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Chemiluminescent Determination of Esterases in Monocytes

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Esterase from monocytes promotes the hydrolysis of 2-methyl-1-propenylbenzoate (MPB) yielding 2-methyl-1-propenol, which is oxidized by horseradish peroxidase/H_2O_2 producing triplet acetone. The chemiluminescence of this reaction can be enhanced by the addition of 9,10-dibromoanthracene-2-sulphonate. The non-specific esterase present in monocytes is responsible for MPB hydrolysis, since (a) the chemiluminescence of the reaction was inhibited by fluoride, and (b) cells that do not contain a significant amount of non-specific esterases, e.g. lymphocytes and neutrophils, did not trigger light emission. The analytical application of this reaction is considered. © 1998 John Wiley & Sons, Ltd.

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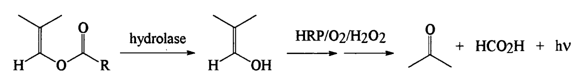
INTRODUCTION

Enzymatic action upon specific substrates yielding chemiluminescence has been extensively used as a method of determination of several substances of clinical interest. From DNA probing, together with ELISA techniques, to direct detection of important analytes for diagnosis, the number of commercial kits based on chemiluminescence has grown exponentially during the last decade (1).

We have recently described a chemiluminescent system that is triggered by hydrolases upon 2-methyl-1-propenyl esters, in the presence of H_2O_2 and horseradish peroxidase (HRP) (2, 3).



Following the release of 2-methyl-1-propenol, HRP catalyses its oxidation to acetone and formic acid, a reaction which was thoroughly studied by Cilento and co-workers (4). At the expense of dissolved oxygen and hydrogen peroxide, the reaction produces acetone phosphorescence, an emission that can be enhanced by addition of fluorescent acceptors, such as 9,10-dibromoanthracene-2-sulphonate (DBAS).

We have shown that liver esterase hydrolyses the 2-methyl-1-propenylbenzoate (MPB), triggering chemiluminescence when in the presence of HRP/H_2O_2 (3). Moreover, the light intensity correlates with the concentration of esterase. Likewise, the

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hydrolysis of 2-methyl-1-propenyllaurate by porcine pancreas lipase elicited light emission when connected to HRP/H_2O_2 enzymatic system (2, 5).

Since HRP can be replaced by other peroxidases (6), the activity of intracellular myeloperoxidase has also been explored as a chemiluminescent marker to differentiate between myeloid and lymphoid blasts from leukaemic patients (7).

Similarly, we demonstrate here the possibility of detecting mononuclear cells in a heterogeneous population from blood extracts by taking advantage of their comparatively high esterase activity (8). Furthermore, we foresee an application for this system in the detection of monocytic leukaemias of the FAB-M5 type.

MATERIALS AND METHODS

HRP (EC 1.11.1.7) type I and VI, Histopaque and Dextran were from Sigma Chemical Co. Hydrogen peroxide was from Aldrich. The synthesis of 2-methyl-1-propenyl benzoate (MPB) was as described previously (2). All other reagents were high purity commercial samples from Sigma, Merck and Aldrich.

Isolation of leukocytes

Monocytes were isolated from individuals with no major haemogram abnormalities but exhibiting moderate monocytosis (1000–1500 cells/mm^3). Human monocytes and lymphocytes were isolated by Ficoll–Hypaque (d = 1.057 g/mL) gradient. After Ficoll–Hypaque treatment, the cells were washed twice with PBS-Dulbecco medium without glucose. After mononuclear cell isolation the monocyte/lymphocyte relationship was determined by cytospin preparations of isolated cells and was generally in a ratio between 1:4 and 1:3.

Some experiments (See Fig. 6) were carried out with monocytes from a patient with a myelodysplastic syndrome with a white cell count of 51,000 cells/mm^3, from which 52% were predominantly mature monocytes and less than 1% lymphocytes. In this case, after mononuclear cell isolation, the population of monocytes was practically 100%. Lymphocytes were obtained from a patient with chronic lymphocytic leukaemia and from healthy donors after monocyte adhesion. Granulocytes were isolated from a healthy donor by a double gradient of Ficoll–Hypaque (d = 1.057 and 1.119 g/mL).

