Chapter 26

CFU-C in Agar

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1. Introduction

Agar culture systems for the clonal growth and differentiation of hemopoietic cells were first described 20 yr ago (*1*). The progenitor cells that developed into colonies in agar after several days of culture in the presence of a source of hemopoietic growth factor (*2,3*) were initially called "Colony Forming Units in Culture" (CFU-C). They are found in bone marrow, spleen, blood, fetal liver, and yolk sac. It was subsequently demonstrated that the CFU-C population was heterogeneous and contained progenitors giving rise to granulocyte/macrophage (CFU-GM), granulocyte (CFU-G), and macrophage (CFU-M) colonies. Progenitor cells of other lineages (erythroid and megakaryocytic) have also been similarly demonstrated in hemopoietic organs.

The progressive identification of different types of progenitors, the demonstration of their hierarchical distribution, the purification of the growth factors regulating their proliferation and differentiation, and the results of other in vitro and in vivo experiments led to a schematic three-compartment model for hemopoiesis involving:

1. The stem cell compartment, with extensive self-renewal and commitment potential.
2. A committed progenitor cell compartment that is heterogeneous and hierarchically distributed from multipotential to unipotential progenitors. The differentiation of cells in this compartment is associated with a progressive loss of proliferative potential and is dependent upon the presence of specific growth factors.
3. A compartment of maturing and mature cells, composed of morphologically identifiable cells restricted to one lineage, endowed with very limited proliferative potential. These cells quickly acquire the phenotypic and functional properties of circulating mature cells (Fig. 1).

The in vitro culture technique (*1*) described in this chapter has provided much information on the hemopoietic system over the last 20 yr. It will remain useful for:

a. Determining the frequencies of different types of progenitors and their modification in vitro or in vivo by different agents.
b. The screening of various substances for their effects on progenitor cell proliferation and/or differentiation.
c. The identification and isolation of hemopoietic stem cells and the analysis of their growth factor requirements.

2. Materials

1. Media:
   a. Alpha (α) medium stock solution: dissolve the powder for 1 L of medium in 177 mL of deionized distilled water (DDW), filter through a 0.22 μm sterile filter and store at –20°C (*see* Note 1).
   b. x2 alpha medium:

Alpha stock solution	32 mL
Glutamine solution (200 m*M*)	2 mL
Fetal calf serum (*see* Note 2)	40 mL
Sodium bicarbonate (5.6% w/v solution)	8mL
Gentamycin solution (10 mg/mL)	0.5 mL
DDW	17.5 mL

   Mix thoroughly, filter through a 0.22 μm filter, and store at 4°C for less than 1 wk.
   c. x1 Medium for cell collection and dilution: x2 alpha medium diluted with an equal volume of water and adjusted to pH 7.35.

