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Highly sensitive and reliable chemiluminescence method for the assay of superoxide dismutase in human erythrocytes

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Superoxide dismutase in human erythrocytes was assayed by the inhibition of highly diluted erythrocyte lysates on 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one-dependent luminescence induced by the hypoxanthine-xanthine oxidase system. Our chemiluminescence procedure gave 95-times higher sensitivity than the cytochrome *c* method. The concentration of superoxide dismutase in erythrocytes of Down's syndrome patients was approx. 1.8-times higher than that of normal humans.

2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one; Chemiluminescence; Superoxide dismutase; Enzyme assay; Erythrocyte; Down's syndrome

1. INTRODUCTION

Recently, many clinicians have directed their attention to the superoxide dismutase (SOD) levels in human erythrocytes of patients with various diseases. Cu-Zn SOD in erythrocytes of Down's syndrome patients has been reported to be present in higher levels compared to normal humans [1–3]. On the other hand, erythrocytes of patients with Fanconi's anemia [4], Duchenne muscle dystrophy [5] and rheumatic arthritis [6] have low levels of Cu-Zn SOD. These results were obtained using different methods without regard to interference by protein contaminants. Oyanagui [3] has pointed out that a simple and sensitive method with a minimum of interference by proteins is needed for SOD assays in crude samples.

We have reported a simple and sensitive luminescence method for the determination of O_2^- generation in activated leukocyte systems and in the hypoxanthine-xanthine oxidase system, using a cypridina luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one [7]. The present paper describes a highly sensitive luminescence method for estimating SOD concentrations in human erythrocytes, according to the inhibitory effect of erythrocyte lysates on 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one-dependent luminescence induced by the hypoxanthine-xanthine oxidase system (an O_2^--generating system).

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2. MATERIALS AND METHODS

2.1. *Chemicals and enzymes*

2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one hydrochloride (MCLA) was synthesized from 2-amino-5-(*p*-methoxyphenyl)pyrazine. The detailed procedure will be published elsewhere [8]. The compound was dissolved in doubly distilled water and stored at −80°C until needed. The MCLA concentration was based upon $\epsilon(430\ \text{nm}) = 9600\ M^{-1} \cdot cm^{-1}$. Horse heart cytochrome *c* (type IV), SOD (from bovine erythrocytes, 3000 U/mg protein) and xanthine oxidase (XOD, grade III) were purchased from Sigma. Hypoxanthine was a product of Wako. Bovine serum albumin (BSA, fatty acid- and globulin-free) was obtained from Sigma. To avoid the possible contamination of SOD (Cu-Zn SOD) with commercial BSA, the BSA (450 mg) was purified by passing through a Sephadex G-100 column (1.4 cm × 1.0 m) using 10 mM Tris-HCl buffer at pH 7.4 as eluant. The main fraction obtained was then lyophilized.

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2.2. *Preparation of erythrocyte lysates*

The procedure was essentially the same as that of Oyanagui [3]. Erythrocyte lysates, obtained by chloroform-ethanol treatment (Tsuchihashi method), were diluted with water to a final concentration of 1/1000 of whole blood. Further dilution was carried out with ethanol (0.25%).

2.3. *Assay of XOD*

Enzyme activity was determined in glycine-HCl buffer at pH 8.8 and at 37°C, using hypoxanthine as a substrate [10]. One unit of enzyme was defined as the amount of the enzyme which catalyzed an increase in absorbance at 290 nm of 0.001/30 min.

2.4. *Assay of SOD activity*

2.4.1. Cytochrome *c* method

This followed the original method of McCord and Fridovich [11]. The reaction mixture contained 1×10^{-5} M cytochrome *c*, 5×10^{-5} M xanthine, XOD (350 U), SOD (10–1000 ng/ml), 1×10^{-4} M EDTA and 50 mM potassium phosphate buffer at pH 7.8, in a total volume of 3 ml. The reaction was initiated by the addition of XOD and the absorption at 550 nm was continuously recorded on a Hitachi spectrometer (model 200-10) at 25°C.

2.4.2. Chemiluminescence method

The standard reaction mixture contained 1×10^{-7} M MCLA, 5×10^{-5} M hypoxanthine, XOD (6.5 U), SOD (0.2–20 ng/ml) or none, and 50 mM Tris-HCl buffer containing 0.1 mM EDTA at pH 7.8, in a total volume of 3.0 ml. Chemiluminescence measurement was initiated by the addition of MCLA to the standard incubation mixture excluding XOD, continued for 4 min without additive and for an additional 4 min after the addition of XOD. Chemiluminescence was measured using a luminescence reader (Aloka, BLR102) at 25°C.

2.5. *Assay of protein in erythrocyte lysates*

Protein content was determined by the method of Lowry et al. [12].

