ULTRASTRUCTURAL ANALYSIS OF HEMATOPOIETIC COLONIES DERIVED FROM HUMAN PERIPHERAL BLOOD

A Newly Developed Method

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ABSTRACT

The ultrastructure of granulocyte colonies derived from normal human peripheral blood leukocytes cultured in semisolid media has been studied by a new method developed for this purpose. Fixation, dehydration, and embedding of the whole content of the Petri dish resulted in a block of Epon containing colonies made up of cells with the spatial orientation of those observed in living cultures. This permitted serial sectioning through entire colonies. Cell maturation in vitro appeared to parallel that of normal marrow. However, even the most mature cells retained cytoplasmic characteristics of more immature cells. This was particularly true for eosinophils which only rarely possessed granules with electron-dense crystalline “cores,” a feature typical for mature eosinophils. In addition to the normal-appearing hematopoietic cells found within colonies, very large round or spindle-shaped cells were present between colonies and firmly attached to the bottom of the culture dish. Although the histochemical and functional characterization of these cells awaits further study, it is suggested that they are related to histiocytes or macrophages.

The technique described here should prove valuable in studies of the development, differentiation, and interaction of many types of cells.

An ever increasing number of studies utilize the soft-agar tissue culture technique to elucidate various aspects of hematopoiesis. Though the potential value of in vitro colony formation for the analysis of mechanisms underlyng normal differentiation and maturation of hematopoietic cells was realized at the outset, the recent demonstration, that not only bone-marrow, but even peripheral blood of healthy subjects contain precursor cells which can give rise to granulocyte colonies in semisolid media (4, 5, 7) has added impetus to these investigations. Unfortunately, because of the physical properties of the culture media, morphological studies have lagged behind, and it is not yet known to what extent the cells grown in vitro resemble those that mature in vivo. This gap in knowledge warrants filling since, presently, the soft-agar tissue culture technique is beginning to be applied to the study of clinical conditions associated with hematologic aberrations. To date, with the exception of the report by Van Noord et al. (11) electron microscopy has been carried out only on cultures suspended in fixation and embedding media. This has resulted in mixing of cells derived from different colonies and loss of intercellular relationships within the same colony. Therefore,
seemed important to develop a method which would facilitate morphologic and histochemical analyses of intact colonies. The present communication reports results obtained by a method whereby the entire content of a Petri dish is fixed in situ. Embedding is carried out in a manner to yield a “block” containing intact colonies. These can be selected with a low-power lens and thin-sectioned for electron microscopy by standard methods. The preservation is such that morphologic criteria which pertain to freshly aspirated human marrow can be applied to the cultured cells. In addition, variants of cells not found in normal bone marrow, but commonly seen in the cultures, will be described.

MATERIALS AND METHODS
For most studies, cultures consisted of $10^6$ cells from theuffy coat of peripheral blood or “purified” lymphocytes (13) in 1 ml 0.3% agar (Difco-Bacto-agar, Difco Laboratories, Detroit, Mich.) dissolved in McCoy’s medium containing 15% fetal calf serum. This was placed on a feeder layer of buffy coat cells in 0.5% agar essentially as described by others (10). Cultures were fixed at various time intervals by addition of 0.6 ml, 3% phosphate- or cacodylate-buffered glutaraldehyde to the surface of the agar. The dishes were returned to the 37°C CO2 incubator for an additional 10–48 h. After fixation they were brought to room temperature and washed twice for 10 min each time with 4.0 ml of 0.1 M phosphate or cacodylate buffer, pH 7.4, containing 7% sucrose. The buffer was added rapidly along the side of the Petri dish by means of a Pasteur pipette. The force of the stream and the density of the sucrose caused the agar to separate from the bottom of the dish. After complete removal of the buffered sucrose (the agar disk being deflected with a spatula to permit a Pasteur pipette to withdraw all of the buffer from beneath it), cultures were postfixed in 2% osmium tetroxide for 1 h at 4°C. After four 5-min washes in 0.85% saline, they were transferred to aluminum foil dishes (Fig. 1) (Milk test-evaporating dish, diam = 57 mm, SGA Scientific, Inc., Bloomfield, N. J.). Transfer was accomplished during the fourth saline wash by placing the aluminum foil dish upside down over the Petri dish and quickly inverting both. A fifth saline wash with gentle agitation was important to achieve removal of the reduced osmium and to assure transparency of the final “block” (compare Figs. 2a and b). During transfer,

1 We are indebted to the laboratories of Dr. Bayard Clarkson and Dr. Robert A. Good at the Sloan-Kettering Institute, New York for helping us to set up the culture method. Dr. Yataganis and Dr. P. L’Esperance provided us with the initial methyl cellulose and soft-agar cultures respectively. Both media are equally suited for embedding by the technique described here.
the culture layer occasionally separated from the feeder layer whereupon the latter was either embedded separately or discarded.

