Identification and Conditions for Selective Expression of Megakaryocytic Markers in Friend Erythroleukemia Cells

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Friend murine erythroleukemia cells (MELCs) have been re-evaluated in terms of their nature and potential pathways of differentiation. MELC induced with 5 mmol/L hexamethyl-bisacetamide (HMBA), in addition to expression of known markers of the erythroid phenotype, were also found to exhibit traits of the megakaryocytic lineage. Erythroid differentiation was shown by the typical synthesis and accumulation of hemoglobin (Hb); megakaryoblastoid differentiation of MELCs upon induction was shown by increased specific activity of acetylcholinesterase (AChE). Incubation of MELCs with 5 mmol/L HMBA in RPMI supplemented with 1% fetal calf serum (FCS) (instead of the usual 5%), induced cells to selectively express high levels of AChE (up to ~170 mU/mg protein) with little activation of Hb synthesis (less than 5% B+ cells). The increase in AChE levels was a general phenomenon affecting the whole cell population and approached its maximum within 3 days of incubation with the inducer. Subsequently, MELCs become committed to terminal division, undergoing growth arrest and expression of the megakaryocytic phenotype even after the removal of HMBA. There were no appreciable changes of basal AChE levels in MELCs that were either made resistant to HMBA or treated with 0.1 mmol/L hemin that activated differentiated erythroid function without commitment. Phorbol 12-myristate 13-acetate (PMA), known to repress induced Hb synthesis in these cells, did not prevent the full increase in AChE when incubated with MELCs 2 days before HMBA addition. HMBA-induced MELCs always underwent AChE increase that was more or less pronounced depending on the low or high serum content in culture, respectively. Conversely, Hb expression was permitted only when MELCs were transferred in the late phase or at the end of commitment from low to high serum media. Variations of FCS content in culture media proved to be a simple and reliable approach to change the MELC response to inducers and to modulate expression of either megakaryocytic or mixed erythromegakaryocytic phenotype. These findings suggested that MELC might be considered, at least, as a bipotential model of differentiation to be used for studies on regulation of either megakaryocytic or erythroid markers and on competition between the two hematopoietic lineages. In this regard, it was intriguing that AChE levels attained under selective induction (low serum) were always higher than under conditions allowing coexpression of both AChE and Hb (high serum). Moreover, MELCs were also found to bind the specific rat-antimouse platelet monoclonal antibody 4A5. Flow cytometry experiments have shown that both uninduced and especially PMA-treated MELCs were prompted to express 4A5-positivity, which increased progressively during 4 days of culture. On the contrary, HMBA-induced cultures showed no appreciable binding of the antibody within the same period. Despite differences in the modulation of AChE and platelet antigen, both of these megakaryocytic markers were expressed constitutively and independently from Hb. The fact that Hb, AChE, and platelet antigen could be elicited, whether alternatively or concurrently, in the bulk of MELC population was strongly against the supposed unipotentiality of these cells.

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tence of independent pathways through which MELC phenotypes might be modulated and augment the general knowledge of this well-known yet not thoroughly explained cell model of differentiation.

MATERIALS AND METHODS

MELCs (745/A strain and subclones) were propagated in RPMI medium (GIBCO Laboratories, Paisley, Scotland) supplemented with 5% fetal calf serum (FCS; Boehringer Mannheim, Mannheim, Germany) at 37°C in a 6% CO₂ humidified atmosphere. Subclones, obtained by limiting dilution of the main strain, were tested for response to inducers by the benzidine test for Hb and benzidine-positive (B⁺) cells were scored by means of a light microscopy. Subclones used for these experiments were chosen on the basis of their high susceptibility to synthesize and accumulate Hb upon induction (70% to 80% B⁺).

Inducers used were HMBA; DMSO (Janssen Chimica, Beerse, Belgium); hemin (Sigma Chemical Co, St Louis, MO). Phorbol 12-myristate 13-acetate (PMA) was also used as an inhibitor of induced terminal erythroid maturation.

The standard protocol for induced differentiation of MELCs involves incubation of cells (at a density of 5 to 15 × 10⁶/mL) for 5 days in the presence of a suitable agent in RPMI supplemented with 5% FCS. Changes in either length of incubation, cell density, percentage of FCS in the medium or other culture conditions are specified in the legends to figures and tables. Clones of MELCs made resistant to HMBA were obtained by repeated passage of the cell line in the presence of the inducer. Acquired HMBA-resistance was assessed by measuring either growth rates or benzidine positivity upon induction with 5 mmol/L HMBA.

