Proc. Nat. Acad. Sci. USA
Vol. 72, No, 5, pp. 1744–1748, May 1975

Growth of Mouse Megakaryocyte Colonies *In Vitro*

(bone marrow culture/colony-stimulating factor/hemopoietic colonies/conditioned media)

D. METCALF*, H. R. MACDONALD, N. ODARTCHENKO, AND B. SORDAT

Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland

Communicated by F. M. Burnet, February 18, 1975

ABSTRACT **Mouse bone marrow and spleen cells formed pure or mixed colonies of up to 80 megakaryocytes in agar cultures after stimulation by medium conditioned by activated mouse lymphoid cells. Megakaryocytes were identified on the basis of their morphology, polyploid mitoses and DNA content, and high cytoplasmic content of acetylcholinesterase. Megakaryocyte colony-forming cells were relatively small with a peak sedimentation velocity of 4.2 mm/hr. Spleen, lymph node, and thymus cells produced the factor stimulating megakaryocyte proliferation after culture in medium containing 2-mercaptoethanol, with or without added mitogens or allogeneic spleen cells. Peak activity in conditioning medium was associated with the small lymphocyte fractions in mouse spleen.**

Culture systems are now available for the clonal growth in semisolid medium of neutrophilic granulocytes and macrophages (1, 2), eosinophil-like cells (3), and erythropoietic cells (4). Mitogen-activated mouse lymphoid cells have been shown to produce large amounts of the colony-stimulating factor that stimulates granulocytic and macrophage colony growth (5, 6) and the special type of colony-stimulating factor that stimulates eosinophil colony formation (3). The present studies have shown that lymphocyte-conditioned medium is also able to stimulate clonal growth of megakaryocytes *in vitro*.

MATERIALS AND METHODS

Marrow Culture Technique. C_{57}BL/6 marrow cells from two-month-old mice were cultured in 35 mm plastic petri dishes (7). The agar-medium used was a mixture of 4 parts of double strength modified Eagle's medium, 1 part of 3% trypticase soy broth, and 5 parts of 0.6% Difco bacto-agar (the last boiled for 2 min and held at 37°). The composition of the double strength medium was: Dulbecco's modified Eagle's Medium HG Instant Tissue Culture powder H-21 (13.47 g) (Grand Island Biological Co. New York); double-glass-distilled water 215 ml; 3 ml of L-asparagine (20 μg/ml); 1.5 ml of DEAE-dextran (75 μg/ml) (Pharmacia, Sweden, molecular weight = 2×10^6, intrinsic viscosity = 0.70); 0.575 ml of penicillin (200 units/ml); 0.375 ml of streptomycin (200 units/ml); 175 ml of $NaHCO_3$ (2.8% w/v); 250 of unheated fetal calf serum.

In routine cultures, sufficient marrow cells were added to the agar-medium to give a concentration of 75,000 cells per ml and 1 ml volumes of the cell suspension in agar-medium were pipetted into petri dishes containing 0.2 ml of lymphocyte-conditioned medium or material containing colony-stimulating factor. After mixing, culture dishes were allowed to gel and were incubated for 7 days in a fully humidified atmosphere of 10% CO_2 in air.

Preparation of Lymphocyte Conditioned Media. Mouse spleen cells were incubated for 7–14 days at concentrations of 5 to 20 $\times$ 10^6/ml in modified Dulbecco's medium (8) containing final concentrations of 2% fetal calf serum and 50 μM 2-mercaptoethanol. Media were harvested after centrifugation and stored for testing at −20°. The most commonly used lymphocyte cultures were (*a*) mixed spleen cultures containing 25 $\times$ 10^6 C_{57}BL spleen cells plus 25 $\times$ 10^6 strain DBA spleen cells (preirradiated *in vitro* with 1000 rads) or (*b*) 25 $\times$ 10^6 C_{57}BL spleen cells in 20 ml of medium.

Scoring of Cultures. Cultures were scored for colony formation by microscopy at ×40. All aggregates containing large cells were removed intact using a fine pasteur pipette, placed on microscope slides, allowed to dry, and stained with 0.6% orcein in 60% acetic acid. Megakaryocytes were examined at ×400 and the number and size of megakaryocyte colonies was recorded. Aggregates containing two or more megakaryocytes were scored as pure or mixed colonies. The diameter of the large cells was in the range of 60–70 μm, with some very large ones slightly above 80 μm.

