A Liquid Culture System for Murine Megakaryocyte Progenitor Cells

By Toshiro Nagasawa, Masaki Nakazawa, and Tsukasa Abe

A liquid culture system is described for murine megakaryocyte progenitor cells (CFU-M) in the presence of pokeweed-mitogen-stimulated spleen-cell conditioned medium. There were dose-related responses between the number of CFU-M developed and the number of cells cultured and the dosage of conditioned medium in this liquid culture system. Murine CFU-M were abundantly cloned in this system and the plating efficiency was similar in comparison with that in a plasma clot system. The acetylcholinesterase-positive colonies (more than 4 acetylcholinesterase-positive cells) were clearly seen on day 3 of culture, and they reached a maximum (80.5 ± 10.7/2 × 10⁶ cells) on day 7 of culture. Ultrastructural analyses of megakaryocytic maturation in this system showed that a few megakaryocytes produced platelets that were released in the culture medium on day 5 of culture. This liquid culture system is suitable for the study of the dynamic process of the megakaryocyte-platelet system.

Previous studies have shown that murine megakaryocyte progenitor cells (colony-forming unit megakaryocyte, CFU-M) can be grown in either plasma clot or semisolid agar systems in the presence of suitable stimulating factors. Although these systems are useful to study the proliferation of megakaryocyte from CFU-M, it is, however, difficult to separate intact megakaryocytes and platelets from these culture systems.

We describe a liquid culture system for murine CFU-M that may facilitate ultrastructural, cytochemical, and biochemical analyses of the megakaryocyte-platelet system. In the present report, the liquid culture method and the characteristics of cultured murine CFU-M in the system are described.

Materials and Methods

Animals

BDF, female mice (6–10 wk), obtained from Japan Jackson Laboratories, were employed in all studies.

Preparation of Bone Marrow Cells

Mice were killed by cervical dislocation. Bone marrow cells were aseptically obtained from femurs by flushing the bone marrow cavity with NCTC 109 medium (Microbiological Associate, Walkersville, Md.) supplemented with 5% fetal calf serum (FCS, Flow Laboratories, Rockville, Md.). The bone marrow cells were repeatedly dispersed into a single-cell suspension through 22-gauge needles. The suspensions were prepared at a concentration of 2 × 10⁶ cells/ml.

Conditioned Medium

Spleen cells from BDF, mice at a concentration of 5 × 10⁶/ml were cultured in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 15% FCS and 5% of a 1:10 dilution of reconstituted pokeweed mitogen (PWM, Grand Island Biological Co.). Thirty milliliters of spleen cell suspension were cultured in a 90 mm × 15 mm plastic culture dish (Terumo Co., Japan) in a high humidity 5% CO₂–95% air tissue culture incubator at 37°C for 7 days. Then, the spleen cells were removed by centrifugation at 2000 g for 20 min at 4°C and the supernatant was filtered through a 0.45 μm filter (Millipore Corp., Bedford, Mass.), and stored at −20°C until used.

Liquid Culture System

The following materials were used for the liquid culture system: (1) Bovine serum albumin solution: Bovine serum albumin fraction V (BSA, Sigma Chemical Co., St. Louis, Mo.) was used. An 8% (w/v) BSA stock solution was prepared by the method described by McLeod et al.2 (2) L-Asparagine solution: l-asparagine (Sigma Chemical Co.) was dissolved in NCTC 109 at a concentration of 2 mg/ml, filtered through a 0.45 μm filter, and stored at −20°C until used. This stock solution was diluted to 1:10 by NCTC 109 before being added to the culture system. (3) Bovine embryo extract: Bovine embryo extract solution (BEE) was purchased from Flow Laboratories, distributed in 2-ml amounts, and stored at −20°C until used. (4) α-medium: α-medium was obtained from Flow Laboratories. (5) Supplemented Eagle’s minimum essential medium with Hank’s balanced salt solution (supplemented HMEM): The composition of supplemented HMEM was Dulbecco’s modified Eagle’s medium (Flow Laboratories) 0.086 g, Hank’s balanced salts solution without sodium bicarbonate (Flow Laboratories) 0.99 g, sodium pyruvate (Flow Laboratories) 0.011 g, 1 ml of MEM nonessential amino acids (Grand Island Biological Co., 10 mM, 100X concentrate), 1 ml of L-glutamine (Grand Island Biological Co., 200 mM, 100X concentrate), 0.89 ml of 7% (w/v) NaHCO₃ solution, 15 ml of FCS, and 83 ml of double distilled water.

Fixation and Staining of Megakaryocyte

For the fixation of megakaryocytic colonies and solitary megakaryocytes, which grew on the bottom of the culture dish, 1 ml of 7.5% glutaraldehyde (TAAB Laboratories) in phosphate-buffered

From the Division of Hematology, Institute of Clinical Medicine, The University of Tsukuba, Ibaraki, Japan.

