CLONAL GROWTH OF HAMSTER FREE ALVEOLAR CELLS IN SOFT AGAR*

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Among mammalian cells, the ability to proliferate and to form colonies in semisolid medium is generally limited to two kinds of cells, malignant (transformed) cells and cells that belong to the hemopoietic system. The latter include the progenitor cells of erythroid series (1) and the bone marrow hemopoietic colony-forming cells in culture that are considered to be the progenitors of the myeloid series (2, 3).

A class of mouse peritoneal exudate cells capable of clonal growth in soft agar medium has recently been described (4–6). Their requirements for optimal growth in vitro resemble those of the bone marrow hemopoietic colony-forming cells. However, peritoneal colony-forming cells have a long lag period (7–14 days) before beginning proliferation and produce only mononuclear phagocytes (4). Since peritoneal colony-forming cells are not present in the peritoneal cavity of mice unless the mice are first given thioglycollate medium or other stimulants locally, they are either "activated" resident peritoneal cells or cells attracted to the site from other parts of the body after stimulation (5).

The free alveolar cell population consists mainly of macrophages which are believed to be derived from multipotent hemopoietic stem cells present in bone marrow (7–10). Even under normal laboratory conditions, the alveolar space is constantly exposed to air that contains various particulate materials, microorganisms and other substances that may act as stimulants. We therefore speculated that even without any additional stimulation, free alveolar cells might contain a class of colony-forming cells similar to that seen in stimulated peritoneal cavities. To test this possibility, we plated hamster free alveolar cells in soft agar containing a source of colony-stimulating factor. Hamsters were used since a reasonable number of free alveolar cells could be obtained from a single animal. Bone marrow cells from the femurs of these hamsters were also grown under identical culture conditions for comparison. A limited study was also carried out with free alveolar cells obtained from mice and rats.

Materials and Methods

Animals. Male syrian golden hamsters weighing 100–130 g were obtained from Engels Laboratory Animal Inc., Farmersburg, Ind. Rats (Wistar) weighing 150 g were obtained from National

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Laboratory Animal Co., Creve Coeur, Mo., and 8- to 12-wk old C3H/He mice weighing 20-25 g from the Jewish Hospital, St. Louis, Mo.

Free Alveolar Cells. The hamsters were first anesthetized with Nembutal and then killed by exsanguination. To obtain free alveolar cells the method of Brain and Frank was used (11). A sterile polyethylene tube was inserted through the trachea and the lungs were washed internally four times with 5 ml saline (0.15 M NaCl solution). We recovered 1–5 × 10^6 cells/hamster. They consisted of 90% macrophages and 5–8% polymorphonuclear cells with a few lymphocytes and other types of cells (ciliated cells, etc.).

Bone Marrow Cells. The femurs were cleaned, their ends cut off, and, using an 18 gauge needle attached to a 3 ml syringe, the marrow cavity was repeatedly aspirated with alpha medium (12).

Assay for Colony-Forming Cells. The techniques and medium used for the soft agar culture were similar to those for mouse bone marrow and peritoneal exudate cells (5) except that a different conditioned medium was used. Unless otherwise stated, medium conditioned by baby hamster kidney (BHK)[1] cells was used as the standard source of colony-stimulating factor. The agar culture medium consisted of 0.3% wt/vol Noble agar (Difco Laboratories, Detroit, Mich.) in alpha medium (Flow Laboratories, Inc., Rockville, Md.), 10% (vol/vol) fetal calf serum, 5% (vol/vol) horse serum (Flow Laboratories, Inc.), and 10% (vol/vol) BHK-cell-conditioned medium. Alveolar or marrow cells were cultured in 1 ml agar medium in 35 mm tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Cultures were kept at 37°C in a humidified incubator in 10% CO_2 in air. Counting of colonies, defined here as cell aggregates containing more than 50 cells, was performed on unstained cultures with an inverted microscope. Alveolar cells were also grown in liquid medium to see whether they could form colonies on the surface of 35 mm plastic tissue culture dishes or glass cover slips placed in tissue culture dishes. The liquid medium contained all the ingredients present in agar medium except that agar was omitted.

