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Assessment of monocytic component in acute myelomonocytic and monocytic/monoblastic leukemias by a chemiluminescent assay

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Introduction: Classically, the monocytic component of acute myelomonocytic (FAB-M4) and acute monocytic/monoblastic (FAB-M5) leukemias is demonstrated by nonspecific esterase positivity in cytochemical stainings. We have previously demonstrated that non-specific esterases from normal monocytes can be determined by a chemiluminescent method. In the present study, we investigated whether this assay can also determine the monocytic component of FAB-M4 and FAB-M5 and distinguish these acute myeloid leukemia (AML) categories.

Materials and methods: Bone marrow samples were obtained from 66 patients with AML (M0, two cases; M1, 12 cases; M2, 13 cases; M3, 10 cases; M4, 11 cases; M5, 12 cases; M6, two cases; M7, four cases). Cells were incubated with a standard reaction mixture and chemiluminescence was measured for 10 min. Two parameters were assessed, the peak (PLE) and the integrated light emission (ILE).

Results: Both PLE and ILE were higher in FAB-M4 and FAB-M5 subtypes compared to other AML subtypes ($P<0.001$). In addition, the classification of AML cases into FAB-M4, FAB-M5 and nonmonocytic subtypes based on ILE analysis was concordant with α-naphthyl acetate esterase (ANAE) in 97% of cases (kappa coefficient 0.94, $P<0.001$).

Conclusions: These findings indicate that this chemiluminescent assay was able to determine the monocytic component of FAB-M4 and FAB-M5 cells, and the classification of AML subtypes based on chemiluminescent analysis strongly agreed with the cytochemical ANAE-staining. In conclusion, this chemiluminescent assay is a simple, fast and objective method, which may be useful as an alternative tool in the differential diagnosis of AML subtypes.
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Introduction

The diagnosis of acute leukemia is based on clinical information and on the analysis of peripheral blood and bone marrow (BM) smears. According to the widely adopted French–American–British (FAB) classification,[1,2] acute leukemia is diagnosed when at least 30% of the total nucleated cells in the BM are blasts, or if there is erythroid predominance in the BM, or if the characteristic morphological features of acute promyelocytic leukemia are present. In contrast, acute leukemia is diagnosed by the World Health Organization (WHO) classification when at least 20% of the total nucleated cells in the BM are blasts.[3] Acute leukemias are divided into acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), depending on the nature of leukemic blasts. The revised FAB criteria subdivide acute myeloid leukemias into eight different subtypes (M0–M7), depending on their degree of maturation and differentiation.[2] FAB-M0 represents the AML subtype with minimal evidence of myeloid differentiation, while FAB-M7 AML represents the acute megakaryoblastic leukemia.

The FAB-M4 (acute myelomonocytic leukemia) AML represents a subtype of AML where both granulocytic and monocytic differentiations are present. The diagnosis is based on the demonstration of such commitments, based on typical cytochemical reactions. The granulocytic component must be at least 20% of nonerythroid cells, and the monocytic component must

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be between 20 and 80%. The staining with chloroacetate esterase (CAE) indicates the granulocytic component, while the α-naphthyl acetate esterase (ANAE) staining demonstrates the monocytic commitment of blast cells. A large number of monocytic cells ($\geqslant 5 \times 10^9$/l) in the peripheral blood may be present.[3]

In addition, the FAB-M5 (acute monocytic/monoblastic leukemia) is an AML subtype where the monocytic component of BM nonerythroid cells is greater than 80%. In acute monoblastic leukemia (FAB-M5a), the majority of monocytic cells are monoblasts (in the BM and/or peripheral blood), while in acute monocytic leukemia (FAB-M5b), the majority of monocytic cells are promonocytes ($\geqslant$80%).[3] This commitment is demonstrated by the strong positivity of monoblasts for nonspecific esterases, such as ANAE, α-naphthyl butyrate esterase (ANBE), naphthol AS acetate esterase (NASA) and naphthol AS-D acetate esterase (NASDA). In these cytochemical stainings, the monoblast esterase activity is fluoride-inhibited; however, the demonstration of inhibition by fluoride is not necessary in the case of ANAE and ANBE, since cells of granulocytic lineage are negative or weak in these stainings. As in FAB-M4, an elevated number of circulating monocytic cells may be observed in FAB-M5.