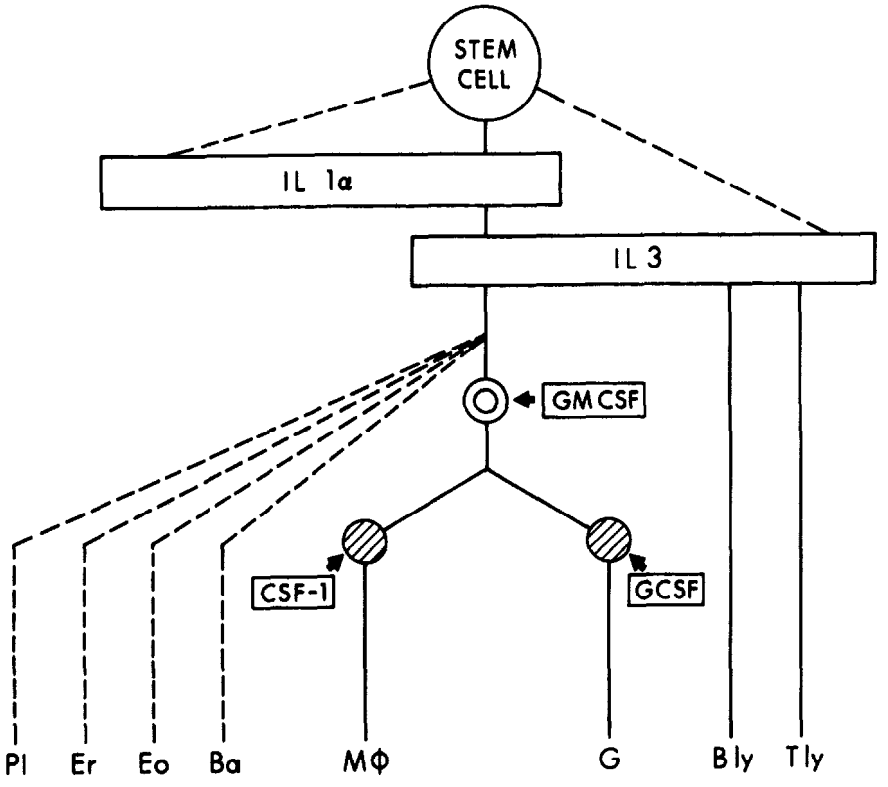

Fig. 1. Schematic representation of the hemopoietic system emphasizing granulocyte and macrophage production. IL1 acts on very primitive hemopoietic cells, both directly on primitive progenitors and indirectly through the stimulation of the production of other growth factors by accessory cells. IL-1 alone does not stimulate hemopoietic cell proliferation. Furthermore, the proliferation of very primitive cells is not stimulated by either IL-1, IL-3, GM-CSF or CSF-1 alone. Combinations of IL-3, GM-CSF and CSF-1 with IL-1 are required (*see* Chapter 23). IL-3 is a multipotential growth factor, active on all hemopoietic cell lineages. GM-CSF, CSF-1 (M-CSF), and G-CSF act on granulocyte or macrophage progenitor cells and maturing or mature granulocytes and macrophages. They not only stimulate proliferation and differentiation, but also survival and activation of functional properties of the mature cells of these lineages. (Pl): platelets; (Er) erythrocytes; (Eo) eosinophils: (Ba) basophils; (Mø) monocytes-macrophages; (G) granulocytes; (Bly) B lymphocytes; (Tly) T lymphocytes.

2. 1% Agar Solution: In a 500 mL conical flask, mix 2 g of agar with 200 mL of DDW. Heat with stirring to boiling; allow to cool and reboil a second time. Allow to cool to 50°C (*see* Note 3).
3. 0.66% Agar Solution: Add 33 mL of prewarmed DDW to 66 mL of 1% agar at 50°C and reboil once.
4. Ficoll Paque.
5. Gas Mix: 7% O_2, 10% CO_2, 83% N_2.

3. Methods

3.1. Bone Marrow Cell Collection

3.1.1. Mouse Bone Marrow

Sterilize surgical instruments by boiling or in 70% ethanol.

1. Kill the mice by cervical dislocation (*see* Note 4). Sterilize the skin with ethanol and, using sterile scissors, remove the muscles from the femur and around the knee. Disarticulate at ball and knee joints. Cut the lower extremity of the femur at the epiphyse-cartilage junction.
2. Using a 3 mL sterile plastic syringe with a 23-gage needle, flush the femoral cavity several times with medium, collecting cells and medium in a 5 mL sterile plastic tube.
3. After flushing all femurs, disperse the cells to form a single cell suspension (*see* Note 5).
4. Count the white cells and adjust the cell concentration, as desired, with medium.

3.1.2. Human Bone Marrow

1. Samples are aspirated from the posterior iliac crest or sternum in syringes previously rinsed with preservative-free heparin diluted 1:10 in heparinized (50–100 U/mL) x1 medium.
2. Carefully layer this aspirate on a Ficoll-Paque cushion and centrifuge at 400*g* for 30 min at 15°C (*4*). The mononuclear cells are aspirated from the interface, washed two times by centrifiguration in x1 medium and diluted to the desired concentration prior to plating as described below.

3.2. Plating Procedure

1. Number the Petri dishes (35 mm Falcon) and add growth factors (GF:0.15 mL/dish) (*see* Note 6) according to the appropriate experimental protocol. Each experimental condition must be set up at least in triplicate, depending on the expected progenitor cell frequency.
2. Calculate the total volume of agar-medium needed for underlayers and overlayers according to the total number of dishes to be used. In order to have enough agar medium for all dishes, prepare the underlayer and overlayer agar-medium mix for 10 more dishes than required in the protocol, e.g., for 50 dishes:

GF		2 x α medium, 37–40°C	Agar, 37–39°C	Cells
Underlayer	+ +	30 mL	30 mL (1%)	–
Overlayer	–	15 mL	15 mL (0.6%)	+ +

3. Underlayer: Keeping the pool of agar medium at 37°C, distribute 1 mL/dish in all dishes using a pipet or an automatic pipeting device. Allow the underlayer to gel at room temperature for 5–15 min.
4. Overlayer: Prepare the agar medium (0.33% final) and quickly add the calculated volume of cell suspension, mix thoroughly (*see* Note 7) and dispense 0.5 mL/dish keeping the temperature of the agar medium pool at 37°C (*see* Note 8) and swirling the dishes as you proceed to ensure even distribution.
5. Allow the overlayer to gel at room temperature and incubate immediately.

