Growth of Human Bone Marrow in Liquid Culture

By David W. Golde and Martin J. Cline

A system for the cultivation of normal and malignant human bone marrow cells in liquid medium is described. The apparatus used is an in vitro diffusion chamber in which cells grow in suspension and upon a dialysis membrane. Proliferation and maturation of normal granulocytes and macrophages were sustained in culture for several weeks in the absence of exogenous stimulating substances. Cell replication was documented by a rise in viable counts, $^3$H-thymidine incorporation, and labeled mitoses. The entire maturational sequence of the granulocyte and macrophage series was identified in culture, and the cells were characterized by light and electron microscopy, cytochemical properties, phagocytic ability, and the presence of surface receptors for IgG. Nucleated red cell precursors were observed in some cultures for up to 11 days. Malignant cells from patients with various hematologic neoplasms were also successfully cultured. With this system human myeloma cells were maintained in culture for up to 3 wk on primary explant, and continued to synthesize immunoglobulin. The in vitro diffusion chamber technique permits the cultivation of normal human bone marrow cells in liquid medium and provides a convenient means for studying normal and neoplastic hematopoietic cell differentiation and function in short-term culture.

DEVELOPMENT OF THE SEMISOLID SYSTEM for cultivation of murine and human bone marrow has provided information critical to the current understanding of marrow physiology and the control of leukopoiesis. This technique defines the colony-forming cell as a progenitor of granulocyte and macrophage colonies and permits the assay of colony-stimulating factors (CSF) in urine and serum. Using agar or methylcellulose as a supporting matrix, hematopoietic precursor cells may be cloned from normal human bone marrow and from peripheral blood and bone marrow of some patients with hematologic neoplasms. However, semisolid supporting media limit cell mobility and cellular interactions which may be important in vivo. Also, the agar colony system presents technical difficulties in cell retrieval, thereby restricting cytochemical and functional studies of cells.

Previous attempts to culture human bone marrow in liquid suspension have generally been unsatisfactory in that the goal of sustained proliferation and differentiation has not been achieved. In this report we describe a system for...
the cultivation of human hematopoietic cells in liquid medium using an in vitro diffusion apparatus. The technique permits the culture of normal human bone marrow cells for several weeks in the absence of exogenous stimulating factors. It also provides a convenient means for studying neoplastic hematopoietic cell differentiation and function in short-term cultures.

MATERIALS AND METHODS

Cell Culture

Bone marrow was obtained from normal volunteers, patients with hematologic malignancies, and hematologically normal patients with localized tumors. Marrow (4-6 ml) obtained by sternal or iliac puncture was mixed thoroughly with approximately 300 U of heparin (Lipo-Hepin, Riker) and then aspirated through progressively smaller-bore needles (No. 18-24). After the addition of an equal volume of 3% dextran in saline and sedimentation for 1 hr at room temperature, the cells in the supernate were collected by centrifugation at 150 g. The red cells were lysed with 9 ml of 0.83% ammonium chloride and 1 ml Tris buffer, pH 7.65, or by hypotonic lysis with distilled water. The nucleated cells were washed twice in complete tissue culture medium and the viable cell counts were performed in a hemocytometer with trypan blue as diluent.

The culture apparatus (Fig. 1) consisted of a stemmed glass bulb mounted in a white rubber stopper in a 120-ml Erlenmeyer flask. Visking dialysis tubing previously boiled in sodium bicarbonate solution and rinsed thoroughly in distilled water was held in place over the glass bulb by a band of Tygon. An air vent was provided for gas exchange, and the entire apparatus was autoclaved.

The inner chamber was filled with 1 ml of cell suspension containing 0.1 to 6 × 10⁶ (usually 3 × 10⁶) nucleated cells. Culture medium was added to the outer flask (approximately 50 ml) and the level adjusted to the height of the cell suspension to prevent a net volume change. Cultures were incubated at 37°C in a humidified environment with 7.5% CO₂. Media used were CMRL 1066 and McCoy's 5A (Grand Island Biological Co.), with 15% fetal calf serum and 100 units of penicillin and 50 µg of streptomycin per ml. Fresh medium was not added during the culture period, and a single lot of fetal calf serum was used for all experiments.

