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Establishment of Megakaryoblastic Cell Lines by Coinfection of Abelson Murine Leukemia Virus and Recombinant SV40-retrovirus

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Murine embryonic cells including yolk sac prepared from 8-day embryos were co-infected with Abelson murine leukemia virus (A-MuLV) and/or a recombinant retrovirus containing large T and small t antigens, and early region of simian virus 40 (M-SV40). By coinfection with A-MuLV and M-SV40, megakaryoblastic cells were obtained in addition to mast cells and fibroblastic cells. However, following infection with A-MuLV or M-SV40 alone, no megakaryoblastic cells were detected, although mast cells and/or fibroblastic cells developed. The same results were obtained in several experiments. By single-cell transfer, 6 acetyl-cholinesterase (AchE)-positive clonal cell lines were established. Characteristics of megakaryocytes, such as AchE, glycoproteins IIb and IIIa, and platelet peroxidase were detected in two representative cells (C1 and C8). More significant changes expressing differentiation were observed following treatment with phorbol myristate acetate or pokeweed mitogen-stimulated murine spleen cell conditioned medium, although release of platelets was not observed. This is the first report showing development of megakaryocytic cells as the result of coinfection with retroviruses.

Key words: Megakaryoblastic cell line — Abelson virus — SV40-retrovirus — Megakaryocytopoiesis — Mast cell

Megakaryocytopoiesis is responsible for the production of platelets, which are essential to the initiation of hemostasis and the maintenance of the vascular system. Permanent cell lines of megakaryocytic lineage would be an efficient tool in the investigation of mechanisms regulating megakaryocytopoiesis, especially in their biochemical analysis. Up to the present, several rat and human megakaryoblastic leukemia cell lines have been established.[1–3)]

We attempted to establish new hemopoietic cell lines by infection with retroviruses. Abelson murine leukemia virus (A-MuLV) was shown to induce a variety of hemopoietic tumors *in vivo*, and the transformation of lymphoid cells, fibroblastic cells and mast cells *in vitro*.[4–6)] In addition, it is well known that the early region encoding the large T and small t antigens of SV40 induces the transformation of cells.[7)] As the target of retroviruses, we chose murine embryo with yolk sac, where hemopoiesis was first observed from the 8th day of gestation.[8)] At this stage pluripotent hemopoietic stem cells and granulocyte-macrophage precursor cells were already present.[9)] By coinfection of murine 8-day embryonic cells with A-MuLV and M-SV40, we obtained megakaryocytic cell lines, as well as mast cell and fibroblastic cell lines. These cell lines were identified as megakaryocytic by examination of various specific markers.

MATERIALS AND METHODS

Preparation of Murine Embryonic Cell Suspension Whole 8th-day embryos of inbred pregnant ICR mice (Shizuoka Experimental Animal Center, Shizuoka) were teased with forceps, and were digested with 0.05% trypsin and 0.025% ethylenediaminetetraacetic acid (EDTA). The suspension was repeatedly aspirated with a Pasteur pipette, followed by passage through a stainless steel mesh. The cell suspension thus obtained was used to establish cell lines.

Viruses NIH 3T3 cells producing A-MuLV with Moloney-MuLV (M-MuLV) helper virus, a gift from Dr. N. Rosenberg (Univ. of Tuft), were

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maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). The culture medium conditioned by these cells was harvested and centrifuged at 1,500*g* for 10 min. The supernatant was Millipore-filtered and stored at −80° prior to use as the A-MuLV stock.

The murine retrovirus vector pEVXT[7] containing the SV40 early region, and large T and a small t antigen sequences, was obtained from Dr. M. Botchan (Univ. of California, Berkeley). The retrovirus packaging cell line $\phi 2$[10] was obtained from Dr. R. Mulligan (Whitehead Institute). The pEVXT was transfected into $\phi 2$ cells using calcium phosphate[11] with pSV2 neo[12] as a coselectable marker. The transfected $\phi 2$ cells were selected with 800 μg/ml of geneticin (G418, Gibco Laboratories). Twenty G418-resistant clones were isolated, and screened for the production of recombinant virus (M-SV40) by focus formation in Rat 2 cells.[13] As a result, a high-titer virus-producing clone, pEVXT14, was obtained and maintained in DMEM with 10% FCS. The viral stock was obtained in the same manner as described above. Both viral stock solutions were assayed by focus formation in Rat 2 cells. A-MuLV and M-SV40 stocks had titers of 3.7×10^4 and 1.1×10^3 focus-forming units/ml, respectively.