Velocity Sedimentation Separation. The technique used has been described in detail elsewhere (9, 14).

DNA Measurements. Microspectrophotometric examination of Feulgen-stained megakaryocyte and macrophage colony cells was performed using a Zeiss UMSP I (Carl Zeiss, Oberkochen, Germany) (10) at a wavelength of 560 mm with a measurement field of 1 μm. Reproducibility of measurements on a single cell was between 1 and 2%.

Chromosome Preparations. Aggregates containing large cells were placed with a minimal amount of surrounding agar on a preheated microscope slide, two to five drops of hot (70–80°) tap water were delivered perpendicularly from a height of 35 cm directly on the aggregates. After air-drying of the water, slides were fixed with acetic acid/methyl alcohol (1:3) and stained with 2% toluidine blue.

RESULTS

Cultures were prepared containing 75,000 C_{57}BL marrow cells and 0.2 ml of medium conditioned by C_{57}BL spleen cells. Although megakaryocytes were present in small numbers in the marrow cell suspensions cultured, inspection of cultures

* To whom reprint requests should be addressed at Walter and Eliza Hall Institute, Royal Melbourne Hospital, P.O. 3050, Australia.

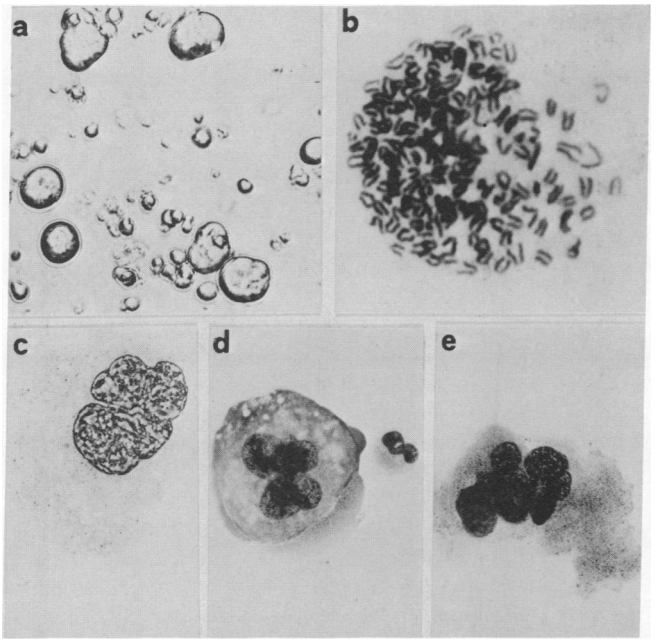

FIG. 1. (a) Portion of a mixed colony *in situ* containing megakaryocytes and smaller cells (phase contrast ×100); (b) A polyploid mitosis (chromosome number approximately 160) from a vinblastine-treated 4-day megakaryocyte colony (toluidine blue ×1250); (c) (d) (e) Three different maturation stages of colony megakaryocytes. Note granulocyte in (d) for size comparison (Leishman ×500).

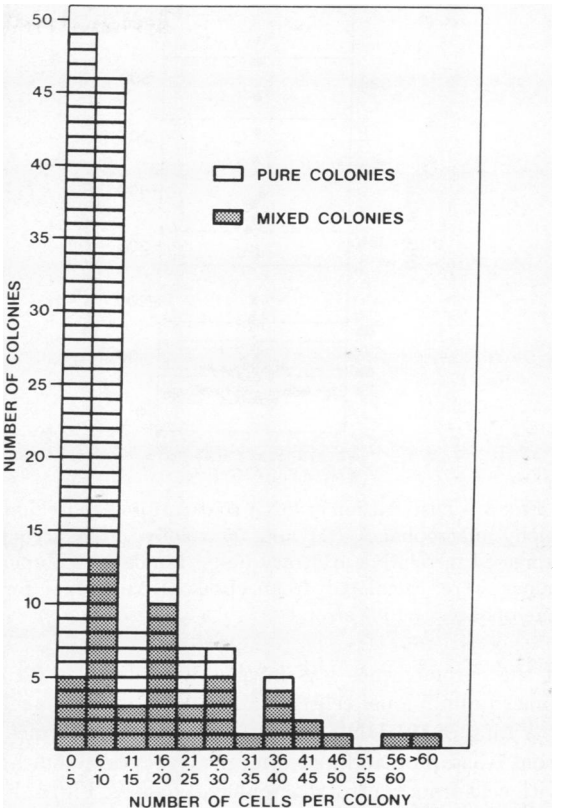

FIG. 2. Number of megakaryocytes in 142 sequentially sampled pure and mixed megakaryocytic colonies in 7-day cultures of mouse bone marrow cells.