Preparation of Conditioned Medium. BHK cells were seeded in 75 cm^2 Falcon culture flasks at 1 × 10^6 cells in 10 ml alpha medium containing 10% fetal calf serum. 7 days later, the medium was removed and centrifuged at 400 *g* for 10 min and then filtered through a 0.22 μm filter and stored frozen. Conditioned media from other established cell lines were obtained by the same procedure. To prepare a hamster spleen or kidney cell-conditioned medium, spleens or kidneys were minced with scissors and then the minces were pressed through a fine stainless steel screen. Spleen or kidney cells were plated at 2 × 10^7 cells in 10 ml medium. The medium was harvested 7 days later and processed as above. Conditioned media from the fibroblasts of 15- to 19-day old rat and mouse embryos were prepared as described previously (5). BHK cells were obtained from Dr. B. Weiss, Washington University, St. Louis, Mo., and human embryo kidney cells from Microbiological Associates, Inc., Bethesda, Md.,

Studies of Phagocytosis. Colonies from the culture dishes were removed with a Pasteur pipette under a dissecting microscope and suspended in alpha medium containing 10% fetal calf serum. A suspension of single cells was prepared by passing the colonies several times through an 18-gauge needle attached to a syringe. Cells were allowed to attach to the surface of tissue culture dishes overnight at 37°C in an incubator. Nonadherent cells and agar were then washed off with warm medium. Heat-killed yeast suspension along with guinea pig complement was added to the dishes and the incubation continued for 30 min. Free yeast cells were washed off with alpha medium.

Test for the Presence of IgG Receptor. Antibody-coated sheep red blood cells (SRBC) were added to the dishes containing adherent cells obtained from alveolar cell cultures. After 30 min of further incubation, SRBC not bound or ingested by cells were washed off from the dishes with warm medium. Antisera to SRBC were prepared in AKR mice 10 days after a single intraperitoneal injection of washed SRBC.

Morphology. For rapid classification, colonies were stained with 0.6% orcein in 60% acetic acid and cells examined at times 400 magnification. For high resolution light and electron microscopy, cultures were prepared by a modification of the method of Zucher-Franklin (13). The Petri dishes were flooded with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, and returned to the incubator overnight. After primary fixation, the glutaraldehyde was replaced with two 10 min

[1] *Abbreviation used in this paper:* BHK, baby hamster kidney.

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changes of buffer, followed by fixation in 1% OsO_4 in cacodylate buffer for 90 min. The agar was dehydrated with successive changes of increasing concentrations of acetone in the Petri dish to 80% acetone then transferred intact to a polypropylene container, where dehydration was completed and the agar infiltrated with epoxy resin (14) overnight. Individual clones in agar were dissected from the infiltrated specimen, transferred to Beam (Ernest F. Fullam, Inc., Schenectady, N. Y.) capsules containing fresh epoxy, and the blocks polymerized for 12 h at 37°C and for 48 h at 60°C. 1 μm sections were stained for light microscopy with toluidine blue. Thin sections were stained for electron microscopy with uranyl acetate and lead citrate.

Results

Development of Colonies in Bone Marrow Cell Culture. When 5×10^4 nucleated bone marrow cells were plated per dish, the replication of cells began within 24 h. By day 7, most of the colonies contained more than 500 cells. Degeneration of cells in colonies usually occurred after 9 or 10 days of incubation. The incidence of colony-forming cells in bone marrow cultures counted on day 7 was 25 ± 15 (SD)/5×10^4 cells. As with mouse bone marrow cultures three types of colonies, granulocytic, macrophage, and mixed type (15), were observed in hamster bone marrow cell cultures. There were some bone marrow cells that did not begin multiplication until 3–4 days after initiation of the cultures. These cells rarely formed aggregates of more than 50 cells, however, and the cells in these aggregates degenerated at the same time as those in large colonies.