Virus Infection Murine embryonic cells (1×10^7 cells/ml) were incubated in DMEM with 10% FCS in the presence of 8 μg/ml of Polybrene (Sigma Chemical, St. Louis, Missouri), in order to facilitate virus adsorption on the cells. After 5 hr of incubation at 37°, the culture medium was removed by centrifugation, and the cell pellet was resuspended at a concentration of 5×10^7 cells/ml in DMEM. A mixture of A-MuLV (2 ml) and M-SV40 (2 ml) was added to 0.2 ml of the cell suspension. After 2 hr of incubation at 37°, the virus suspension was removed by centrifugation, and replaced with fresh DMEM containing 10% FCS. The virus-infected cells were seeded on 35 mm plastic dishes at a concentration of 2×10^6 cells/3 ml/dish. Half the culture medium was replenished every 3–5 days. In addition, the cells were transferred at appropriate intervals to avoid overgrowth.

Cell Cloning and Establishment of Cell Lines After about 8 weeks in culture, acetylcholinesterase (AchE)-positive cells were detected among the virus-infected non-adherent cells. One thousand non-adherent cells were plated onto 35 mm plastic dishes in DMEM supplemented with 15% FCS and 1.2% methylcellulose (Fisher Scientific Co., Pittsburgh, Pennsylvania). Single isolated cells were transferred to each well of a flat-bottomed microtiter plate (Sumitomo Bakelite Co., Tokyo) containing 150 μl of DMEM with 10% FCS, using a fine Pasteur pipette attached to a micromanipulator (Narishige Scientific Instrument Lab., Tokyo) under direct microscopic visualization. Each growing colony was transferred to a well of a 24-well plate (Sumitomo Bakelite Co.) and thence eventually to 25 cm^2 culture flasks.

Cloned cell lines were expanded and frozen. The frozen cells could be successfully thawed.

Light and Fluorescence Microscopy Representative clones, designated C1 and C8, were spun in a Shandon cytocentrifuge (Cytospin; Shandon Southern, Sewickley, Pennsylvania), and were used for cytochemical and immunofluorescence staining. Preparations were stained with May-Grünwald-Giemsa, and for acethylcholine-esterase as previously described.[14] Immunofluorescence staining for T-antigen of SV40 was carried out as follows. Briefly, cytocentrifuge preparations of C1 and C8 cells were fixed with methanol and incubated with monoclonal antibody against SV40 T-antigen (Oncogene Science, Inc., Malvern, Pennsylvania) at 37° for 30 min. After extensive rinsing in PBS, the slides were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cooper Biomedical, Inc., New York) at 37° for 30 min and then thoroughly rinsed. The preparations were observed with a fluorescence microscope (Nikon Co., Tokyo).

Electron Microscopy Cultured cells were divided into two aliquots, one of which was fixed for 1 hr at 4° with 2.5% glutaraldehyde for subsequent ultrastructural and myeloperoxidase examinations. The other aliquot was washed with PBS and fixed in a mixture of 2% paraformaldehyde, 0.5% glutaraldehyde and 1% tannic acid for detection of platelet peroxidase (PPO).[15] All specimens were post-fixed with osmium tetroxide, dehydrated and embedded in epoxy resin.

Northern Blot Analysis Total cellular RNA was extracted from 1×10^8 C1 cells using 10m*M* vanadyl-ribonucleoside complexes (Bethesda Research Laboratories, Inc., Massachusetts). Poly-$(A)^+$ RNA was prepared by one cycle of chromatography on oligo(dT)-cellulose (Collaborative Research, Inc., Type 3, Lexington, Massachusetts); electrophoresed on 1% denaturing formaldehyde agarose gels, transferred to nitrocellulose filters,[16] and hybridized with ^{32}P-labeled v-*abl* probes (Oncogene Science, New York).