after 1–2 days of incubation failed to detect large cells and such megakaryocytes presumably had disintegrated. Small aggregates of newly formed large cells were first detected at 3–4 days of incubation, and at 7 days of incubation 2 to 10 aggregates containing very large cells were present. Two types of aggregates were observed: (*a*) loose aggregates of 2 to 40 cells of uniformly large size ("pure colonies"), clearly separated from the large numbers of granulocytic, mixed, and macrophage colonies which also had developed in the culture dish; or (*b*), small colonies with the general morphology of granulocytic colonies containing, in addition, up to 80 large cells ("mixed colonies") (Fig. 1a). A mixed cell population of this type was never observed in macrophage colonies. Continued culture beyond 7 days failed to increase the size of the pure colonies and the cells in most disintegrated between 7–10 days of incubation. Some mixed colonies showed a progressive increase in the number of large cells between 7 and 10 days of incubation.

In most cultures, "pure" large cell aggregates outnumbered "mixed" large cell aggregates by a ratio of 2:1. A size distribution analysis of the number of large cells in such aggregates (Fig. 2) indicated that at 7 days pure aggregates contained from 2 to 40 large cells and usually contained fewer than 10 cells. Mixed aggregates usually contained more large cells, the number varying from 2 to 80 cells.

The large cells were opaque with smoothly rounded cytoplasmic projections and had a prominent bulge overlying the nucleus. Often, after 7–10 days of incubation such cells were surrounded by a faintly opaque halo of material apparently shed from the cytoplasm. In orcein-stained preparations the large cells had the morphology of megakaryocytes with a single, usually multilobulated nucleus, and abundant cytoplasm. In cultures containing carbon, no phagocytosis by these large cells was observed, in sharp contrast to the behavior of macrophages in adjacent colonies. Megakaryocytes also differed from colony macrophages in being nonadherent to the culture dish. Cytocentrifuged preparations, stained with Leishman, confirmed the megakaryocytic morphology of the large cells (Fig. 1c, d, and e). However, cells with the fully mature morphology of "platelet-shedding" megakaryocytes were not seen, nor were obvious platelets seen in the surrounding agar.

Acetylcholinesterase shows significant activity only in megakaryocytes in marrow populations. Megakaryocyte colonies and control granulocytic and macrophage colonies were placed intact on microscope slides and treated according to the technique of Karnovsky and Roots (11) after cold acetone fixation (12). The cytoplasm of granulocytic and macrophage cells did not stain, while that of colony megakaryocytes showed an intense brownish copper ferrocyanide stain, similar to that of megakaryocytes in control smears prepared from normal mouse bone marrow.

Mitotic activity was observed in aggregates of early megakaryocytes at days 3 and 4 of incubation (maximum mitotic index 16% on day 3). After day 5, mitoses were rarely observed in pure megakaryocytic colonies but were occasionally seen in mixed colonies. Vinblastine (1 μg) was added to 4-day cultures of C_{57}BL marrow cells, and 3 hr later metaphase preparations were made. Small numbers of polyploid mitoses were observed in megakaryocytic cells and an example of an octoploid mitosis is shown in Fig. 1b. The developmental stage

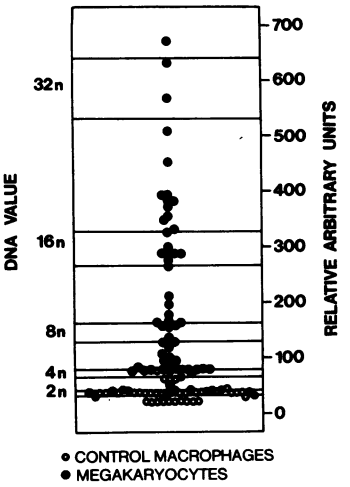

FIG. 3. Distribution of DNA content for 46 individual control colony macrophages (○) and 55 colony megakaryocytes (●) expressed in relative arbitrary units. Limits for polyploid DNA values were calculated from observed 2n values for control macrophages ±10% error.

of the chromosomes was invariably uniform in all chromosomes in individual cells, arguing strongly against cell fusion.

