytes still float freely over the monolayer; an early lymphocytic aggregate is seen in the center of the field. Unfixed, unstained culture. × 100.

FIGURE 2 2 day old culture of lymph node cells of Sprague-Dawley rats on Sprague-Dawley monolayer after the free, nonaggregated lymphocytes were washed away. A small aggregate is more clearly seen than in Fig. 1. Unfixed, unstained culture. × 100.

"Passaging"

The free nonadherent cells were transferred to new monolayers at various intervals after plating. The cells were collected in the medium by gentle pipetting and centrifuged at 1000 rpm for 5 min; they were then resuspended in medium and plated on a new monolayer. All cultures were incubated at 37° C in 7% CO_2 in air, in a humidified incubator.

Fixation and Staining

For light microscopy the plates were washed twice with saline, fixed with absolute methanol, and stained with methyl green-pyronin (2). For electron microscopy, 4% glutaraldehyde (in 0.1 M cacodylate buffer pH 7.4 with 0.1% Alcian blue) was added to saline-washed cultures. Cultures were fixed for 4 hr at 4° C, washed with 7.5% sucrose in cacodylate buffer, and postfixed in *s*-collidine buffered osmium tetroxide for 1 hr. The postfixed aggregates were scraped from the Petri dishes, dehydrated, and embedded in Epon 812. Thick sections were stained with alkaline Toluidine blue. Thin sections were stained with uranyl acetate and lead tartrate.

RESULTS

Aggregations of Lymphocytes on Rat Embryo Monolayers

Collections of small lymphocytes in numerous sites on the monolayer were evident as early as 1 day after a suspension of lymphocytes had been added to a monolayer (Fig. 1). Continued accumulation of lymphocytes occurred until the 3rd or 4th day, when the aggregates were apparent to the naked eye. Removal of the lymphocytes remaining in suspension 20–25 hr after they had been plated

left small aggregates of lymphocytes distributed over the monolayer (Figs. 2, 3). These aggregates were ideal for observations in living cultures. Free lymphoid cells collected during the first 3 days of culture retained the capacity to aggregate when transferred to new monolayers.

Transformation of Small Lymphocytes to Blast Cells in the Aggregates

Although at 24 hr the aggregates were composed entirely of lymphocytes (Fig. 2), by the 2nd day a small number of larger cells had appeared (Fig. 4). A sharp increase in the number of these cells was observed between the 3rd and 5th day (Fig. 5), and by the 6th day the aggregates were composed predominantly of cells characterized by their large size and basophilic cytoplasm. The large lymphoid cells continued to multiply, expanding the boundaries of the aggregates. Even in cultures in which the free lymphocytes were washed away on the 1st or 2nd day, an extensive population of lymphoid cells developed, spread over the monolayer, and seeded into the medium (Fig. 6). No lysis of the monolayer occurred under these conditions.

The cells observed in the cultures from the 8th day were of several types. The commonest, regularly obtained in cultures from normal Sprague-Dawley rats, was a pyroninophilic lymphoid cell (7–12.5 μ in diameter) (Fig. 6), smaller than the typical cell (14–19 μ in diameter) of the in vitro graft reaction (1–3), but with more cytoplasm and a larger nucleus than a small lymphocyte. Occasionally histiocytes (1) arose in the aggregations. The frequency of differentiation to a predominantly histiocytic culture was increased when the initiating cells were obtained from rats immunized against horse serum or ovalbumin (1).

When Sprague-Dawley lymphocytes were placed on monolayers derived from Lewis rat embryos, aggregates formed. In the case of Wistar embryo monolayers, there was lysis of the monolayers. Lewis lymphocytes aggregated on Sprague-Dawley monolayers but caused lysis of Wistar monolayers. In preliminary experiments in which Lewis lymphocytes were cultured on Lewis monolayers, neither aggregation nor lysis occurred. Similar results were obtained when lymphocytes from inbred Wistar rat lymphocytes were cultured on syngeneic monolayers.