Stimulatory substances added to the cell chamber included purified human urinary CSF (0.1 ml/ml, kindly provided by Dr. R. Stanley, Walter and Eliza Hall Institute, Mel-
GROWTH OF BONE MARROW IN CULTURE

borne), human urinary erythropoietin (0.2 to 1.0 unit/ml, provided by the National Heart and Lung Institute), a dialysate prepared from lysed peripheral white blood cells from a patient with chronic myelocytic leukemia, and conditioned medium from pure cultures of human fibroblasts or neutrophils and monocytes prepared by the method of Boyum and grown for 3-6 days in medium containing 20% human AB serum.

Bone marrow and peripheral blood from patients with myelocytic leukemia in the blastic or chronic phase, acute lymphocytic leukemia, acute myelocytic leukemia, multiple myeloma, and macroglobulinemia were cultured in a similar manner.

An alternative culture apparatus (Fig. 2) consisted of an inner chamber containing one cell population (A) and separated from the outer chamber by a Millipore filter (pore size 0.45 μ). Bone marrow cells (B) were placed in the outer chamber, which was closed off with dialysis membrane and incubated under the conditions described below.

Bone marrow was also cultured in a semi-solid medium using standard techniques. Two to $5 \times 10^5$ cells were plated in McCoy's medium is 0.3% agar over a base layer containing 0.5% agar and various cell populations or conditioned medium.

Cell Retrieval

Cells in suspension were collected by piercing the membrane with a #22 needle and aspirating the contents into a syringe. The membrane either was removed from the bulb, fixed in methanol, stained with Giemsa, and mounted intact on a glass slide, or was incubated for 10 min with 0.25% trypsin in buffered saline and the adherent cells removed with a rubber policeman. The viable cells in suspension and from the membrane were separately counted in trypan blue and deposited on glass slides with a cytocentrifuge (Shandon, Inc.) for staining or autoradiography. The usual stain was Giemsa. In selected instances the cells were stained for peroxidase or PAS, or with methyl green pyronin. Differential counts were performed on at least 100 cells.

Autoradiographs were prepared on samples labeled with 3H-thymidine (specific activity 2 Ci/mmole) at a concentration of 1 μCi/ml of cell suspension. Cultures were usually pulsed 1 hr before harvest, but in some experiments cells were incubated with tritiated thymidine at the initiation of culture, then washed and grown in medium containing $10^{-5}$ molar “cold” thymidine.

Slides coated with Kodak NTB emulsion were exposed for 5-7 days, developed, and stained with Giemsa. Labeling indices were expressed as percent cells labeled.

Cells were prepared for electron microscopy by fixation in 1.5% glutaraldehyde in com-

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**Fig. 2. Double chamber diffusion culture apparatus.**
plete culture medium held at 4°C for 24–48 hr. After fixation, the cells were collected by centrifugation and the marrow was postfixed with osmium, dehydrated with graded alcohols, and embedded in Araldite. Specimens were examined with a Siemens 1A electron microscope.

Examination for the presence of macrophage surface receptors for IgG was by techniques previously described using human erythrocytes coated with anti-D.12,13

RESULTS

Normal Bone Marrow

Pattern of Growth: In cultures of normal marrow, proliferating cells occurred both suspended in the culture medium and on the surface of the dialysis membrane. In suspension the cells appeared singly or in small clusters of 3–20 cells. Cells on the membrane formed cords, sheets, or heaped-up aggregates (Fig. 3). In cultures up to 5–6 days of age, granulocytes and young macrophages were the principal constituents of the aggregates. In older cultures macrophages predominated and multinucleate cells appeared to form a syncytium. After day 3, the membrane-adherent cells usually comprised 80% or more of the total population.

Cell Number: The variation of viable cell counts with time is illustrated in Fig. 4A. A common pattern of growth was that of falling cell numbers until approximately day 4, followed by a rise and then a second decline. During the period of increase in cell numbers, evidence of cell proliferation was seen in 3H-thymidine incorporation and mitoses.