For all subsequent steps, the foil dishes containing the free-floating agar disks were kept on a rotator (Scientific Industries, Inc., Springfield, Mass.) (Fig. 1) to enhance circulation of media above as well as below the agar disk. Dehydration was accomplished by two 15-min changes of 70 and 95% ethanol followed by 10 and 15 min 100% ethanol. At each alcohol change, the agar was again dislodged with a spatula from the aluminum foil dish to permit circulation below the cultures. In propylene oxide, the agar became buoyant and care had to be taken that the surface did not dry upon contact with the air. The same precaution was taken during the two succeeding washes of 30 min each in 2:1 and 1:2 propylene oxide to Epon. The solutions were added in excess quantity to allow for evaporation of the propylene oxide. After two 1-h changes in 100% Epon 50% A:50%B (6), fresh Epon was poured into the dishes (and the specimens were polymerized at 37°C (~16 h), 45°C (~8 h), and 60°C (~16 h). After polymerization, the aluminum foil could be peeled from the Epon block with ease. The embedded colonies were scanned with either a dissecting or a low-power conventional microscope and were scored and counted (Fig. 2 a). Selected colonies were cut from the block with a jeweler’s saw (Fig. 2 b) and mounted for thick sectioning by routine methods. For the studies reported here, ribbons of sections obtained with an LKB Ultrotome (LKB Instruments, Inc., Rockville, Md.) were placed on slit Parlodion-covered grids so that serial sections through an entire colony could be studied. Contrast was enhanced with uranyl acetate and lead citrate, and the sections were examined with an Elmiskop I electron microscope.

RESULTS

Although precise quantitation of the number of cells within each colony was not possible, the total number of colonies and their apparent size was identical before and after the embedding procedure. Figs. 3 a and b illustrate two colonies photographed through the intact Epon block before any sectioning. These appear identical to published illustrations of colonies in fresh soft-agar cultures viewed with an inverted microscope (8, 9). Even the character of the colony, whether it was “loose” or “tight” was left undisturbed. In addition, the degree of osmiophilia permitted some prediction as to the type of cells which would be found on thin sectioning of the colony; eosinophils were most osmiophilic, and colonies consisting of agranular primitive cells had the least contrast. Phase photomicrographs of thick (~1 μm) sections at different levels of two large, 21-day old colonies are shown in Figs. 4 a and b. As in other ultrastructural studies, areas of interest were selected for thin sectioning. Fig. 4 c shows a randomly selected low-power electron micrograph of such a colony. The relationship of the cells to each other is similar to that seen in marrow

**FIGURE 1**  Rotator, showing two aluminum foil dishes containing agar disks suspended in embedding media.

**FIGURE 2 a**  Agar disc (black) within Epon block (transparent). Circles represent scored colonies. Two colonies have been removed (open squares).

**FIGURE 2 b**  Methyl cellulose disk in Epon, polymerized within the Petri dish. The total area of the block corresponds to the area of the disk. More than 15 colonies have been removed.
FIGURE 4 a  Thick section through the periphery of a 21-day old colony which appears to consist of eosinophilic myelocytes and metamyelocytes as judged by the large osmiophilic granules. × 1,200.

FIGURE 4 b  Detail of a large 21-day old colony showing neutrophilic myelocytes and metamyelocytes. Many nuclei are multilobulated (arrow). × 1,200.