Rates of MELC growth were determined by estimating the increase in cell number within 0 to 6 days after inoculation. Cell viability was assayed by trypan blue exclusion. Protein concentrations were determined according to Bradford.

Extraction of MELCs was performed on cells washed twice with PBS and suspended in 0.5 to 2.5 mL of 0.87% NH₄Cl containing 0.2% Triton X-100. Cell suspensions were sonicated at 100 Watts for 30 seconds on ice and then centrifuged at 11,000 rpm for 1 minute in a Minifuge (Beckman Instruments, Irvine, CA). The resulting supernatants were used for assays.

AChE activity was determined spectrophotometrically at 405 nm according to the DTNB method using 5 mmol/L acetylthiocholine iodide (Boehringer Mannheim) as the substrate. Activity was expressed in milliunits, i.e., the amount of enzyme that catalyzes the conversion of 1 nanomole of acetylthiocholine per minute, at 25°C.

Hb concentrations were measured from the visible absorption spectra typical of oxyhemoglobin. The A₅₅₂ values were multiplied by 0.1221 to give Hb concentration in milligrams per milliliter.

Cytoconcentrufugates were obtained onto a glass slide by centrifugation of 1 mL of cell suspension with 10 to 20 × 10⁶ cells at 1,500 rpm for 10 minutes (Minifuge GL; Heraeus-Christ, Osterode am Harz, Germany), at 20°C. Glasses were air-dried and then stained with AChE according to the copper-thiocoline method. The reagent was prepared fresh every time and incubated with the slides for about 4 hours at room temperature. Cells were counterstained with Papanicolaou’s solution 1a (Merck, Darmstadt, Germany).

Specificity to the murine megakaryocyte lineage was evaluated with the aid of the specific rat-antimurine platelet monoclonal antibody (MoAb) 4A5. This antibody, although not tested with other tissues, was previously characterized as a selective label of murine blood platelets and marrow megakaryocytes. Cells (~ 1 × 10⁶) were incubated in phosphate-buffered saline (PBS) added with 1% bovine serum albumin (BSA) for 60 minutes at 0°C with a preparation of biotin-conjugated 4A5 IgG (20 μg/mL). Cells were then incubated with streptavidin-R phycoerythrin conjugate for 60 minutes at 4°C. Positive cells were identified by flow cytometry on FACScan flow cytometer (Becton Dickinson, Mountain View, CA) after gating for nonspecific fluorescence measured in control tubes (cells processed as experimental ones but without biotin-4A5).

RESULTS

The standard protocol adopted for MELC commitment to terminal maturation involved the incubation of cells in RPMI plus 5% FCS and 5 mmol/L HMBA (or 1.5% DMSO). Under these conditions the expression of both Hb and AChE was stimulated to various degrees depending on the clone used. Induced cells showed a progressive reduction in growth rates and concurrent accumulation of Hb, approaching a maximum by the fifth day of culture. Percentages of hemoglobinized cells, shown by the benzidine test, rose from ≤1% in untreated cultures to approximately 60% to 80% (corresponding to ~80 to 110 μg Hb/mg cell protein). AChE specific activity also increased from a basal level of 15 to 25 mU/mg protein in control cultures to 80 to 120 mU/mg protein in differentiated cultures after 5 days of incubation with 5 mmol/L HMBA.

However, the effects of the inducer on both Hb and AChE increases were found to change as a function of the FCS concentration in the culture medium. As shown in Fig 1, the amount of Hb accumulated by HMBA-treated MELCs correlated directly with the increase in FCS concentration.
Fig 2. Cytochemical determination of AChE in either uninduced (A) and 5 mmol/L HMBA-induced (B) MELCs. Cells were incubated for a 4-day period and then procured and washed with PBS. The cells were centrifuged onto glass slides, air dried, and stained for AChE activity according to the copper-thiocoline method (original magnification x 400).