Cell Type: The absolute number and relative percentage of nucleated red cell precursors generally declined progressively from the initiation of culture, as shown by the representative experiment in Table 1. In some experiments, however, large, basophilic megaloblasts (Fig. 5) were observed at day 4, and orthochromic normoblasts with clear hemoglobinization were observed in culture as late as day 11.

Up to day 4 or 5, granulocytes at or beyond the myelocyte stage predomi-
nated in culture; thereafter, the proportion of immature and mature macrophages increased progressively and by day 8–14 were the predominant cells (Table 1). Small numbers of lymphocytes persisted in culture for up to 4 wk, and rare megakaryocytes were found during the first week.

In culture the entire maturational sequence of cells of the granulocytic series (Fig. 6) was identified, including eosinophils and basophils. Polymorphonuclear leukocytes were observed in cultures as old as 28 days. These cells maintained their peroxidase activity and were functionally capable of phagocytosis. Large net increases in eosinophils were observed in several cultures, but the conditions favoring eosinophil proliferation were not determined.

The macrophages were demonstrated to have surface receptors for IgG globulin, as evidenced by rosette formation with anti-D-sensitized red cells. Mature macrophages phagocytized microorganisms actively (Fig. 7), and erythrophagocytosis was prominent in some cultures.

An immature macrophage precursor was identified which had an eccentric
<table>
<thead>
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<th>Day of culture</th>
<th>Blasts</th>
<th>Myelocytes</th>
<th>Promyelocytes</th>
<th>Myelocytes to PMN</th>
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<th>Lymphocytes</th>
<th>Plasma cells</th>
<th>Macrophages</th>
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round or oblong nucleus, dispersed chromatin, and a prominent nucleolus; it had peroxidase activity limited to the Golgi zone and was poorly phagocytic (Fig. 8). This cell had many of the features described for the mouse promonocyte. Mature macrophages were often multinucleate and normally peroxidase negative.

Macrophage ultrastructure was well preserved, as seen in the electron microscope.

**Labeling Indices:** Representative labeling indices from a single normal subject obtained after a 1-hr exposure of cultured bone marrow cells to tritiated thymidine are shown in Table 2. The labeling index was high during the first week of culture and thereafter declined. During the first week of culture the predominant cells labeled included undifferentiated blast cells and cells resembling medium lymphocytes. After the first week, the predominant labeled cells were immature macrophages.

After brief exposure to tritiated thymidine and subsequent cultivation in medium containing “cold” thymidine for 4–14 days, nuclear labeling was observed in mature neutrophils and macrophages and, rarely, in late normoblasts—indicating that these nondividing cells had matured from earlier dividing precursors. Labeled mitoses were also observed in these cultures.

**Influence of Culture Conditions on Normal Marrow:** Cell proliferation occurred in both McCoy's medium and CMRL 1066. Variation in the concentration of cells put into culture had no effect on the differential cell count at day 6–7. The number of cells in culture at one week was a straight-line function of the initial cell concentration in the range of 0.5 to $5 \times 10^6$/ml. Addition of human AB serum (0.1 ml/ml) had no influence on cell numbers at day 6 or 7 of culture. The effect of various potential stimulatory substances on cell counts is shown in Table 3. Allowing for variability between cultures, no consistent effect of erythropoietin, urinary CFS, or white cell lysate on total cell counts could be documented.

In some studies, normal bone marrow was cultured in a double-chamber system separated from another cell line by a cell-impermeable Millipore filter.
The method of culture was based on the supposition that substances elaborated by one cell population, A, would pass through the filter to influence the growth of the marrow population, B. Populations of A cells were chosen which had a known effect on the growth of the same bone marrow in the agar culture system. The following observations were made: Pure populations of monocytes, neutrophils, lymphocytes, and fibroblasts or mixed peripheral blood leukocytes had no stimulatory effect on the growth of bone marrow in the double chamber system, whereas mixed leukocytes, monocytes, and to a lesser extent neutrophils had a definite effect on clonogenic potential in agar (Table 4). It was noted that the maximal stimulatory activity in the agar system was obtained with underlays containing pure populations of monocytes. Neonatal fibroblasts and phytohemagglutinin-stimulated lymphocytes were without effect.