Development of Colonies in Alveolar Cell Culture. In sharp contrast to bone marrow cells in culture, the first division of all cells in alveolar cell cultures did not take place until after 4–8 days of incubation. Colonies began to appear after 10–12 days and increased in number with time up to 18–21 days. As a standard procedure therefore, colonies were counted on day 21. The length of the initial lag period, the rate of cell proliferation, and the size of colony were dependent on the number of cells originally plated in the dish. Alveolar cells which were plated in higher concentrations had a tendency to divide earlier than those plated in lower concentrations. The morphology of alveolar cell colonies generally resembled that of bone marrow macrophage colonies (Fig. 1). The fraction of free alveolar cells which was capable of forming colonies in soft agar was fairly constant among different individual hamsters that were housed in two separate animal facilities and tested over a period of 3 mo. It ranged from 24 to 183 colonies/10^3 cells with an average value of 81. Enumeration of alveolar colony-forming cells indicated a linear relationship between the number of alveolar cells plated and the number of colonies (Fig. 2), suggesting a single cell origin of the alveolar colonies.

Since mouse peritoneal colony-forming cells can form colonies in liquid culture as well as in agar medium (16), between 10^3 to 10^5 hamster alveolar cells in 1 ml of complete medium without agar were seeded in 35 mm tissue culture dishes with or without cover glasses to see whether they also form colonies on a glass or plastic surface. Unlike mouse peritoneal cells, the majority of hamster cells did not adhere tightly to the bottom of the dishes and they rarely formed discrete colonies. However, there was a definite increase in the number of cells in these plates with time.

Identification of Cells Present in Alveolar Cells Colonies. Various times after incubation, cell aggregates from alveolar and bone marrow cultures were

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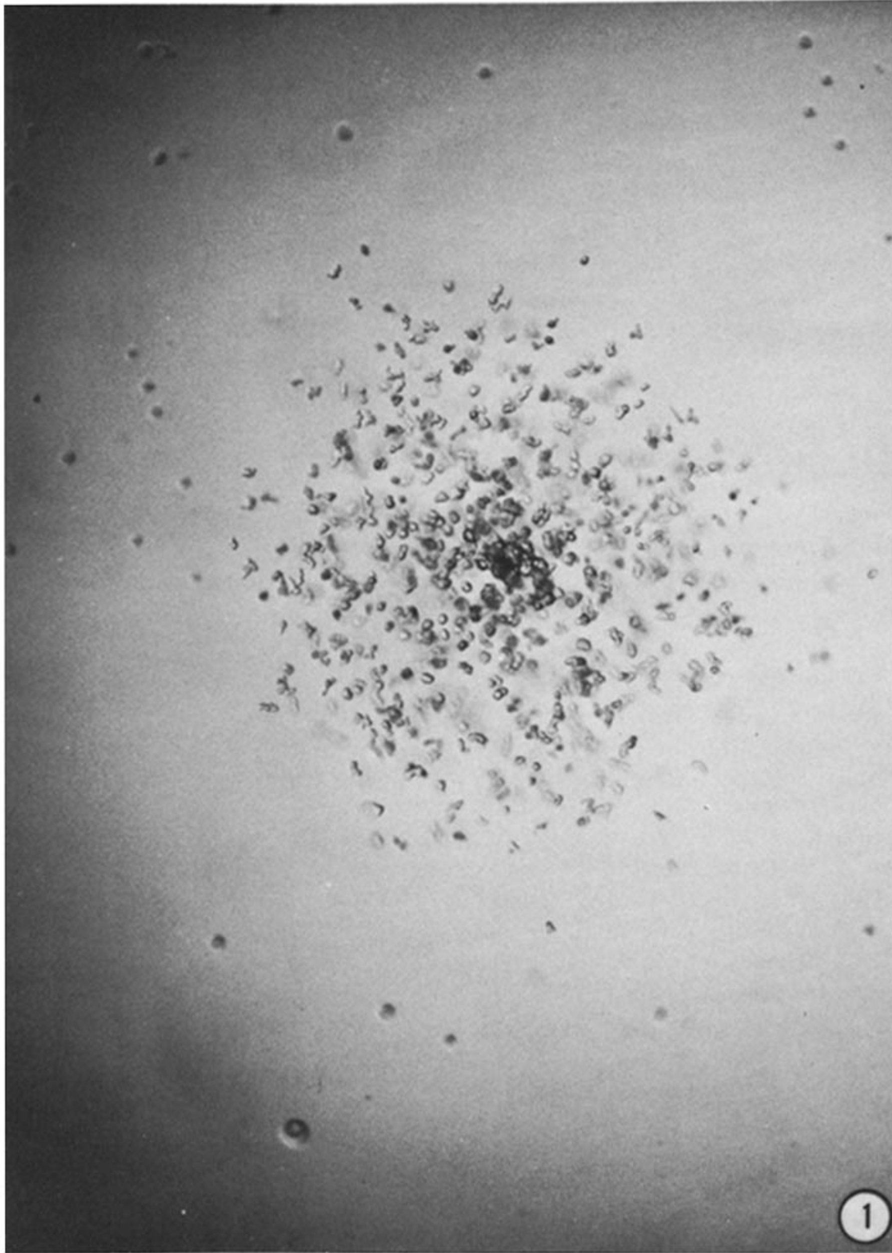