Western Blot Analysis C1 cells (2×10^7 cells/1.6 ml) and human platelets (final conc. 0.5 mg/ml) as a control were treated with 1% Triton X-100/5 m*M* EDTA solution and centrifuged. The supernatants (100 μl) were incubated for 2 hr at 4° with rabbit antibodies raised against human platelet membrane (40 μg/ml), which were shown to react mainly with platelet glycoproteins (GP)IIb and IIIa and weakly with GPIb and myocin.[17] Goat anti-rabbit IgG (40 μg/ml, TAGO Inc., Burlin-

game, California) was added to the solutions and incubation was continued for another 2 hr. Immunoprecipitates were spun down with PBS and dissolved in 10 μl of Laemmli's sample buffer.[18] Whole samples were subjected to sodium dodecylsulfate gel electrophoresis, and separated proteins were transferred to a nitrocellulose membrane.[17, 19] GPIIb and IIIa were immuno-stained with the antibody against platelet membrane. The antibody used here was confirmed to react with GPIIb and IIIa from rat platelets (data not shown).

Results

Establishment of Cell Lines After about 2 weeks of coinfection of 8-day embryonic cells with A-MuLV and M-SV40, mast cells (mast-2) appeared among the adherent cells, and have now been maintained for more than 9 months. At 4 weeks, non-adherent cells appeared among the adherent cells in some flasks. Within a further 2 weeks, AchE-positive cells were identified among the non-adherent cells derived from a flask, and the number of positive cells reached 70–80% of the total cells at 12 weeks. Nine clonal cell lines were established from 50 single cell cultures, 6 of which were AchE-positive; the rest were fibroblastic clones. Two AchE-positive clones, termed C1 and C8,and one fibroblastic clone, termed C9, grew predominantly and we used them in further experiments.

From the cultures infected with A-MuLV alone, mast cells (mast-1) and fibroblastic cells appeared. The cultures infected with M-SV40 alone yielded only fibroblastic cells (T-fibroblastic cells). Both these mast cells and fibroblastic cells have grown for more than a year in the DMEM with 10% FCS. Hemopoietic cells other than mast cells were not obtained from the embryonic-cell cultures infected with A-MuLV or M-SV40 alone.

These experiments were repeated three times and gave similar results.

Table I. The Expression of A-MuLV-genes and/or T-antigen in, and the Release of Viruses from Cell Lines

Name of cells	Expression		Release of viruses
	A-MuLV-gene	T-antigen	
C1	+	+	+
C8	+	+	+
C9	+	+	+
mast-1	+	−	+
mast-2	+	−	+
T-Fib	−	+	−

C1 and C8 cells (megakaryoblastic cells), C9 cells (fibroblastic cells) and mast-1 cells were obtained from murine 8-day embryonic cells by coinfection with A-MuLV and M-SV40. Mast-2 cells and T-Fib (fibroblasts) were obtained from the murine 8-day embryonic cells following infection by A-MuLV or M-SV40 alone. The expression of A-MuLV genes or T-antigen in these cells was investigated by a northern blotting analysis with cDNA complementary to the v-*abl* sequence or by using an immunofluorescence staining technique with monoclonal antibody against SV40 T-antigen, respectively. The release of viruses from these cells was examined by focus formation in Rat 2 cells; positive (+), negative (−).

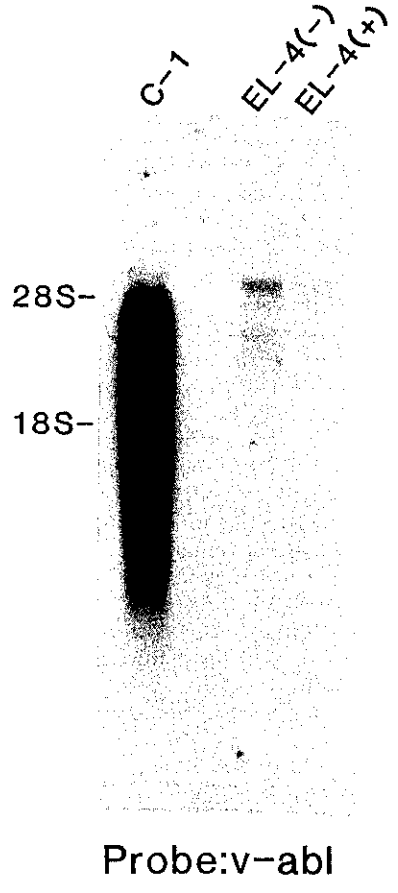

Fig. 1. Northern blot analysis. The expression of A-MuLV-gene was examined. Poly(A)-selected mRNAs (10 μg) from C1 cells and EL-4 cells stimulated with (+) or without (−) pokeweed mitogen were electrophoresed on agarose gel, transferred to nitrocellulose filters and hybridized with ^{32}P-labeled DNA probes of v-*abl*.