Supported in part by a Grant from Idiopathic Disorders of Hematopoietic Organ Research Committee, the Ministry of Health and Welfare, Japan.

Submitted December 30, 1980; accepted September 23, 1981.

Address reprint requests to Toshiro Nagasawa, M.D., Institute of Clinical Medicine, The University of Tsukuba, 1-1-1, Tenno-dai, Sakura-mura, Niihari-gun, Ibaraki, 305 Japan.

© 1982 by Grune & Stratton, Inc.
0006-4971/82/5902-0007$1.00/0

Blood, Vol. 59, No. 2 (February), 1982
Characteristics of CFU-M in Liquid Culture System

Murine megakaryocyte progenitor cells were abundantly cloned in this liquid culture system. Figure 1 shows a photograph of acetylcholinesterase-positive colonies (megakaryocytic colonies) under the inverted phase microscope. As shown in Fig. 2, megakaryocytic colonies (25.1 ± 4.1/2x10^5 cells, mean±SD, n=10) were clearly identified on day 3 of culture. The numbers of colonies reached a maximum (60.5 ± 10.7/2x10^5 cells) on day 7 and then decreased in number to 38.1 ± 6.7/2x10^5 cells on day 10 of culture.

Bone marrow cells (2x10^6 cells) prior to culturing contained approximately 330 ± 104 (n=15) megakaryocytes. These initial megakaryocytes were gradually decreased in number to 98 ± 68/2x10^5 cells on day 7 of culture, while the initial cells were sustained in the same culture medium other than pokeweed mitogen stimulated conditioned medium.

Platelet Counts

A culture medium on day 5 was gently removed without fixation of cultured cells. Platelet counting in the culture medium was carried out by a phase microscope.

Electron Microscopy

Platelets were obtained from culture medium on day 5 by centrifugation at 1500 g for 20 min. All the cultured cells containing megakaryocytes on day 7 were recovered from the dish with an addition of 2 ml of 2% xylocaine (Fujisawa Pharmaceutical Co., Japan) without fixation. The cells containing megakaryocytes were harvested by centrifugation at 1500 g for 15 min. The ultrastructural analyses of platelets and megakaryocytes were performed by transmission electron microscope using routine techniques. To distinguish technical artifacts from changes produced by in vitro culture, the electronmicroscopic pictures of a megakaryocyte freshly removed from bone marrow and also megakaryocyte sustained for 7 days in the same culture media other than pokeweed mitogen stimulated conditioned media were prepared.

RESULTS
There was a linear relationship between the number of colonies developed and the dosage of conditioned medium added.

The Ultrastructure of Platelets Released in Culture Medium

Platelets were first seen in the culture medium on day 5 of culture. The number of platelets in the culture medium was approximately 10,000–20,000/ml. The platelets were approximately 2.0–5.0 μm in diameter. Electron microscopic analyses of the size distribution for 1000 platelets generated from cultured megakaryocytes were performed and the size distribution was compared with that of normal platelets (Fig. 5).

With respect to size distribution, the cultured platelets were 802 ± 31, 1114 ± 52, 1300 ± 74, and 720 ± 56 (n=10), respectively (Fig. 2), indicating a fourfold increase in the number of megakaryocytes during culture.

Most colonies in the liquid culture system contained 4–16 megakaryocytes, which are small colonies in comparison to the number developed in a plasma clot system (32–128 megakaryocytes) in our laboratory.

A clear linear relationship between the number of cells seeded and the number of colonies developed was found over the range tested from $0.5 \times 10^3$ to $2 \times 10^3$ cells (Fig. 3).

The dose-response of CFU-M to the spleen-cell conditioned medium is shown in Fig. 4. No colonies were detected in the absence of conditioned medium.

![Graph](image)

**Fig. 3.** Relationship between the number of cells plated and the number of CFU-M (O–O) and the total number of megakaryocytes (single cells and the colony forming cells) (O---O) developed. The values represent the means ± SD, n = 4.

![Graph](image)

**Fig. 4.** Relationship of the number of CFU-M (O–O) and the total number of megakaryocytes (single cells and colony forming cells) (O---O) per $2 \times 10^5$ cells to concentration (%) of pokeweed-mitogen-stimulated conditioned medium added to liquid culture system. When the amount of conditioned medium was over 10%, the dosage of α-medium was reduced. The values represent the means ± SD, n = 4.