A total of 101 DNA measurements were performed on cells from 7-day pure and mixed megakaryocytic colonies together with cells from control macrophage colonies. Fig. 3 shows that the DNA values for the majority of the megakaryocytes ranged from 4 to 32 n as assessed from the predominantly 2 n reference values of control macrophages (n = haploid DNA

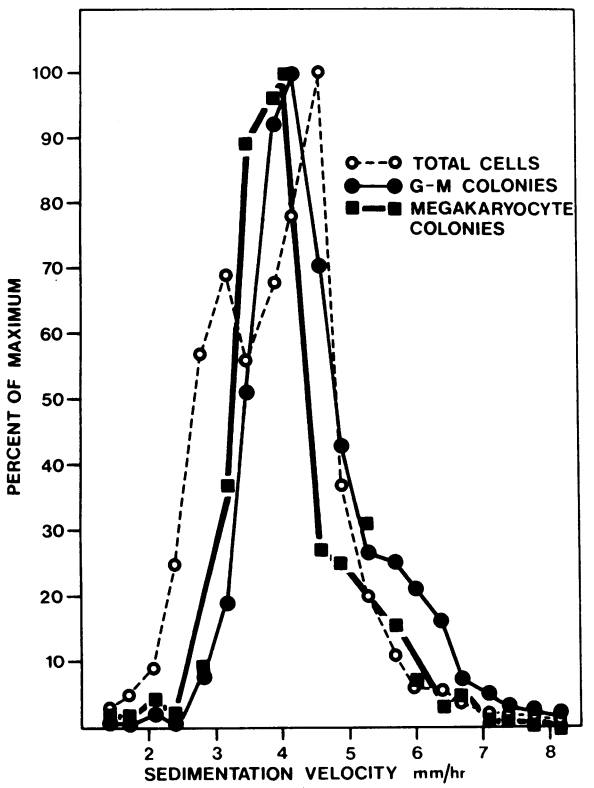

FIG. 4. Velocity sedimentation segregation of mouse bone marrow cells showing distribution of cells forming granulocytic and macrophage colonies (G-M) and megakaryocyte colonies.

TABLE 1. *Relationship between number of marrow cells cultured and number and size of megakaryocyte colonies developing*

Number of cells per dish	Mean no. megakaryocyte colonies	Megakaryocyte colonies per 10^5 marrow cells	Mean colony size, no. of cells
400,000	37 ± 3	9.3	14 ± 12
200,000	14 ± 8	7.0	10 ± 9
100,000	6 ± 2	6.4	10 ± 7
50,000	3 ± 1	4.8	5 ± 4
25,000	1 ± 1	3.0	4 ± 3

All cultures contained 0.2 ml of spleen lymphocyte conditioned medium. Colonies were scored at day 7 of incubation. Data are from four replicate cultures, ±SD.

content). The values observed are similar to those reported by others for rat marrow megakaryocytes (13). The intermediate DNA values observed for some colony megakaryocytes were consistent with the low level of mitotic activity observed in the mixed megakaryotic colonies that were sampled.

Nature of Cells Generating Megakaryocyte Aggregates. C_{57}BL marrow cells were fractionated according to cell volume using velocity sedimentation separation. Fifty thousand cells from each fraction were cultured in replicate with 0.2 ml of C_{57}BL spleen cell conditioned medium and scored at day 7. The results of five experiments were in close agreement and data from one such experiment are shown in Fig. 4. The cells forming granulocytic and macrophage colonies segregated as a single major peak (sedimentation velocity 4.2 mm/hr) (14). Cells forming megakaryocyte aggregates also segregated as a single major peak with the same peak sedimentation velocity (4.2 mm/hr), although the curve was slightly displaced to the small cell regions (slower sedimentation velocity) compared with that for granulocytic and macrophage colony-forming cells. Because of their large size, megakaryocytes sediment extremely rapidly in the sedimentation chamber and can be recovered from the cone fractions harvested prior to the collection of the major marrow cell fractions. However, cultures of these fractions failed to develop megakaryocyte aggregates. Mixing experiments were performed in which cell fractions containing megakaryocytic colony-forming cells were cocultured with fractions lacking such cells. No evidence was obtained of any inhibitory activity of such latter fractions on megakaryocyte colony formation.