FIGURE 4 c  Low-power electron micrograph showing a representative field of a colony which would appear on thick section as the one illustrated in Fig. 4 a. The large osmiophilic granules and gray inclusions (arrows) are characteristic for eosinophilic myelocytes and metamyelocytes. × 2,800.
biopsies. After mitosis the cells appear to move apart leaving no visible intercellular connections or bridges. Systematic study of colonies fixed at various time intervals, described in greater detail elsewhere (14), showed that on day 5 in vitro, most colonies consisted of primitive cells (Fig. 5). These had an irregular contour and a small nucleo-cytoplasmic ratio; the nucleus showed little heterochromatin and usually had a large nucleolus; the cytoplasm was replete with polysomes and numerous profiles of rough ER; granules were inconspicuous or absent. It is probably correct to refer to this cell as a myeloblast, although myeloblasts obtained from normal marrow have a more regular, round contour. By day 15 in vitro, few colonies consisted of pure myeloblasts, although occasional primitive precursors were still seen in colonies which had developed granulated cells. A representative field showing eosinophilic myelocytes in a 15-day old culture is illustrated in Fig. 6. All cells appeared to be approximately at the same stage of maturation. In general, the cells had a large Golgi zone which showed budding of granules at the convex surface, suggesting that the cell had entered the myelocyte stage of development. In 15-day old cultures none of the granules showed crystalloids characteristic of granules seen in mature human eosinophils; there were numerous polysomes and a moderate amount of rough ER. In addition, the majority of the cells had large gray inclusions which occasionally measured more than 1 \( \mu \text{m} \) in diameter and were either uniformly electron dense or somewhat denser at the periphery (Figs. 6 and 7). Most of the inclusions were membrane-bounded, but in many instances a limiting membrane could not be resolved. The presence of more than one of these dense bodies within one membrane-bounded space suggested that coalescence of the structures may have taken place (inset in Fig. 7). Although the inclusions resembled those seen in the immature cells of patients with eosinophilia (12), it should be noted that the nucleus of the cells had the chromatin distribution of mature granulocytes and that it often lacked a nucleolus. Thus, there appeared to be a discrepancy between nuclear and cytoplasmic maturation. Cultures fixed between days 15-21 showed predominantly colonies consisting of either neutrophilic or eosinophilic metamyelocytes (Figs. 8 and 9) with a few more primitive cells in their midst. Mixed colonies containing both eosinophils and neutrophils were not encountered. The nuclei of the most mature cells were multilobed and lacked nucleoli. The cytoplasm revealed only a few slender mitochondria, and at times, abundant glycogen. The neutrophilic granules resembled those of mature peripheral blood polymorphonuclear leukocytes in size and shape (Fig. 8). On the other hand, eosinophils never achieved full maturation. Although their nuclei had a mature chromatin pattern and some of the granules had differentiated to show a central crystallloid (Fig. 9) the majority of the granules had elliptical or angular shapes, but remained uniformly electron dense. The cells also retained more rough ER and polysomes than mature eosinophils in circulating blood.

In addition to the cells which could be identified as belonging to the well accepted hematopoietic series, very large cells were found which are not seen in freshly obtained normal human blood or marrow (Fig. 10). The majority of these cells were spherical, measured up to 20 \( \mu \text{m} \) in diameter, and had an eccentric nucleus. Their most striking feature was an abundance of membrane-bounded inclusions, uniform in electron density and size. Originally such cells were only occasionally seen in areas between or at the periphery of colonies where they occurred singly or in small numbers apparently without undergoing division. At a later time, it was discovered that similar cells were attached to the Petri dish beneath the feeder layer. The attached cells were not removed with the agar disk and required separate embedding if their ultrastructure was to be studied. Though many of the adherent cells were spindle shaped and measured up to 600 \( \mu \text{m} \) in length, their nuclei and cytoplasmic inclusions were identical in morphology to those found in the intercolonial areas of the culture layer. When the attached cells were stained with routine blood stains, such as Wright's or Giemsa, and viewed by light microscopy, some of the inclusions appeared as basophilic granules, but the bulk of the cytoplasm assumed a pink hue.

DISCUSSION

The method described here, developed specifically for the ultrastructural study of colonies grown in soft-agar cultures, has become routine in our laboratory. To date, more than 100 colonies from 25 specimens have been subjected to electron microscopy with satisfactory results. Preliminary experiments have also shown that the method lends itself to histochemical and autoradiographic analyses of the cultured cells. On the basis of these observations, it seems reasonable to conclude that granulocyte colonies grown from precursors circu-
**FIGURE 5** Primitive cell in a 5-day old colony believed to represent a myeloblast. Granulation is sparse and nonspecific (Gr). Polysomes and profiles of rough ER abound. The nucleus displays a large nucleolus (Nu). × 12,000.

**FIGURE 6** Detail of a relatively mature eosinophil colony. Note that the cell on the right has a well differentiated multilobed nucleus, whereas the cell on the left still retains a large nucleous (Nu). There are many large gray inclusions (Gl), uniformly osmiophilic granules, as well as granules with crystalline cores (arrows). The Golgi zone has remained large. × 5,000.
FIGURE 7 Promyelocyte, showing the large gray inclusions (GI) to better advantage. The chromatin distribution and the presence of a nucleolus (Nu) indicate immaturity of the nucleus. The inclusions appear to be more electron dense at the periphery, and their membrane is often difficult to resolve. The inset shows an example of two inclusions within one membrane-bounded space. × 11,000.

FIGURE 8 Mature polymorphonuclear leukocyte, showing multilobed nucleus (N). × 7,000.

FIGURE 9 Detail of an eosinophil, showing several granules with a crystalline core (arrows). × 40,000.
FIGURE 10 Example of a macrophage or histiocyte which can be seen in between colonies or attached to the Petri dishes. Note the relatively small undifferentiated nucleus with a large nucleolus (Nu). The cytoplasm is replete with gray inclusions measuring ± 750 nm in diameter. The large Golgi zone (G) is surrounded by membrane-bounded spaces which contain innumerable thin processes (P). Similar processes cover the surface of the cell. Mitochondria (M), glycogen particles, and small electron-dense granules are interspersed between the gray inclusions. × 3,000.

