Spectrophotometric Determination of Leukocytes in Blood

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The determination of leukocyte concentration in human blood depending on the detection of oxidized o-dianisidine in acidic solution is studied. The oxidation of o-dianisidine was carried out by peroxidase enzymes found in leukocytes. The reaction was stopped by the addition of 4N H₂SO₄ to the solution, and a very stable, colored o-dianisidine derivative was obtained. The calibration graph was plotted with the

recorded absorbance values at 400 nm assigned to the y-axis, and leukocyte counts in 1-mL blood samples to the x-axis. The equation of the calibration graph was $y=0.0025x+0.0904$, with a correlation coefficient of $R=0.994$. The coefficient of variation and P -value of the method were 4.00% and 0.05%, respectively. J. Clin. Lab. Anal. 16:233–236, 2002. © 2002 Wiley-Liss, Inc.

Key words: leukocyte; o-dianisidine; human blood; spectrophotometry

INTRODUCTION

The combination of peroxidase enzymes, hydrogen peroxide, and halide or thiocyanate ions forms an antimicrobial system in leukocytes; monocytes, neutrophils, eosinophils, and exocrine secretions (including milk, tears, and saliva) are leukocyte cells (1–3). Myeloperoxidase is a hydrogen peroxide oxidoreductase that is specifically found in mammalian granulocytic leukocytes, including polymorphonuclear leukocytes, monocytes, basophils, and eosinophils (4–8). Myeloperoxidase catalyses the formation of hypochlorous acid from hydrogen peroxide and chlorine ions. In the phagocytic vacuole, hypochlorous acid can react further, forming secondary chlorinating agents, such as chlorine, N-chloroamines, and N-chloroamides (9–11). The ability of human neutrophils and monocytes to generate hydroxyl radicals through a myeloperoxidase-dependent mechanism has already been determined (12,13). The heme enzyme myeloperoxidase oxidizes bromide, iodide, thiocyanate, as well as chloride. However, chloride is generally accepted to be a physiological substrate of myeloperoxidase because it is present at high concentrations in plasma (100–140 mM) compared to thiocyanate (20–120 μ M), bromide (20–100 μ M), and iodide (<1 μ M) (14–16). Human eosinophil peroxidase oxidizes chloride ions less

efficiently than does myeloperoxidase, and it preferentially oxidizes bromide to a halogenating intermediate in the presence of at least a 1,000-fold excess of chloride (17–20).

There is accumulating evidence that cellular redox status is an important parameter for lipoxygenase activation in B cells. Selenium-dependent peroxidases are responsible for the suppression of cellular 5-lipoxygenase activity in B lymphocytes and immature myeloid cells. However, the relationship between cellular peroxide tone, glutathione peroxidase function, and catalytic activity of 5-lipoxygenase in various cell types is unclear. In intact cells, peroxide levels are considered to be under the control of peroxidases (21–24). T lymphocytes are the specific source of interleukin-4 and interferon gamma. The regulation of cytokin

Grant sponsor: Research Foundation of Yildiz Technical University, Istanbul, Turkey.

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Received 14 December 2001; Accepted 2 June 2002

DOI 10.1002/jcla.10049

Published online in Wiley InterScience (www.interscience.wiley.com).

biosynthesis in the cells and signaling is redox-dependent (23–26).

Spectrophotometric methods based on the detection of enzyme activities for the measurement of leukocytes in body fluids and tissues have been reported. The quantitation of polymorphonuclear leukocytes in tissues has been carried out via the detection of myeloperoxidase activity using o-dianisidine as a substrate (27). The determination of eosinophils and neutrophils in lung tissue has been performed by detection of eosinophil peroxidase and myeloperoxidase activities (4). Tests to determine the concentration of leukocytes in urine based on detection of esterase activity (28), and in other body fluids based on elastase activity, using many types of substrates, have been described (29–33).

In this study we found that the oxidized form of o-dianisidine was very stable in acidic pH when oxidized by redox enzymes of leukocytes. The concentration of leukocytes was determined by measuring the maximum absorbance of this product at 400 nm.

MATERIALS AND METHODS

Materials

Triton X-100 and o-dianisidine was purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals used were all analytical grade. The o-dianisidine (0.1264 g) was dissolved in about 10 mL distilled water by the addition of 1:2 diluted HCl. The volume was adjusted to 20 mL with water, and 0.53 mM o-dianisidine stock solution was obtained (27). Before use, the o-dianisidine assay solution was prepared by the addition of 2 mL stock solution and 2 μ L 30% H_2O_2 to 98 mL 50 mM PBS, pH 6.

Methods

Isolation and lysis of leukocytes

After the enumeration of human blood cells using the automatic cell counter, the red blood cells were lysed by the ammonium chloride method, and the leukocytes were isolated using the density gradient method. Hence, 2-mL ammonium chloride lysing solution was added to 100 μ L of blood samples in 5-mL polystyrene tubes, mixed by vortex, and incubated at room temperature for 10 min. The erythrocytes were lysed and the leukocyte cells were pelleted by centrifugation at 350 g for 5 min. The supernatant was decanted and cells were washed with 1 mL 10 mM PBS buffer, pH 7.3–7.4, containing 0.9% NaCl. The obtained pellets were suspended in 1 mL of this buffer (34). After mixing, a suitable amount of leukocyte suspension was transferred to a 5-mL test tube, 50 μ L 0.2% (v/v) Triton X-100 was pipetted into

the samples, and the leukocytes were lysed for about 5 min (35).

Spectrophotometric assay

The reaction was started by the addition of 1,800 μ L freshly prepared o-dianisidine assay solution to the lysed leukocyte samples. After 10 min of incubation time at 25°C, the reaction was stopped by the addition of 150 μ L 4N H_2SO_4 . The spectra of oxidized o-dianisidine were recorded before and after addition of H_2SO_4 by use of a Shimadzu model 150 02 spectrophotometer.

RESULTS AND DISCUSSION

Figure 1 shows the absorption spectra of the oxidized o-dianisidine in PBS buffer at neutral pH and in the acidic solution. The addition of H_2SO_4 caused a shift of the absorption maxima through the short wavelengths. The oxidized o-dianisidine was very stable in the acidic solution, so the daily recorded absorption spectra did not change for 1 week. Therefore, the absorption maxima of oxidized substrate appearing at 400 nm in acidic conditions can be utilized to determine leukocyte concentration in blood.

The calibration graph was obtained with the absorbance recorded at 400 nm to the y-axis, and leukocyte counts in 1-ml blood samples to the x-axis (Fig. 2). The equation of the calibration graph was $y = 0.0025x + 0.0904$, with a correlation coefficient of $R = 0.994$. The minimum measurable absorbance difference of 0.01 corresponded to about four cells per

Fig. 1. The absorption spectra of oxidized o-dianisidine in neutral (—) and acidic (· · ·) solutions.

Fig. 2. Calibration curve.

Fig. 3. Comparison of leukocyte counts of human blood samples as determined by the automatic cell counter and the spectrophotometric method.

milliliter at the standard curve. The coefficient of variation and P -value were 4.00% and 0.05%, respectively. The results obtained using the spectrophotometric method and automatic cell counter are compared in Fig. 3.

Calculations of absolute cell numbers in tissue based on peroxidase measurements may be affected by variations in the peroxidase content between cells, depending on their degree of maturation or state of activation. However, this may not be a major factor in practice (4). In this study, despite the different cell sizes and oxidative enzyme contents of the leukocytes in blood (21–26), good statistical criteria were obtained.

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