PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 587-590 (1976)

In Vitro Colony Assay for a New Class of Megakaryocyte Precursor: Colony-Forming Unit Megakaryocyte (CFU-M)[1] (39265)

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Following initial reports on the *in vitro* production of megakaryocytes from early precursors in mouse marrow cultured in agar (1) and marmoset marrow in liquid culture (2), two recent studies report the successful growth of megakaryocyte colonies from mouse marrow in agar cultures (3, 4). Since the megakaryocyte precursor compartment remains almost a complete enigma, *in vitro* cell colony assays for this compartment are important analytical tools for examining its composition. Agar cultures, however, cannot presently be used to identify and quantitate megakaryocyte colonies *in situ*. This report presents a modified plasma culture system (5) used successfuly to grow megakaryocyte colonies from mouse marrow. It has permitted the quantitation of these colonies *in situ* in microtiter cultures following their staining for acetylcholinesterase, which has been shown to be a cytochemical marker for rodent megakaryocytes (6, 7). A class of megakaryocyte precursor has been assayed in this system entitled the colony-forming unit, megakaryocyte, or CFU-M.

Materials and methods. Femoral bone marrow cells were obtained from B6D2F1 (C57Bl/6 × DBA/2) female mice (Jackson Lab.) by flushing each femur with 1 ml of phosphate-buffered saline (PBS) supplemented with 2% fetal calf serum (FCS, Flow, Rockville, Maryland). An appropriate dilution of cells in 0.1 ml of PBS was added to a solution containing 0.2 ml of FCS, 0.1 ml of conditioned medium (CM), 0.1 ml of bovine embryo extract (Gibco) diluted 1:4 in L-15 and 0.4 ml of Leibovitz (L-15) tissue culture medium (Gibco). Following the addition of 0.1 ml of bovine citrated plasma (Gibco) and thorough mixing, 0.1-ml aliquots were placed in wells of polyvinyl microtiter plates (Cooke Engineering, Alexandria, Virginia), which were then placed in 100-mm culture plates (Falcon) with a reservoir of water and cultured at 37° in 7.5% CO_2 in air at 100% humidity. CM, which gave equivalent results, has been obtained from mouse L-cells (LCM) and mitogen-stimulated mouse spleen cell cultures. The latter were obtained 4 days after culturing B6D2F1 mouse spleen cells at 10^6/ml in alpha-MEM with 10% FCS to which was added phytohemagglutinin (PHA) (Difco, Detroit, Mich.) to a final concentration of 12 μg/ml or pokeweed mitogen (PWM) (Gibco) to a final dilution of 1:320.

At various times, plasma clots were rimmed and groups of three transferred to 25 × 75-mm glass slides, covered with a piece of PBS-moistened filter paper, fixed for 10 min with 5% glutaraldehyde (Electron Microscopy, Inc.), pressed tightly to the slides, and rinsed (1 min) in 0.1 *M* sodium phosphate. The "direct-coloring" thiocholine method of Karnovsky and Roots (8) was then used to stain for acetylcholinesterase activity following a 3-hr incubation at room temperature in a solution consisting of: 30 mg of acetylthiocholine iodide, 45 ml of 0.1 *M* sodium phosphate, 6 ml of 30 m*M* copper sulfate, 3 ml of 0.1 *M* sodium citrate, and 6 ml of 5 m*M* potassium ferricyanide. Following a 1-min rinse in 0.1 *M* sodium phosphate and postfixation in absolute methanol for 10 min and 50% methanol for 30 sec, slides were counterstained in Harris' hematoxylin (Fisher), "blued" for 2 to 3 min in running tap water, then air-dried, and mounted in Eukitt. The final preparations were scanned microscopically at 500× for acetylcholinesterase-positive colonies containing four or more positive cells. The specificity of mouse megakaryocytes for ac-

[1] Supported by USPHS Grant No. CA13053 from the NCI.

etylcholinesterase was checked in samples of normal marrow collected on glass slides or harvested from plasma cultures immediately after clot formation and stained for acetylcholinesterase as described above, which revealed that the only large cells that were acetylcholinesterase-positive were also morphologically recognizable as megakaryocytes.