A survey of marrow populations from 2-month-old C_{57}BL, C_3H, DBA, SJL, and NZB mice showed that the frequencies of cells forming megakaryocyte aggregates were approximately similar in these strains, varying from 2 to 15/10^5 cells. A roughly linear relationship was observed between the number of marrow cells cultured and the number of megakaryocyte colonies developing. However, as shown in the example in Table 1, a slight but consistent departure from linearity was observed when cultures contained more than 100,000 cells, and colonies in such cultures grew to a larger average size.

Spleen cell suspensions also contained megakaryocyte colony-forming cells but in a lower frequency than marrow (1–5/10^6 cells). An exception was the SJL spleen, which often contained 2–10 colony-forming cells per 10^5 spleen cells. Cultures of thymus, lymph node, peritoneal, and pleural cells failed to develop megakaryocyte aggregates but such cells did

TABLE 2. *Capacity of various tissues to produce conditioned media capable of stimulating megakaryocyte colony formation*

Tissue used to condition medium*	Mean number of colonies stimulated†		
	Neutrophilic and/or macrophage	Eosinophilic	Megakaryocytic
C_{57}BL spleen cells	48	12	3
	136	20	6
C_{57}BL spleen cells + irradiated DBA spleen cells	136	24	9
	96	20	8
C_{57}BL lymph node cells	44	6	0
	35	2	0
C_{57}BL lymph node cells + irradiated DBA spleen cells	300	20	8
	172	48	9
C_{57}BL thymus cells	0	0	0
	0	0	0
C_{57}BL thymus cells + irradiated DBA spleen cells	43	4	3
	96	16	5
C_{57}BL peritoneal cells	21	0	0
	18	0	0
C_{57}BL peritoneal cells + irradiated DBA spleen cells	82	0	0
	70	0	1
C_{57}BL pleural cells	11	0	0
C_{57}BL marrow cells	1	0	0
	1	0	0
C_{57}BL femur shaft cells	36	0	0
	52	0	0
C_{57}BL lung	124	0	0
	116	0	0
C_{57}BL heart	96	0	0
	176	0	0
C_{57}BL kidney	24	0	0
	12	0	0

* All cultures contained 50 μM mercaptoethanol and were incubated for 7 days. Cells were cultured at concentrations of 5×10^6/4 ml of culture; minced tissues were: 1 lung, 1 heart, 1 kidney, or 1 femur per 4 ml of culture.

† Calculated numbers of colonies stimulated by 0.2 ml of medium. Mean data from duplicate cultures.

not inhibit the formation of megakaryocyte aggregates by bone marrow cells.

Production of Conditioned Media Stimulating Megakaryocyte Growth. The following materials containing colony-stimulating factor (15) were found to be inactive in stimulating megakaryocyte colony formation: human serum and urine; mouse serum before or after the injection of endotoxin; extracts of mouse lung, salivary gland, kidney, and whole embryo; media conditioned by mouse lung, kidney, heart, and femur shaft. Media conditioned by C_{57}BL spleen, lymph node, thymic, or marrow cells similarly failed to stimulate megakaryocyte proliferation. However, mouse spleen cells cultured for 7–14 days in the presence of 50 μM mercaptoethanol regularly produced media with the capacity to stimulate megakaryocyte colony formation (Table 2). Additional mitogenic stimulation achieved by the mixed culture of C_{57}BL spleen cells with equal numbers of irradiated DBA spleen cells or the addition of pokeweed mitogen (0.2 ml 1:15 dilution/4 ml; Grand Island Biological Co., New York) or phytohemagglutinin (0.1 ml 1:10 dilution/ml; Wellcome, London) did not further increase the activity of the conditioned media.