Moreover, the FAB-M6 (acute erythroid leukemia) is an AML subtype where $\geqslant$50% of the entire nucleated cells in the BM are erythroid precursors and at least 20% of the nonerythroid cells are myeloblasts.[1–3]

We have recently demonstrated that the nonspecific esterase activity of monocytes can be determined by a chemiluminescent method.[4] In this assay, esterases from monocytes promote the hydrolysis of 2-methyl-1-propenylbenzoate (MPB), yielding 2-methyl-1-propenol. Horseradish peroxidase (HRP) in the presence of H_2O_2 catalyzes the oxidation of 2-methyl-1-propenol into acetone and formic acid. This reaction produces acetone phosphorescence at the expense of dissolved oxygen and H_2O_2, an emission that may be enhanced by the addition of fluorescent acceptors, such as 9,10-dibromoanthracene-2-sulfonate (DBAS), and measured in a luminometer.[4]

In the present study, we investigated whether this chemiluminescent assay was able to determine the nonspecific esterase activity of FAB-M4 and FAB-M5 AML blast cells, and hence whether it was able to diagnose these AML categories.

Materials and methods

Patients

BM samples from 66 patients with the diagnosis of *de novo* AML were studied at diagnosis. In total, 40 patients were male and 26 were female, ranging in age from 2 to 90 years (median, 41 years). Diagnosis of AML was based on clinical and laboratory criteria. The myeloid nature of blast cells was demonstrated in BM blast cells by the myeloperoxidase (MPO) cytochemical reaction by light microscopy[1] or by the detection of MPO by immunocytochemistry[5] and the presence of other myeloid markers by flow cytometry, as previously described.[6] According to the FAB criteria,[1,2] two cases were classified as M0, 12 cases as M1, 13 cases as M2, 10 cases as M3, 11 cases as M4, 12 cases as M5, two cases as M6 and four cases as M7. The diagnosis of M4 and M5 categories was based on the demonstration of the monocytic component by the ANAE cytochemical staining on BM smears.[7] Acute biphenotypic leukemia cases were excluded from the analysis. All BM samples were collected after informed consent.

Isolation of BM blast cells

BM samples were obtained by posterior iliac crest aspiration into EDTA-containing tubes and mononuclear cells isolated by density gradient centrifugation and kept frozen (−80°C) until the day of the experiment.

Reagents

All chemicals used in this study were analytical-grade reagents. Hydrogen peroxide was obtained from Peróxidos do Brasil (São José dos Campos, SP, Brazil), and the sodium salt of 9,10-dibromoanthracene-2-sulfonate (DBAS) and 2-methyl-1-propenylbenzoate (MPB) stock solutions were prepared in absolute ethanol, as previously described.[8] HRP type I was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Chemiluminescent assay

BM mononuclear cells were thawed at the day of the experiment, washed twice, resuspended in Dulbecco's buffer pH 7.4 (38 mmol/l phosphate buffer) and adjusted for a final concentration of 10^6 cells/ml. One million mononuclear cells were then added to the standard reaction mixture (1 mmol/l MPB, 2.77 U HRP type I, 7.4×10^{-5} mol/l H_2O_2 and 1.62×10^{-5} mol/l DBAS) at 37°C in 1 ml of final volume. The reaction was carried out in a BioOrbit® model 1251 luminometer (Turku, Finland). Two parameters of chemiluminescence were measured, peak light emission (PLE) and integrated light emission (ILE), which represents the area under the curve (AUC) for luminescence, for a 10-min analysis period. The values of PLE and ILE for each individual were the mean of three independent measures. The reactions were also performed in the presence of sodium fluoride (final concentration, 5 mM) added to the standard reaction mixture in identical experimental conditions.