We concluded that under the conditions employed, the liquid culture system did not show a definite requirement for externally supplied CSF.
Table 2. Labeling Indices After Incubation With $^3$H-Thymidine

<table>
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<tr>
<th>Day of culture</th>
<th>% Cells labeled Membrane</th>
<th>% Cells labeled Supernatant</th>
<th>Total number of cells labeled</th>
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<td>7</td>
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<td>$1.9 \times 10^6$</td>
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<tr>
<td>18</td>
<td>1</td>
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<td>$0.18 \times 10^5$</td>
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**Culture of Abnormal Bone Marrow and Blood**

In certain pathologic marrow cultures the ratio of membrane-adherent and nonadherent cells was reversed in that more cells were found in suspension than on the membrane. This phenomenon was observed in some cultures of multiple myeloma, macroglobulinemia, and acute leukemia. Also, increases in cell counts with some stimulator were noted in cultures of abnormal bone marrow (Table 3), although it was not determined whether this represented a nutritional effect or true stimulation of growth.

**Iron Deficiency Anemia:** Nucleated cell numbers from one patient with iron deficiency increased fourfold in 7 days. At that time 13% of the cells were identified as red cell precursors.

**Chronic Myelocytic Leukemia:** The pattern of growth of cultures inoculated with peripheral blood from one of two patients with chronic myelocytic leukemia in relapse is seen in Fig. 4B. The cell number doubled over the course of 1 wk. At 2 wk, granulocytes constituted the majority of cells, but by 3 wk of culture morphologically normal macrophages were the predominant cell.

**Acute Leukemia:** Marrow or peripheral blood was cultured from four patients with acute leukemia. Malignant myeloblasts from patients with acute myelogenous leukemia grew in culture, and population doubling was noted in one culture at 7 days. These cells frequently showed signs of toxicity such as vacuole formation, and their persistence in culture was variable (Fig. 4B). In one culture initiated with peripheral blood myeloblasts, mature neutrophils comprised the majority of cells in culture at 25 days. Acute lymphocytic leukemia cells could also be cultured and maintained their PAS positivity. They had a high labeling index (30%) at 11 days.

**Hodgkin's Disease:** An unusual bone marrow culture was obtained from a patient with Hodgkin's disease not histologically affecting the marrow. Nucleated cell counts exceeded initial value by sixfold at 12 days of culture, and the population was composed almost entirely of morphologically normal macrophages.

**Multiple Myeloma:** Bone marrow from five patients with multiple myeloma was cultured. Malignant plasma cells persisted in cultures for at least 3 wk. They produced immunoglobulin, stained normally with methyl green pyronin, and retained their typical ultrastructure as viewed by electron microscopy. The pattern of change of cell numbers with time in one culture of
myeloma bone marrow is shown in Fig. 4B. Proliferation of plasma cells as evidenced by tritiated thymidine incorporation into plasmablasts and cells resembling medium lymphocytes was observed for up to 3 wk. Mature plasma cells incorporated tritiated thymidine only rarely and were never observed in mitosis. The characteristics of plasma cell proliferation in vitro are being reported separately.18

DISCUSSION

Human bone marrow may be studied in vitro using semisolid support cultures and with diffusion chambers implanted into animals.17,18 Attempts to culture human bone marrow in liquid suspension have generally been unsuccessful in terms of sustaining cell replication.19,20 Sumner et al.21 have used an in vitro diffusion chamber technique, initially described by Mishell and later modified by Marbrook,22 to culture murine bone marrow. They observed proliferation and differentiation of mouse granulocytes and macrophages for up to 10 days. We have modified and adapted the in vitro diffusion system to the culture of normal human marrow and malignant human hematopoietic cells.

Data obtained in normal marrow cultures indicate that replicating cells could be maintained in culture for up to 4 wk. Proliferation was demonstrated by a rise in viable cell counts, by incorporation of 3H-thymidine, and by labeled mitoses. Maturation along several cell lines was documented by a transfer of nuclear label to nondividing cells.