When monocytes were sonicated, a vibra cell of Sonics & Materials Ins. Danbury CT (USA) model VC 50T was used. The cells were disrupted by two pulses of 10 seconds each. The sample was kept on ice to minimize loss of enzyme activity. After sonication the material was used without any further procedure, or else it was centrifuged at 6000 *g* for 30 minutes at 4°C.

The time course of chemiluminescence was followed in a BioOrbit model 1251 luminometer (Turku, Finland). MPB stock solutions were prepared in absolute ethanol. Unless otherwise stated, the reaction was monitored in 38 mmol/L phosphate buffer, pH 7.4. The standard reaction mixture was 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.

RESULTS AND DISCUSSION

Luminescence could be observed when monocytes were incubated with $MPB/H_2O_2/HRP$ in phosphate buffer pH 7.4 in the presence of DBAS (Fig. 1). The conditions of pH and phosphate molarity of the reac-

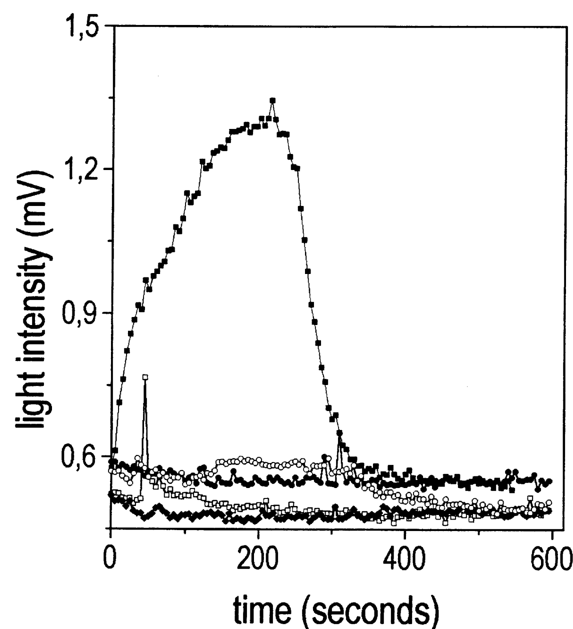

Figure 1. Light emission from the monocytes (6.4×10^5 cells/mL)/MPB/H_2O_2/HRP (type I)/DBAS system (■). The controls without HRP (□), H_2O_2 (◆), MPB (○) and monocytes (•) were also shown

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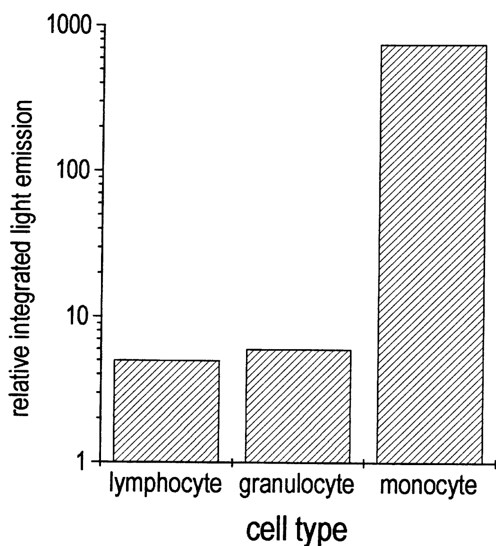

Figure 2. Integrated light emission when 2.7×10^6 monocytes, lymphocytes or granulocytes/mL were incubated with MPB/H_2O_2 (10 μmol/L)/HRP (type VI, 1 μmol/L)/ DBAS (7.9 μmol/L)