FIG. 1. An alveolar cell colony in soft agar after incubation for 21 days. × 40.

picked, stained, and examined. Both mononuclear cells resembling macrophages and polymorphonuclear cells were present in bone marrow cell cultures during the first 8 days of culture. In contrast, no polymorphonuclear cells were ever observed in alveolar cell cultures even between day 5 to day 10 when cell aggregates were still quite small.

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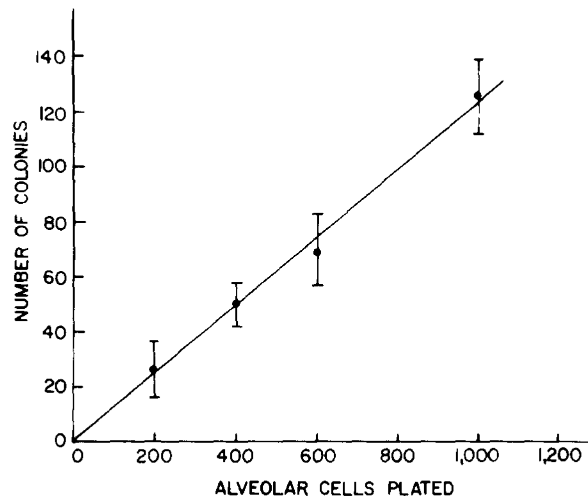

FIG. 2. Number of colonies formed as a function of alveolar cells plated. Error bars are 1 SD.

In 1-μm thick sections the cells comprising the alveolar cell colonies ranged up to 25 μm long. Their outline was markedly irregular with many pseudopodia. Their nuclei were eccentrically placed and generally oval or indented, often with an irregular or crenated outline. Their cytoplasm was abundant and filled with vacuoles (Fig. 3 *a*). Electron microscopy (Fig. 3 *b*) showed the presence of a distinct cytocenter adjacent to a nuclear indentation and containing the centriole and a large Golgi zone. The endoplasm surrounding the cytocenter contained short lamellae of rough endoplasmic reticulum, numerous mitochondria, and many membrane-bound vacuoles. The vacuoles contained clumps of dense granular material as well as fine branching threads of a substance which resembled the surrounding agar, suggesting an endocytic origin of the vacuoles. Numerous coated vesicles at the periphery of the cells also suggested active endocytosis. Only very rare, dense membrane-bound granules were present. Fibrils of two types were seen in the cytoplasm. In the central region, near the Golgi zone, there were numerous 70–90 Å filaments, which tended to be in parallel arrays. Ectoplasmic projections devoid of organelles usually contained a feltwork of very fine filaments.