Electron Microscopy

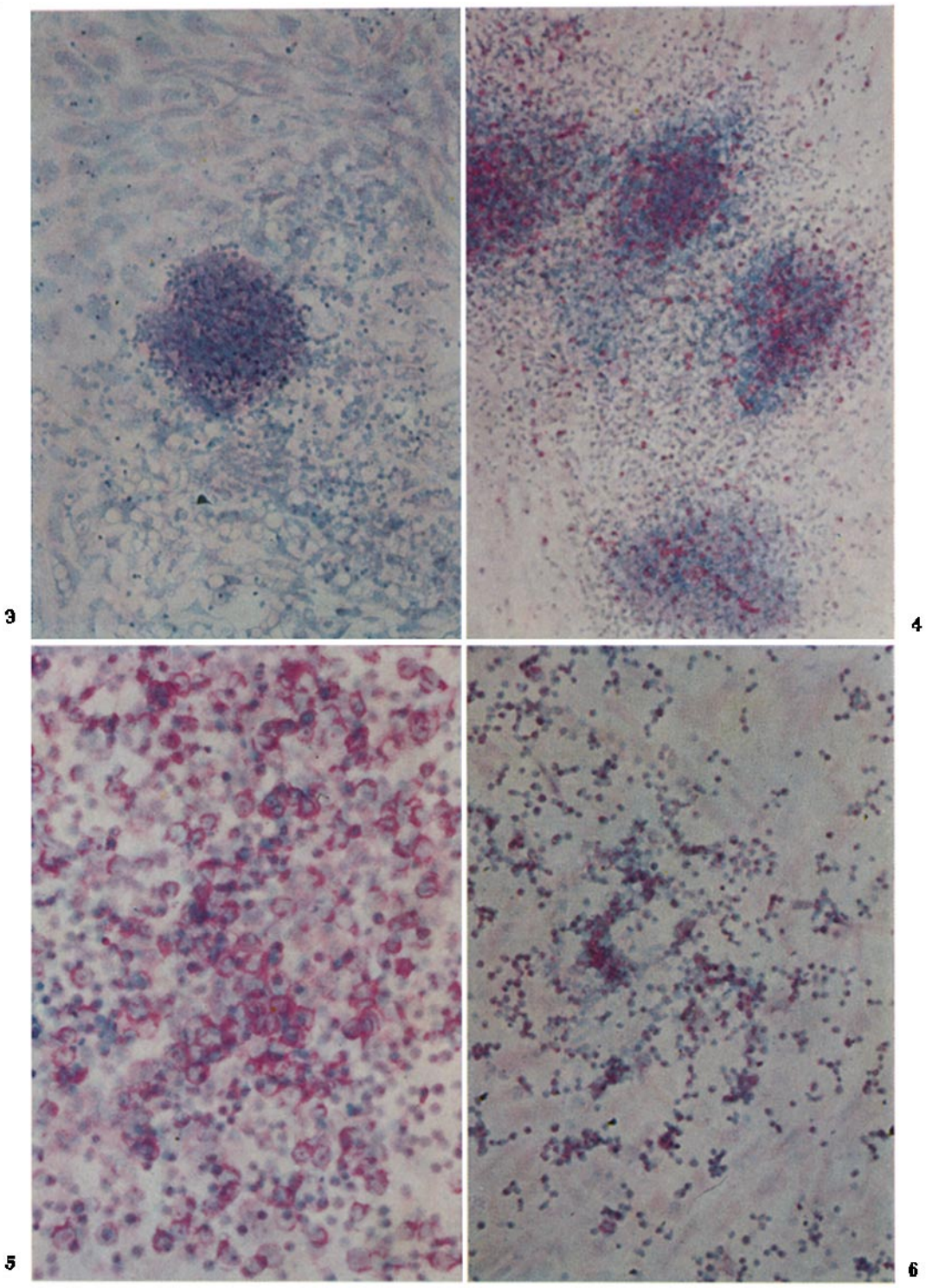
In order to better define the characteristics of the cellular transformations of the lymphocytes on the monolayer, electron microscopic studies were carried out on one set of cultures. Lymph node cells of Sprague-Dawley rats were plated on Sprague-Dawley monolayers, and free cells were

FIGURE 3 Early lymphocytic aggregate. This culture was fixed 20 hr after plating a suspension of lymph node cells of Sprague-Dawley rats on Sprague-Dawley monolayer. The aggregate is composed entirely of lymphocytes. No pyroninophilic cells are present. Methanol-fixed. Methyl green-pyronin stain. × 135.

FIGURE 4 Appearance of blast cells with pyroninophilic cytoplasm in aggregates. These aggregates formed after transfer of free nonaggregated suspension of lymphocytes from a 71 hr old culture to a new monolayer of Sprague-Dawley fibroblasts. The culture was fixed 50 hr after transfer. Methanol-fixed. Methyl green-pyronin stain. × 135.

FIGURE 5 Blast cells in aggregates after 3 days. This culture was established as a third transfer of nonaggregated cells and was fixed 3 days after plating on the monolayer. Many cells with large nuclei and intensely pyroninophilic cytoplasm are evident. Methanol fixed. Methyl green-pyronin stain. × 270.

FIGURE 6 Secondary formation of lymphoid cells from blast cells. In this culture the free, nonaggregated lymphocytes were washed away 25 hr after establishing the culture. Cultures were kept for a period of 9 days and then fixed. The cells are smaller than the blast cells but retain moderate pyroninophilia. These cells are found dispersed over the monolayer rather than in aggregates typical of younger cultures. Methanol fixed. Methyl green-pyronin stain. × 135.



