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Chemiluminescent Determination of Leukocyte Alkaline Phosphatase: An Advantageous Alternative to the Cytochemical Assay

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The determination of leukocyte alkaline phosphatase (LAP) is used as an aid to diagnose many diseases in the laboratory. For example, it can be used to distinguish chronic myeloid leukemia (CML) from other myeloproliferative disorders (particularly myelofibrosis and polycythemia) and leukemoid reactions (LR). Traditionally, this test is performed with the use of subjective cytochemical assays that assign a score to the level of LAP. Here we present a nonsubjective, quantitative, sensitive, and inexpensive chemiluminescent technique that determines LAP based on the commercial reagent Immulite® (AMPPD). To validate this methodology, intact leukocytes obtained from 32 healthy subjects, nine CML patients, and nine LR patients were submitted to the optimized protocol. By measuring the light emission elicited by four concentrations of neutrophils, we were able to estimate the activity of LAP per cell (the slope of the curve obtained by linear regression). A high linear correlation was found between the chemiluminescent result (slope) and the cytochemical score. The slope for healthy individuals ranged between 0.61 and 8.49 (10^{-5} mV.s/cell), with a median of 2.04 (10^{-5} mV.s/cell). These results were statistically different from those of CML patients (range = 0.07–1.75, median = 0.79) and LR patients (range = 3.84–47.24, median = 9.58; $P < 0.05$). J. Clin. Lab. Anal. 21:91–96, 2007.

Key words: chemiluminescence; leukocyte alkaline phosphatase; Immulite® (AMPPD); chronic myeloid leukemia; leukemoid reactions

INTRODUCTION

Leukocyte alkaline phosphatase (LAP) is a monophosphoesterase that is normally contained in the mature neutrophil granulocytes of healthy individuals (1). The cytochemical LAP assay score is based on the intensity of staining (judged visually) and the number of positive granules in the cytoplasm of neutrophils (2). Since this mode of assessment is subjective, each laboratory must establish its own normal range, and experienced personnel are required. Moreover, this staining techni-

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que has poor reproducibility and is laborious and time-consuming.

LAP activity assessed by cytochemical reactions may be used in laboratory diagnosis to discriminate between CML and diseases such as polycythemia vera, leukocytosis, and leukemoid reactions (LR), which are secondary to infection or neoplasia (3). Despite the greater availability of molecular analysis, LAP assays continue to be used in a two-step workup of findings in the complete blood count that make CML a diagnostic consideration. In the usual strategy, suspect hematological data are first assessed in the light of the patient's history, physical examination, and LAP results. The combined information is then used to determine whether to use bone marrow biopsy, cytogenetics, or molecular analysis (4). Although it is not decisive for this diagnosis, a decrease or lack of LAP activity in blood neutrophils supports the clinical and morphological diagnosis of CML. In fact, the mRNA for LAP is undetectable in the neutrophils of more than 95% of patients with CML (1). A cytogenetic and molecular determination of Philadelphia chromosome and bcr-abl translocation is conclusive for the diagnosis of this disease.

The determination of LAP may also be used for other purposes, such as to evaluate neutrophilic function (5), discriminate chronic neutrophilic leukemia (6), and as complementary laboratory criteria to discriminate between polycythemia vera and secondary polycythemia (7). In addition, the LAP assay has been proposed as a means of distinguishing between malignant and benign paraproteinemias (8), as a predictor of the metastatic state of breast and colorectal cancer (9), and as a prognostic test for lung cancer (10). It is possible that further applications for LAP determination will be discovered in the future.

In addition to its importance in cytochemical assays, alkaline phosphatase (AP) is also a commonly used enzyme in immunoenzymatic techniques that achieve the required sensitivity with the use of chemiluminescent substrates such as 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy) phenyl-1,2-dioxetane (AMPPD). This is a phosphate-protected dioxetane compound that upon enzymatic hydrolysis becomes unstable and decomposes, generating prolonged light emission. With this sensitive method, attomoles of AP can be detected (11). Currently, AMPPD is the main substrate in commercial chemiluminescence-based kits, such as the Immulite® system, used in automated enzyme-linked immunosorbent assays (ELISAs) (12).

