Acetylcholinesterase in Murine Erythroleukemia (Friend) Cells: Evidence for Megakaryocyte-like Expression and Potential Growth-Regulatory Role of Enzyme Activity

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Features of true acetylcholinesterase (AChE) regulation during growth and differentiation of Friend murine erythroleukemia cells (MELC) have been investigated with respect to other erythroid and nonerythroid murine elements. Enzyme levels of uninduced MELC were in between the very low AChE contents of erythroid cells and the huge amounts of enzyme activity exhibited by megakaryocytes and platelets. After MELC commitment to terminal division, the enzyme-specific activity increased largely, approaching values that were much closer to those of thrombocytotic than of normal erythroid elements. The bulk of AChE activity in MELC, megakaryocytes, and platelets was found to be located in the cytosol as a free-soluble form. Moreover, during incubation, MELC actively released large amounts of AChE into the medium, like it occurs in murine thrombocytes. Conversely, the enzyme of the erythroid elements was mainly associated with the membranes and was not released extracellularly.

The main recognized function of true acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AChE) is that of terminating neurotransmission by hydrolyzing acetylcholine at cholinergic synapses in the central and peripheral nervous tissues. However, the enzyme has also been reported in a variety of noncholinergic tissues of several animal species in which it has been suspected to play roles presumably unrelated to neurotransmission. Indeed, significant amounts of AChE were detected in human erythrocytes and T lymphocytes, murine thrombocytotic cells, WRL-10A cultured fibroblasts, and several erythroleukemia cell lines. In all these cells, the enzyme might exert alternative and still undetermined catalytic activities possibly acting on substrates different from acetylcholine.

The regulation and role of AChE in noncholinergic systems can be suitably studied in Friend murine erythroleukemia cells (MELC). These are virus-transformed hematopoietic elements, presumably proerythroblasts, that can undergo partial maturation to normoblast-like cells when cultured with 1.5% dimethyl sulfoxide (DMSO) or 5 mmol/L hexamethylene-bisacetamide (HMBA). These compounds are known to induce the arrest of cell proliferation and the contemporary expression of hemoglobin and other specialized proteins according to a sequence of events typically encountered in normal erythropoiesis. AChE, which is a constitutive enzyme of undifferentiated MELC, increases significantly during induced differentiation of HMBA- or DMSO-treated cultures. Because the increase in enzyme activity precedes that of hemoglobin, AChE has been proposed as an early marker of differentiation in both the MELC and normal murine erythroid systems.

However, in rodents, contrary to what is observed in humans, AChE activity is very low in erythrocytes and quite high in megakaryocytes and platelets, for which it represents a distinctive marker. Therefore, we decided to investigate the regulation of AChE in MELC and verify whether enzyme induction can actually be ascribed to erythroid maturation.

The results of the present study seem to cast doubt on the merely erythroid nature of AChE regulation in MELC, which appeared, conversely, to exhibit the typical enzyme expression of murine megakaryocytic cells.

MATERIALS AND METHODS

MELC (745/A strain; originally obtained by Dr C. Friend and kindly provided by Dr G. B. Rossi, Istituto Superiore di Sanità, Rome, Italy) were grown in suspension using RPMI medium (GIBCO Laboratories, Paisley, UK) supplemented with 5% fetal calf serum (FCS; Boehringer Mannheim, Mannheim, Germany) at 37°C in 6% CO2 humidified atmosphere. For terminal maturation experiments, cell suspensions (5 to 10 x 10⁶/mL) were incubated for 5 days in the presence of 0.5 mmol/L HMBA or 1.5% DMSO (Janssen Chimica, Beersel, Belgium). MELC have been also treated with 0.1 mmol/L l-hemin (Sigma Chemical Co, St Louis, MO) for the same length of time.

