Improved Techniques for Liquid Culture of Human and Mouse Bone Marrow

By Martin J. Cline and David W. Golde

Previous studies using the in vitro diffusion chamber (Marbrook) have shown that bone marrow grown in this system will undergo limited stem cell replication and differentiation to mature granulocytes and mononuclear phagocytes. A series of studies with modified culture systems was initiated to improve cell production and committed stem cell (CFU-C) proliferation in vitro. Introduction of a continuous-flow system and a migration technique providing means of egress for mature neutrophils resulted in substantially improved performance. CFU-C were found to be capable of migration through a 3-μm pore membrane. These studies indicated that membrane surface area, culture medium circulation, and mature cell egress were among the conditions that could be optimized for maximum hematopoietic cell proliferation in suspension culture. The present observations also suggested that large-scale in vitro growth of mammalian bone marrow may be feasible.

Two basic techniques have been used to grow human and murine bone marrow in vitro: cell cloning in semisolid supporting matrices such as agar, methyl cellulose, and plasma clots, and growth of cells in suspension culture in tubes and on dialysis membranes. In addition, cocultivation of bone marrow and thymus cells on glass surfaces has recently been reported to permit prolonged growth and stem cell maintenance.

Semisolid and suspension culture systems allow the growth of precursor cells committed to granulocyte and mononuclear phagocyte differentiation as well as erythroid precursors. The semisolid culture technique provides a useful assay of the relative or absolute numbers of granulocyte–monocyte stem cells (CFU-C) and of committed erythroid progenitors (CFU-E). The suspension culture system is useful for examining cellular proliferation and differentiation, hematopoietic cell interactions, morphology, cytochemistry, and cell function. Analysis of hematopoietic response to stimulatory hormones such as erythropoietin and colony-stimulating activity (CSA) is best accomplished with semisolid supporting matrices, although suspension cultures of hematopoietic tissues also show dependence on added CSA (mouse bone marrow) and erythropoietin (mouse and human bone marrow).

While the semisolid cloning technique is a potent analytical tool, it is not suitable as a cell production system. Both the semisolid matrix and suspension culture techniques are characterized by limited stem cell self-renewal capacity. Ultimately, all cellular proliferation ceases, and the cultures consist of a population of viable and functional but nondividing macrophages.
The present studies were undertaken with the objectives of improving the suspension culture methods to allow greater cellular proliferation and better preservation of CFU-C. Several modifications of the standard in vitro diffusion chamber system were examined. A diffusion–migration culture technique was developed that supports considerably augmented granulopoiesis and allows for mature cell egress and collection.

**MATERIALS AND METHODS**

**Medium**
McCoy's 5A medium (Grand Island Biological Co.), supplemented with 15% fetal calf serum, nonessential amino acids, penicillin (100 U/ml), and streptomycin (50 μg/ml), was made up at double strength and diluted with double strength agar for semisolid culture or with distilled water for the liquid culture system.

**Colony-stimulating Activity (CSA) Preparation**
A gravid mouse uterus extract containing potent CSA was prepared from 16- to 20-day embryos, pregnant uteri, and membranes of white Swiss-Webster mice as previously described.

For most experiments, the extract was used at 100 μl/ml, a concentration of the plateau portion of the dose–response curve which produced maximal growth of marrow colonies in agar and maximal cell numbers at 5 days in liquid culture. For human marrow culture, conditioned medium was prepared from normal peripheral blood leukocytes and used at 100 μl/ml.

**Standard Liquid Culture System**
White Swiss-Webster mice were used in all mouse experiments. A pool of bone marrow cells was prepared from the femora of 2- to 3-mo-old female mice, suspended in complete medium at 3 x 10^6/ml, and inoculated into Marbrook-type diffusion cultures in 1-ml volumes. Cells were cultured at 37°C in a humidified atmosphere of 7.5% CO_2 in air for 5-20 days. Bone marrow suspensions obtained from normal human volunteers were cultured under the same conditions.

