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p-Iodophenol-enhanced luminol chemiluminescent assay applied to discrimination between acute lymphoblastic and minimally differentiated acute myeloid (FAB-M0) or acute megakaryoblastic (FAB-M7) leukemias

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***Introduction*:** In this report, we propose the application of the *p*-iodophenol-enhanced luminol chemiluminescent technique to the determination of peroxidase (myeloperoxidase and/or platelet peroxidase) activity in blasts of minimally differentiated acute myeloblastic leukemia (AML-M0) and acute megakaryoblastic leukemia (AML-M7).
***Methods*:** The frozen blast cells from 29 patients were thawed and submitted to the optimized protocol.
***Results*:** All cases of AML-M7 and AML-M1 exhibited integrated light emission greater than 73 (10^2 mV × s), which was the arbitrary cutoff point set for the discrimination between AML and acute lymphoblastic leukemia (ALL) (mean + 3 × s.d. of ALL samples, $n = 10$). In addition, five out of seven cases of AML-M0 showed results above the cutoff point.
***Conclusion*:** This highly sensitive enhanced chemiluminescent technique may be applied to discriminate between ALL and AML-M7 or AML-M1 cases, and most AML-M0 cases. It is very simple, cheap and easy to perform compared to other procedures used to measure MPO activity in AML-leukemias including AML-M7 and AML-M0.
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Introduction

The WHO classification of acute leukemias takes into account clinical, morphological, cytochemical, immunophenotypic and genetic features.[1] Immunophenotypic studies are essential for the characterization of minimally differentiated acute myeloid leukemia (AML-M0, FAB classification) and acute megakaryoblastic leukemia (AML-M7, FAB classification), since the cytochemical reaction for myeloperoxidase (MPO) is negative.[2] Electron microscopy cytochemistry is able to detect extremely low MPO activity in small granules, endoplasmic reticulum, Golgi area and nuclear membrane of AML-M0 blasts, and in nuclear membrane and endoplasmic reticulum of AML-M7 blasts. However, this methodology is unsuitable for clinical purposes.

Chemiluminescent techniques are routinely employed for enzymatic determination in biomedical laboratory, especially when sensitivity is crucially important.[3] Our research group has been working with the synthesis of substrates and the development of chemiluminescent assays for the determination of enzymatic activity. Specifically, our interest is focused on enzymes that might be used as labels for the identification and discrimination of acute leukemias. In this regard, we have proposed the compound 2-methyl-1-propenyl-benzoate as a chemiluminescent substrate to measure the esterase activity of monocytic components to discriminate FAB-M4 and M5 from other acute myeloid leukemias.[4] In addition, we have demonstrated the use of the enol ether of isobutyraldehyde to differentiate acute lymphoid leukemia (ALL) and AML. The latter technique enabled the characterization of myeloid cells, including minimally differentiated AML (FAB-M0).[5] However, its prompt application in hematology laboratories as a complementary test was hindered by the lack of commercial source of this substrate.

In this report, we propose a new highly sensitive enhanced chemiluminescent technique based on a commercially available substrate (*p*-iodophenol-enhanced luminol) for the determination of MPO activity in blasts of minimally differentiated AML-M0 and AML-M7.

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Materials and methods

Chemicals: All the reagents used for buffer preparation were of analytical grade. Protein determination kit was purchased from Doles (Brazil). Hydrogen peroxide 30% was purchased from Merck (Brazil). Myeloperoxidase (E.C. 1.11.1.7), luminol, *p*-iodophenol, *p*-bromophenol, *p*-phenylphenol and Ficoll–Hypaque were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Patients: Bone marrow or peripheral blood samples from 29 patients were selected for the study. AML and ALL were diagnosed when $\geqslant$30% myeloblasts or lymphoblasts were found in the bone marrow. The cytochemical profiles were determined for myeloperoxidase and α-naphthyl acetate esterase activities. According to the FAB classification, 10 cases were classified as acute lymphoblastic leukemia and 19 as AML, including seven AML-MO, six AML-M1 and six AML-M7 subtypes.