3. RESULTS AND DISCUSSION

3.1. *MCLA-dependent luminescence*

MCLA in the standard incubation mixture excluding XOD emitted light in the visible region, as does CLA [7]. Such luminescence may originate from the auto-oxidation of MCLA, i.e. non-specific luminescence. When XOD was added to this system, the luminescence increased rapidly, reached a maximum at 2 min after the addition of XOD and remained constant for an additional 3 min. The luminescence in the system containing XOD decreases with increasing SOD concentration. However, the non-specific luminescence remains almost constant for 10 min after the addition of MCLA and is not significantly influenced by SOD. These results are shown in fig.1.

The XOD-induced luminescence (I_0), expressed in terms of light intensity (counts/min), can be calculated by subtraction of the non-specific light intensity at 8 min after the addition of MCLA from the light intensity at 4 min after the addition of XOD. The same incubation experiments, except that SOD was present in both the experimental and control systems, were carried out and XOD-induced luminescence (I_i) was calculated in the same manner as in the absence of SOD. The percent of SOD-dependent inhibition on XOD-induced luminescence (I_0) could be calculated from eqn 1, and was then plotted vs SOD concentration to obtain the standard curve.

$$\% \text{ inhibition} = \frac{I_0 - I_i}{I_0} \times 100 \quad (1)$$

The SOD concentration required for 50% inhibition of XOD-induced luminescence was then compared with that obtained by the cytochrome *c* method. As shown in fig.2A, the luminescence method has 95-times higher sensitivity than the cytochrome *c* procedure. XOD-induced luminescence was also inhibited by purified BSA at concentrations above 5 μg/ml of the reaction mixture (fig.2B). To ascertain the contamination of SOD with BSA, either a purified BSA sample or commercial BSA with or without 1 mM KCN (an in-

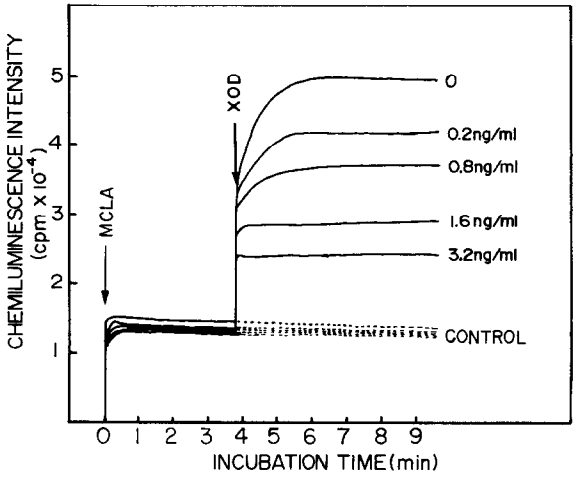

Fig.1. Effect of SOD on MCLA-dependent luminescence. The standard reaction mixture with or without SOD (ng/ml) was used. Incubation conditions are given in the text. Arrow indicates the time at which MCLA or XOD was added.

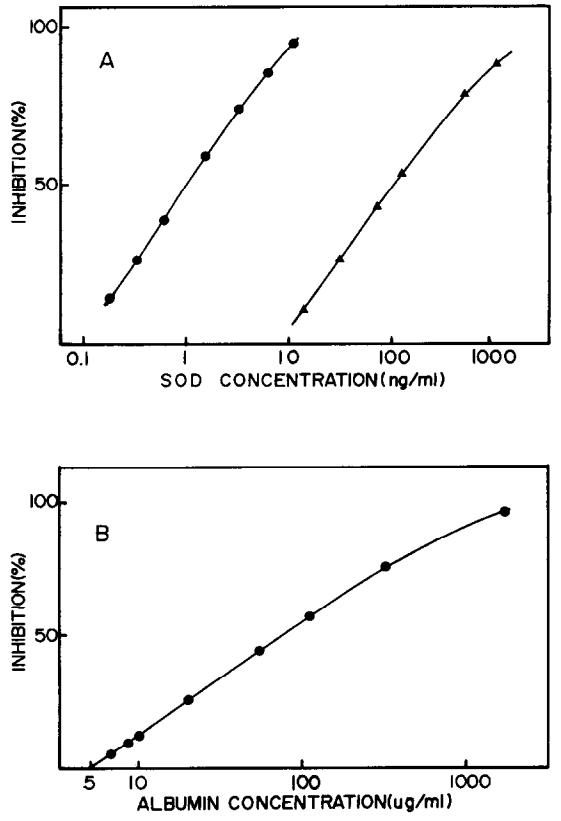

Fig.2. (A) Comparison of the luminescence and cytochrome *c* methods. The standard and conventional reaction mixtures were used for the luminecence (●) and cytochrome *c* methods (▲). Under the incubation conditions described in the text, luminescence and cytochrome *c* reduction were measured and corrected for controls. The percent inhibition by SOD at various concentrations was calculated in the usual way. (B) Effect of bovine serum albumin on XOD-induced luminescence. Reaction mixture essentially the same as in (A), except that BSA was used instead of SOD. The BSA concentration is expressed as μg/ml of the reaction mixture.