At the termination of culture, suspensions were incubated with 3H-thymidine, 1 μCi/ml (specific activity 2 Ci/m mole) for 1 hr. Cells were retrieved from both the medium and the dialysis membrane for total viable cell counts, differential counts of Giemsa-stained cells prepared with a cytocentrifuge (Shandon Instruments Co.), 3H-thymidine autoradiography, and subculture in agar as described below.

**Assay of Agar Colony-forming Cells (CFU-C)**
Cultures were assayed for CFU-C by a double-layer agar technique. The system consisted of a semisolid nutrient 0.5%, agar underlay of 1 ml containing 0.1 ml CSA, and a 1-ml 0.3% agar overlay containing hematopoietic cells in a 35-mm plastic petri dish (Falcon Plastics). Duplicate or triplicate dishes were incubated in 7.5% CO_2 and colonies containing at least 50 cells were counted in the upper layer at 7 days (mouse) or 11 days (human).

Assays of CFU-C were commenced on the day the liquid cultures were started and repeated at intervals thereafter. The initial agar assay culture contained 5 x 10^4 mouse or 2 x 10^5 human bone marrow cells. Subsequently, the cell number plated in agar was adjusted to give colony counts in the range of 100-200 per dish.

**Variations in Culture Conditions**

**Optimal surface area.** The standard Marbrook diffusion chamber is shown schematically in Fig. 1A. In the standard system, 3 x 10^6 bone marrow cells were placed directly on a circle of dialysis membrane with a diameter of 7.5 mm. CSA was added to the cell compartment. In initial experiments, the cell number was kept constant and the area of dialysis membrane was varied. Alternatively, the area was kept constant and the size of the cell inoculum was varied.

**Continuous-flow system.** In the standard Marbrook diffusion chamber, both the cell and the external compartments are static and are left undisturbed from inoculation until termination of
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Fig. 1. Schematic representation of diffusion chamber systems for growth of bone marrow cells in suspension culture. (A) Standard Marbrook system in which cells grow in suspension and upon a dialysis membrane. (B) System modified to permit migration of mature cells. Bone marrow cell suspension rests on a 3-μm Nuclepore membrane. The inner cell compartment is enclosed in a second concentric compartment sealed by dialysis membrane. These two compartments contain CSA. (C) System modified to permit continuous recirculation of external culture medium. 1—Cap for gas exchange; 2—cell compartment volume 1 ml; 3—dialysis membrane; 4—Nuclepore membrane; 5—external dialysis compartment; 6—external tissue culture compartment.

The culture. We have devised an alternative culture system in which the external compartment medium is continuously circulated from a large external reservoir by means of a Holter pump at the rate of 175–225 ml/hr. Medium in the reservoir is changed every 3 days. The cell compartment containing cells and CSA is undisturbed until harvest. The design is illustrated in Fig. 1C.

An alternative to this system was one in which a standard chamber (Fig. 1A) was used, but the medium in the external compartment was kept in continuous motion by means of a stirring bar and an external magnet.

Cell migration methods. In the standard diffusion culture technique (Fig. 1A), mature cells cannot leave the system unless the cell compartment is periodically sampled. A variation was constructed in which the cell compartment was sealed with a Nuclepore membrane of 3-μm pore size. In turn, this was separated from the large volume external compartment by a concentric tube scaled with dialysis membrane. By this means, a double compartment was created as shown in
Fig. 1B. The mature cells could migrate through the Nuclepore filter and be collected in the dialysis compartment. The dialysis membrane retained these cells and also kept added CSA in contact with cells in a relatively small volume that was not diluted by the large amount of culture medium in the external compartment.

Variations in this technique included Nuclepore membranes with different pore sizes (0.2-3.0 μ), and a combination of this migration technique with the continuous-flow system illustrated in Fig. 1C. Another variable was to collect cells from the dialysis compartment at intervals, or to leave the cells in situ until termination of culture.