Subclones were obtained by limiting dilution of the cell line; isolated clones were then tested for their response to inducers by the benzidine test for hemoglobin, as follows. Cells were washed and resuspended with phosphate-buffered saline (PBS) at a density of about 0.75 x 10⁶/mL. Then, 0.1 mL of the suspension was mixed with 50 μL of 0.5 mol/L acetic acid containing 0.2% benzidine and 0.2% hydrogen peroxide. After incubation for 4 min at 37°C without light, 0.1 mL of 0.3% benzidine was added, and the reaction was stopped by the addition of 1 mL of 0.15 mol/L sodium bicarbonate. The absorbance at 540 nm was measured, and clones showing an absorbance over 0.2 were considered positive.

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was freshly prepared and incubated with the slides for 4 to 6 days after inoculation. Cell viability was assessed by the trypan blue exclusion test.

AChE activity was determined spectrophotometrically at 405 nm according to the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) method using 5 mmol/L acetylthiocholine iodide (Boehringer Mannheim) as the substrate. Activity was expressed as millinits, i.e., the amount of enzyme that catalyzes the conversion of 1 nmol/L of acetylthiocholine per minute at 25°C. The enzyme has also been assayed in the presence of 50 μmol/L ethopropazine-HCl or 10 μmol/L BW284C51 (Sigma), which inhibit true AChE without affecting butryrycholinesterase activity.

Proteins were determined according to Bradford.

Cyto centrifugates were prepared on a glass slide by spinning cell suspensions (10 to 20 x 10^6 cells/mL) at 1,500 rpm for 10 minutes (Minifuge GL; Heraeus-Christ, Osterode, Germany) at 20°C. Glasses were air-dried and then stained for AChE, without fixation, following the copper-thiocoline method. The reagent mixture was freshly prepared and incubated with the slides for 4 to 5 hours at room temperature. Cells were eventually counterstained with Papanicolaou's solution 1a (E. Merck, Darmstadt, Germany).

Spleen cell suspensions were obtained from mice (CBA strain) made anemic with phenylhydrazine, as described by Krystal. Total spleen cells were separated by centrifugation for 30 minutes at 70g on Ficoll-Paque (Pharmacia, Uppsala, Sweden). Cell populations enriched in erythroblasts and normoblasts were recovered at the interface and bottom of the tube, respectively.

Lymphocytes were recovered after separation of normal spleen cells on Ficoll-Paque and contaminating erythrocytes were eliminated by two cycles of lysis with 0.87% ammonium chloride.

Murine megakaryocytes were collected from bone marrow basically as described by Rabellino et al. Low-density (< 1.055 g/mL) cell fractions, obtained from centrifugation on discontinuous Percoll gradients, were further enriched in megakaryocytes by sedimentation through a discontinuous bovine serum albumin (BSA) gradient (refractive indexes: 1.343/1.340/1.336, from the bottom to the top). The resulting megakaryocyte population (partially contaminated by myeloid cells and occasional eosinophils) was 80% to 85% pure by cell number. Platelets were prepared by differential centrifugation of whole mouse blood collected with 0.2 mol/L Na2 EDTA (50 μL/mL whole blood) by centrifugation in the thrombocytic versus other hematopoietic lin-

| Table 1. AChE Specific Activity in Murine Normal and Neoplastic Cells |
|-----------------------------|-----------------------------|
| Cells*                      | AChE Specific Activities (mU/mg protein ± SD)* |
| Uninduced MELC              | 15.34 ± 2.4                 |
| HMBA-induced MELC           | 70.11 ± 7.5                 |
| DMSO-induced MELC           | 86.54 ± 16.9                |
| Immature erythroid elements | 0.68 ± 0.06                 |
| Erythrocytes                | 0.05 ± 0.05                 |
| Megakaryocytes              | 368.36 ± 6.9                |
| Platelets                   | 417.77 ± 42.8               |
| Transformed lymphocytes (RI)| Undetectable                |
| Spleen lymphocytes          | 6.15 ± 0.11                 |

MELC were cultured for 5 days with or without the inducer. Other murine elements were collected according to the procedures reported in Materials and Methods.