Isolation of blast cells and immunophenotyping: Blast cells from bone marrow or peripheral blood were isolated in a Ficoll–Hypaque gradient and kept frozen at −80°C in DMSO. Immunophenotyping was performed on fresh cells or cryopreserved cells with a panel of monoclonal antibodies by direct immunofluorescence and analyzed by flow cytometry in a FACScan (Becton Dickinson, San Jose, CA, USA). All antibodies were purchased from Becton Dickinson except for CD61, CD117 and MPO (Dako AS, Denmark) and CD42b (Pharmingen International). All blasts were initially tested for the following antigens: CD45, CD2, CD33, CD13, HLA-DR, CD117, anti-MPO, CD34, CD42a, CD10, CD19, cIgM, TdT, kappa and lambda-chains. Samples, not definitely classified with the first panel of antibodies, were tested against a second panel which included: CD1, cCD3, CD5, CD7, cCD22, CD4, CD8, TCR-1, CD16, CD57, CD11b, CD14, CD15, CD42b and CD61. Cell Quest software (Becton Dickinson) was used for data acquisition and analysis. Results were considered positive if 20% or more blast cells expressed a particular antigen.

Myeloperoxidase determined by immunocytochemistry: Cytospin slides from bone marrow or peripheral blood blast cells previously isolated on a Ficoll–Hypaque gradient were stained for the detection of myeloperoxidase antigen (clone MPO-7, Dako A/S, Denmark), using the peroxidase:antiperoxidase procedure as previously described.[5]

Chemiluminescent assay for determination of peroxidase activity of blast cells: The *p*-iodophenol-enhanced chemiluminescent assay for determination of MPO and PPO activity of blast cells was adapted from the technique previously described for horseradish peroxidase determination.[6] It was optimized by varying experimental parameters such as type and concentration of buffer, luminol, enhancers, strength of hydrogen peroxide, pH and temperature (data not shown). Commercial MPO was used in these experiments and the best results were obtained with Tris-HCl buffer 0.2 mol/l, hydrogen peroxide 67 μmol/l, luminol 34 μmol/l, pH 8.7, 25°C and a final volume of 750 μl. The assays were triggered by the addition of hydrogen peroxide and reactions were carried out in a BIOOrbit model 1251 luminometer (Turku, Finland). A typical emission profile for luminol oxidation catalyzed by MPO with or without the enhancer is presented in Figure 1. *p*-Phenylphenol (*p*-PP), *p*-bromophenol (*p*-BP) and *p*-iodophenol (*p*-IP), which are commonly used as enhancers in luminol assays, were compared. The stock solutions of enhancers were prepared in ethanol and the final concentration of ethanol in the assays was 0.4 mol/l or less. The best results were obtained with *p*-IP and the effect was concentration dependent (Figure 2). Above 5 mmol/l a suppression of the chemiluminescence by all enhancers was verified.

Neutrophils from healthy donors and separated by Ficoll–Hypaque centrifugation were used to test the sensitivity of the assay. In this case, all experimental parameters were as above, except that MPO was