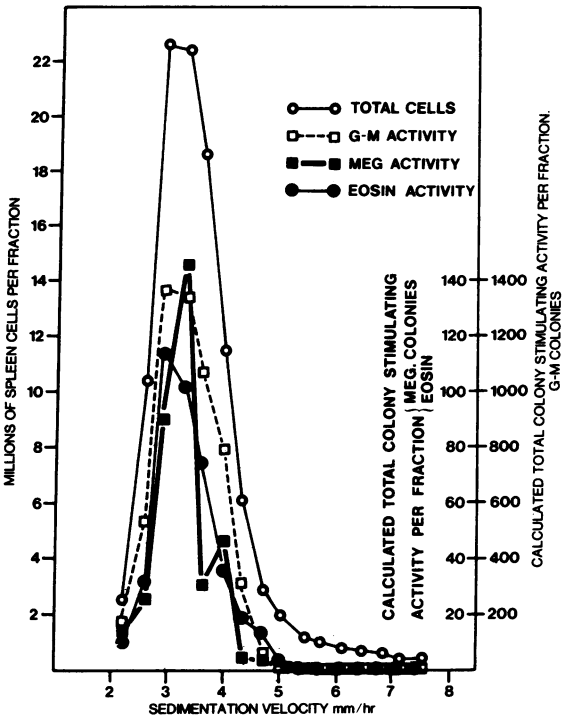

FIG. 5. Velocity sedimentation separation of mouse spleen cells showing distribution of cells which on co-cultivation with irradiated allogeneic cells produced the factors stimulating neutrophilic granulocyte and macrophage (G-M), eosinophil (EOSIN), and megakaryocyte (MEG) colony formation by mouse bone marrow cells. Colony stimulating activity is calculated as total activity for fraction. Also shown is the distribution of total nucleated cells per fraction.

In contrast, culture of C_{57}BL lymph node or thymic cells in the presence of mercaptoethanol did not result in the development of megakaryocyte stimulating activity, although such activity did develop when irradiated allogeneic spleen cells were co-cultured with these cells. C_{57}BL peritoneal cells, cultured with or without mercaptoethanol and allogeneic cells, failed to produce conditioned media with a capacity to stimulate megakaryocyte proliferation. In fact, mixture of C_{57}BL peritoneal cells with C_{57}BL spleen cells suppressed the capacity of the latter cells to produce active conditioned media. Addition of mercaptoethanol to cultures of non-lymphoid tissues such as marrow cells, lung, femur shaft, heart, or kidney failed to produce media with megakaryocyte-stimulating activity.

C_{57}BL spleen cells were fractionated by velocity sedimentation and 2×10^6 cells from each fraction were co-cultivated in 4 ml of medium containing mercaptoethanol with 2×10^6 DBA spleen cells, irradiated *in vitro* with 1000 rads. Irradiated DBA spleen cells alone were found to be unable to produce active media. After 14 days of incubation the supernatant fluids were harvested and assayed for their capacity to stimulate neutrophilic, macrophage, eosinophilic, and megakaryocytic colony formation. The results of one such experiment are shown in Fig. 5. In agreement with previous data showing that mitogen-stimulated lymphocytes are a rich source of the

factors stimulating neutrophilic and macrophage colonies (5, 6) and the sole source of the factor stimulating eosinophil colony formation (3), the peak colony stimulating activity for these colony types coincided with the peak of small lymphocytes in the spleen cell fractions (peak activity 2.9–3.3 mm/hr). Megakaryocyte colony-stimulating activity was also maximal in media harvested from cultures of the same spleen cell fractions, suggesting strongly that spleen small lymphocytes were also the cells producing the material stimulating megakaryocyte proliferation.

In all these experiments the capacity of a conditioned medium to stimulate megakaryocyte proliferation was invariably associated with a capacity to stimulate eosinophil colony formation.

The active factor in spleen lymphocyte conditioned medium stimulating megakaryocytic colony formation was non-dialyzable and was inactivated progressively by heating to 65° or higher temperatures.

DISCUSSION

The giant cells present in the pure or mixed aggregates grown from mouse marrow cells appear to be typical mouse megakaryocytes because of their characteristic morphology, polyploid mitoses and DNA content, and their strong reactivity for acetylcholinesterase. Preliminary electron microscopic studies have also indicated that these cells have a morphology consistent with that of megakaryocytes. The presence of similar cells has been reported in agar cultures of mouse marrow cells (16). However, it is possible that megakaryocytes developing *in vitro* may not develop the full cytoplasmic maturation associated with extensive platelet production.