Our aim was to investigate the possibility of developing a sensitive and nonsubjective chemiluminescent assay for the determination of LAP activity using the AMPPD substrate. Using the Immulite® substrate, we were able to detect LAP activity in 500 neutrophils/mL in healthy individuals. A strong correlation was obtained between the classic cytochemical and AMPPD-chemiluminescent assays, and all studied cases of CML were distinguished from LR.

MATERIALS AND METHODS

All of the reagents used in this study were of analytical grade. The Leucognost-Alpa® kit for cytochemical determination of LAP activity was purchased from Merck (Darmstadt, Germany). Immulite® substrate was purchased from DPC (Los Angeles, CA), and Dextran T500 was obtained from Pharmacia Amersham (Uppsala, Sweden). Ficoll-Hypaque 1077® was purchased from Sigma Diagnostics (St. Louis, MO).

Leukocytes Isolation

Total leukocytes were isolated from blood collected with heparin (EDTA inhibits LAP activity) by dextran hemosedimentation. After isolation the leukocytes were washed twice, resuspended in 0.9% NaCl, and counted with a hemocytometer (13). Peripheral blood mononuclear (PBMC) and polymorphonuclear (PMN) cells were separated on a Ficoll-Hypaque® gradient, washed twice, and resuspended in 0.9% NaCl.

Analytical Protocol

The reaction mixture consisted of 100 μL of Immulite® reagent, 50 μL of leukocyte suspension, and deionized water to a final volume of 500 μL. The reaction was triggered by the Immulite® reagent and measured for 15 min at 25°C in a BioOrbit 1251 luminometer (Turku, Finland). The integrated light emission was used as analytical parameter. The assays were performed in triplicate at each dilution. The cytochemical LAP score was determined as previously described (2).

Patients

After the study was approved by the Brazilian Ethics Committee and informed consent was obtained from the subjects, peripheral blood samples were collected in evacuated heparinized tubes from 32 normal healthy volunteers, nine CML patients (at diagnosis or not responding to hydroxyurea/α-interferon treatment), and nine LR patients. All of the LR patients had white cell counts higher than 25×10^6 per mL.

Statistics

The results were expressed as the median and 25th and 75th percentiles. Differences in LAP activity among

control, CML, and LR groups were tested by Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks, followed by Dunn's method for pairwise multiple comparison, with $P \leq 0.05$.

RESULTS

We first demonstrated that LAP, like the antibody-conjugated AP widely used in immunoenzymatic tests, was able to catalyze the hydrolysis of AMPPD substrate, leading to light emission. Figure 1 shows a typical light emission profile obtained with neutrophils from a healthy donor. Since this is a new application for a commercial and optimized substrate for ELISA, we performed kinetic studies to find the appropriate experimental conditions to measure LAP activity. Figure 2 shows the correlation between substrate concentration and light emission for a fixed number of neutrophils. Substrate saturation (above 15%) was chosen because it guarantees that the light emission depends only on LAP activity. Under these conditions, a high linear correlation was obtained between the number of cells and the chemiluminescent signal (Fig. 3). The detection limit was 500 neutrophils/mL (signal-to-noise ratio (SNR) = 3) and the technique was linear in the range of 500–10,000 neutrophils/mL.

In addition to being sensitive, this technique has a high specificity, since AP is not found in peripheral mononuclear cells. In fact, neither a pure preparation of neutrophils nor the extraction of LAP from these cells was necessary; hence, as stated above, cells were isolated by dextran hemosedimentation and total leukocytes were used in the assay. Experiments performed with mononuclear cells isolated by the Ficoll-Hypaque® density gradient confirmed the absence of AP in these cells (data not shown).

Leukocytes obtained from 32 healthy subjects recruited from among blood donors at the local blood bank (Hemonúcleo Regional de Araraquara, SP) were used to establish a normal range for the chemiluminescent assay. For each sample, four different concentrations of leukocytes were analyzed. In addition to