Cells obtained from 15- to 25-day old alveolar cell colonies were allowed to attach to tissue culture plate surfaces overnight and those which adhered to the surface were tested for the ability to phagocytize yeast particles and for the presence of IgG receptors. About 30% of cells adhered to the surface of the dishes and 40–60% of these adherent cells took up yeast particles. When antibody-coated SRBC were added to the dishes about 80% of these adherent cells had three or more SRBC on their surface.

Requirement for BHK-Cell-Conditioned Medium. Both mouse bone marrow hemopoietic colony-forming cells in culture and peritoneal exudate colony-forming cells are known to require a colony-stimulating factor for initiating and sustaining growth in agar (5). When BHK-cell-conditioned medium was omitted from the agar medium, no colony formation was observed in either hamster

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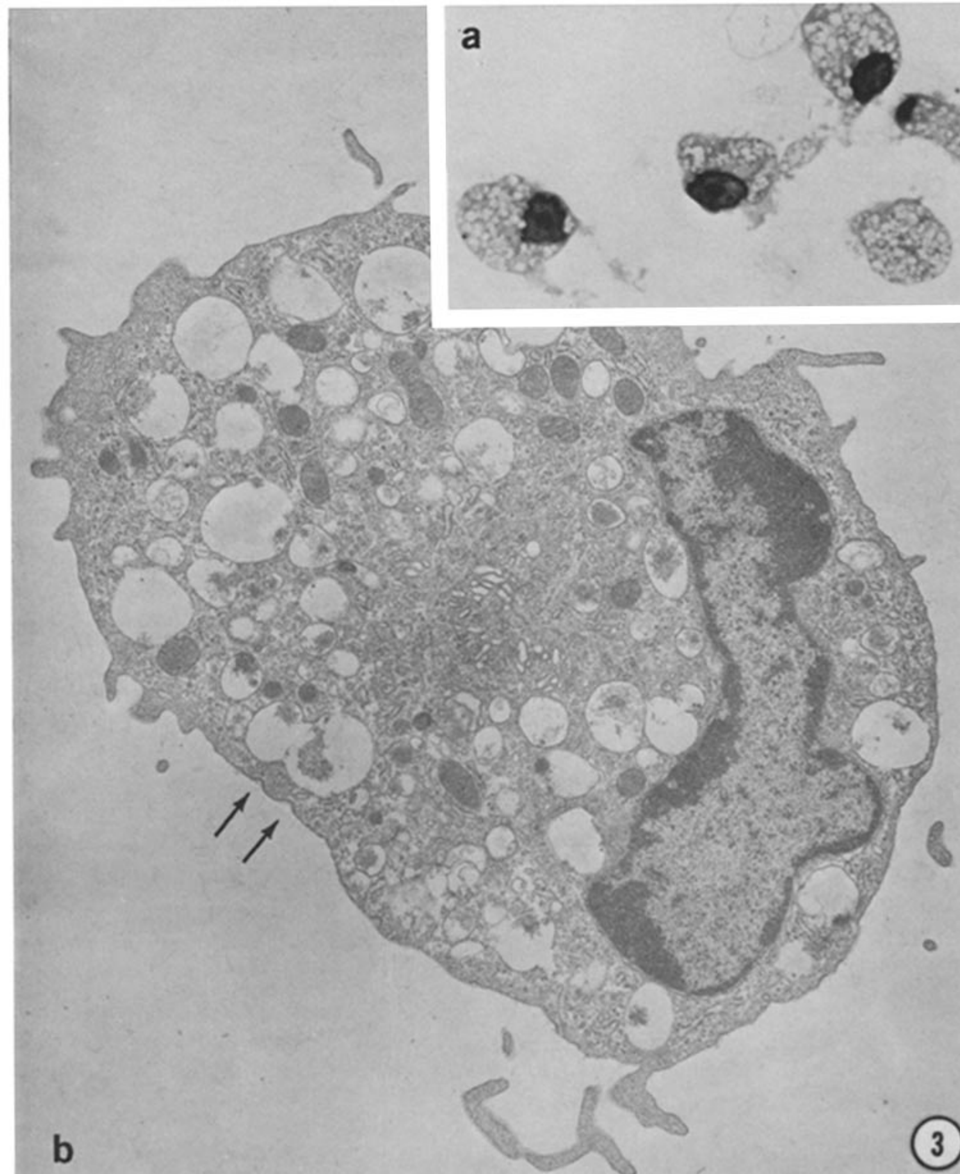