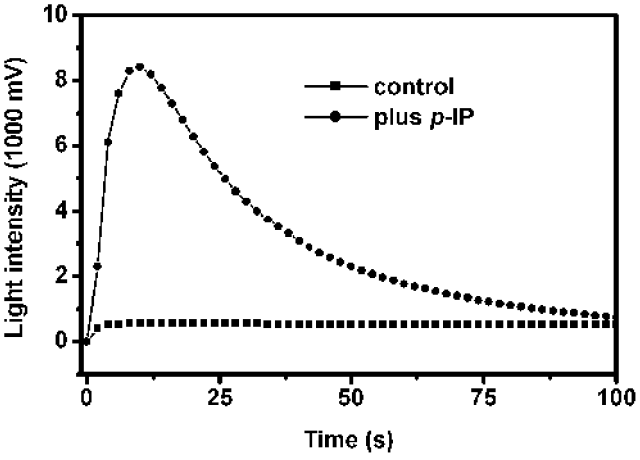

Figure 1 Typical light emission profile of MPO/H_2O_2/luminol system and the reaction enhanced by the addition of *p*-IP. Reaction conditions: H_2O_2 67 μmol/l; luminol 34 μmol/l; MPO 65 mU/l; *p*-IP 25 μmol/l; Tris-HCl buffer 0.2 mol/l, pH 8.2; 25°C; final volume 750 μl.

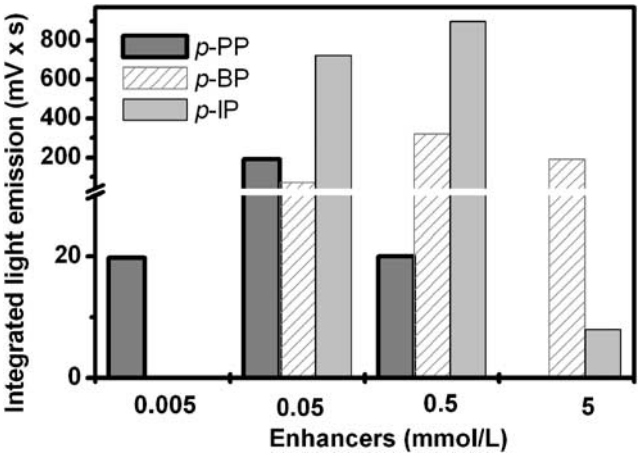

Figure 2 Effect of the enhancers and their concentration on ILE: *p*-PP; *p*-BP; *p*-IP. Reaction conditions: H_2O_2 67 μmol/l; luminol 34 μmol/l; MPO 65 mU/l; Tris-HCl buffer 0.2 mol/l, pH 8.7; 25°C; final volume 750 μl. ILE measured over 5 min.

replaced by a known number of neutrophils. The reactions were triggered by adding hydrogen peroxide and the integrated light emission measured over 5 min. It was possible to detect the MPO activity present in less than 100 neutrophils/assay (data not shown).

Results

Leukemic cells analysis

The frozen blast cells (see methods) were thawed on the day of the assay, washed three times, counted and kept in Dulbecco's buffer, pH 7.4. A known number of cells (10^5 cells/assay) were submitted to the optimized chemiluminescent assay as above, with 0.5 mmol/l *p*-IP. Table 1 presents the results of integrated light emission (ILE) and a complete panel of tests for classification of AML-M0, AML-M1 and AML-M7 cases. Blast cells from AML-M0 and AML-M7 patients did not stain for MPO by light cytochemistry. The number of positive MPO cells detected by immunocytochemistry or flow-cell cytometry with anti-MPO was $<3\%$. In contrast, all cases of AML-M7 and AML-M1 exhibited ILE greater than 73 (10^2 mV $\times$ s), the arbitrary cutoff point set for the discrimination between AML and ALL (mean + 3 s.d. of the ALL samples). On the other hand, five out of seven cases of AML-M0 showed results above the cutoff point.

Discussion

Substituted phenols are well-known to enhance the chemiluminescence of luminol during its oxidation catalyzed by horseradish peroxidase. These compounds improve the analytical characteristics of the reaction by increasing the signal and decreasing the back ground light emission.[6] In this study, we obtained similar results using either purified MPO or isolated neutrophils.