3.3. Incubation Procedure

1. Transfer the dishes to grids over a thin DDW layer in plastic boxes (Fig. 2) (for a detailed description, *see* Chapter 23).
2. Seal the boxes with plastic tape and using holes in the cover (one as an inlet, the other as an outlet) gas with a 7% O_2, 10% CO_2, 83% N_2 (*5*) gas mixture for 20 min, at a flow rate of 3 L/min. At the end of this time, the medium in the dishes should look slightly orange.
3. Immediately seal the two holes in the cover with plastic tape.
4. Incubate at 37°C in the dark for 7–10 d depending on the type of progenitor cell colony.
5. Use a dissecting microscope at x20 magnification to count colonies (>50 cell/aggregate) and clusters (4–50 cells/aggregate).

3.4. Morphological Analysis of Colony Cells

Procedures for whole plate staining are time consuming and give poor results with the agar system. For morphological studies, we suggest a collagen gel culture technique that allows good quality *in situ* staining of colonies (*6*):

1. Prepare a collagen solution from rat tail tendons according to the technique of Lanotte and Schor (*6,7, see* Chapter 19, this vol.). Briefly, the tail tendons are cut out, dropped in absolute ethanol for sterilization and transferred to 0.5*M* acetic acid for depolymerization. When all the collagen is solubilized, it is dialyzed against distilled water, filtered through a 0.22 µm sterile filter and sterilely stored at 4°C.
2. The culture medium mix contains:

x2 Medium	25%
Collagen solution	25%
FCS	20%
x1 Medium	20 or 25%
Cell suspension	5%
Growth factors	5% (or added separately to the dishes)