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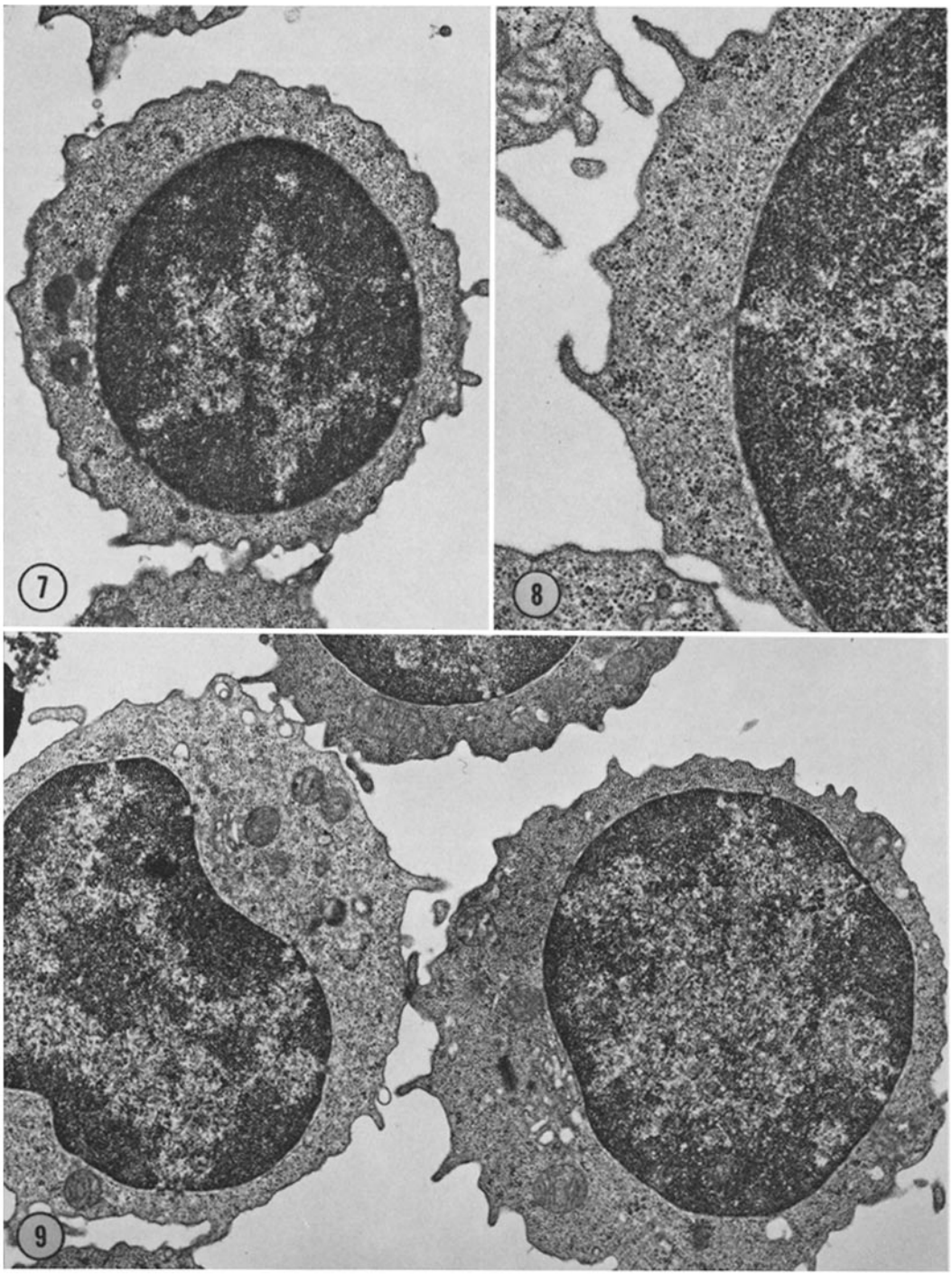

FIGURE 7 A typical small lymphocyte present in the population of free cells after 24 hr in culture. The nucleus is dense with a small, irregular, central pale area. The rim of cytoplasm contains a few nondescript dense bodies and dispersed ribosomes. × 15,000.

FIGURE 8 A higher magnification of a cell similar to that in Fig. 7. The ribosomes are characteristically dispersed. No other organelles are present in this section. A few pseudopods jut from the surface of the cell. × 25,500.

FIGURE 9 Portions of three small lymphocytes from the same culture as the cells in Figs. 7 and 8. The cell to the left has somewhat paler cytoplasm than do the other two cells, and its nucleus is deformed. Golgi lamellae are evident in an indentation of the nuclei, and several mitochondria are present. This cell may represent a very early phase of the postulated transition from small lymphocyte to blast cell. The cell on the right also contains a Golgi apparatus, and a centriole is present as well. × 12,000.

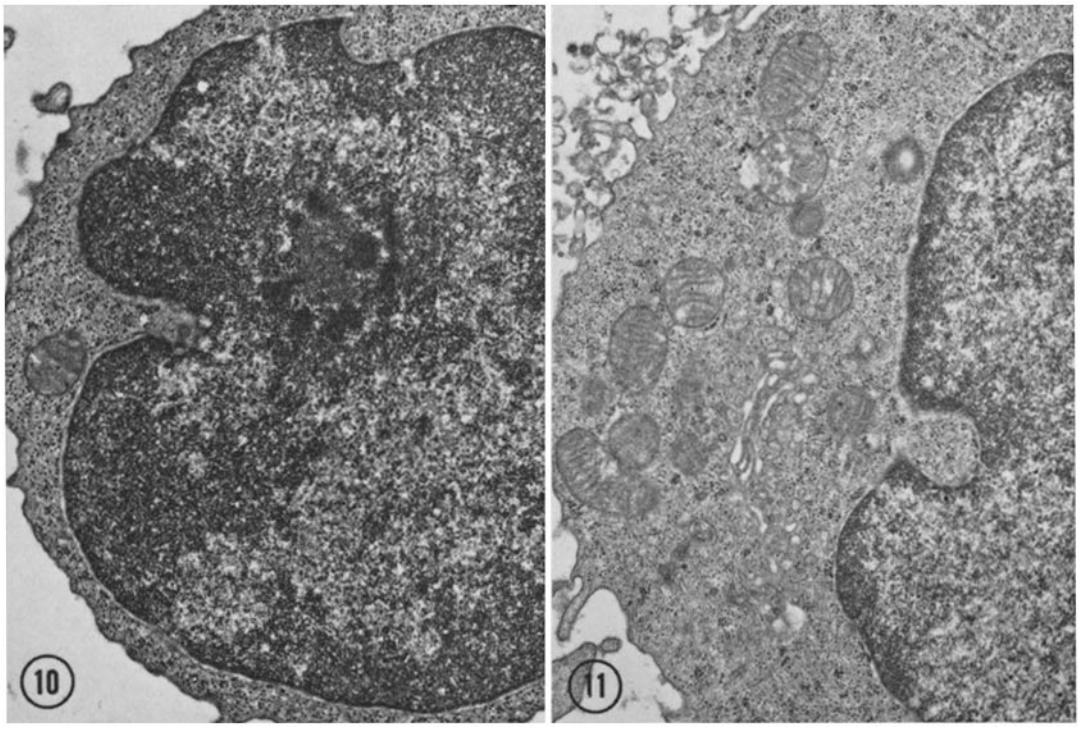

FIGURE 10 A small lymphocyte from the same culture as the cells in the previous electron micrograph with intranuclear body that is probably a nucleolus. × 16,000.