Statistical analysis

The nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison test was used to evaluate the chemiluminescence emission among different AML subtypes. Concordance between chemiluminescence and ANAE was tested by the kappa coefficient of agreement. A P value of <0.05 was considered to be statistically significant.

Results

Peak light emission

PLE was taken in triplicate and the results of different measures varied less than 5% for each individual. PLE was significantly higher in FAB-M5 AML cases (median, 3.4 mV; range, 1.5–14) in comparison to other AML cases (FAB-M0/M1, 0.3 mV, 0.1–1.6, $P<0.001$; FAB-M2, 0.8 mV, 0.1–2.0, $P<0.01$; FAB-M3, 0.7 mV, 0.2–1.0, $P<0.001$; FAB-M6/M7, 0.3 mV, 0.2–0.8, $P<0.001$; Figure 1). However, the difference in PLE between the FAB-M5 and FAB-M4 cases (1.8 mV, 0.9–3.1) was not statistically significant. In addition, PLE was significantly higher in FAB-M4 AML cases in comparison to FAB-M0/M1 ($P<0.001$) and FAB-M6/M7 cases ($P<0.001$; Figure 1). However, Dunn's multiple comparison test was unable to demonstrate significant differences between FAB-M4 and FAB-M2, FAB-M3 and FAB-M5 when multiple comparisons were employed (Figure 1).

Integrated light emission

ILE was also taken in triplicate and variation was less than 5% in each sample. The ILE was also significantly higher in FAB-M5 cases (38.4 mV, 23.1–387.8) in comparison to other AMLs (FAB-M0/M1, 2.3 mV, 0.5–4.1, $P<0.001$; FAB-M2, 6.6 mV, 0.4–8.9, $P<0.01$; FAB-M3, 3.9 mV, 2.0–6.9, $P<0.001$; FAB-M6/M7, 3.3 mV, 0.8–5.8, $P<0.001$; Figure 2), but not in comparison to FAB-M4 (13.4 mV, 8.2–22.9). In addition, the ILE was also significantly higher in M4 AML cases when compared to FAB-M0/M1 ($P<0.001$), FAB-M3 ($P<0.05$) and FAB-M6/M7 cases ($P<0.05$; Figure 2). Dunn's multiple comparison test failed to demonstrate statistic differences between FAB-M4 and FAB-M2 and FAB-M5 cases (Figure 2).

Fluoride inhibition

In acute monocytic/monoblastic leukemia (FAB-M5) samples, the chemiluminescence emission was completely inhibited by the addition of sodium fluoride to the standard reaction mixture (Figure 3). Moreover, sodium fluoride was able to inhibit more than 70% of chemiluminescence in acute myelomonocytic (FAB-M4) leukemia cells. For other AML cells, fluoride inhibition was less than 20%.

ILE vs *FAB classification*

We also investigated whether analysis of ILE was efficient in classifying AML cases into FAB-M4, FAB-M5 and nonmonocytic AML subtypes. Two different

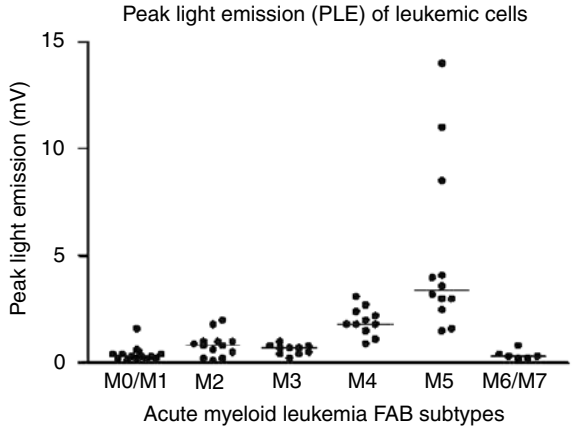

Figure 1 Esterase activity of leukemic myeloid cells of different FAB subtypes measured by the PLE in a luminometer. FAB-M5 cases presented significantly higher PLE in comparison to other AMLs, but not in comparison to M4. PLE was also higher in FAB-M4 cases in comparison to FAB-M0/M1 and FAB-M6/M7 cases. Bars represent median values for each group.