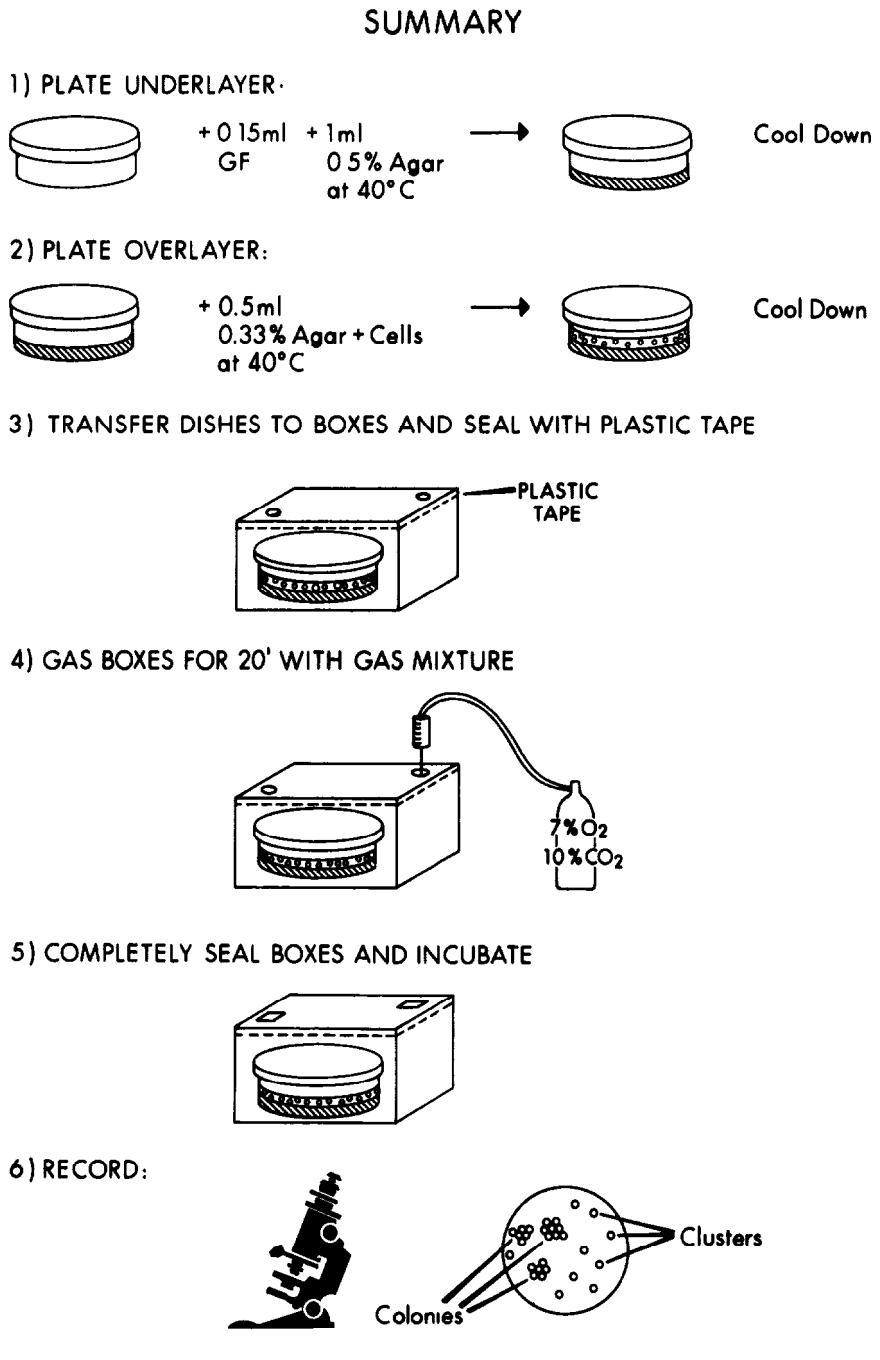

Fig. 2. Schematic summary of plating and incubation procedure.

All reagents, including the mixed medium, must be kept at 4°C until distribution in dishes. Polymerization of collagen (gel formation) occurs when the temperature rises above 15°C.

3. Incubate as for agar cultures.
4. To prepare the gel for staining, it must be transferred from the plate to a glass slide, then dried and fixed. This part of the method is delicate and is described in detail in the figure in ref. *8*.
5. The dried gels yield a very thin film of collagen containing the colonies and clusters. Standard staining and most cytochemical reactions are as easily performed on the dried gel as on a smear and allow easy cellular identification (*8*).

4. Notes

1. The Alpha medium stock solution must be prepared without sodium bicarbonate in order to avoid amino acid precipitation upon thawing.
2. Several batches of fetal calf serum must be tested in this culture system in order to eliminate batches with inhibitory activity.
3. Agar must not be sterilized by autoclaving. Care must be taken to avoid burning during preparation. Discard the preparation if burning occurs.
4. For mouse bone marrow cells, even if a low number of cells is needed, it is necessary to use cells pooled from three different mice.
5. The cell suspension has to be carefully dispersed to single cells by repeated and patient pipeting. Before diluting for culture, tiny cell aggregates and debris may be eliminated by allowing them to settle under unit gravity.
6. If several growth factors are to be added in the same dish, be careful not to contaminate pipet tips with other growth factors. When dispensing the underlayer, avoid touching the dishes or the dispensed growth factor.
7. A homogeneous cell distribution in the agar mix culture medium is necessary for reliable results; because of its high viscosity, it requires careful mixing.
8. To prevent premature gelling, the temperature of the agar medium mix must be kept between 37 and 40°C. A previously adjusted water bath is very useful for this purpose. Gelling of the agar underlayer must be complete before addition of the overlayer and the overlayer must gel before incubation at 37°C. Approximately 5 min at 20°C is required for gel formation.

References

1. Bradley, T. R. and Metcalf, D. (1966) The growth of mouse bone marrow cells *in vitro*. *Austalian Journal of Experimental Biology and Medical Science* **44,** 287–299.
2. Metcalf, D. (1986) The molecular biology and functions of the granulocyte macrophage-stimulating factors. *Blood* **67,** 257–264.
3. Clark, S. C. and Kamen, R. (1987) The human hematopoietic colony stimulating factors. *Science* **236,** 1229–1237.
4. Boyum, A. (1968) Isolation of leukocytes from human blood. *Scandinavian Journal of Clinical and Laboratory Investigation* **21: Supp. 97,** 31–50.
5. Bradley, R. R., Hodgson, G. S., and Rosendaal, M. (1987) The effects of oxygen tension on haemopoietic and fibroblast cell proliferation *in vitro*. *J. Cell Physiol.* **94,** 517–522.
6. Lanotte, M., Schor, S., and Dexter, T. M. (1981) Collagen gels as a matrix for haemopoiesis. *Journal of Cellular Physiology* **106,** 269–277.
7. Schor, S. L. and Court, J. (1979) Different mechanisms in the attachment of cells to native and denatured collagen. *Journal of Cell Science* **38,** 267–281.
8. Lanotte, M. (1984) Terminal differentiation of hemopoietic cell clones cultured in tridimensional collagen matrix *in situ* cell morphology and enzyme histochemistry analysis. *Biology of the Cell* **50,** 107–120.