FIGURE 11 A large lymphocyte present in the population of free cells after 24 hr in culture. Not only is more cytoplasm present than is seen in a small lymphocyte, but the mitochondria are increased in number, and some of the ribosomes are clumped. The nucleus shows some alteration of the peripheral dense chromatin, but rarefaction of the central chromatin is not noticeable. × 15,000.

harvested after 24 hr. A sample was taken for electron microscopy, the cells were replated on Sprague-Dawley monolayers, and cultures were fixed at 24-hr intervals thereafter. The small lymphocyte (Figs. 7–10) was the predominant but by no means the exclusive cell in both the lymph node cell suspensions and the thoracic duct lymph used to initiate the cultures. This cell type was characterized by a basically round nucleus with a relatively thin rim of cytoplasm (Figs. 7, 9, 10). The dense chromatin of the nucleus was most concentrated at the periphery but extended well into the central nuclear region (Figs. 7, 9); a nucleolus was only rarely discernible (Fig. 10). When the section was such that any appreciable eccentricity in the distribution of cytoplasm was evident, a few mitochondria and a small Golgi apparatus were usually present in the cytoplasmic cap (Fig. 9). Single ribosomes were spread rather evenly through the cytoplasm (Fig. 8). These cells exhibited a notably greater density of the cytoplasmic ground substance than did other larger cells types. Typically, moderate numbers of stubby pseudopods sprouted from the surface of the small lymphocyte (Figs. 8, 9).

The basophilic blast cell (Figs. 12, 15, 17) that developed within the aggregates differed from the small lymphocyte in almost every structural aspect. The blast cells were two or three times the size of a small lymphocyte; they were irregularly polygonal with an ample endowment of ribosome-rich cytoplasm. The blast cell nucleus was large and rarely as symmetrical as that of the small lymphocyte. The chromatin was thinly dispersed through the major portion of the nucleus with only a narrow, discontinuous rim of con-

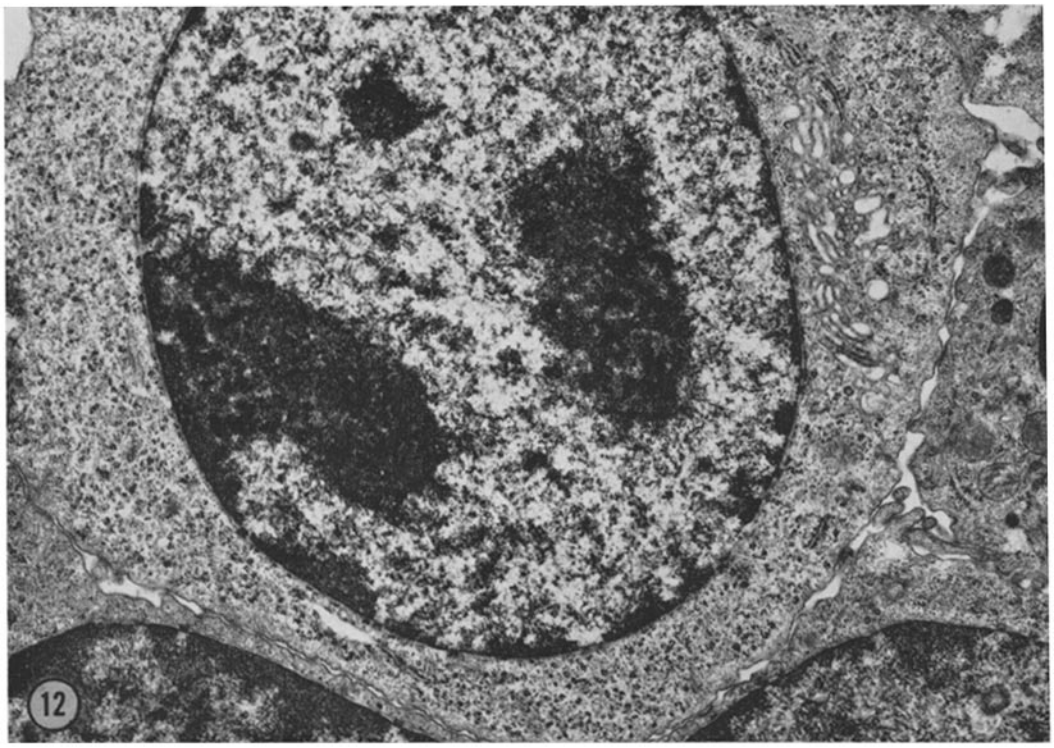

FIGURE 12 A blast cell from a culture 96 hr after replating. The nucleus is typical with its thin, interrupted rim of dense chromatin and prominent nucleoli. A well-developed Golgi with lamellae and vacuoles is present, and the ribosomes are essentially all arranged in clumps. A few shreds of ribosome-studded endoplasmic reticulum are evident. Although the cell surface is irregular, no pseudopods are apparent. × 14,500.