Expression of A-MuLV- and/or M-SV40-genes We investigated whether the C1, C8 and C9 continuous cell lines were truly infected with A-MuLV and/or M-SV40, producing these viruses. By immunofluorescence staining, using a monoclonal antibody, T-antigen was detected in C1, C8, C9 and T-fibroblastic cells (Table I). The expression of A-MuLV-gene, using a northern blot analysis with cDNA complementary to the v-*abl* sequence, was identified in C1, C8, C9, mast-1 and mast-2 cells. As shown in Fig. 1, A-MuLV transcripts were recognized in C1 cells but not in EL-4 cells. It was found that supernatants from cultures of C1, C8, C9, mast-1 and mast-2 cells were able to transform Rat 2 cells, indicating that virus was released by these cell lines.

Morphology and Cytochemistry of C1 Cells Approximately 70–80% of the total cells were positive for AchE to various degrees, although most of them were strongly positive (Fig. 2). The attached C1 cells were small and uniform in size, possessed basophilic agranular cytoplasm and a single round nucleus, and displayed a high nucleus-to-cytoplasm ratio. On treatment with 16n*M* phorbolmyristate acetate (PMA) or 10% (v/v) pokeweed mitogen-stimulated murine spleen cell-conditioned medium which was prepared in serum-free culture (PWM-SPCM),[20] the C1 cells became more mature, although platelet release was not detected.

Ultrastructural Morphology and Cytochemistry of C1 Cells C1 cells were round and measured approximately 20 μm in diameter, had round or oval nuclei, and were predominantly euchromatic, with one or two large nucleoli. The cytoplasm contained a well-developed Golgi apparatus, many mitochondria and short rough endoplasmic reticulum, but a demarcation membrane system was not observed. A few small cytoplasmic granules with cores were also observed. The nuclear envelope and rough endoplasmic reticulum were positive for PPO (Fig. 3). On the other hand, myeloperoxidase (MPO) was not detected in the cells. These findings confirmed the cells to be megakaryocytes. On culture with PWM-SPCM, increase in cell size up to 40 μm in diameter was observed in 20–30% of C1 cells (Fig. 4). The nuclei of these cells were irregularly lobulated or multiple.

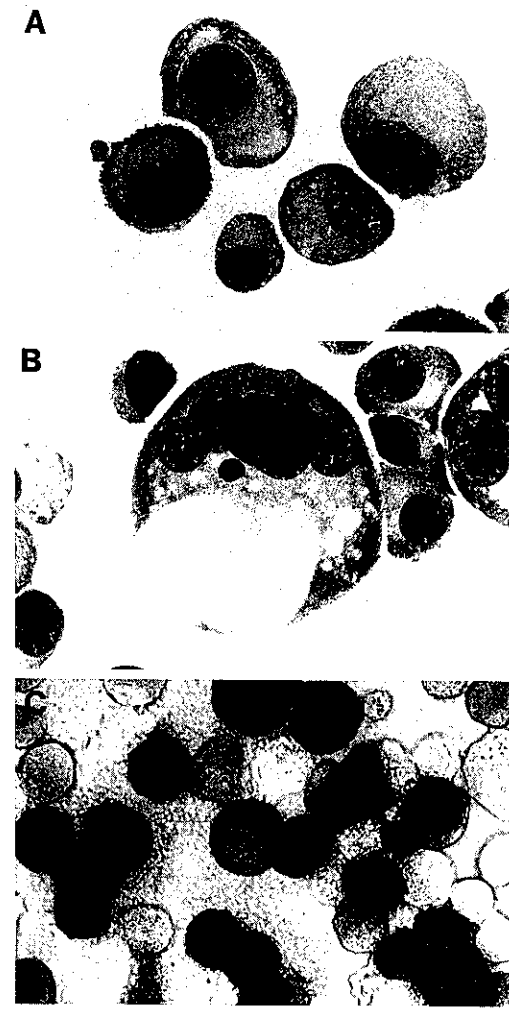

Fig. 2. May-Grünwald-Giemsa and acetylcholinesterase staining. Cytocentrifuge samples of C1 cells were stained with May-Grünwald-Giemsa (A, B) and stained for acetylcholinesterase (C). C1 cells treated without (A, C) or with PMA (B).