Results. The examination of plasma clots during the first 2 days of culture revealed the presence of individual megakaryocytes that resembled those previously described in agar cultures of mouse marrow (3) and that were acetylcholinesterase-positive. By Day 3, however, we observed the first colonies of morphologically recognizable megakaryocytes that were also positive for acetylcholinesterase as shown in Fig. 1. These colonies appeared with increasing frequency thereafter. The maximum number of colonies occurred after 4 days in culture; most had disappeared by Day 7 regardless of the type of conditioned medium used (Fig. 2).

Colony size ranged from 3 to 12 megakaryocytes with a mean of about 6 and was a function, to some extent, of the dose and type of conditioned medium used and the time at which the cultures were assayed. Both relatively large (>30 μm) and small (<10 μm) acetylcholinesterase-positive cells were observed in the colonies with predominantly more of the large cells present at later times in culture. It was also observed that early colonies did not stain nearly as brightly for acetylcholinesterase as those which were more mature.

The relationship between the number of megakaryocyte colonies formed and the number of cells initially plated was determined by plating increasing numbers of nucleated bone marrow cells and scoring the number of acetylcholinesterase-positive colonies present in cultures with PWM-CM 4 days later.

The number of colonies formed varied linearly with the number of cells plated between doses of 0.1 and 2 $\times$ 10^5 cells per culture with the slope of the line indicating that a single colony was formed for every

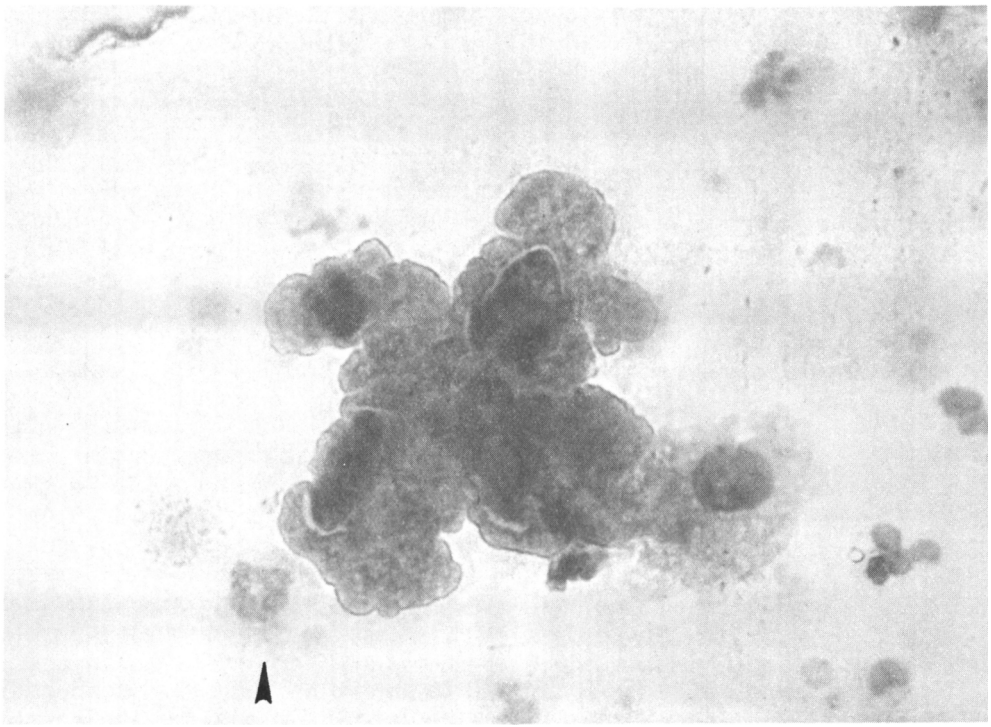

FIG. 1. Photomicrograph of an acetylcholinesterase-positive megakaryocyte colony from mouse bone marrow after 4 days in the plasma culture system. Note myeloid cell at arrow. Magnification 400×.

10,000 nucleated mouse marrow cells initially plated (Fig. 3).

Discussion. The results presented in this report describe an accurate and reproducible assay for a new class of megakaryocyte precursor, the CFU-M. This system permits the histochemical demonstration of megakaryocyte colonies *in situ* in microtiter cultures and makes their quantitation less equivocal and certainly more rapid. The use of microtiter cultures in this assay has the added advantage of requiring very small amounts of conditioning factors and other stimulants. This is a distinct advantage, since some of these materials are difficult to prepare in large quantities and may be somewhat unstable.