*Values of AChE specific activities were the mean ± SD of three separate experiments. **See Materials and Methods.
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Cultures of murine transformed lymphocytes (RI) were virtually deficient in enzyme activity, while normal spleen lymphocytes, slightly contaminated by megakaryocytes, showed a specific activity of about 6 mU/mg protein.

The enzyme activity in all of the samples listed above was due to true AChE because it was abolished by the addition of specific enzyme inhibitors (see Materials and Methods) to the assay mixture.

Cytochemistry and intracellular distribution of AChE. The marked increase in AChE occurring during MELC-induced maturation was morphologically shown by the specific staining of cells with the copper-thiocholine reagent. MELC treated with 5 mmol/L HMBA (Fig 1B) were heavily stained, with respect to uninduced cultures (Fig 1A), because of the presence of many copper grains engulfing the entire cytoplasmic space.

In agreement with these findings, approximately 80% of total AChE activity, from both induced and uninduced MELC (Table 2), was readily extracted by homogeneization with 10 mmol/L Tris-HCl buffer, pH 7.4, and concentrated in the postmicrosomal supernate. Some activity (less than 20%) was found to be associated with the particulate fraction (spun down at 100,000g) and solubilized with the aid of 0.2% Triton X-100. On the contrary, the bulk of AChE activity in normal murine erythrocytes, taken as the control, appeared to be almost entirely associated with the cell membranes.

Megakaryocytes and platelets did not show such striking differences as above between the soluble and bound AChE, although the amount of free cytosolic activity (around 60%) was still prevalent over that associated with the membrane fraction.

Changes in AChE specific activity during MELC growth in culture. AChE specific activity (mU/mg protein) was determined in MELC extracts during a 6-day period of culture (Fig 2). Enzyme levels were steadily around 7 mU/mg protein throughout the logarithmic phase (days 1 through 3). Thereafter, contemporary to the decline of cell growth, AChE specific activity increased progressively, attaining a value of about 23 mU/mg protein at day 6, when culture reached a saturation density of 2.85 × 10⁶/mL. One day after the inoculation of resting cells into fresh medium, AChE levels dropped again to about 7 mU/mg protein.

The inverse relationship existing between intracellular AChE activities and cell growth rates was confirmed by the constitutive enzyme levels of several MELC subclones (Table 3). These were isolated and chosen on the basis of their relative mass reached within a 5-day period of culture. Clones attaining a saturation density higher than 4 × 10⁶ cells/mL were assigned to the so-called fast-growing group. Clones attaining a saturation density lower than 3.2 × 10⁶ cells/mL were assigned to the so-called slow-growing group. AChE specific activities were found to be significantly lower in fast-growing versus slow-growing MELC subclones.

Effects of inducers of MELC erythroid maturation on AChE levels. Changes in AChE levels were determined in cultures submitted to several inducers of erythroid maturation (Fig 3). The enzyme reached specific activities of about 70 and 90 mU/mg protein when MELC were treated for 5 days with 5 mmol/L HMBA or 1.5% DMSO, respectively. By the way, DMSO was always found to be more effective than HMBA in raising AChE-specific activity, even though the yield in benzidine-positive MELC was constantly lower. These treatments, as expected, also induced terminal erythroid differentiation, as shown by the increase in benzidine-positive cells and the decrease in cell growth rates. On the

![Fig 1](https://example.com/image1.png)

**Fig 1.** Cytochemical determination of AChE in uninduced (A) and 5 mmol/L HMBA-induced (B) MELC. Aliquots of each cell suspension were taken on day 5 of incubation and cells were washed with PBS. The cytospins were prepared (see Materials and Methods) onto glass slides, which were air-dried and then stained for AChE activity according to the copper-thiocholine method for about 5 hours at room temperature. Pictures (original magnification ×1,000) were taken using a green filter to convert copper red deposits into dark grains.
from the culture at each day of incubation. Values reported were the mean of two separate experiments.