The nucleoli were small, and the cytoplasm was widened. Granules with cores were sparse but more prominent than in PWM-SPCM-untreated cells. These findings again indicated that the C1 cells cultured in the presence of PWM-SPCM were more mature than those without it.

Surface Marker for C1 Cells GPIIb and IIIa, which are prominent membrane components

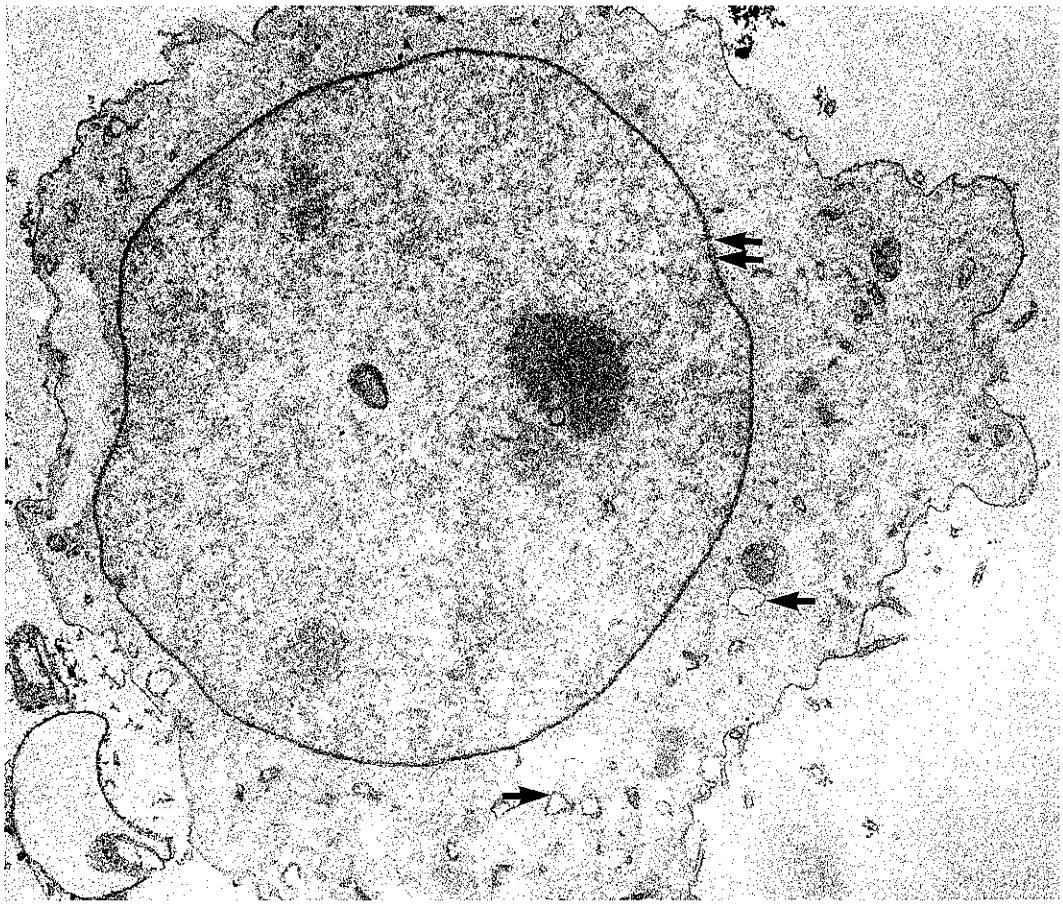

Fig. 3. Electron microscopic morphology of the C1 cells. Platelet peroxidase reaction in the C1 cell. The nuclear envelope (double arrows) is positive for this reaction, as well as the short rough endoplasmic reticulum (arrow). No counter-staining. ×7,400.

of platelets and megakaryocytic lineage cells, were examined by immunoblotting techniques. In the membrane fraction of C1 cells, two bands were recognized whose molecular weights corresponded with those of GPIIb and IIIa (Fig. 5).