hibitor of Cu-Zn SOD [9]) was assayed by the chemiluminescence method. The results obtained showed no contamination of SOD with the purified BSA and commercial BSA. Thus, BSA itself has some inhibitory effect on XOD-induced luminescence. Even if an SOD sample is contaminated with BSA, albumin at less than 5 μg/ml would not interfere with the SOD assay by the luminescence method. Furthermore, no inhibitory effect was observed with 4×10^{-6} M glutathione, 3×10^{-8} M ascorbate, 10^{-5} M uric acid or 6×10^{-4} M glucose. The concentrations of glutathione, ascorbate, uric acid and glucose in a 3×10^4-fold dilution of erythrocyte lysates, corresponding to about 6×10^4-fold dilution of whole blood, were reported to be 8.7×10^{-8}, 7.4×10^{-10}, 3.8×10^{-9} and 1×10^{-7} M, respectively [13]. Protein in a 3×10^4-fold dilution of erythrocyte lysates treated according to the Tsuchihashi method corresponded to 0.3 μg/ml. Thus, each of these compounds in such diluted erythrocyte lysates would not interfere with the SOD assay by the luminescence method.

3.2. *SOD in erythrocyte lysates*

To assay SOD in samples, samples corresponding to 6×10^4- and 10^3-fold dilutions of whole blood were used for the chemiluminescence and cytochrome *c* methods, respectively. Each sample was added to the reaction mixture, instead of SOD, and assayed as % inhibition on lumines-

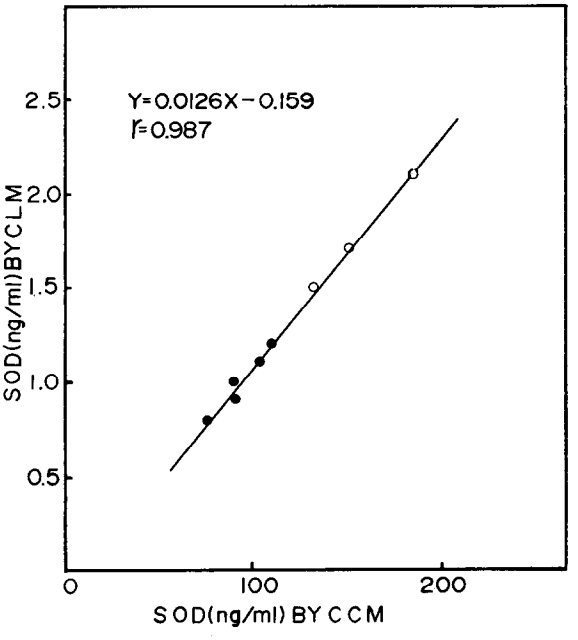

Fig.3. Correlation of the luminescence method (CLM) with the cytochrome *c* method (CCM). Erythrocyte lysates, normal human (●) or Down's syndrome patient (○), corresponding to 6×10^4-fold and 1×10^3-fold dilutions of whole blood, were used for the luminescence and cytochrome *c* methods, respectively. Reaction mixture and incubation conditions essentially as in fig.2A, except that erythrocyte lysates were used instead of SOD. SOD concentration in erythrocyte lysates was calculated with % of inhibition by known SOD, using the data in fig.2A, and expressed as ng/ml of the reaction mixture.

Table 1

Recovery test of added SOD on erythrocyte lysates by the luminescence method

Expt no.	Added SOD (ng/ml)	Erythrocyte lysate (ng/ml)		
		Theoretical	Measured	Recovery (%)
1	0		1.1	(100)
	0.2	1.3	1.2	92
	0.5	1.6	1.7	106
	1.0	2.1	2.1	100
2	0		0.6	(100)
	0.2	0.8	0.76	95
	0.4	1.0	0.98	98
	0.6	1.2	1.2	100

cence or cytochrome *c* reduction, as shown in fig.2A. Five normal humans, aged 22–40 years, and three Down's syndrome patients, aged 1–5 years, donated blood which was assayed in terms of bovine erythrocyte Cu-Zn SOD. As shown in fig.3, there was a good correlation between the values obtained by both luminescence and cytochrome *c* methods. With the former, SOD in erythrocyte lysates corresponding to a 6×10^4-fold dilution of whole blood amounted to 1.0 ± 0.14 ng/ml for normal humans and 1.83 ± 0.21 ng/ml for the patients. With the latter, SOD in erythrocyte lysates corresponding to a 10^3-fold dilution of whole blood was 94 ± 13.2 ng/ml for normal humans and 155 ± 22.7 ng/ml for the patients. Using a different technique, Oyanagui [3] has also reported that the augmentation of SOD activity in erythrocytes from the patients is 56%.

To investigate the recovery of SOD, SOD was loaded on erythrocyte lysates prepared in two different dilutions and assayed by the chemiluminescence method. This method gave good recovery with erythrocyte lysates (table 1). Another cypridina luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (CLA) [7], can also be used instead of MCLA for assaying SOD in erythrocyte lysates. The MCLA method is, however, 4.5-times more sensitive than the CLA method.

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