Megakaryocyte colonies developed in two forms—aggregates composed solely of megakaryocytes, and mixed aggregates containing cells with a continuous size spectrum, the smallest of which resembled granulocytic cells. The nature of these latter cells requires further investigation. Since the velocity sedimentation data indicated that typical megakaryocyte colony-forming cells were not much larger than small lymphocytes, it is possible that mixed colonies represent the full cellular sequence involved in the generation of mature megakaryocytes. The "pure" megakaryocyte colonies may represent progeny of more mature cells in the sequence, capable of only two or three divisions.

The frequency of megakaryocytic colony-forming cells in the marrow (2–15/10^5 cells) is approximately one-tenth of that of other *in vitro* colony-forming cells and is in general agreement with the relative infrequency of megakaryocytes in marrow populations. The size of megakaryocyte colonies was small compared with that of granulocytic or macrophage colonies grown in agar. This is also in agreement with the apparently restricted capacity for proliferation of this cell lineage as judged from the small size of megakaryocytic colonies developing from marrow cells in the spleens of irradiated mice (17). However, the conditioned media used had relatively weak activity and possibly the frequency and size of megakaryocyte colonies might be increased by stronger stimulation.

Megakaryocyte proliferation appeared to require stimulation by a factor produced by mitogen-activated lymphoid cells, the specific cells involved probably being small lymphocytes. The consistent association in lymphocyte-conditioned media of the capacity to stimulate both eosinophil and megakaryocyte proliferation requires further investigation. While it seems improbable that platelet production *in vivo* should be dependent on regulation by lymphocyte products, increased levels of megakaryocytes are common in the spleens of mice bearing antigenic tumors and in NZB mice showing chronic autoimmune stimulation of lymphopoiesis. The two cell lineages may therefore have unsuspected functional interrelationships.

The present cloning system represents a powerful new tool for analyzing the nature and control of megakaryocyte formation.

We are indebted to Dr. C. Leuchtenberger, Miss H. Campbell, Dr. S. Fakan, Mr. T. Keneklis, and Miss L. Wallace for technical assistance in these experiments. We are indebted to Dr. H. Cottier, Bern, for the use of the Zeiss UMSP I instrument. This work was supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria; the National Cancer Institute, Washington, Contract no. NO1-CB-3584; The World Health Organization, and the Swiss National Foundation for Scientific Research.

1. Bradley, T. R. & Metcalf, D. (1966) *Aust. J. Exp. Biol. Med. Sci.* **44**, 287–300.
2. Ichikawa, Y., Pluznik, D. H. & Sachs, L. (1966) *Proc. Nat. Acad. Sci. USA* **56**, 488–495.
3. Metcalf, D., Parker, J., Chester, H. M. & Kincade, P. W. (1974) *J. Cell. Physiol.* **84**, 275–290.
4. Stephenson, J. R., Axelrad, A. A., McLeod, D. L. & Shreeve, M. M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1542–1546.
5. Parker, J. W. & Metcalf, D. (1974) *J. Immunol.* **112**, 502–510.
6. Parker, J. W. & Metcalf, D. (1974) *Immunology* **26**, 1039–1049.
7. Metcalf, D. (1970) *J. Cell. Physiol.* **76**, 89–100.
8. Cerottini, J. C., Engers, H. D., MacDonald, H. R. & Brunner, K. T. (1974) *J. Exp. Med.* **140**, 703–717.
9. Miller, R. G. & Phillips, R. A. (1969) *J. Cell. Physiol.* **73**, 191–201.
10. Sordat, M., Sordat, B., Cottier, H., Hess, M. W., Riedwyl, H., Chanana, A. & Cronkite, E. P. (1972) *Exp. Cell Res.* **70**, 145–153.
11. Karnovsky, M. J. & Roots, L. (1964) *J. Histochem. Cytochem.* **12**, 219–221.
12. Jackson, C. W. (1973) *Blood* **42**, 413–421.
13. Paulus, J. M. (1968) *Exp. Cell Res.* **53**, 310–313.
14. Metcalf, D. & MacDonald, H. R. (1975) *J. Cell. Physiol.*, in press.
15. Metcalf, D. (1973) *Exp. Hematol.* **1**, 185–201.
16. Nakeff, A., Van Noord, M. J. & Blansjaar, H. (1974) *J. Ultrastruct. Res.* **49**, 1–10.
17. Metcalf, D. & Moore, M. A. S. (1971) *Haemopoietic Cells* (North Holland, Amsterdam).