C8 cells exhibited a less differentiated phenotype than C1 cells, and further differed from them in the following respects: their doubling time was longer than that of C1 cells (about 48 hr); they became firmly attached to the flask and took up an elongated morphology; the number of attached cells was larger; and PPO activity was not detected (data not shown).

These data demonstrated that C8 cells were more immature megakaryoblasts than C1 cells.

Discussion

By coinfection with A-MuLV and M-SV40, murine megakaryoblastic cell lines were established from embryonic cells. The results were reproducible in several repeated experiments. The cells clearly showed the characteristics of megakaryocytic cell lineage, such as AchE activity, GPIIb-IIIa, and PPO reaction. Among them, AchE is a specific and early marker of the thrombocytic series of rodent hemopoietic cells.[14, 21, 22] However, other

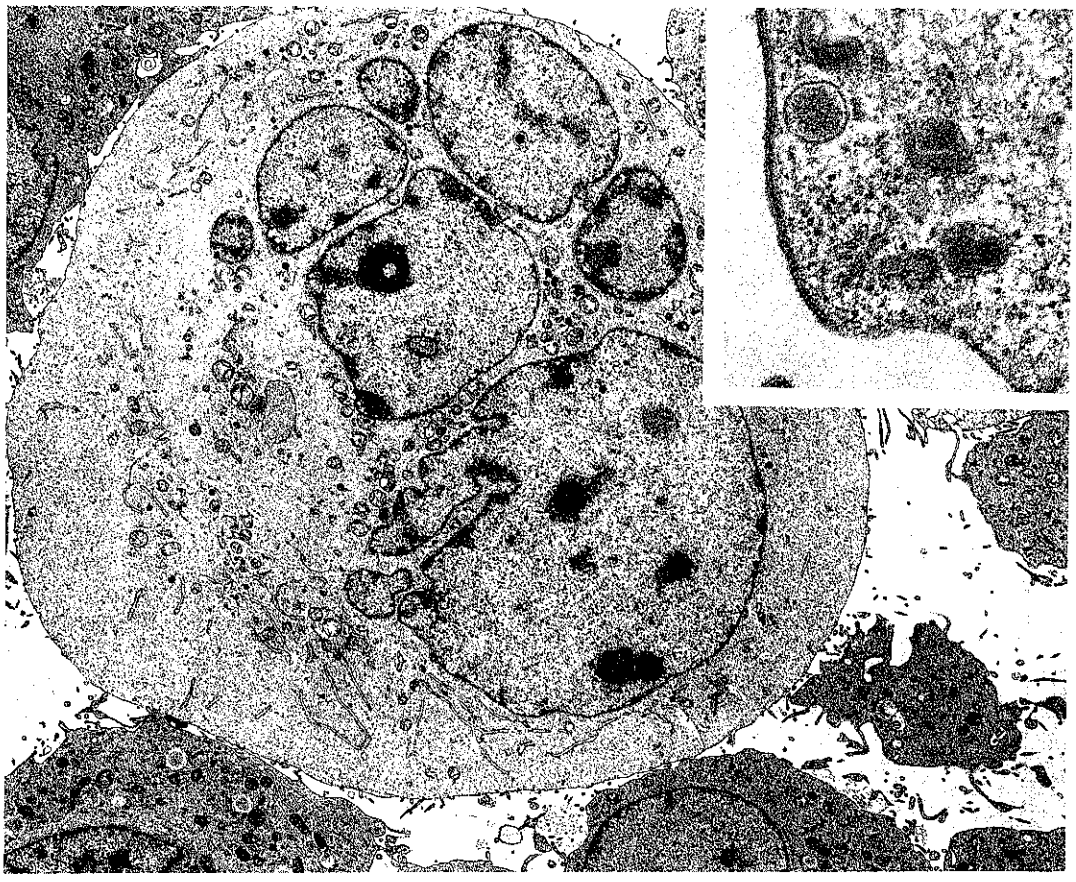

Fig. 4. Electron microscopic morphology of C1 cells cultured with PWM-SPCM. The multinuclear cells have small nucleoli and a wide cytoplasm. UA-LC stain (×3,100). The insert shows small granules with a central core (×52,000).