**Release of AChE into the medium.** AChE activity in MELC cultures was not associated only with cells. A significant amount of the enzyme was found to accumulate in the culture medium along with time of incubation. Extracellular accumulation of the enzyme was the result of active secretion, as shown by a time-course experiment (Fig 4, insert) in which the process appeared to be temperature-dependent, being steadily active at 37°C and inhibited at 4°C. Uninduced MELC released AChE at a constant rate of about 3.4 mU/h/mg cell protein within 12 hours of incubation, accounting for three to four times the intracellular enzyme content. The amount of AChE delivered extracellularly depended closely on time and conditions of culture. Within a 5-day period (Fig 4), the release of hemin-treated culture was equivalent to the control. DMSO-induced MELC released huge amounts of AChE activity, as compared with control and even with HMBA-induced cells. No significant release of AChE was observed with normal immature erythroid elements from the spleens of anemic mice.

Inhibition of cell mass increase by pure bovine AChE. Cultures have been incubated with a medium containing increasing amounts of pure bovine AChE. The bovine enzyme was chosen to replace pure murine AChE, which was not commercially available. The aim of the experiment was to monitor the growth of uninduced MELC in the presence of extracellular true AChE as it occurred in...
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At day 5 of incubation, cultures containing 50, 125, and 250 μM of medium attained approximately 84%, 78%, and 45% of control cell mass (Table 4). The inhibitory effect was evident since day 1 of incubation with the enzyme (data not shown) and was not due to cell death, as confirmed by the trypan blue exclusion test performed daily on each culture. It is worth mentioning that in this experiment the amounts of bovine AChE used were not much different than those usually accumulated in a 5-day-old medium of DMSO-induced cultures (100 to 150 μM/mL).

DISCUSSION

MELC have been found to express true AChE activity at levels higher by far than other murine normal and neoplastic hematopoietic cells, apart from megakaryocytes and platelets that are specifically marked by large amounts of the enzyme.17,28 The possibility that MELC AChE might be due to viral infection per se seems to be ruled out by the fact that cultured fibroblasts infected with the Friend virus failed to show appreciable increase in enzyme activity.15 Therefore, we can reasonably assume that AChE represents a constitutive protein of MELC phenotype and that changes in enzyme-specific activity may reflect intrinsic metabolic needs of cells, along with growth and differentiation.

The bulk of AChE activity in MELC was found to be mainly soluble and localized in the cytosol, whereas the significantly lower activities detected in normal erythroid cells were primarily associated with membranes. Such a mirror-like enzyme distribution between MELC and their supposed normal erythroid counterpart suggested that, in these systems, AChE was under the regulation of two completely distinct mechanisms. This hypothesis was supported by the finding that uninduced and especially HMBA- or DMSO-induced MELC delivered substantial amounts of AChE into the medium by an active secretory process. The release of AChE is not typical of murine erythroid cells, as the spleens of anemic mice. Instead, megakaryocytes and platelets are the murine blood elements known to release large quantities of AChE,5 which has been inferred to act in vivo as a regulator of thrombopoietic precursor proliferation.39

Moreover, experiments with inducers have clearly indicated that: (1) the induced synthesis of hemoglobin as typically expressed in the erythroid phenotype, and the increase in AChE activity were distinct and largely unrelated metabolic events; and (2) the two processes could be dissected by treating MELC cultures with hemin, which activated differentiated erythroid functions, without affecting either intracellular content or release of AChE.

All these data, together with the reported isolation of MELC subclones, which were prone to AChE increase but not to induced hemoglobin synthesis,15 argue against the hypothesis that the enzyme might serve as a specific marker of erythroid maturation in the mouse. Rather, they point to AChE expression as the result of a megakaryocyte-like program residing in uninduced MELC and enhanced greatly after culture treatments with either HMBA or, particularly, DMSO. These compounds will commit MELC to terminal division and simultaneously promote the activation of multiple differentiated functions, among which hemoglobin synthesis and increase in AChE activity should be regarded as the coexpressed markers of the erythrocytic and megakaryocytic lineages, respectively. As regards these two hematopoietic lineages, it is worth recalling that they may have common precursors;30 moreover, the existence of burst forming-unit megakaryocytic, erythroid cells (BFU-ME) was proved in the mouse by chromosome analysis of mixed erythroid-megakaryocytic colonies grown in vitro.31 Therefore, it is not unlikely that target cells of virus infection might have been progenitor cells still sharing bipotential or multipotential properties. Actually, the polycytemic strain of Friend virus has been reported to infect either megakaryoblasts or uncommitted hematopoietic elements.32