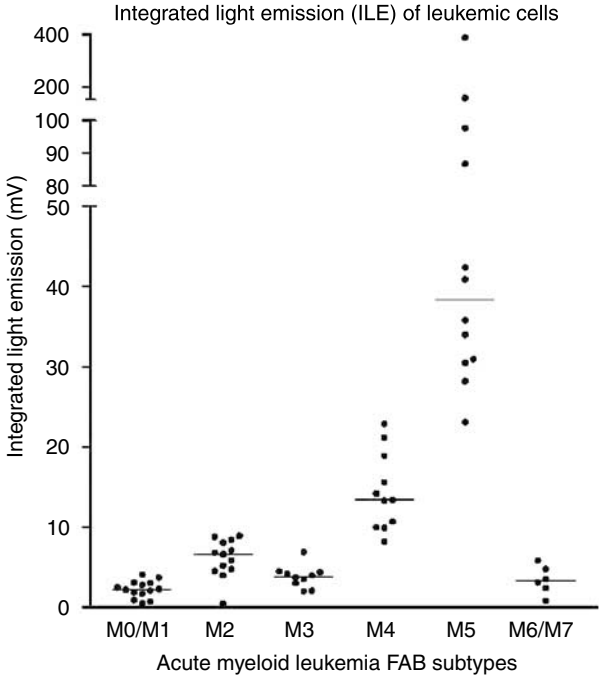

Figure 2 Esterase activity of leukemic myeloid cells of different FAB subtypes measured by ILE in a luminometer. FAB-M5 cases presented significantly higher ILE in comparison to other AMLs, but not in comparison to M4. PLE was also higher in FAB-M4 cases in comparison to FAB-M0/M1, FAB-M3 and FAB-M6/M7 cases. Bars represent median values for each group.

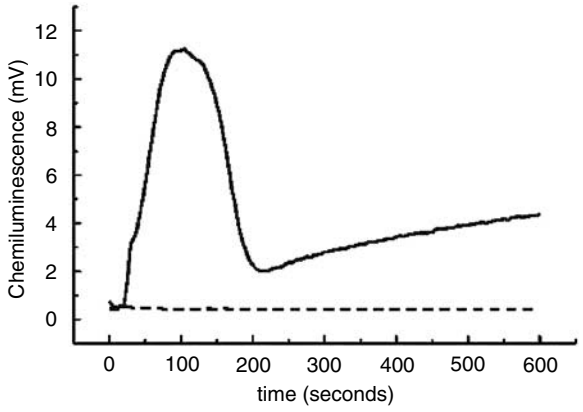

Figure 3 Pattern of chemiluminescence (mV) as a function of time (s) measured in a luminometer representing the esterase activity of a FAB-M5 AML. Reactions were carried out in the absence (black line) and in the presence (gray line) of sodium fluoride, demonstrating the fluoride-inhibited chemiluminescence.

Table 1 Classification of acute myeloid leukemia cases in FAB-M4, FAB-M5 and nonmonocytic subtype using the ANAE staining and chemiluminescence*

ANAE	*Chemiluminescent assay*			*Total*
	FAB-M4	*FAB-M5*	*Nonmonocytic*	
FAB-M4	9	0	2	11
FAB-M5	0	12	0	12
Nonmonocytic	0	0	43	43
Total	9	12	45	66

*Kappa coefficient of agreement, 0.94 ($P<0.001$).

reference values were set (10.0 and 23.0 mV). Cases with ILE values less than 10.0 mV were classified as nonmonocytic, cases with ILE values between 10.0 and 23.0 mV as FAB-M4, and cases with ILE values greater than 23.0 mV as FAB-M5. According to these parameters, nine cases were categorized as FAB-M4, 12 cases as FAB-M5 and 43 cases as nonmonocytic AML, with an overall agreement of 97% (kappa coefficient of agreement, 0.04; $P<0.001$). Two cases of FAB-M4 were classified as nonmonocytic by this method (Table 1).