Despite the advances in immunophenotyping assays and molecular genetics, MPO cytochemical activity is widely used as an analytical tool to classify acute leukemias. Discrimination between AML and ALL is crucially important, since the prognostic and therapeutic procedures are totally dependent on this classification. The peroxidase activity (MPO and PPO) is also a determinant criterion in the diagnosis of minimally differentiated AML and acute megakaryoblastic leukemia.[2] The low level of MPO and PPO activity in these leukemia subtypes cannot be detected by conventional cytochemistry or immunocytochemistry, and hence electronic microscopy cytochemistry is the only analytical tool available in these cases.[7] Here, using the optimized technique, it was possible to detect the MPO activity present in less than 100 neutrophils/assay. Regarding leukemia blast cells, it was possible to distinguish between ALL and AML, including AML-M7 and most cases of AML-M0. The finding of two cases of AML-M0 with ILE results below the cutoff point is not unexpected. In fact, Eguchi *et al*.[8] have demonstrated by ultrastructural studies that the MPO activity was negative in one-fifth of AML-M0 cases. It is also important to consider that it was possible, for the first time, to measure the peroxidase activity of PPO, which is present in the acute megakaryoblastic leukemia. The only way to determine this enzyme is usually through electronic microscopy cytochemistry.[2] Finally, the analytical protocol described here does not require

Table 1 Results of ILE and FAB classification of acute leukemias

Patient no.	*FAB*	*Bone marrow (% blasts)*	*ILE (10^2 mV $\times$ s)*	*Markers %*		
				CD13	*CD33*	*Other*
1	M0	100	150	44	90	HLA-DR+, CD117+, CD34−, MPO−[a]
2	M0	96	300	92	93	HLA-DR+, CD117−, CD34+, CD42a−, MPO−
3	M0	96	2850	92	11	HLA-DR+, CD117+, CD34+, MPO−, CD42a−, MPO−
4	M0	89	5600	52	6	HLA-DR+, CD117+, CD34+, CD42a−, MPO−
5	M0	98	745	77	67	HLA-DR+, CD117+, CD34+, CD42a−, MPO−
6	M0	90	9	52	74	HLA-DR−, CD117−, CD34+, CD42a−, MPO−
7	M0	95	25	74	45	HLA-DR+, CD117+, CD34+, CD42a−, MPO−
8	M1	98	5500	88	89	HLA-DR+, CD117+, CD34+, CD42a−, MPO+
9	M1	91	8800	3	75	HLA-DR+, CD117+, CD34+, CD42a−, MPO+
10	M1	96	6900	92	87	HLA-DR+, CD117+, CD34+, CD42a−, MPO+
11	M1	94	5100	26	94	HLA-DR+, CD117+, CD34−, CD42a−, MPO+
12	M1	92	5000	70	82	HLA-DR+, CD117+, CD34+, CD42a−, MPO+
13	M1	91	4100	11	95	HLA-DR+, CD117+, CD34−, CD42a−, MPO+
14	M7	59	1100	10	22	HLA-DR−, CD117−, CD34−, CD42a+, CD61+, MPO−
15	M7	39	1550	94	73	HLA-DR+, CD117+, CD34+, CD42a+, CD61+, MPO−
16	M7	90	1670	30	48	HLA-DR+, CD117+, CD34+, CD42a+, CD42b+, CD61−, MPO−
17	M7	72	300	78	86	HLA-DR−, CD117+, CD34+, CD42a−, CD61+, MPO−
18	M7	86	700	84	95	HLA-DR−, CD117−, CD34−, CD42a−, CD61+, MPO−
19	M7	27[b]	3500	19	55	HLA-DR+, CD117+, CD34−, CD42a−, CD61+, MPO−

[a]MPO determined by flow-cell cytometry or immunocytochemistry.
[b]32% of blasts in peripheral blood.
ILE was measured over 5 min using the optimized protocol (see Materials and methods).
The cutoff point set for the discrimination between AML and ALL was 73 (10^2 mV $\times$ s), which is the mean + 3 s.d. of the ALL cases ($n = 10$).

expertise in cell morphology recognition, is fast (each assay takes less than 20 min from thawing the samples up to completing the luminometer analysis), objective, and can be performed with inexpensive commercial chemicals.

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