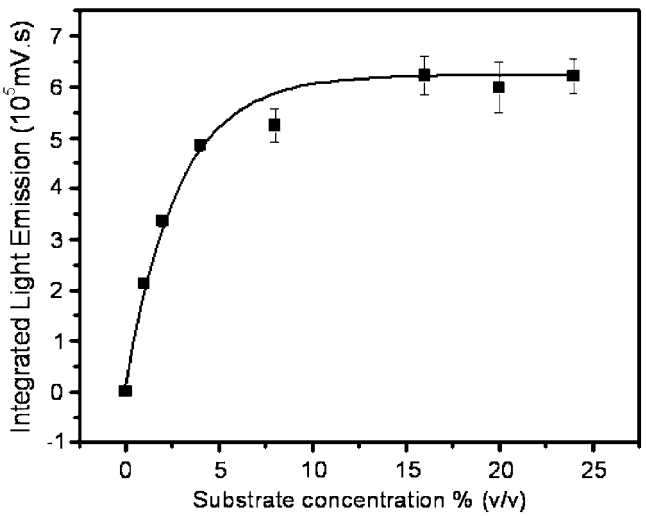

Fig. 2. Effect of Immulite® concentration on integrated light emission for a fixed number of neutrophils (6×104 cells/mL). See Materials and Methods for the analytical protocol. The data are reported as the mean and SEM of triplicates.

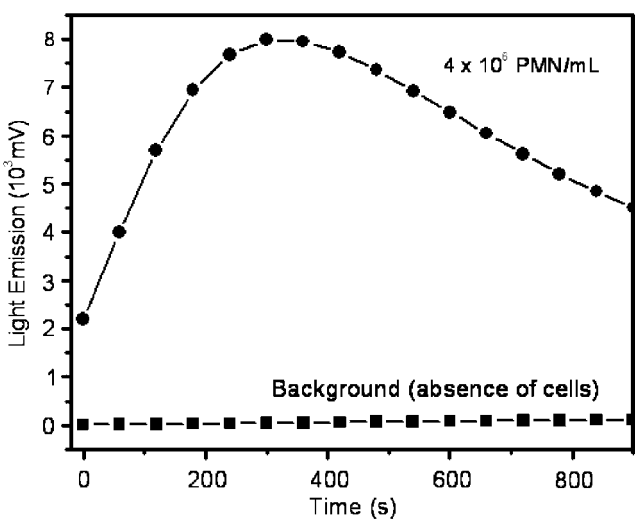

Fig. 1. Typical kinetic profile of light emission promoted by LAP-catalyzed AMPPD hydrolysis. See Materials and Methods for the analytical protocol.

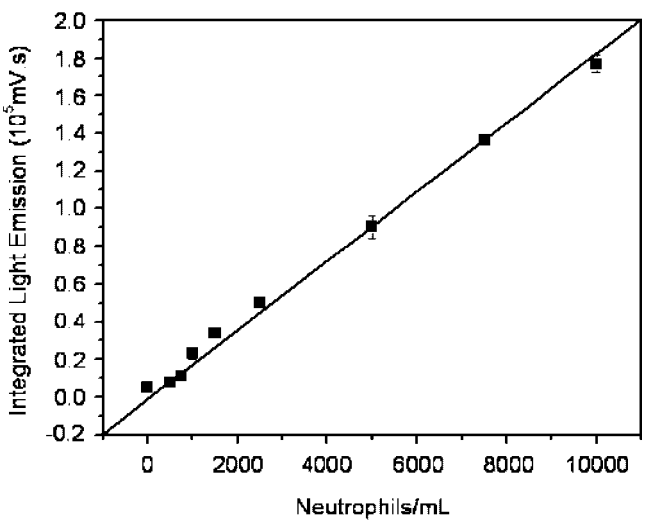

Fig. 3. Correlation between number of neutrophils and integrated light emission. See Materials and Methods for the analytical protocol. The data are reported as the mean and SEM of triplicates (r = 0.9994, $P < 0.0001$, $N = 9$).

ensuring that the activity of LAP was the only variable, the curve obtained for each subject allowed the exact calculation of LAP activity per cell (the slope of the curve obtained by linear regression, Microcal Origin® software). This slope was used directly as the analytical parameter. Figure 4 shows typical plots of data from two patients with very different levels of LAP, resulting in lines with high and low gradients.

Since the chemiluminescent technique was developed as an alternative to the cytochemical assay, for comparison we also performed the latter method and obtained LAP scores for healthy individuals. A high linear correlation was found between the chemiluminescent slope and the cytochemical score (Fig. 5).