Fig 3. Effect of initial cell density on the relative increase of AChE by MELCs. Cell suspensions were initially adjusted to various densities within a range of 0.035 to 1.05 x 10^6 cells/mL and incubated for 4 days in RPMI supplemented with 1% FCS and 5 mmol/L HMBA. AChE specific activity of induced MELCs was determined after 4 days of incubation and reported as a percentage of maximum. Symbols shown (∆, ●) represent two different experiments.

within the range of 1% to 5% FCS; subsequently it leveled off and remained fairly constant around 100 μg/mg protein. AChE specific activity increased sharply to yield ≈170 mU/mg protein at 1% and 2.5% FCS. At higher serum concentrations, enzyme levels were partially reduced compared with the maximum, yet they were maintained at over 100 mU/mg protein. HMBA-induced MELCs incubated at 1% FCS were exceedingly high in AChE whereas Hb content was very low (10 μg Hb/mg corresponding to less than 5% B' cells). This finding indicated that under conditions of restricted FCS content, the megakaryocyte-like and erythroid differentiation processes of MELCs might be dissociated. None of the control cultures (without HMBA) showed appreciable changes in either AChE and Hb at any of the FCS concentrations used (1% to 10%), implying that the increase in AChE was due to MELC interaction with the inducer. Statistical analyses of data (see legend for detailed comments) has shown that Hb levels at 1%, 2.5%, and 5% were significantly different from each other. With regard to AChE, the levels attained at 1% to 2.5% diverged significantly from those at 7.5% to 10% to confirm a slight inhibitory effect of serum on AChE increase. Instead, enzyme activities at 5% FCS were borderline between values at lower and higher serum concentrations from which they did not differ significantly. However, other experiments performed with 11 distinct MELC subclones, which were induced specifically at either 1% or 5% FCS, showed that enzyme activities of the two groups differed significantly (Student's t test for matched samples; P < .0001). The mean value at low serum was 23.3% higher than at high serum. This suggested that full AChE expression was more favored when it was selectively induced rather than coinduced with Hb.

Beyond the relative content of AChE and Hb in differentiated MELCs, there were additional aspects of marker expression deserving further comment. The expression of Hb in individual single cells of HMBA-treated cultures is an all-or-none process; therefore, variations in the Hb content of the induced population are a close reflection of the percentage of cells which have been committed to terminal erythroid maturation. In contrast, AChE is a constitutive protein of undifferentiated MELCs and its increased expression upon induction appeared to involve the majority of the population to the same degree. As shown in Fig 2, virtually all of the cells in HMBA-induced cultures at 1% FCS displayed an intense and widespread distribution of copper grains representing the substantial increase in AChE activity, as compared with control. The estimated percentage of B' cells in this experiment approached 4%.

In other experiments, we asked whether induced AChE increase might be affected by the cell density at which MELCs were initially seeded in the presence of the inducer. Cell suspensions have been adjusted within the range of 0.03 to 1.05 x 10^6 cells/mL and incubated for 4 days in RPMI supplemented with 1% FCS and 5 mmol/L HMBA. Values of AChE specific activity (Fig 3) were found to correlate directly with the initial cell densities up to 0.25 x 10^6/mL, suggesting that AChE accumulation was promoted by cell-
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Fig 4. Time-course of AChE increase and commitment of MELCs by 5 mmol/L HMBA at 1% FCS in the culture medium. Cells were initially seeded at a density of 0.2 x 10⁶/mL in the presence of HMBA. At various intervals, aliquots of cell suspensions were processed immediately for AChE assay (●, —) to follow the time course of enzyme increase. Other aliquots of cell suspensions were used for commitment experiments as follows. Cells, washed free of HMBA-containing medium, were resuspended with an equal volume of fresh RPMI plus 1% FCS and incubated up to day 4 when cultures were assayed for AChE specific activity (○, →). Cultures that were treated for 3 days with HMBA and then transferred to fresh media without the inducer were followed up to day 6. At each interval, MELCs were counted in a Burker chamber to assess increase in cell number and calculate PDLs, reported in the text. Results are the means ± SD of three separate experiments.

To determine the time-course of AChE increase upon induction, MELCs were incubated for various intervals in the presence of 5 mmol/L HMBA and 1% FCS in RPMI. At the times indicated, aliquots of MELCs were taken and processed to determine AChE specific activity (Fig 4). During the first day of incubation with HMBA, AChE was maintained close to the basal level. Thereafter, AChE increased steadily up to 90 mU/mg protein on day 3 and approached a maximum by day 4. Along with the time-course experiment, other aliquots of the MELC suspension were withdrawn separately and cells were transferred to a fresh medium without HMBA. MELCs were then processed on the following days of incubation to ascertain whether enzyme levels of HMBA-induced cultures were affected by removal of the inducer. Results reported in Fig 4 showed that during the first 2 days of incubation with HMBA, the transfer of cells to fresh medium without inducer did not change enzyme levels attained just before HMBA removal. Rather, the cells that were withdrawn after 3 days of incubation with HMBA and transferred to fresh medium without inducer, showed a significant burst in enzyme activity approaching 150 mU/mg protein. Such a high level of AChE was attained just after 24 hours of incubation in fresh medium and was maintained constantly for the following 2 days.