FIG. 3. Cells from 21-day old hamster alveolar cell colony. (*a*) Light micrograph shows ameboid cells with eccentric nuclei and vacuolated cytoplasm. Toluidine blue, × 1,000. (*b*) Electron micrograph showing a cytocenter containing a large Golgi zone with numerous vacuoles and mitochondria arranged peripherally. There are coated vesicles at the cell membrane (*arrows*). × 8,000.

alveolar or bone marrow cultures. However, clusters of 10–30 cells were often observed in dishes containing more than 5×10^4 bone marrow cells and 2–10 cell clusters in alveolar cell culture containing more than 10^4 cells. These findings suggested that both alveolar and bone marrow cell populations contain cells that

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produce a limited amount of colony-stimulating factor.

To test whether alveolar cells require BHK-cell-conditioned medium for continuous replication, cells from 18-day old alveolar cell cultures were obtained and subcultured in fresh agar medium with or without conditioned medium. Cells plated in fresh agar medium supplemented with conditioned medium continued to proliferate whereas cells in agar medium devoid of conditioned medium failed to replicate, indicating BHK-cell-conditioned medium is also required for sustaining the growth of alveolar cells.

Effect of Various Conditioned Media. Media conditioned by cells other than BHK cells were tested for their ability to stimulate the growth of hamster alveolar and bone marrow cells. The results from several separate experiments were summarized and are shown in Table I. Various conditioned media not only gave a different incidence of colony formation but also influenced the morphology and size of colonies formed. For instance, medium conditioned by hamster spleen cells was as good as medium conditioned by BHK cells when the incidence of colony formation was compared. However, the alveolar cell colonies formed in the presence of the former contained fewer cells and the cells were more tightly packed than those stimulated with BHK-cell-conditioned medium. Some media conditioned by cells from other species also stimulate the growth of both hamster alveolar and bone marrow cells.

Colony Formation by Alveolar Cells from Mice and Rats. Free alveolar cells from mice and rats were obtained by the same technique used to obtain hamster cells. Cells from bronchial washing of four to five mice were pooled and plated in both agar and liquid culture. L-cell-conditioned medium was used as the source of colony-stimulating factor. Like mouse peritoneal exudate colony-forming cells, mouse alveolar cells formed colonies equally well in both agar and liquid

TABLE I

Comparison of Different Conditioned Media for their Colony-Stimulating Activity on Hamster Cells

Cells used to condition medium	Colony-stimulating activity	
	For alveolar cells	For bone marrow cells
None	—	—
Hamster		
BHK cells	++++	++++
Spleen cells	++++	++++
Kidney cells	++	++
V79 (lung fibroblasts)	++	++
Rat		
Embryo fibroblasts	+	+
Mouse		
L cells	+++	+++
Embryo fibroblasts (C3H/He)	+	+
Human		
Embryo kidney cells	–	–

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cultures. The growth kinetics of alveolar cells and the gross morphology of colonies formed were not distinguishable from those of peritoneal cells reported previously (5, 16). They had an initial lag period of 7–14 days in agar and 4–8 days in liquid culture. Mouse alveolar cell populations contained, on the average, 4.5% (2–10%) cells that were capable of clonal growth in agar and liquid cultures.