![Graph](image)

**Fig. 5.** Comparison of platelet size distributions in normal platelets (A) and in platelets generated from culture megakaryocytes in vitro (B). The longest diameter of 1000 platelets were measured under the electron microscope.
LIQUID CULTURE SYSTEM FOR MURINE CFU-M

253

Maturation of Megakaryocyte in Liquid Culture System

The maturation of murine CFU-M in the liquid culture system was studied by electron microscopy. Cells with a detectable demarcation membrane were defined as megakaryocytes without acetylcholinesterase staining. The ultrastructural characteristics of megakaryocytes from day 7 of culture, when the number of CFU-M reached a maximum, could be classified into three types as follows.

Type I (early immature megakaryocyte). The megakaryocyte was small, 15–20 μm in diameter, and had an unlobulated nucleus with a few nucleoli. A few golgi apparatus and the early stage of the demarcation membrane system (DMS) in the cytoplasm were evident (Fig. 7). However, only a few dense granules were seen. This is the youngest megakaryocyte we recognized by electron microscopy.

Type II (immature megakaryocyte). The megakaryocyte of this type was bigger, 20–40 μm in diameter, and had a multilobed nucleus. Chromatin was condensed into nuclear membrane. There were a few dense granules, numerous mitochondria, and rare glycogen granules in the cytoplasm. DMS was developed at several sites along the plasma membrane (Fig. 8 A and B).

Type III (mature megakaryocyte). The megakaryocyte of this type was 30–40 μm in diameter. There were many dense granules and abundant mitochondria. DMS was dilated and extended from multiple sites of the periphery of the megakaryocytes toward the central portion. Platelet fields were observed in the cytoplasm (Fig. 9).

The percentages of types I, II, and III on day 7 of culture were 18.5%, 66.1%, and 15.4%, respectively. Most megakaryocytes in the liquid culture system were immature, while a few megakaryocytes had developed to full cytoplasmic maturation.

Figure 10 shows an electronmicroscopic picture of megakaryocytes that were freshly removed and immediately fixed in same manner. Figure 11 shows an electronmicroscopic picture of initial megakaryocytes that were sustained for 7 days in the same conditioned media other than pokeweed-mitogen-stimulated conditioned media. The degenerative changes, a marginal condensation of chromatin and a swelling of peripheral cytoplasmic zone, were seen in the initial megakaryocytes. These degenerative changes were rarely seen in the megakaryocytes generated in vitro.

DISCUSSION

It is known that the platelets of mammals originate from the cytoplasm of mature megakaryocytes in vivo. Furthermore, there have been a few reports that platelets were clearly visible surrounding the mega-
Fig. 8. (A) Ultrastructural analysis of immature megakaryocytes (type II) from day 7 cultures (×5200). Maximum diameter is 30 μm. The demarcation membrane system (DM) develops in local areas and a few dense granula (G) are seen. (B) High magnification of demarcation membrane system (DM) shown in Fig. 8A (×20,000).

karyocytes of an in vitro culture system. An in vitro culture system for the megakaryocyte-platelet system is necessary to establish a system that permits clonal growth and differentiation of megakaryocyte from CFU-M and platelet production by megakaryocytes.

Murine megakaryocyte progenitor cells can proliferate in plasma clot or semisolid agar systems in the presence of various stimulating factors, namely mitogen-stimulated spleen cell conditioned medium, murine myelomonocytic leukemia cell conditioned medium, and erythropoietin. Metcalf and Johnson reported in 1978 that pokeweed-mitogen stimulated mouse spleen cell conditioned medium could stimulate the four types of hematopoietic cells, granulocyte-macrophagic, erythrocytic, eosinophilic, and megakaryocytic progenitor cells. The pokeweed-mitogen stimulated spleen cell conditioned medium used in this study could also stimulate not only megakaryocytic colony formation but also erythrocytic, granulocyte-macrophagic, and eosinophilic colony formation. In this liquid culture system, erythrocytic and megakaryocytic colonies began to grow up on day 3. Granulocytic colonies rapidly increased on days 4 and 5 of culture. After 7 days of culture, granulocytic and megakaryocytic colonies declined, whereas macrophages became predominant in the culture system.

Plasma clot or semisolid agar systems are useful to study the proliferation from CFU-M. However, to study the dynamic process of platelet release from megakaryocytes, the liquid culture system may be superior to the plasma clot or semisolid agar systems. Also, the liquid culture system may facilitate the analysis of characteristic morphology, DNA content, polyploid mitosis, and cytoplasmic maturation. Furthermore, as shown in Figs. 2 and 3, it is also possible to study clonable CFU-M quantitatively in the liquid culture system. However, the number of
cells per megakaryocyte colony were small, indicating that large and loose colonies may be difficult to retain in this liquid culture system.

Another advantage of the liquid culture system is that it is possible to harvest and study the characteristics of platelets released from cultured megakaryocytes. In this liquid culture system, the yield of platelets was not sufficient for biochemical study. However, with further research it should be possible to increase the yield.