Figure 6 shows the results obtained for LAP determination by chemiluminescence in neutrophils of 32 healthy individuals (controls), nine CML patients, and nine LR patients. The slope for healthy individuals ranged between 0.61 and 8.49 (10^{-5} mV.s/cell), with a median of 2.04 (10^{-5} mV.s/cell). These results were statistically different from those of CML patients

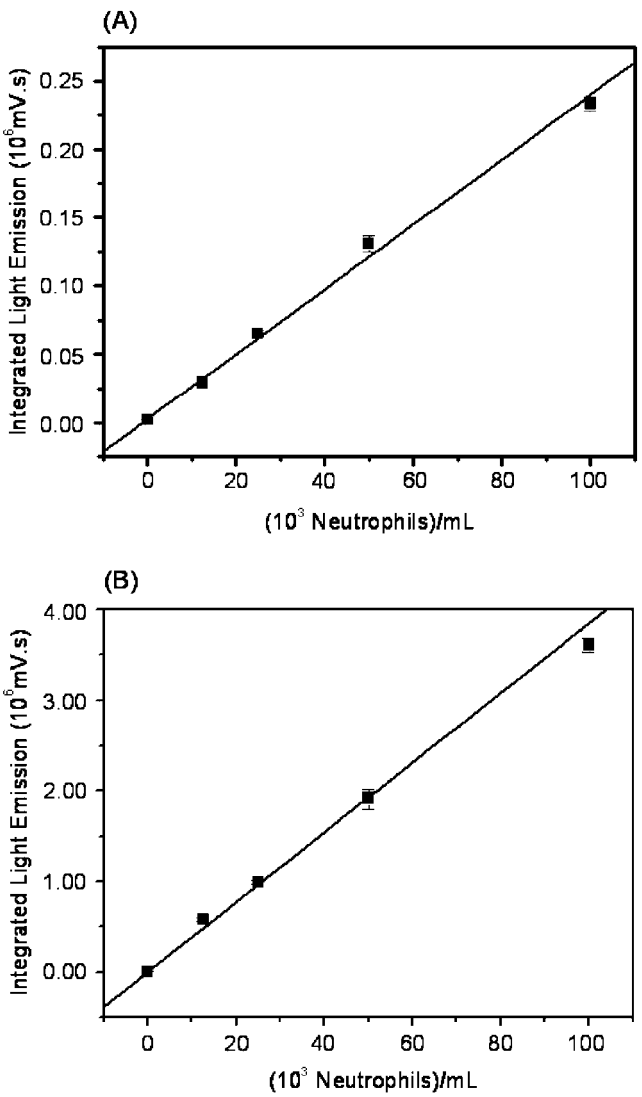

Fig. 4. Acquisition of LAP activity per cell (slope). **A:** Typical case of CML (r = 0.9989, $P < 0.0001$, slope = 0.24×10^{-5} mV.s/cell). **B:** Typical case of LR (r = 0.9966, $P < 0.0002$, slope = 3.85×10^{-5} mV.s/cell). See Materials and Methods for the analytical protocol. The point at zero represents the control in the absence of leukocytes. The data are reported as the mean and SEM of triplicates.

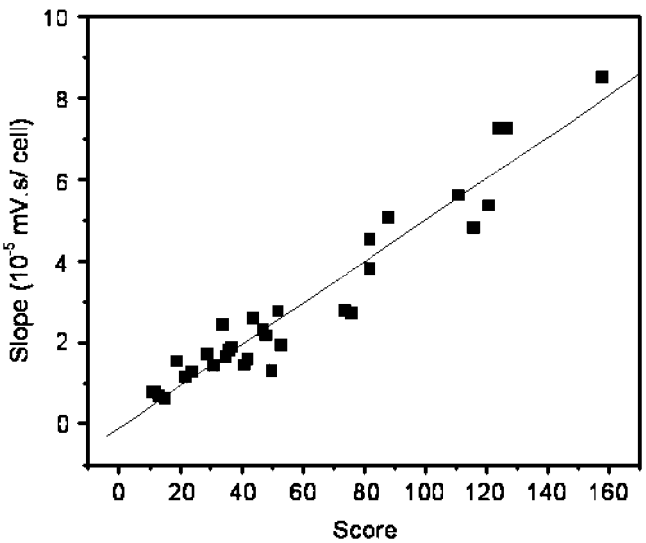

Fig. 5. Cytochemical score vs. chemiluminescent slope. See Materials and Methods for the analytical protocol (r = 0.9618, $P < 0.0001$, $N = 32$).