Alveolar cells obtained from healthy, unstimulated rats also proliferated and formed colonies in agar supplemented with rat embryo fibroblast- or L-cell-conditioned medium. The formation of colonies in liquid culture was not as reliable as that in agar. The fraction of agar colony-forming cells in rat bronchial washings was about 1%.

Discussion

The present study has shown that cells obtained by bronchial lavage from normal healthy hamsters contain cells that are capable of clonal growth in soft agar. The results also show that medium conditioned by BHK cells or other cells is required for the initiation and continuation of alveolar cell proliferation.

The morphologic features of the cells in colonies are those of macrophages. The eccentric nucleus, abundant cytoplasm with pseudopodial projections, the large perinuclear Golgi zone, and the many presumably endocytic vacuoles are all characteristic of macrophages. In vivo alveolar macrophages have large lysosomal granules. These granules contain the residue of inhaled and phagocytized materials to which the cultured cells have not been exposed. Since such phagocytized material as may have been present in the starting cell sample, will have been diluted by subsequent cell divisions, the absence from cultured cells of the dense granules characteristic of alveolar macrophages is not unexpected. Filaments have been described in macrophages by a number of investigators (17–19) which were similar to those seen in the cultured cells. In addition to their morphology, three criteria identified the cells in colonies as mononuclear phagocytes: adherence to glass or plastic, avid phagocytosis, and the presence of gamma globulin receptors.

The ability to form colonies in agar is not limited to alveolar cells obtained from hamsters alone. Cells from both mice and rats formed colonies under appropriate culture conditions, indicating the generality of the phenomenon. These studies also indicate that alveolar colony-forming cells resemble peritoneal colony-forming cells (4, 5) in having a long lag period before the first replication and in producing only mononuclear phagocytes. It would be extremely interesting to find out whether they belong to the same class of cells.

The bone marrow colony-forming cell in culture morphologically resembles a transitional lymphocyte (20). Although alveolar colony-foming cells have not been morphologically identified, it appears that they are a subpopulation of alveolar "macrophages." This follows from the fact that 8% or more of free alveolar cells are colony-forming cells. Except for mature granulocytes, only macrophages were present in the starting cell population in sufficient numbers to account for the colonies. Studies are under way to isolate and characterize this class of colony-forming cells.

Alveolar macrophages from experimental animals and men have been shown

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to divide in vivo (21) and in vitro (22–25). However, to our knowledge, this is the first attempt to grow alveolar cells in soft agar and to quantitate the fraction of cells that have the capacity of extensive proliferaiton. The technique, however, only provides a minimum figure for proliferative cells, since there may be cells that require different conditions for multiplication.

Summary

Free alveolar cells obtained from healthy unstimulated hamsters were tested for their ability to form colonies in soft agar. Every bronchial washing so far tested contained colon-forming cells. The average plating efficiency was 8.1% (2.4–18.3%). Alveolar colony-forming cells were characterized by having a long initial lag period (4–8 days) and only mononuclear phagocytes were found in the colony. Medium conditioned by baby hamster kidney cells or other cells was required for the initiation and maintenance of their growth. Alveolar cells from normal mice and rats also formed colonies under appropriate culture conditions.