During the 6 days of incubation in culture, variations in cell number were also monitored, at each interval, with the aid of a Burker chamber. MELC growth in the presence of HMBA was found to continue at a reduced rate, as compared with control, up to day 3 (population doublings [PDLs] calculated for HMBA-treated and controls were 2.3 and 3.1, respectively). Thereafter, induced cells underwent growth arrest (PDL, 0.39 over the last 4 days of incubation) independently of either the presence of HMBA or density at which they were inoculated into fresh medium. These results were in keeping with a process of HMBA-induced commitment of MELCs to terminal maturation through the megakaryocytic rather than erythrocytic pathway.

Effects of FCS content in the medium on expression of differentiation markers in HMBA-induced MELCs have been investigated further. MELCs were preincubated at 1% FCS with 5 mmol/L HMBA for 2 days, that is a time insufficient for commitment. Thereafter, the cell suspension was split into two parts that were spun down and resuspended with fresh medium containing 5 mmol/L HMBA and either 1% or 5% FCS. Three days later, MELCs maintained at 1% FCS with the inducer showed the typical large increase in AChE to reach a mean specific activity of 98.4 mU/mg protein, whereas B' cells were less than 5% (Fig 5). On the...
Contrary, HMBA-induced MELCs that were transferred from (74% B+ cells) also exhibiting increased AChE specific ac-

Activity and Benzidine Positivity of MELCs

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>AChE Specific Activity (mU/mg protein)*</th>
<th>B+ Cells (%)</th>
</tr>
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<tr>
<td>With 5% FCS</td>
<td></td>
<td></td>
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<tr>
<td>MELCs, uninduced</td>
<td>18.8 ± 7 (9)</td>
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<tr>
<td>MELCs + 1 mmol/L HMBA</td>
<td>24.7 (1)</td>
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<td>MELCs + 2.5 mmol/L HMBA</td>
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<td>MELCs + 5 mmol/L HMBA</td>
<td>117.4 ± 30 (5)</td>
<td>83.7</td>
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<td>MELCs + 1.5% DMSO</td>
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<td>MELCs + 0.1 mmol/L hemin</td>
<td>24.8 ± 7 (2)</td>
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<td>MELCs + 100 ng/mL PMA</td>
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<td>141.9 ± 20 (4)</td>
<td>48</td>
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<tr>
<td>HMBA-resistant MELCst, uninduced</td>
<td>8.7 (1)</td>
<td>≥1</td>
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<tr>
<td>HMBA-resistant MELCs + 5 mmol/L HMBA</td>
<td>19.8 (1)</td>
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<td>With 1% FCS</td>
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<td>MELCs, uninduced</td>
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<td>MELCs + 1 mmol/L HMBA</td>
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<td>MELCs + 5 mmol/L, HMBA</td>
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<tr>
<td>MELCs + 5 mmol/L HMBA</td>
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<td>MELCs + 1.5% DMSO</td>
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<td>≤1</td>
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<tr>
<td>MELCs + 100 ng/mL PMA</td>
<td>37.9 (1)</td>
<td>≤1</td>
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<td>MELCs + 100 ng/mL PMA + 2.5 mmol/L HMBA</td>
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<td>≥1</td>
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<tr>
<td>MELCs + 100 ng/mL PMA + 5 mmol/L HMBA</td>
<td>137.9 ± 4 (2)</td>
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<td>HMBA-resistant MELCst, uninduced</td>
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<tr>
<td>HMBA-resistant MELCs + 5 mmol/L HMBA</td>
<td>12.1 (1)</td>
<td>≤1</td>
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</table>

* AChE specific activity was determined after 4 days of incubation and values were the means ± SD. Number of experiments is shown in parentheses.
† PMA was added 2 days before incubation with HMBA.
‡ HMBA-resistant MELCs were obtained as described in Materials and Methods.