kinds of cells, especially those involved in neurotransmission are known to contain AchE.[23] Therefore, further detailed studies to fully identify C1 cells were performed. These cells showed the ultrastructural peroxidase activity characteristic of PPO, but not MPO. The antigenic markers, GPIIb and IIIa were also present in their membranes. Slight development of the demarcation membrane system and α-granules were first observed following the addition of PMA and PWM-SPCM. These results indicated that C1 cells were megakaryoblastic cells at an early stage of development that were capable of maturation and differentiation. Following the addition of PMA or PWM-SPCM to the culture medium, features of maturation of C1 were observed. However, complete maturation to the platelet-releasing stage has not yet been attained. It is necessary to examine whether further maturation can be achieved by treatment with certain hemopoietic factor(s) or reagent(s).

The mechanisms involved in establishing hemopoietic cell lines remain to be clarified. It has been reported that at least two oncogenes are needed to transform primary rat embryo fibroblasts into tumorigenic cells[24–26]; namely, one gene having an immortalizing function and another gene capable of inducing malignant cell transformation. Oliff *et al.*[27] have shown that immortal cells, which were derived from Friend murine leukemia virus-infected mice, were converted into growth factor-independent and tumorigenic cells by

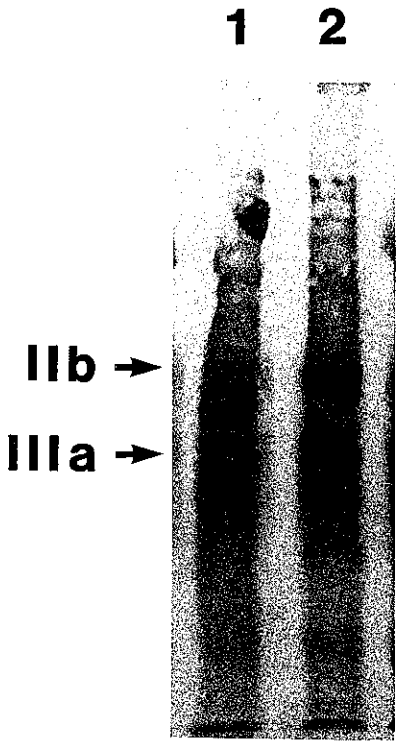

Fig. 5. Western blot analysis. The presence of GPIIb and IIIa in the membranes of C1 cells was investigated. Triton X-100-soluble proteins were isolated from the membranes of C1 cells, subjected to SDS-PAGE, and blotted onto nitrocellulose membranes. The blot was incubated with antiserum against GPIIb and IIIa and detected with a second antibody as described in "Materials and Methods." C1 cells (lane 1) and human platelets (lane 2) were used as controls.

infection with A-MuLV. Using coinfection with A-MuLV and M-SV40, we succeeded in inducing the transformation of megakaryoblastic cells, as well as mast cell and fibroblastic cells. It is well known that A-MuLV causes various hemopoietic tumors *in vivo*, including myelomonocytic leukemias, T-cell lymphomas and mastocytomas, but this virus primarily induces the transformation of B lymphocyte lineage cells *in vivo* and *in vitro*.[6] Recently, transformation studies *in vitro* demonstrated that A-MuLV induced interleukin 3 (IL-3)-independent mast cell lines.[4,5] SV40 is not oncogenic in its natural host species, and rarely induces stable transformation in murine cells. However, a previous paper[7] revealed that recombinant SV40 retrovirus was highly efficient in the transformation of mouse rat cells.

As target cells, murine 8-day whole embryonic cells were used for the following two reasons; murine hemopoiesis is first observed in the yolk sac at this stage,[8] and actively growing feeder layers at this stage may be required for the completion of stable transformation. As was expected, feeder layer-rich cultures were obtained, and these seemed to be indispensable to our success. Megakaryocytic cell line establishment was not successful when we used 13-day fetal liver cells or adult bone marrow cells (data not shown).

This is the first report to describe establishment of murine megakaryoblastic cell lines. In addition, these were the first megakaryoblastic cell lines to be established by means of infection with retroviruses, and they may become useful tools in the study of megakaryopoiesis. Furthermore, transformation by coinfection with retroviruses may provide some clues in the analysis of oncogenesis in hemopoietic cells.

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