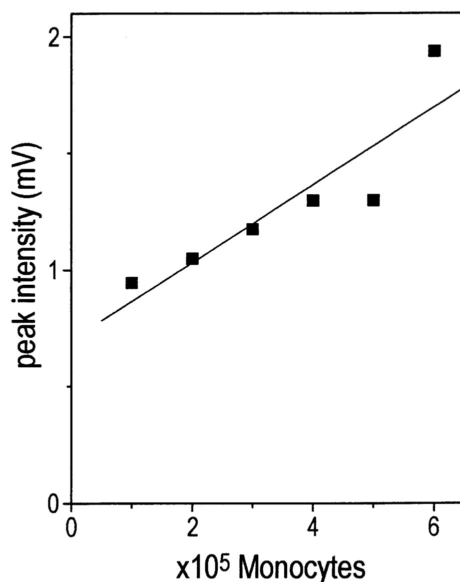

Figure 3. Correlation between maximum light emission and number of monocytes in the monocyte/MPB/H_2O_2/ HRP (type I)/DBAS system. All points were taken from the same batch of monocytes

tion are compatible with the maintenance of blood cells and esterase and HRP activities. Due to the relatively high liposolubility of MPB, it is assumed that the ester crosses the monocyte membrane and it is hydrolysed by intracellular esterases. After hydrolysis, the generated 2-methyl-1-propenol must also have free access to the extracellular medium, suffering oxidation by the added HRP. Since monocytes are poor in myeloperoxidase, the intracellular oxidation of 2-methyl-1-propenol was not expected as a major process. Indeed, control experiments without addition of HRP revealed no light emission.

Leukocyte esterase activities are usually determined by hydrolysis of α-naphthylacetate (or butyrate) and naphthol-AS-D chloroacetate (8). The first is used to measure an activity associated to non-specific esterases and gives positive test in monocytes and its precursors, while the second is used for the identification of granulocytes. The existing esterase activity in monocytes is known to be inhibited by addition of inorganic fluoride, while the one found in granulocytes is not (8). In order to identify the hydrolase activity, NaF was added in the MPB/ monocyte/H_2O_2/HRP/DBAS system. The possibility of fluoride catalysing hydrolysis of MPB was excluded, since there was no light emission triggered by NaF in the absence of monocytes. It was attested by the strong inhibition of the chemiluminescence of the

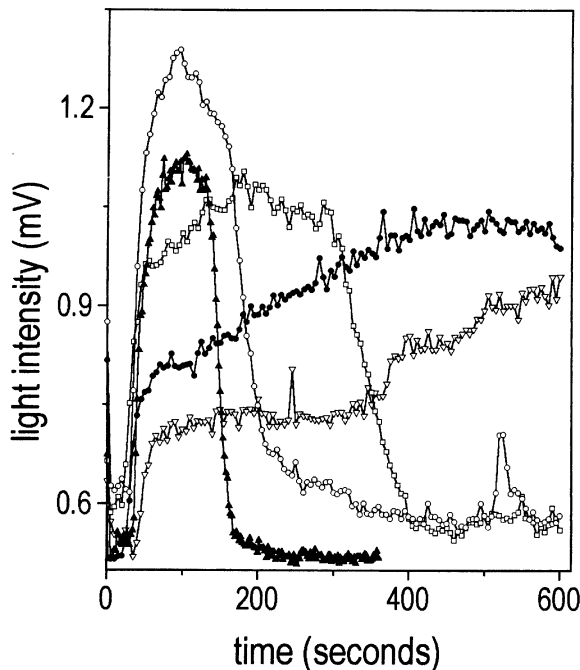

Figure 4. Effect of number of monocytes (2.6×10^5, ▽; 3.7×10^5, •; 6.3×10^5, □; 7.5×10^5, ○; 10×10^5, ▲) in the light emission kinetics of the monocyte/MPB/H_2O_2/HRP (type I)/DBAS system