RESULTS

Optimal Surface Area

A series of experiments was performed to determine the optimal surface of the dialysis membrane for growth of bone marrow cells in suspension culture. Human or mouse bone marrow cells at 3 × 10⁶/ml in volumes of 1 ml were inoculated into Marbrook-type flasks in which the diameter of the cell compartment varied between 7.5 and 19 mm. Mouse CSA or human leukocyte-conditioned medium was included in the cell chamber. After 5 days (human) or 6 days (mouse), cultures were terminated and total and differential cell counts determined. When the cell volume was kept constant and the dialysis membrane area was altered, a number of other characteristics of the culture system were changed as well; i.e., the height to volume ratio of culture fluid over the cells and the area of fluid available for gas exchange all varied with changes in membrane area. As shown in Fig. 2, the optimal geometry for growth of mouse marrow was found to occur with either 12- or 15-mm diameter membranes (Fig. 2A). For human marrow 12-mm membranes were best. Under these conditions of culture, optimal cell numbers per unit surface area were 2.65 × 10⁴/cu mm and 1.70 to 2.65 × 10⁴/cu mm for human and mouse bone marrow, respectively. The standard Marbrook system utilizing 3 × 10⁶ mouse marrow cells in a chamber of 7.5-mm internal diameter was roughly one-quarter as effective in supporting cell numbers as the 12- or 15-mm chamber. Variation in the area of dialysis membrane was less critical for the growth of human bone marrow cells.

The effect of a constant surface area on the growth of human bone marrow cells is shown in Table 1. As observed previously,9 the number of viable human hematopoietic cells present at 5–7 days of culture approximated a linear function of the number of marrow cells inoculated between 5 and 30 × 10⁶/ml when the geometry of the system was kept constant. There was nonlinearity with downward divergence of the curve only at higher inoculation densities.

<table>
<thead>
<tr>
<th>Marrow Cell Inoculum (x 10⁶)</th>
<th>Cell No. at Harvest (x 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>20</td>
<td>19.5 ± 1.5</td>
</tr>
<tr>
<td>30</td>
<td>31.0 ± 1.0</td>
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</tbody>
</table>
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Fig. 2. Effect of variation of surface area of dialysis membrane on the growth at 7 days of a constant number (3 x 10^6) of bone marrow cells. (A) Mouse bone marrow. (B) Human bone marrow. ○—○, total cell counts; △—△, differentiated neutrophilic granulocytes; ●—●, blast cells.

Stationary Versus Continuous-Flow Systems

Mouse bone marrow cells (3 x 10^6/ml) were inoculated into the inner chamber of standard Marbrook flasks (stationary cultures, Fig. 1A) or into the continuous-flow system described in Methods (Fig. 1C). At intervals, total and differential cell numbers and CFU-C were determined. As shown in Fig. 3, preservation of blast cells and granulocytes was substantially better in the continuous-flow system throughout an 18-day period of culture. Greater increases in CFU-C (Fig. 4) and in 3H-thymidine labeling (Table 2) through day 10 were seen in the continuous-flow cultures. In such cultures, the numbers of CFU-C at 5 days were about 30-fold greater than the numbers at initiation of incubation. These cultural advantages were also observed when larger surface area cultures were used with the continuous-flow system.
Fig. 3. The growth of mouse bone marrow in stationary (S) and continuous-flow (F) diffusion chambers was compared. Total cell numbers (total height of the bars), numbers of blast cells (solid bars), granulocytes (diagonal stripes), macrophages (stippled bars), and other cell types (horizontal bars) were determined.