Discussion

The monocytic component of FAB-M4 and FAB-M5 AML is usually assessed by the demonstration of nonspecific esterase activity by cytochemical reactions, such as ANAE and ANBE.[1,7] In FAB-M4 AML, the monocytic component is between 20 and 80% of BM nonerythroid cells, while in FAB-M5 AML, monocytic commitment is present in over 80% of BM nonerythroid cells.

In the present study, we evaluated the ability of a chemiluminescent assay to determine the nonspecific esterase activity of the FAB-M4 and FAB-M5 AML blast cells and its efficiency in identifying these AML categories. Esterases from monocytic cells promote the hydrolysis of MPB in 2-methyl-1-propenol, which can be catalyzed by HRP into triplet acetone.[4] The reaction produces acetone phosphorescence that, enhanced by DBAS, may be measured by a luminometer.

We demonstrated that the chemiluminescent method is able to determine esterase activity of monocytic leukemic cells and hence to demonstrate the monocytic component of FAB-M4 and FAB-M5 AML. Using ILE, which was found to be more sensitive to detect esterase activity from blast cells than PLE, classification of AML cases into FAB-M4, FAB-M5 and nonmonocytic subtypes was concordant with ANAE classification in 97% of the cases (kappa coefficient of agreement, 0.94). However, two cases of FAB-M4 were categorized as nonmonocytic by this method. This fact may be explained by different reasons. First, the monocytic component of M4 AML is variable, ranging from 20 to 80%, and it may be difficult to differentiate FAB-M4 AML cases with monocytic commitment near 20% from M2 AML cases with some degree of monocytic component (lower than 20%) or with nonspecific light emission. However, the fluoride-inhibited pattern of chemiluminescence does not support the latter hypothesis (Figure 3) and may serve to differentiate cases near the threshold. FAB-M4 cases had a high fluoride inhibition of luminescence (over 70%) in comparison to other nonmonocytic AMLs (less than 20% of inhibition). Finally, the intensity of esterase activity is variable in FAB-M4 blast cells, as is usually noticed in ANAE reactions. Hence, lower contents of esterase in FAB-M4 blast cells may lead to lower chemiluminescent activity.

On the other hand, in the present study, frozen cells were analyzed and this procedure could impair or reduce the esterase activity of leukemic cells. However, some cases were studied before and up to 1 year after freezing, and the results did not change over time (data not shown).

A new classification of acute leukemias has been recently proposed by the WHO.[3] The WHO classification highlights the cytogenetic abnormalities for the categorization of *de novo* AMLs. However, those *de novo* AML cases without recurrent cytogenetic alterations are classified in a manner similar to that proposed by FAB.[1,2] FAB-M4 is named acute myelomonocytic leukemia and FAB-M5 is named acute monocytic/monoblastic leukemia, and the criteria adopted for categorization are not different from FAB.[3]

Until now, it is not known if the WHO classification will be as popular as the FAB classification among hematologists. However, even if time favors the WHO classification, the demonstration of the monocytic component of blast cells will not be neglected. Assays demonstrating esterase activity of myeloid blast cells will certainly have a part in future classifications of AMLs.

It is important to observe that nonspecific esterase stainings[7] are laborious, time-consuming (takes approximately 3.0 h from staining until reading the smears), and may be difficult to interpret since it is a subjective method. Since the chemiluminescent assay is simple, fast (each assay takes less than 30 min from thawing the samples up to completing the luminometer analysis) and

objective, this new technique is advantageous and may be a useful tool in the diagnosis of monocytic leukemias. In addition, the luminometer is not very expensive, is available in most laboratories for clinical analysis, and is used in different luminescent assays.

In conclusion, the chemiluminescent assay herein analyzed was able to demonstrate the monocytic component of FAB-M4 and FAB-M5 AML, and may be a helpful tool in the differential diagnosis of AML subtypes. Prospective studies with larger series of patients are needed to validate the accuracy of this method in the diagnosis of FAB-M4 and FAB-M5 AMLs and in the differential diagnosis between these leukemia categories.

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