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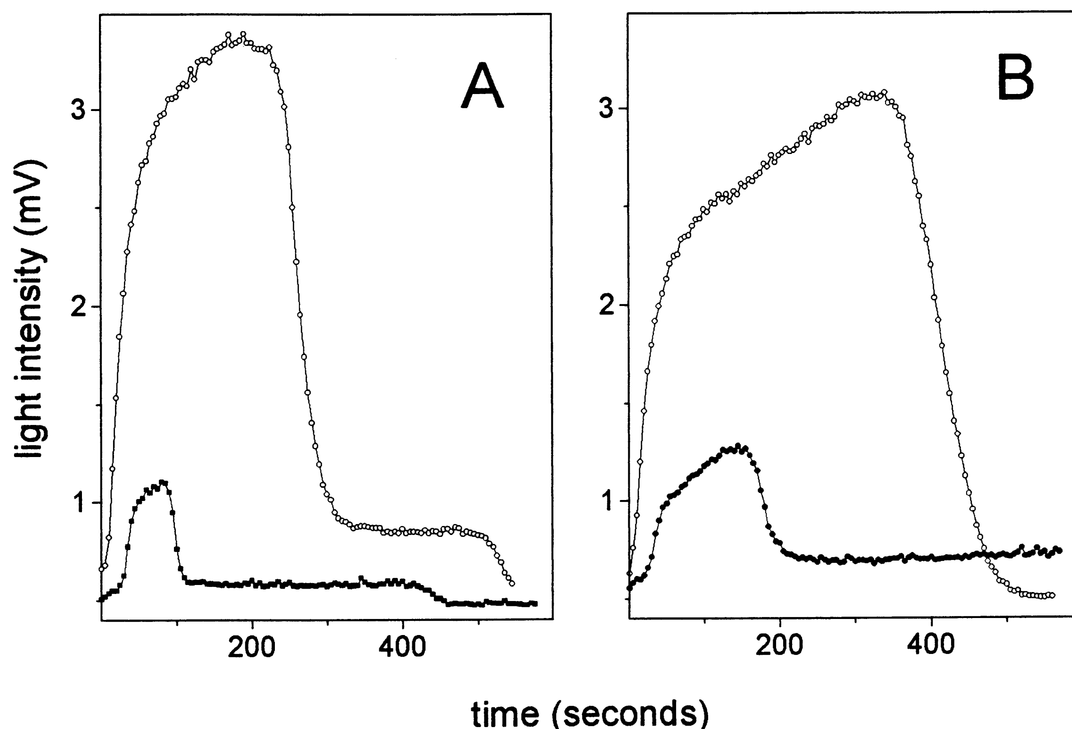

Figure 5. Effect of monocyte disruption by sonication in the monocyte/MPB/H_2O_2/HRP(type I)/DBAS system; (○) intact monocytes and (•) disrupted monocytes with (A) 8.6×10^5 cells or (B) 5.7×10^5 cells

complete reaction system in the presence of fluoride that non-specific esterases are responsible for the chemiluminescence. The light emission was totally suppressed by the addition of 2 mmol/L NaF, while partial inhibition was observed with lower concentrations (data not shown). Control experiments without monocytes, HRP, H_2O_2 or substrate did not render chemiluminescence (Fig. 1).

The minor variation observed in light kinetics may be resultant of individual cellular variations and differences in the monocyte:lymphocyte ratio from the mononuclear cells preparation. In general, a monophasic and simple kinetic was observed (Fig. 1). The monocyte:lymphocyte ratio is important, since lymphocytes may act as an internal filter due to light scattering without contributing to the total esterase activity. Indeed, neither lymphocytes nor polymorphonuclear leukocytes were able to trigger chemiluminescence from MPB/H_2O_2/HRP/DBAS (Fig. 2).

The chemiluminescence intensity of the reaction is directly related to the number of added mononuclear cells in a narrow range, approximately 2.5–4.5 $\times 10^5$ cells/assay (Fig. 3). At different numbers of cells, the kinetics suffered great profile variations (Fig. 4), possibly reflecting esterase activity vs. light suppression.

Monocytes submitted to sonication triggered less intense emission with maximal intensity shift to shorter times compared to intact cells (Fig. 5). The disruption of the cellular membrane and exposure of the substrate to the released enzyme may be the cause of the time course shortening of the reaction. Additionally, a partial esterase inactivation might have occurred during sonication due to vibration or heat. Centrifugation of sonicated cells did not affect the kinetics or the intensity of light. The possibility that some quencher had been released by sonication was also discarded, since adding intact cells to disrupted cells resulted in mixed kinetics (data not shown).