Table 2. Comparison of $^3$H-Thymidine Labeling of Mouse Bone Marrow Cells in Stationary and Continuous-Flow Diffusion Chambers and Migration Chambers

<table>
<thead>
<tr>
<th>Period of Culture (days)</th>
<th>Stationary</th>
<th>Continuous Flow</th>
<th>Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inner Chamber</td>
</tr>
<tr>
<td>3</td>
<td>11 (9–13)*</td>
<td>16 (9–18)</td>
<td>46 (43–48)</td>
</tr>
<tr>
<td>5</td>
<td>30 (20–44)</td>
<td>25 (16–21)</td>
<td>38 (33–43)</td>
</tr>
<tr>
<td>7</td>
<td>15 (7–20)</td>
<td>12 (4–22)</td>
<td>40 (32–47)</td>
</tr>
<tr>
<td>10</td>
<td>6 (1–12)</td>
<td>10 (5–15)</td>
<td>—</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>1</td>
<td>—</td>
</tr>
</tbody>
</table>

|                          |            |                 | Inner Chamber | Outer Chamber |
| 3                        | 2.7 ± 0.8† | 6.4 ± 2.0       | 5.4 ± 2.1 | 2.0 ± 0.9 |
| 5                        | 2.8 ± 0.3  | 6.6 ± 2.9       | 8.2 ± 3.0 | 11.1 ± 3.1 |
| 7                        | 1.9 ± 1.4  | 4.4 ± 2.5       | 7.0 ± 4.4 | 14.1 ± 10.2 |
| 10                       | 0.7 ± 0.4  | 1.9 ± 1.1       | —         | —         |
| 18                       | 0          | 0.1 ± 0.02      | —         | —         |

* These values represent the labeling index (%).
† These values represent the total labeled cells ($\times 10^2 \pm 2$ SD).
The better performance of the continuous-flow cultures was not due simply to movement of tissue culture medium over the external surface of the dialysis membrane since continuous stirring of the external fluid produced only marginal increases in cell numbers.

Cell Migration

In the bone marrow of the intact animal or man, granulocytes and mononuclear phagocytes normally leave the sinusoids to enter the blood stream as the cells mature to the PMN leukocyte and monocyte stage. In semisolid matrix cultures, mature cells are also capable of limited mobility. However, in the standard diffusion chamber system, mature and intermediate granulocytes and mononuclear phagocytes are retained in contact with proliferating younger cells. In order to simulate more closely in vivo conditions in the suspension culture system, a Nuclepore membrane with holes of sufficient size to permit egress of motile cells was used to support the marrow cell suspensions (Fig. 1B). Colony-stimulating activity was added to both the cell compartment and to the dialysis compartment into which the cells escaped after traversing the pores of the Nuclepore membrane. In the usual experiment, the cultures were terminated at intervals and total and differential cell counts, and numbers of CFU-C were determined in both compartments. As shown in Fig. 5A, total cells in the inner cell chamber fell during the first 3 days of culture and then remained stable through day 7. Simultaneously, the number of cells in the outer dialysis compartment increased progressively with time. With the 3-μm pore size filters, the majority, but not all, of the blast cells were retained in the inner cell compartment (Fig. 5B), whereas the great majority of intermediate and mature granulocytes migrated into the outer compartment. In this system, the numbers of CFU-C increased strikingly above the levels present at initiation of culture (Fig. 5C). Initially, most CFU-C were present in the inner chamber, but with time more than half were found in the outer chamber. These observations indicated that CFU-C were capable of migration through a 3-μm pore filter. The high rates of cellular proliferation in the migration system were reflected by the percentage and total number of labeled cells after a 1-hr pulse of 3H-thymidine (Table 2).

In another series of experiments, the migration system was combined with the continuous-flow system (Fig. 1B and 1C). Essentially the same pattern of cell movement was observed as in the stationary migration system but the numbers of cells, and especially of CFU-C, were greater in the continuous-flow migration system than in the stationary migration system.