The finding that early colonies were weakly acetylcholinesterase-positive may reflect their degree of cellular proliferation, since Jackson (9) has postulated that acetylcholinesterase activity of megakaryocytes *in vivo* may be related inversely to their degree of cellular proliferation and directly to their rate of cytoplasmic maturation.

The number of megakaryocytes in an average colony is relatively small when compared, for instance, to granulocyte colonies *in vitro*. This may be an intrinsic property of megakaryocyte clonal growth with its complex interplay of mitosis and endomitosis, since megakaryocyte colonies formed *in vivo* in the spleen colony assay also contain only small numbers of megakaryocytes.

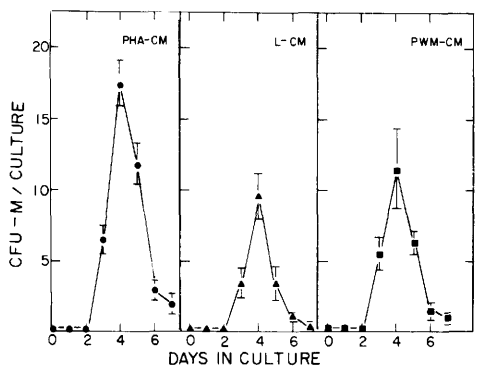

FIG. 2. Number of megakaryocyte colonies (CFU-M) as a function of time in culture after plating 1×10^5 nucleated mouse marrow cells per culture with the addition of 10% CM from either mouse L-cells or mouse spleen cell cultures stimulated with either PHA or PWM (see text). Each point is the average of three to five experiments with three to six replicate cultures in each experiment. Errors shown represent ±1 SE.

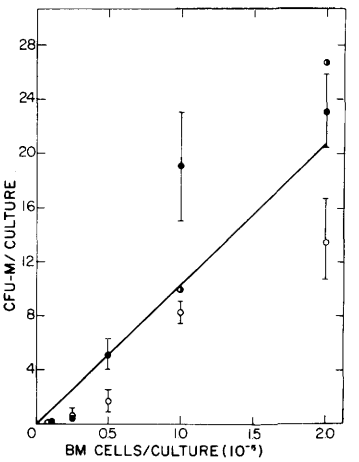

FIG. 3. Number of megakaryocyte colonies (CFU-M) as a function of the number of nucleated mouse marrow cells plated. Each symbol is a separate experiment with each point the average of two to six replicate cultures. Errors shown represent ±1 SE.

Mononuclear phagocytes constitute the only other large hematopoietic cell reported to appear in substantial numbers in semisolid culture systems, which could possibly be confused with megakaryocytes. The latter have been shown to be distinct from mononuclear phagocytes in agar cultures on the basis of morphologic staining, response to thrombocytopoietic serum (3), and electron microscopy (10), as well as polyploid mitoses, DNA content, and acetylcholinesterase staining (4). In addition, we have been able to show that colonies of mononuclear phagocytes (11) grown in the plasma culture system and stained for acetylcholinesterase as described above were clearly acetylcholinesterase-negative.

Preliminary results suggest that addition of thrombocytopoietic mouse serum, obtained as described by Nakeff and Roozendaal (12), influences the appearance of CFU-M in cultures of normal mouse marrow. This confirms similar findings previously reported for the agar culture system (3) and makes it possible to use the plasma culture system to assay for humoral controllers of new platelet production. Furthermore, the use of this assay as a significant new cellular probe with which to examine

the differentiation potential of the pluripotent hematopoietic stem cell into the megakaryocyte-platelet system and to describe very early events occurring in the morphologically-unrecognized megakaryocyte precursor compartment remains intriguing.

Summary. A plasma culture system has been used successfully to grow and quantitate megakaryocyte colonies from mouse bone marrow following their staining for acetylcholinesterase activity *in situ*. Colonies averaging about six acetylcholinesterase-positive cells appear with a peak incidence after 4 days in culture with a plating efficiency of one colony formed for every 10^4 nucleated cells plated.

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Received September 16, 1975. P.S.E.B.M. 1976, Vol. 151.