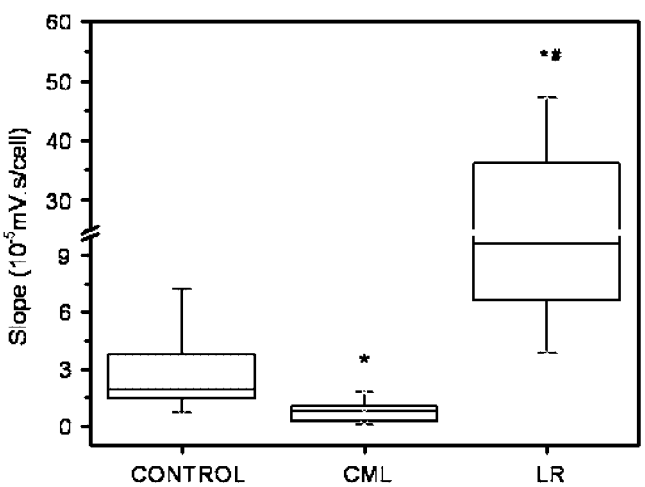

Fig. 6. Slopes obtained for the control group and CML and LR patients. See Materials and Methods for the analytical protocol. Box chart (25^{th}, median, and 75^{th} percentiles), whiskers (5^{th} and 95^{th} percentiles), $^*P < 0.05$ control vs. CML and LR, $\sharp P < 0.05$ LR vs. CML.

(range = 0.07–1.75, median = 0.79) and LR patients (range = 3.84–47.24, median = 9.58; $P < 0.05$).

DISCUSSION

APs include various isoenzymes that are able to hydrolyze phosphate from various phosphomonoesters under alkaline conditions. These isoenzymes are widely distributed in several tissues, including bone, liver, placenta, and granulocytes, and as such they are very useful as biological markers of several pathological states (14). In oncohematology there are many applications for LAP activity determination (6–10), for example to aid in differentiating CML from other myeloproliferative diseases (particularly myelofibrosis and polycythemia vera) or inflammatory/infectious diseases (3). The cytochemical technique for LAP determination is performed on fixed blood smears. LAP, which is present in mature neutrophils, catalyzes the hydrolysis of 1-naphthyl-phosphate sodium salt. The liberated naphthyl is coupled to a diazonium salt, leading to the formation of an insoluble dye at the LAP site. The colored product of the reaction is present only in the final mature stages of granulopoiesis (2). This method is semiquantitative, time-consuming, and subjective, and varies greatly among laboratories.

Our research group has been working on synthesizing substrates and developing very specific and sensitive chemiluminescent assays for enzymatic activity determination in leukocytes and blast cells for laboratory diagnosis of certain leukemias (15–18). In this study we developed a new application for the widely used AMPPD chemiluminescent substrate as an alternative to the cytochemical assay for LAP. In congruity with immunoenzymatic techniques, the AMPPD method was able to detect the enzyme present in intact neutrophils and showed a high sensitivity. Moreover, as a quantitative assay, this technique was not dependent on subjective criteria such as cell-morphology recognition. The high correlation observed in a double-blind assay between the conventional cytochemical test performed by an experienced analyst and the chemiluminescent assay is a clear indication that this protocol may be an excellent alternative to the former morphology-based method. In this study, all cases of LR were distinguished from CML when a cutoff value for LAP activity was set arbitrarily at 2.40 (10^{-5} mV.s/cell; mean+3 SD of the CML slope values).

A flow cytometry assay for LAP determination was previously described; however, it is an expensive technique and requires a specific antibody for enzyme recognition (19). As a consequence, although the cytochemical assay is antiquated and subjective, it continues to be used widely in epidemiological studies to screen for CML (20) or as a CML evolution marker (21). Normally, hematological data, including LAP results, are first assessed together with clinical data. Then the results are used to determine the need for subsequent bone marrow biopsy, cytogenetics, and molecular analysis. In other words, the LAP score can be used as a "gatekeeper" to avoid the use of more-invasive and costly procedures (4). In this context, the proposed AMPPD-dependent chemiluminescent technique is a quantitative, sensitive, and selective assay for LAP activity. In addition, it does not require expertise in cell-morphology recognition or enzyme extraction, it is fast and objective, and it can be performed with inexpensive commercial chemicals.

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