In a further series of experiments, mouse bone marrow (3 × 10⁶ cells) was inoculated into migration chambers with 3-μm Nuclepore filters. The cells in the inner chamber were left undisturbed while the cells in the outer chamber were harvested at days 3, 5, and 7 and the medium in this chamber replenished at these intervals. Cell accumulation in the outer chamber increased with time and the total numbers of migrating cells harvested over 7 days was greater than if the cultures were left undisturbed for that period of time (Fig. 6). As before, the principal migrating cells were neutrophils, with a small percentage of macrophages and blast cells. It was not determined whether the poorer performance
Fig. 5. Mouse bone marrow cells (3 x 10^6) were cultured on 3-μm Nuclepore membranes as illustrated in Fig. 1B. (A) Cell numbers were determined at intervals of culture: e—e total cells in both inner and outer chambers; o—o cells in the inner chamber. The height of the vertical lines indicates the numbers of cells which have migrated through the Nuclepore into the dialysis compartment (No. 5 of Fig. 1B). (B) Blast cell numbers were determined at intervals: e—e, total blast cells in both inner and outer chambers; o—o, blast cells in inner chamber. The height of the vertical lines indicates the numbers of blast cells which have traversed the Nuclepore. (C) CFU-C were determined at intervals: e—e, total CFU-C in both chambers; o—o, CFU-C in the inner chamber. The height of the vertical bars indicates the number of CFU-C which have traversed the Nuclepore.
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Fig. 6. Mouse bone marrow cells (3 x 10⁶) were cultured in migration chambers. Cultures were left undisturbed and terminated at intervals to count total cells accumulating in the outer chamber (e—o) or the cells of the outer chamber were collected at 3, 5, and 7 days while the cells in the inner chamber were left undisturbed for continued culture. The total number of cells harvested is indicated by the open circles (○—○).

of the undisturbed cultures was due to less cell proliferation or greater mature cell death over a period of 7 days.

DISCUSSION

The standard in vitro diffusion system of the type developed by Marbrook for the cultivation of lymphocytes is also useful for growing hematopoietic cells. The system permits easy cell retrieval and assessment of cellular differentiation, cytochemistry, and function. However, in standard diffusion culture systems the number of stem cells falls relatively rapidly. The present investigations indicate that substantial improvements in the standard liquid system can be achieved by adapting it to continuous flow and by permitting egress of the maturing granulocytes. With such modifications, there is greater cell proliferation and expansion of the population of committed stem cells (CFU-C).

Among the factors demonstrated to affect bone marrow proliferation in the Marbrook chamber are the geometry of the system, including the surface area of the membrane, circulation of culture medium, and removal of mature leukocytes. Membrane surface area and culture medium modifications probably relate to nutritional requirements typical of other tissue culture systems. Thus, cell density, metabolic waste removal, and nutrient supply may be idealized for hematopoietic cell growth in suspension culture. Improvement in cell production and stem cell replication in the migration chamber cultures is also apparent. With this technique, mature leukocytes migrate through a Nuclepore filter, and the improved cell growth may be due to removal of inhibition from the differentiated neutrophil. However, the inhibitory effect of mature granulocytes may relate to the discharge of lysosomal enzymes or other potentially toxic substances from degenerating neutrophils. Whatever the mechanism, a similar phenomenon has been observed in organ cultures of mouse spleen.
Although the mechanism of increased cell growth in the migration culture system is not known, this technique probably simulates the in vivo situation more closely than any of the presently available marrow culture methods.

The migration culture method also provides a means for studying cell mobility and egress from proliferating compartments. The present studies indicate that the CFU-C is capable of migrating through a 3-μm pore. This observation suggests that the committed stem cell may have considerable deformability and possibly explains the circulation of CFU-C in the blood of normal mice and man.12,18,19

Clearly, a number of further modifications of diffusion cultures can be considered: continuous removal of mature cells, pore sizes which will selectively retain the less motile blast cells, and chemotactic stimuli to attract more effectively the differentiated mature granulocytes and mononuclear phagocytes. These studies are in progress and hopefully will result in more effective means for growing mammalian bone marrow in vitro. A reasonable objective is the prolonged growth of mammalian bone marrow with retention of pluripotent and committed stem cells and high rates of production of differentiated nucleated blood cells in vitro. A realistic, albeit long-term, goal is the in vitro production of hematopoietic cells for therapeutic use.

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REFERENCES

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