In vitro maturation in normal human blood differentiate and mature essentially as those in normal marrow. However, it seemed that under conditions prevailing in the cultures there is some discrepancy in nuclear versus cytoplasmic maturation. This is particularly noteworthy in the case of eosinophils. While the nucleus of these cells had often become multilobed and devoid of a nucleolus, many of the granules lacked the central crystalloid, a feature typical for mature human eosinophils. Moreover, the Golgi zone remained large and there was an excessive amount of rough ER for a cell at the metamyelocyte stage of development. In addition, the eosinophils contained numerous large inclusions (Figs. 4 c, 6, 7), considered to be immature granules by some observers (1). Such inclusions are rare in mature eosinophils of healthy subjects but are frequently seen in patients with severe eosinophilia (12). In addition, there were inclusions which exceeded the size of “immature” granules seen in normal eosinophil precursors (Fig. 7), though their ultrastructure was otherwise similar. On light microscopy of colonies presumed to consist of eosinophils, the large inclusions were basophilic, whereas the normally sized granules had the expected affinity for eosin. Thus, in
cultured cells as well as in freshly obtained bone marrow from patients with eosinophilia, the origin and biochemical nature of such inclusions warrant further study.

Another question to be resolved is the definitive identification of monocytes in these cultures. It is not always easy to distinguish neutrophilic myelocytes from immature monocytes solely by morphologic means. The same difficulty has been encountered by other workers who have studied the cells by light microscopy (3, 4, 8). In fact, perusal of the literature revealed that many workers have referred to these cells as "mononuclear" cells rather than as monocytes (4), a distinction which may be prudent at the present state of our knowledge.

On the other hand, the type of cell illustrated in Fig. 10 probably should be classified as a histiocyte or macrophage on the basis of past experience with this cell type (for a review, see reference 2). This leaves the nature of the homogeneously gray inclusions which fill the cytoplasm of such cells in question. Though there is convincing evidence that mononuclear cells grown in agar are able to ingest this medium (8), it cannot be assumed prima facie that the inclusions contain phagocytosed agar. The fact that in preliminary studies we have not been able to demonstrate the presence of acid phosphatase or peroxidase in these structures, may also speak against their lysosomal nature. Therefore, we favor the hypothesis that the inclusions are of biosynthetic origin, a possibility which is under active investigation at the present time.

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REFERENCES

1. BAINTON, D. F., and M. G. FARQUHAR. 1970. Segregation and packaging of granule enzymes in eosinophilic leukocytes. J. Cell Biol. 45:54–73.
2. BESIS, M. 1973. Living Blood Cells and Their Ultrastructure. Springer-Verlag, New York Inc. Chap. 6.
3. BRADLEY, T. R., and M. A. SUMNER. 1968. Stimulation of mouse bone marrow colony growth in vitro by conditioned medium. Aust. J. Exp. Biol. Med. Sci. 46:607–618.
4. CHERVENICK, P. A., and D. R. BOGGS. 1971. In vitro growth of granulocytic and mononuclear cell colonies from blood of normal individuals. Blood J. Hematol. 27:131–135.
5. KURNICK, J. E., and W. A. ROBINSON. 1971. Colony growth of human peripheral white blood cells in vitro. Blood J. Hematol. 27:136–141.
6. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409.
7. MCCREDIE, K. B., E. M. HERSH, and E. J. FREIREICH. 1971. Cells capable of colony formation in the peripheral blood of man. Science (Wash. D. C.). 171:293–294.
8. METCALF, D., T. R. BRADLEY, and W. ROBINSON. 1967. Analysis of colonies developing in vitro from mouse bone marrow cells stimulated by kidney feeder layers on leukemic serum. J. Cell. Physiol. 69:93–107.
9. METCALF, D., and M. A. S. MOORE. 1971. Haemopoietic Cells. American Elsevier Publishing Co., Inc., New York. 124.
10. PIKE, B. L., and W. A. ROBINSON. 1970. Human bone marrow colony growth in agar gel. J. Cell. Physiol. 76:77–84.
11. VAN NOORD, M. J., N. BLANJAR, and A. NAKEFF. 1973. The processing of mammalian haemopoietic cells in thin layer agar cultures for electron microscopy. Stain Technol. 48:239–246.
12. ZUCKER-FRANKLIN, D. 1974. Eosinophil function and disorders. Adv. Intern. Med. 19:1–25.
13. ZUCKER-FRANKLIN, D. 1974. The percentage of monocytes among "mononuclear" cell fractions obtained from normal human blood. J. Immunol. 112:234–240.
14. ZUCKER-FRANKLIN, D., G. GRUSKY, and P. L’ESPERANCE. 1974. Granulocyte colonies derived from lymphocyte fraction of normal human peripheral blood. Proc. Natl. Acad. Sci. U.S.A. 71:2711–2714.