Results of Table 1 are a summary of AChE modulation under various culture conditions. Of particular interest were data showing that PMA added to MELC cultures 2 days before HMBA did not prevent AChE from increasing at either 1% or 5% FCS. However, erythroid differentiation in MELCs exposed to HMBA in the presence of 5% FCS (83% B+ cells) was efficiently repressed by the PMA treatment (48% B+ cells). Additionally, (1) MELCs made resistant to HMBA did not respond to the inducer and maintained both Hb and AChE at basal levels; (2) concentrations of HMBA required to trigger AChE increases were lower at 1% than 5% FCS; and (3) DMSO was a less effective inducer of AChE than was HMBA.

Additional evidence for the expression of megakaryocytic markers in MELCs was provided by flow cytometry experiments using a biotin-conjugated preparation of the MoAb 4A5, which was specifically raised against a murine platelet antigen. Results of Fig 6 showed the time-course of 4A5 positivity in cultures maintained at 1% FCS and treated in the absence and in the presence of either PMA (100 ng/mL) or HMBA (5 mmol/L). One day after seeding all cultures were virtually unreactive with 4A5, as shown by the coincidence of specific and nonspecific signal, apart from PMA-treated cells that exhibited a partial positivity. At day 2, approximately half of the control cells were found to bind the antibody, whereas most of PMA-treated MELCs were already progressed towards 4A5 positivity; in turn, HMBA-treated cells still showed no appreciable binding. A clear 4A5 positivity of both control and PMA-treated MELCs was attained at day 4, as shown by the shift on the right of the gray area and by the fact that 4A5-positive cells were broadly distributed over the entire population (data not shown). HMBA-treated cells remained substantially negative even at day 4. These results indicated that the platelet antigen recognized by the antibody 4A5 is constitutively expressed by uninduced MELCs during growth in culture. The addition of PMA seemed to accelerate, as compared with control, the antigen expression. On the contrary, the incubation of MELCs with HMBA prevented MELCs from expressing the platelet antigen within a 4-day period. It is worth mentioning that flow cytometry results have been confirmed by experiments performed with several freshly-isolated subclones of MELCs to rule out any problem of dealing with mixed populations.

### DISCUSSION

During the past several years evidence for the potential megakaryocytic nature of MELCs has been accumulating. This includes (1) the ability of MELCs to incorporate serotonin; (2) the constitutive and induced expression of AChE together with the typical intracellular distribution of the enzyme; (3) the active extracellular release of AChE as it occurred in murine megakaryocytes and the inhibitory effect exerted by exogenous AChE on MELC growth; and (4) the finding that activin A–treated MELCs expressed thromboxane A2 synthetase, an enzyme occurring in murine megakaryocytes but not in erythrocytes.

Megakaryocytes and erythrocytes are believed to share common precursors and there are evidences that most erythroblastic and megakaryoblastic cell lines express markers specific to both the erythrocytic and megakaryocytic lineages. Therefore, it is not surprising that MELCs, in addition to erythroid markers, could also show traits of the megakaryocytic lineage as it was reported previously by us and very recently confirmed by the effects of activin A/EDF on erythroid and megakaryocytic differentiation in the
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Fig 6. Time course of 4A5 positivity in control and either PMA- or HMBA-treated MELCs, as determined by flow cytometry. MELCs from a resting culture were diluted up to 1 × 10⁶/mL in RPMI with 1% FCS in the absence (A) or presence of either PMA (100 μg/mL) (B) or HMBA (5 mmol/L) (C). At days 1, 2, and 4 after seeding, the cells were harvested, washed with PBS containing 1% BSA, incubated (1 × 10⁶ cells) with biotin-conjugated 4A5 (20 μg/mL) followed by incubation with streptavidin-R phycoerythrin conjugate, and then analyzed as reported in the Materials and Methods. The specific (with 4A5 antibody) and nonspecific signal (without 4A5 antibody) have been indicated as gray and empty areas, respectively.

MELC line Nevertheless, the practice of using MELCs as an erythroid system alone with little attention paid to markers other than Hb, has hampered a full understanding of this model. Under culture conditions routinely used for MELC differentiation, the synthesis of Hb was always found to be triggered, whereas AChE expression could be either increased (with 5 mmol/L HMBA or 1.5% DMSO as the inducers) or maintained at basal levels (with 0.1 mmol/L hemin). In the former situation, induced MELCs were committed to terminal division and could be regarded as a mixed phenotype coexpressing both megakaryocytic and erythroid markers. In the case of MELCs induced with hemin, these cells were not committed to terminal division and behaved as practically pure erythroid precursors.