In addition to granulopoiesis, the various stages of human macrophage morphogenesis are seen with unusual clarity in the liquid culture system. The early monocyte precursors which are difficult to identify in newly isolated bone marrow are readily identified and characterized in culture. For example, cells with the morphologic and functional properties ascribed to mouse pro-monocytes are abundant at certain phases of culture. Mature cells of the macrophage series appeared morphologically normal by light and electron

<table>
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<th>Subject</th>
<th>Diagnosis</th>
<th>Day</th>
<th>Control</th>
<th>Erythropoietin $\times 10^5$</th>
<th>Urine CSF</th>
<th>WBC Lysate</th>
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<td>7</td>
<td>2.9</td>
<td>1.2</td>
<td>—</td>
<td>3.2</td>
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*Cells in suspension only; membrane-adherent cells not counted.
microscopy and could be identified functionally by the presence of IgG receptors and phagocytosis. Eosinophils were prominent in some cultures and were definitively identified by their characteristic granules seen on electron micrographs. Eosinophil proliferation has also been reported to occur in semisolid cultures.23

Sustained marrow cell proliferation in the absence of exogenous stimulating substances reflects a major difference between the liquid and semisolid systems in that colonies rarely form in agar cultures without a source of CSF. Cell growth in liquid medium may perhaps be stimulated by endogenously produced CSF or by cell contact phenomena.7 Haskill et al. were able to demonstrate in vitro colony growth in the absence of exogenous CSF and defined a role for cell-to-cell interactions in the regulation of granulopoiesis in vitro.24

The pattern of cell growth in the presently described system is similar to that reported for murine suspension cultures.21 Cell counts usually fell during the first 4 days, subsequently rose, and then decreased again. Granulocytes predominated in young cultures, while macrophages comprised the majority of cells in order cultures.

Identification of human erythroid precursors as late as the 11th day of culture is an intriguing finding; although proliferation of red cell precursors was not documented, maturation was clearly observed. Since red cell precursors normally mature in vivo in about 5 days, the erythroid elements in culture at 11 days either matured slowly or arose from stem cells which began their maturational sequence after the initiation of culture.

Marrow and blood cultures from patients with hematologic neoplasms generally showed good growth, and cell production exceeding cell death was observed in several cultures at 7 days. Chronic myelogenous leukemia cells proliferated actively and grew in a pattern similar to that observed with normal cells in that granulopoiesis preceded the predominance of macrophages. Myeloblasts from patients with acute myelogenous leukemia were cultured, and population doubling occurred in 7 days in cells obtained from one patient. Evidence suggesting in vitro maturation of leukemic myeloblasts was obtained in a culture initiated with peripheral blood blasts in which mature polys were observed after 25 days of culture. Other workers have reported apparent maturation of leukemic myeloblasts in vitro.25

Malignant plasma cells from five patients with multiple myeloma were sustained in culture for up to 3 wk on primary explant. The plasmablasts

| Table 4. Effect of Peripheral Blood Leukocyte Populations on Bone Marrow Growth in Agar |
|---------------------------------|-----------------|
| Cells in underlay               | CFC/2 × 10⁶ cells |
| None                            | 0-2             |
| Mixed leukocytes                | 69              |
| Neutrophils*                    | 24              |
| Monocytes*                      | 104             |
| Lymphocytes*                    | 13              |

*These populations were at least 95% pure.
incorporated thymidine. Plasma cells remained morphologically intact and functionally active in terms of immunoglobulin production (immunoglobulin synthesis studies kindly performed by Dr. Sydney Salmon). Neoplastic cells from a patient with macroglobulinemia were cultured and, like the plasma cells, showed a low labeling index.

The liquid culture system described here augments the agar culture technique. Cells grown in liquid culture can readily be retrieved for studies of function, of precursor-progeny relationship, of cytogenetics, and of cytochemical analysis.

ACKNOWLEDGMENT

We are indebted to Mrs. M. A. Sumner for many helpful suggestions and discussions. The authors also wish to acknowledge the excellent technical assistance of Lisken Byers and Tazuko Howard.

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