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References

1. Stephenson, J. R., A. A. Axelrad, D. L. McLead, and M. M. Shreeve. 1971. Induction of colonies of hemoglobin-synthesizing cells by erythropoietin *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1542.
2. Bradley, T. R., and D. Metcalf. 1966. The growth of mouse bone marrow cells *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* **44**:287.
3. Pluznik, D. H., and L. Sachs. 1965. The cloning of normal "mast" cells in tissue culture. *J. Cell. Comp. Physiol.* **66**:319.
4. Lin, H., and C. C. Stewart. 1973. Colony formation by mouse peritoneal exudate cells *in vitro*. *Nat. New Biol.* **243**:176.
5. Lin, H., and C. C. Stewart. 1974. Peritoneal exudate cells. I. Growth requirement of cells capable of forming colonies in soft agar. *J. Cell. Physiol.* **83**:369.
6. Lin, H. 1974. Peritoneal exudate cells. II. Kinetics of appearance of colony-forming cells. *J. Cell. Physiol.* **84**:159.
7. Godleski, J. J., and J. D. Brain. 1972. The origin of alveolar macrophages in mouse radiation chimeras. *J. Exp. Med.* **136**:630.
8. Bowden, D. H., I. Y. R. Adamson, W. G. Grantham, and J. P. Wayatt. 1969. Origin of the lung macrophage. *Arch. Pathol.* **88**:540.
9. Brunstetter, M., J. A. Hardie, R. Schiff, J. P. Lewis, and C. E. Cross. 1971. The origin of pulmonary alveolar macrophages. *Arch. Intern. Med.* **127**:1064.
10. Virolainen, M. 1968. Hematopoietic origin of macrophages as studied by chromosome markers in mice. *J. Exp. Med.* **127**:943.
11. Brain, J. D., and R. Frank. 1973. Alveolar macrophage adhesion: wash electrolyte composition and free cell yield. *J. Appl. Physiol.* **34**:75.
12. Stanners, C. P., G. L. Eliceiri, and H. Green. 1971. Two types of ribosome in mouse-hamster hybrid cells. *Nat. New Biol.* **230**:52.
13. Zucker-Franklin, D., and G. Grusky. 1974. Ultrastructural analysis of hematopoietic colonies derived from human peripheral blood. *J. Cell Biol.* **63**:855.
14. Lockwood, W. R. 1964. A reliable and easily sectioned epoxy embedding medium. *Anat. Rec.* **150**:129.
15. Ichikawa, Y., D. H. Pluznik, and L. Sachs. 1966. *In vitro* control of the development of macrophage and granulocyte colonies. *Proc. Natl. Acad. Sci. U. S. A.* **56**:488.

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16. Stewart, C. C., H. Lin, and C. Adles. 1975. Proliferation and colony forming ability of peritoneal exudate cells in liquid culture. *J. Exp. Med.* **141**:1114.
17. De Petris, S., G. Karlsbad, and B. Pernis. 1962. Filamentous structures in the cytoplasm of normal mononuclear phagocytes. *J. Ultrastruct. Res.* **7**:39.
18. Bhisey, A. N., and J. J. Freed. 1971. Ameboid movement induced in cultured macrophages by colchicine or vinblastine. *Exp. Cell Res.* **64**:419.
19. Carr, I. 1972. The fine structure of microfibrils and microtubules in macrophages and other lymphoreticular cells in relation to cytoplasmic movement. *J. Anat.* **112**:383.
20. Moore, M. A. S., N. Williams, and D. Metcalf. 1972. Purification and characterization of the *in vitro* colony forming cell in monkey hemopoietic tissue. *J. Cell. Physiol.* **79**:283.
21. Evans, M. J., L. J. Cabral, R. J. Stephens, and G. Freeman. 1973. Cell division of alveolar macrophages in rat lung following exposure to NO_2. *Am. J. Pathol.* **70**:199.
22. Bowden, D. H., and I. Y. R. Adamson. 1972. The pulmonary interstitial cells as immediate precursor of the alveolar macrophage. *Am. J. Pathol.* **68**:521.
23. Soderland, S. C., and Y. Naum. 1973. Growth of pulmonary alveolar macrophages *in vitro*. *Nature (Lond.).* **245**:150.
24. Golde, D. W., L. A. Byers, and T. N. Finley. 1974. Proliferative capacity of human alveolar macrophage. *Nature (Lond.).* **247**:373.
25. Pinkett, M. O., C. R. Cowdrey, and P. C. Nowell. 1966. Mixed hematopoietic and pulmonary origin of "alveolar macrophages" as demonstrated by chromosome markers. *Am. J. Pathol.* **48**:859.

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