The coexpression of multiple markers of distinct hematopoietic lineages has widely been described in leukemia cells.33 This so-called lineage promiscuity is especially evident in human erythroleukemia cell lines HEL34 and K562,35,36 whose differentiated phenotypes express properties of more than one lineage, together with a fairly constant association between erythroid and megakaryoblastic markers.37 These considerations and results concerning AChE seem to support the view that MELC are not unipotential in terms of their differentiated phenotype, as proposed initially by Marks et al.38 Conversely, the system, although predominantly erythroid, still retains significant megakaryoblastic features related so far to AChE expression and serotonin incorporation.39 Moreover, under suitable culture conditions, MELC may present even some traits of leukopoietic differentiation.40

The changes in AChE specific activity occurring during MELC growth in culture have disclosed an inverse relationship between enzyme levels and cell proliferation rates. Two additional facts seem to support this finding: (1) slow-growing subclones exhibited constantly higher AChE levels than fast-growing subclones; and (2) large increases in enzyme intracellular levels and release occurred only

Table 4. Inhibitory Effect of Exogenous Bovine AChE on MELC Growth

<table>
<thead>
<tr>
<th>Culture*</th>
<th>Cell Density†%</th>
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<tbody>
<tr>
<td>Control (no AChE added)</td>
<td>3.1 x 10⁶/mL</td>
</tr>
<tr>
<td>Plus 50 μM AChE/mL</td>
<td>2.6 x 10⁶/mL</td>
</tr>
<tr>
<td>Plus 125 μM AChE/mL</td>
<td>2.1 x 10⁶/mL</td>
</tr>
<tr>
<td>Plus 250 μM AChE/mL</td>
<td>1.4 x 10⁶/mL</td>
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*MELC (5 x 10⁵/mL) were incubated in RPMI supplemented with 5% FCS in the presence of increasing amounts of pure AChE preparations from bovine erythrocytes.

†Cell density and cell viability were evaluated at day 5 of incubation. Control cultures contained about 85% viable cells, while MELC treated with 50, 125, and 250 μM bovine AChE/mL approached values of 80%, 95%, and 98%, respectively.
when MELC differentiation was contemporarily accompanied by induction to terminal division. An increase in AChE activity content and release was reported to occur also in WRL-1OA mouse fibroblasts as soon as they reached high saturation densities in suspension cultures. Such a close interaction between components of cholinergic systems and non-neuronal cell growth is consistent with our finding that MELC mass increase was inhibited by exogeneous bovine AChE added to the culture medium. This result supports the suggestion that AChE would actively be synthesized and secreted once MELC growth declines and cells enter their differentiation pathway. The enzyme released might then exert its inhibitory effect on MELC growth acting as a negative autocrine factor. Our conclusions are in line with current hypotheses of cholinergic modulation of murine megakaryocytes as well as the interplay of AChE and acetylcholine. In fact, megakaryocyte colonies appeared to be enhanced by both acetylcholine analogs and cholinesterase inhibitors, whereas they were decreased by the presence of AChE in the culture medium. Moreover, it has also been reported that manipulation of cholinesterase gene expression modulates murine megakaryocyte differentiation in vitro. We believe that the sensitivity of MELC to exogeneous AChE might represent remarkable additional evidence that the system retains properties of the normal murine thrombocytoid phenotype. This conclusion seems to be supported by the recent finding that thromboxane A2 synthase, an enzyme observed in megakaryocytes and platelets but not in erythroid cells, is induced in activin A-treated MELC.

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