When a highly purified monocyte preparation was used it enabled the increase of the amount of monocytes per experiment in a very high monocyte:lymphocyte ratio (Fig. 6). The light emission could be highly intensified by the addition of 7.9 µmol/L DBAS, and approximately 10–15 times more light

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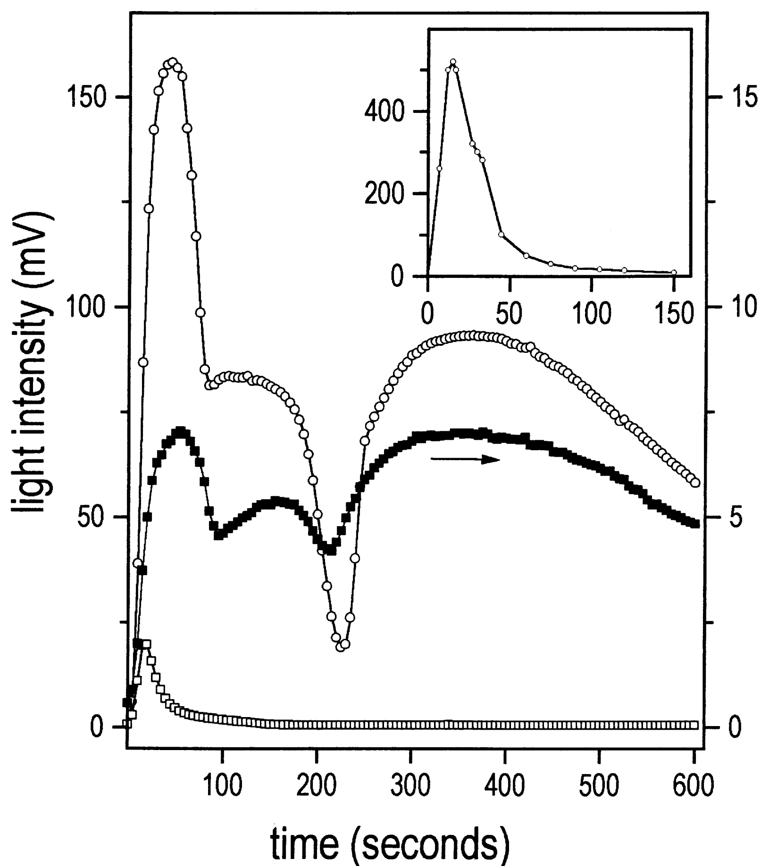

Figure 6. Light emission kinetics from the monocyte (3.2×10^6 cells)/MPB/H_2O_2 (10 μmol/L)/HRP (type VI, 1 μmol/L) system (■). The emission was highly enhanced in the presence of DBAS (7.9 μmol/L) (○) and, still in the presence of DBAS, it was almost totally suppressed by 0.01 mol/L NaF (□). The inset shows the light emission obtained from the monocyte/MPB/H_2O_2/HRP system when submitted to cell disruption by sonication

was observed. A multiphase kinetic was evidenced in both absence and presence of DBAS. This was reproducible and it probably reflects different compartments of esterase to be accessed by the substrate. Part of the light emission, especially the first peak, may be the consequence of extracellular esterases resulting from some cellular lysis during isolation. Although the activity of liver esterases is not necessarily the same as that of monocyte esterase, the time course of the first peak is similar to the previously observed kinetics of free esterase (2). Moreover, prior sonication of monocytes prevented the multiphase kinetic (Fig. 6, inset) with the dominance of one peak at approximately 20 seconds.

In conclusion, MPB can be hydrolysed by non-specific esterases presented in monocytes and the product, 2-methyl-1-propenol, is then oxidized by H_2O_2/HRP, yielding chemiluminescence which can be sensitized by DBAS. Since specific esterases present in granulocytes are apparently unable to cleave MPB, this assay could be used to detect mononuclear cells in heterogeneous blood cell populations or estimate the esterase content in monocyte preparations. Furthermore, it is possible to devise an application of this reaction to distinguish monocytic leukaemias, similar to the chemiluminescent assay for myeloid leukaemias (7).

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