densed chromatin (Figs. 12, 15). The nucleolus was very prominent and in some sections was seen as continuous with the peripheral rim of condensed chromatin (Figs. 12, 15). Two prophase nuclei were observed in the sample studied (Fig. 17). A very characteristic feature of the cytoplasm of the large basophilic cell was the clustered arrangement of ribosomes (Fig. 16). The number of ribosomes per cluster and the patterns varied. Spiral arrays characteristic of ribosomes associated with endoplasmic reticulum were infrequent, and endoplasmic reticulum with associated ribosomes was sparse. A well-developed smooth membrane system in the form of a Golgi apparatus (Fig. 12) was evident. Mitochondria were present in only modest number. Dense, membrane-bounded bodies identifiable as lysosomes (Fig. 14) were infrequent in these cells. Per length of plasma membrane, the blast cells seemed to exhibit fewer pseudopods than small lymphocytes (Fig. 12). Quite fully-developed blasts were observed as early as 72 hr after the initial inoculation of the monolayers.

The third cell frequently seen in the cultures was one that had many of the features that would be predicted for a cell intermediate between a small lymphocyte and a blast cell (Figs. 11, 13). This cell was larger than the small lymphocyte. At lower magnifications both the nucleus and the cytoplasm were seen to be less dense than those of the small lymphocyte, and the cytoplasmic:nuclear ratio was increased (Fig. 13). The nucleus of the larger lymphocyte was often flattened on one side or indented and irregular. A nucleolus was frequently evident, and the distribution of chromatin ranged from a pattern like that of the small lymphocyte to a predominantly dispersed state with peripheral clumping. In many of these cells a well-developed Golgi apparatus was situated over

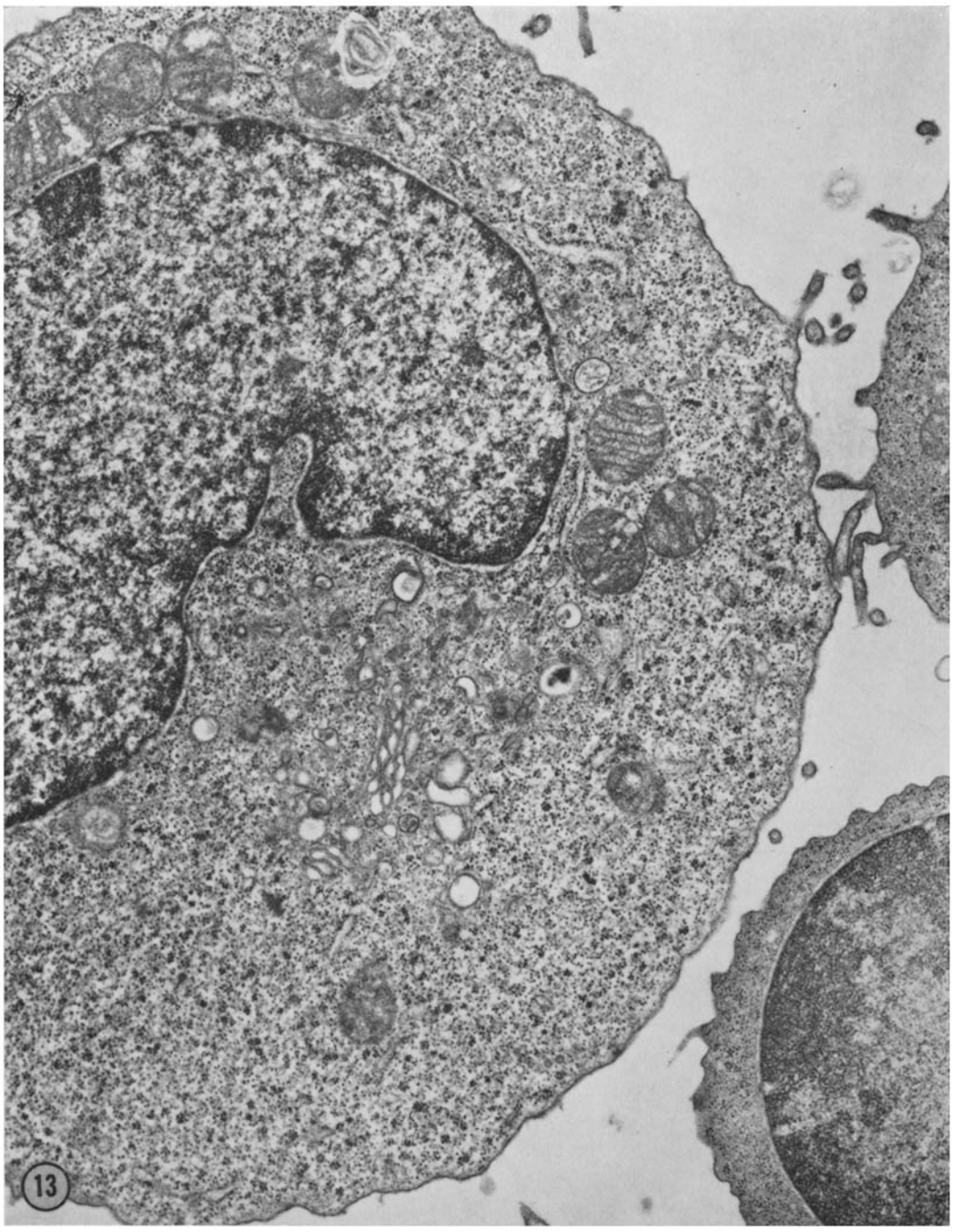

FIGURE 13 A large lymphocyte present in the population of free cells after 24 hr in culture. This large cell has a low nuclear:cytoplasmic ratio. While many of the ribosomes are clumped, there is still a large number of single ribosomes. The peripheral dense chromatin is sparse, but rarefaction of the remainder is only moderate. One mitochondrion contains a myelin figure characteristic of suboptimal glutaraldehyde fixation. × 16,500.