We have shown herein that by decreasing FCS in the culture medium, HMBA-induced MELCs were induced to resume their resident megakaryocytic program with negligible activation of the erythrocyte differentiation pathway. The presence of HMBA was necessary to promote the AChE increase; therefore, it appears that there is a true commitment to terminal maturation involving the expression of differentiated functions and contemporary arrest of cell growth.

It is not clear why low serum concentrations channel induced MELCs toward the megakaryocytic rather than erythroid phenotype. However, there are two possible explanations that we are presently evaluating. The first is that the inducer might interact differently with fast-growing (5% FCS) or slow-growing (1% FCS) cell populations. Two facts might support this assumption: (1) the typical commitment of MELCs by HMBA was reported to require at least one cycle of cellular replication to take place; and (2) as we have previously observed, variations of AChE levels in uninduced MELCs appeared to be inversely related to rates of cellular replication. The second conceivable explanation is that restricted serum conditions may lead to a decrease in factors that act preferentially as inhibitors of megakaryocyte differentiation, like it was reported for transforming growth factor β and platelet factor 4. The inhibitory activity of these factors might be shown by the fact that HMBA-induced MELCs shifted from a plain megakaryocytic (very high AChE and negligible Hb) to a mixed erythromegakaryocytic phenotype (high AChE and high Hb) upon the transfer of cells, whether partially or fully committed, from 1% to 5% serum media (see results of Fig 5). It remains to be established whether, in addition to the lack of physiologic inhibitors that would allow HMBA to selectively turn on the
MELC megakaryocytic program, some positive natural modulator might help the process. However, this possibility seems to be ruled out by preliminary results indicating that 1% FCS could be efficiently replaced in promoting AChE increase by comparable amounts of synthetic serum-free media.

The finding that a large part of either uninduced or PMA-treated MELCs was labeled by the antimurine-platelet MoAb 4A5 indicates that these cells express more than one trait of the megakaryocytic phenotype. Conversely from AChE, the increase in 4A5-positivity appeared to correlate with cell growth rather than HMBA-induced differentiation. Nevertheless, both these megakaryocytic markers must be considered as MELC constitutive proteins whose expression could be elicited independently from Hb. Moreover, because PMA did not interfere with AChE expression while it reduced erythroid commitment, it is conceivable that the two pathways of differentiation were triggered separately by HMBA. It is also of interest that PMA, reported to promote megakaryocyte differentiation in several human cell lines including Dami, HEL, K562, and TF, was also found also to accelerate the expression of murine platelet antigen in MELCs. Owing to the little information available on murine megaglobin markers and lack of probes for their detection, it is difficult and probably too early to compare megaglobinocytic potentiality of MELCs with that of human erythroleukemic cell lines that also express both erythroid and megakaryocytic traits. At any rate, AChE and murine platelet antigen of MELCs were largely expressed in the bulk of MELC population. On the other hand, expression of some of the megaglobinocytic features in pluripotential HEL, K562, and TF, were reported to vary within a wide range and to often involve a limited number of cells.

Ultimately, AChE levels in HMBA-induced MELCs approached values of ~150 to 200 mU/mg protein, which is more than 35% of the enzyme activity present in mature murine megaglobinocytes. Further, we have shown that MELCs constitutively express a murine platelet antigen during growth in culture. In contrast, the amount of Hb accumulated in fully differentiated MELCs did not usually exceed 110 μg/mg protein, which is below 15% of the Hb content in mature erythrocytes. We also have data (Paoletti F, manuscript in preparation) showing that a small yet significant percentage of polyploid cells occurred spontaneously in MELC cultures and that a large part of the population, upon particular treatments, can undergo a large increase in ploidy without any activation of erythroid functions. Taken together, all these facts would imply that MELCs are a much more complex system than previously believed. Particularly, MELCs, because of their clear and spontaneous megaglobinocytic nature, might be paradoxically considered more closely related to precursors of megaglobinocytes than of erythrocytes.

We believe that the identification of suitable conditions for alternatively inducing either megaglobinoblastoid and/or erythroid markers in MELCs may be of help in further understanding of this model and the complex interplay between the erythrocytic and megaglobinocytic lineages.

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REFERENCES

23. McDonald TP, Sullivan PS: Megakaryocytic and erythrocytic cell lines share a common precursor cell. Exp Hematol 21:1316, 1993
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F Paoletti, AM Vannucchi, A Mocali, R Caporale and SA Burstein