The question may arise that the platelets that appeared in the culture medium were generated from the megakaryocytes in the initial bone marrow preparation. In our preliminary experiments, the platelets were not produced in the supernatant, and also, initial megakaryocytes gradually declined during 5 days when $2 \times 10^7$ cells were kept in same cultured media other than the pokeweed-mitogen stimulated spleen cell conditioned medium (Fig. 2). Also, the electronmicroscopic analyses of these megakaryocytes revealed severe degenerative changes and were not capable of active platelet formation (Fig. 11). These degenerative changes were rarely seen in the mega-

Fig. 9. Ultrastructural analysis of mature megakaryocytes (type III) from day 7 cultures ($\times 6600$). Demarcation membrane systems were fully developed, and platelet fields were seen (arrow). Maximum diameter is 35 $\mu$m.

Fig. 10. Section of megakaryocytes (type II) prepared freshly from bone marrow showing well developed demarcation membrane system and granula system ($\times 5200$).

Fig. 11. The electronmicroscopic picture of megakaryocytes (type II) that were sustained in the liquid culture system for 7 days in the absence of pokeweed-mitogen-stimulated conditioned medium ($\times 4600$). The peripheral condensation of chromatin (arrow) and the swelling of peripheral cytoplasmic zone (two arrows) were seen.
Megakaryocytes generated in vitro. These results suggest that platelets appearing on day 5 came from megakaryocytes generated in vitro. However, the possibility remains that initial megakaryocytes produced platelets in the presence of the pokeweed-mitogen-stimulated spleen cell conditioned medium. It is also possible that pokeweed mitogen may sustain the megakaryocytes in the initial preparation.

The platelet size distribution study under an electron microscope revealed that platelets from megakaryocytes generated in vitro varied from 2.0 μm to 5.0 μm and that the percentage of large platelets was greater than that in a population of normal platelets. However, the fragments of megakaryocytes could not be detected in the culture medium. Furthermore, the ultrastructural analyses of cultured platelets (Fig. 6) showed a morphology similar to normal mouse platelets.

The initial preparation of bone marrow cells contained 210 ± 36 megakaryocytes per 2 × 10^5 cells by Romanovsky staining, which is higher than that reported previously. However, the counting of megakaryocytes in this study was performed on the basis of acetylcholinesterase staining, which allowed the inclusion of small megakaryocytes that are morphologically unrecognized by Romanovsky staining. Furthermore, the murine strain used in this study had a slightly higher number of megakaryocytes (0.09%-0.12% of bone marrow cells) even on basis of Romanovsky staining. These may explain higher percentages of megakaryocytes in the initial preparation of bone marrow cells.

Megakaryocytes differentiated from CFU-M in the liquid culture system were classified into three groups on the basis of the development of the demarcation membrane system under an electron microscope. Ebbe and Stohlman classified megakaryocytes in vivo into the presence of the demarcation membrane system. These findings may explain higher percentages of megakaryocytes in the initial preparation of bone marrow cells.

Megakaryocytes in vitro, electronmicroscopic analysis is superior to the Romanovsky staining because most megakaryocytes in the in vitro culture system appear to be in the young stage which is otherwise difficult to recognize.

Electronmicroscopic studies also showed several differences between megakaryocytes developed in vitro and in vivo. In this liquid culture system, most megakaryocytes were immature while a few megakaryocytes had developed to full cytoplasmic maturation. Nakeff et al. observed similar findings in thin-layer agar cultures of mouse bone marrow. The demarcation membrane proliferated in local areas of the cytoplasm and was not extended fully in some megakaryocytes. Also, the granula system may not generate in parallel with the development of the demarcation membrane system. These findings suggest that other stimulating factors may be necessary for full cytoplasmic maturation.

The megakaryocyte-platelet system is regulated by stimulatory and inhibitory processes in vivo, but it is not clear whether a single or multiple factors are involved. Harker, and also Williams, suggested that two factors may be necessary for the proliferation of megakaryocytes in vivo. However, the role of pokeweed-mitogen-stimulated spleen cell conditioned medium has been unknown in vivo, and also, this factor may influence in vitro differentiation of megakaryocytes in a nonphysiologic manner.

On the basis of the present results, the further improvement of the in vitro short-term cloning assay is necessary for extensive platelet production.

REFERENCES

9. Williams N, Jackson H: Regulation of the proliferation of megakaryocytes.


12. Levine RF, Bunn PA Jr, Hazzard KC, Schlam ML: Flow cytometric analysis of megakaryocyte ploidy. Comparison with Feulgen microdensitometry and discovery that 8N is the predominant ploidy class in guinea pig and monkey marrow. Blood 56:210, 1980


A liquid culture system for murine megakaryocyte progenitor cells

T Nagasawa, M Nakazawa and T Abe