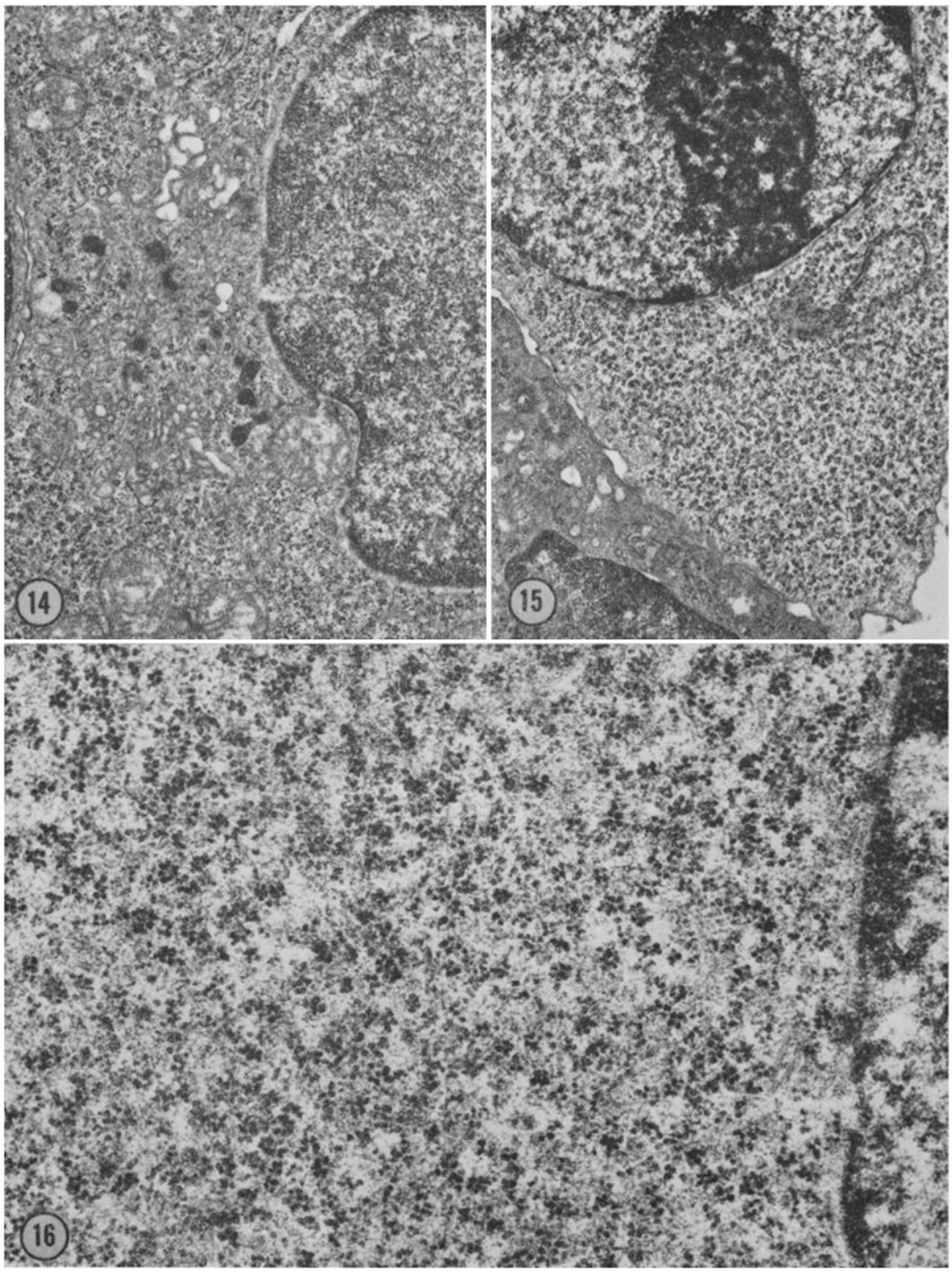

FIGURE 14 The cytocentrum of a cell with a nucleus characteristic of a large lymphocyte but with predominantly clumped ribosomes. The culture was fixed 48 hr after replating. The several dense bodies are presumably lysosomes and represent the largest number of such organelles seen in any cell from the cultures examined. × 15,500.

FIGURE 15 A portion of nucleus and cytoplasm of a blast cell adjacent to a cell of the monolayer 48 hr after replating. This cell is particularly rich in aggregates of ribosomes. × 13,000.

FIGURE 16 At higher magnification the variety of patterns of aggregation of the ribosomes is evident. While a few circular arrangements or rosettes are present, there is considerable variation in both the number of ribosomes per clump and the pattern in which they are arranged. × 45,000.

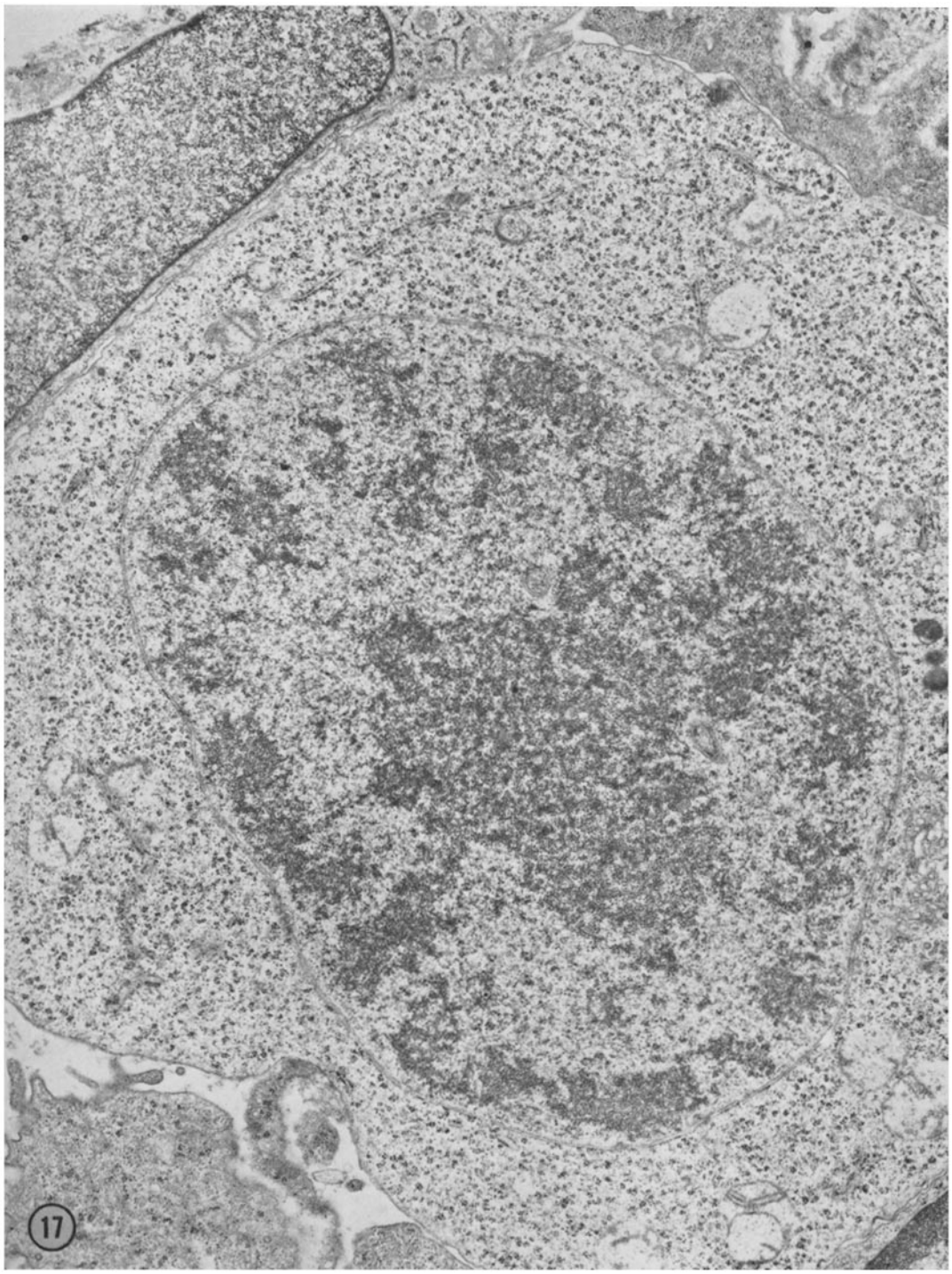

FIGURE 17 One of the two prophase nuclei observed in blast cells 96 hr after replating. The nuclear membrane is still intact. The mitochondrial membranes and the cristae are poorly preserved in this cell, presumably because of suboptimal fixation. It may be noted, however, that the mitochondria of blast cells consistently were less well preserved than mitochondria of neighboring cells of other types. × 14,000.

the flattened or indented aspect of the nucleus. Centrioles were often present near the Golgi apparatus, and several intact mitochondria were frequently present in the vicinity (Figs. 11, 13). Both separated and aggregated ribosomes were present in the large lymphocytes (Fig. 13). Organelles identifiable as lysosomes were rarely present in this cell type.

DISCUSSION

The capacity of lymphoid cells to grow and differentiate on embryonic fibroblast monolayers provides a potentially useful in vitro system for the analysis of cell lineages, of interactions of cell types, and of effects of hormones, metabolites, antimetabolites, and other agents on lymphoid cells. An important aspect of lymphoid biology to come to the fore in recent years is the in vitro transformation of the small lymphocytes to blast cells (6); in this process a seemingly minimal cell consisting of a nucleus with a thin rim of organelle-poor cytoplasm transforms to a cell with a large nucleus and richly endowed with cytoplasm packed with ribosomes. Although phytohemagglutinin (PHA) (7) was the substance that initiated the renaissance of lymphocyte in vitro transformation studies, antigenic stimulation of cells from sensitized donors (8), antiglobulin allotype antibodies (9), a protein extracted from the pokeweed plant (10, 11), allogeneic cells (12), and antileukocyte antisera (13) have all been shown to effect similar transformation of small lymphocytes. Transformation in vitro of cells in the absence of specific inducing agents has also been reported (14). On appropriate fibroblast monolayers, transformation of lymphocytes is a regular phenomenon in the absence of any known inducing agent. Close cell-to-cell contact in PHA agglutinates and monolayer aggregates may be suggested as one possible factor in enhancing transformation.

Several electron microscopic studies of transformation of human lymphocytes in vitro have been reported and comprehensively summarized by Douglas et al. (15) in tabular form. The cell described by most investigators who have employed PHA resembles the intermediate cell type or larger lymphocyte in the monolayer cultures. The PHA-transformed cell has one or more nucleoli in a large nucleus with diffuse chromatin. Ribosomes are increased in number but exhibit only moderate clumping; the Golgi apparatus is usually well developed (17). The prevalence of lipid vacuoles (16) and lysosomes (18, 19) claimed for PHA transformation is not prominent in the monolayer transformation system. In PWM (pokeweed mitogen) stimulated lymphocytes at early stages, the clumping of ribosomes (15) resembles the condition in the blast cells in the monolayer culture. However, with PWM the cells go on to develop an ample rough-surfaced endoplasmic reticulum, a feature that is rare in cells transformed on monolayers.

Careful observation of living cultures leads us to the opinion that the blast cells, as in other transformations, arise from small lymphocytes. The larger lymphocyte seems to fit the morphological requirements for a transitional cell through which the small lymphocyte passes on its way to becoming a blast cell.

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REFERENCES

1. GINSBURG, H. 1965. Growth and differentiation of lymphoid origin on embryo cell monolayers. *In* Methodological Approaches to the Study of Leukaemias. V. Defendi, editor. Wistar Institute Press, Philadelphia, Pa. 4:21.
2. GINSBURG, H., and L. SACHS. 1965. Destruction of mouse and rat embryo cells in tissue culture by lymph node cells from unsensitized rats. *J. Cellular Comp. Physiol.* **66**:199.
3. GINSBURG, H. 1968. Graft versus host reaction in tissue culture. I. Lysis of monolayers of embryo mouse cells from strains differing in the H-2 histocompatibility lecus by rat lymphocytes sensitized in vitro. *Immunology.* **14**: 621.
4. GINSBURG, H., R. W. TYLER, and N. B. EVERETT. 1967. Immunological studies of lymphocytes employing an *in vitro* monolayer system. *Anat. Record.* **157**:247. (Abstr.)
5. GINSBURG, H., and D. LAGUNOFF. 1967. The

in vitro differentiation of mast cells: Cultures of cells from immunized mouse lymph nodes and thoracic duct lymph on fibroblast monolayers. *J. Cell Biol.* **35**:685.

6. ROBBINS, J. H. 1964. Tissue culture studies of the human lymphocyte. Experiments under controlled conditions provide new information on this cell's function and potentiality. *Science.* **146**:1648.
7. NOWELL, P. C. 1960. Phytohemagglutinin: An initiator of mitosis in cultures of normal human leukocytes. *Cancer Res.* **20**:462.
8. PEARMAIN, G., R. R. LYCETTE, and P. H. FITZGERALD. 1963. Tuberculin-induced mitosis in peripheral blood leucocytes. *Lancet.* **1**:637.
9. SELL, S., and P. G. H. GELL. 1965. Studies on rabbit lymphocytes *in vitro*. I. Stimulation of blast transformation with an antiallotype serum. *J. Exptl. Med.* **122**:423.
10. FARNES, P., B. E. BARKER, L. E. BROWNHILL, and H. FANGER. 1964. Mitogenic activity in *Phytolacca americana* (Pokeweed). *Lancet.* **2**:1100.
11. CHESSIN, L. N., J. BORJESON, P. D. WELSH, S. D. DOUGLAS, and H. L. COOPER. 1966. Studies on human peripheral blood lymphocytes *in vitro*. II. Morphological and biochemical studies on the transformation of lymphocytes by pokeweed mitogen. *J. Exptl. Med.* **124**:873.
12. BAIN, B., M. R. VAS, and L. LOWENSTEIN. 1964. The development of large immature mononuclear cells in mixed leukocyte cultures. *Blood.* **23**:108.
13. GRASBECK, R., C. T. NORDMAN, and A. DE LA CHAPELLE. 1964. The leucocyte-mitogenic effect of serum from rabbits immunized with human leucocytes. *Acta Med. Scand. Suppl.* **421**:39.
14. SABESIN, S. M. 1965. Lymphocytes of small mammals: spontaneous transformation in culture to blastoids. *Science.* **149**:1385.
15. DOUGLAS, S. D., P. F. HOFFMAN, J. BORJESON, and L. N. CHESSIN. 1967. Studies on human peripheral blood lymphocytes *in vitro*. III. Fine structural features of lymphocyte transformation by pokeweed mitogen. *J. Immunol.* **98**:17.
16. TANAKA, Y., and T. J. LIDDY. 1966. Lipids and acid phosphatase in cultured lymphocytes of peripheral blood. A histochemical and electron microscopic study. *Lab. Invest.* **15**:455.
17. PARKER, J. W., H. WAKASA, and R. J. LUKES. 1965. The morphologic and cytochemical demonstration of lysosomes in lymphocytes incubated with phytohemagglutinin by electron microscopy. *Lab. Invest.* **14**:1736.
18. HIRSCHHORN, R., J. M. KAPLAN, A. F. GOLDBERG, K. HIRSCHHORN, and G. WEISSMAN. 1965. Acid phosphatase-rich granules in human lymphocytes induced by phytohemagglutinin. *Science.* **147**:55.
19. HIRSCHHORN, R., K. HIRSCHHORN, and G. WEISSMANN. 1967. Appearance of hydrolase rich granules in human lymphocytes induced by phytohemagglutinin and antigens. *Blood.* **30**: 84.