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A simple, sensitive, non-stimulated photon counting system for detection of superoxide anion in whole blood

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Abstract. A simple, sensitive, non-stimulated assay was developed to measure the superoxide anion concentration in whole blood, using an ultra-sensitive chemiluminescence (CL) analyzer and lucigenin amplification. The assay system can be performed without leukocyte isolation or stimulant administration. The blood CL levels of healthy males (362.8 ± 337.7 counts/10 sec) were not different from those of females (335 ± 308.7 counts/10 sec) ($p = 0.64$), whereas the CL levels in whole blood in patients with acute pancreatitis (2522 ± 2014 counts/10 sec) were significantly higher than those of healthy controls ($p < 0.001$). This assay system may be valuable in the future for quantitative measurement of reactive oxygen species in various disorders.
Key words. Superoxide; free radical; reactive oxygen species; chemiluminescence; pancreatitis.

Abbreviations. ROS = reactive oxygen species, CL = chemiluminescence, PMA = phorbol myristate acetate, SOD = superoxide dismutase, WBC = white blood cells, PMNL = polymorphonuclear leukocytes.

Excessive production of reactive oxygen species (ROS), including superoxide anions, peroxides and hydroxyl radicals, is associated with many diseases[1–8]. A number of assays, including determination of chemiluminescence (CL), have been developed to measure the production of ROS. In these measurements, isolation of purified leukocytes is usually required so that specific activity per cell can be determined[9–11]. Moreover, a stimulant such as phorbol myristate acetate (PMA) or opsonized zymosan particles needs to be added to activate the respiratory burst of phagocytes during measurement of CL[12]. With an improvement in sensitivity offered by a new photon detector, free radicals in the peripheral blood can be detected without need of leukocyte isolation[3–8,13–19].

In the current study, we describe a newly-developed assay for detecting superoxide anion in whole blood using an ultra-sensitive CL analyzer. This method is rapid, simple, and sensitive. The amount of superoxide anion in the whole blood of healthy subjects was compared with the level in patients with acute pancreatitis, a disease known to produce excessive ROS[2,16]. This measurement may be potentially useful as a screening test for diseases associated with ROS production.

Materials and methods

Subjects. Between June and September 1994, we screened 189 subjects who were attending for annual health examinations in the National Taiwan University Hospital. Of these, 125 subjects (age: 21–82, 70 males and 55 females) were enrolled as controls. They had been shown by a series of screening tests to be free of major cardiopulmonary, gastrointestinal, and hepatobiliary-pancreatic diseases. None of the healthy subjects had any clinical symptoms such as fever. The total white blood cell (WBC) counts were measured with an electronic cell-counter (Coulter Electronics Ltd., UK) and the total number of polymorphonuclear leukocytes (PMNL) was calculated from differential counts on stained blood films. A separate group of 12 patients with acute pancreatitis (age: 21–78, 6 males and 6 females) were included on the basis of clinical diagnostic criteria including symptoms of epigastralgia, elevation of serum amylase and lipase, and compatible imaging studies. Based on Ranson's criteria[20], the patients were divided into three groups according to the severity of pancreatitis: mild (n = 6), moderate (n = 4), and severe (n = 2). Among these, one patient with severe pancreatitis had fever up to 39 °C and three patients with moderate pancreatitis had mild fever of 38 °C at the time of CL measurement.

Sample preparation and CL determination. For all controls, whole blood samples were obtained with heparinized plastic syringes in the early morning after 12 hours of fasting. For patients with acute pancreatitis, blood samples were obtained on the first day of admission to the emergency service. The tubes of heparinized blood were immediately wrapped in aluminium foil to avoid light exposure and kept in the ice box until testing for ROS, which in general was done within 2 hours. For

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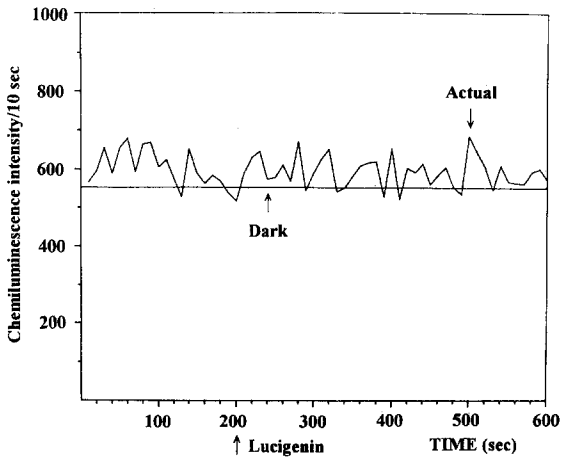

Figure 1. Chemiluminescence (CL) demonstrated in the heparinized blood of a healthy male subject. After subtraction of the dark average (dark) from the actual reading (actual), the net CL level is 40 counts/10 sec.

Table 1. Chemiluminescence levels of the whole blood of healthy subjects and patients with acute pancreatitis.

Status	Chemiluminescence level (counts/10 sec)
Control	
Lucigenin (0.01 mM)	11.9
PBS buffer, pH 7.4	13.0
Healthy subjects	
Male (n = 70, age: 34–82)	362.8 ± 337.7 (24.8–1484)[a]
Female (n = 55, age: 21–73)	335.4 ± 308.7 (11.5–1138)
Total (n = 125, age: 21–82)	348.4 ± 324.0 (11.5–1484)
Acute pancreatitis	
Male (n = 6, age: 21–78)	2537.8 ± 2289.6 (416.4–5454)
Female (n = 6, age: 38–70)	2506.5 ± 1918.5 (1163–6281)
Total (n = 12, age: 21–78)	2522.0 ± 2014.0 (416.4–6281)[b]

[a]All samples were assayed in duplicate and all results are expressed as mean ± standard deviation (range).
[b]$p < 0.005$ vs. healthy subjects by Wilcoxon's rank sum test.

each measurement, 0.2 ml of heparinized blood was added with 0.1 ml of PBS buffer, pH 7.4 in a special chamber unit (Model No. TLU-21, Tohoku Electronic Indust. Co.) including a stainless steel cell with magnetic stirrer and stirrer bar, in an absolutely dark chamber of the Chemiluminescence Analyzing System (Tohoku Electronic Industrial Co., Sendai, Japan). This system contains a photon detector (Model CLD-110), a chemiluminescence counter (Model CLC-10), a water circulator (Model CH-20), and a 32 bit IBM personal computer system. The cooler circulator was connected to the Model CLD-110 photon detector to keep the temperature at 5 °C. The Model CLD-110 is extremely sensitive, and according to the manufacturer's instructions can detect radiant energy as weak as 10^{-15} W. Photon emission from whole blood was counted at 10 second intervals at 37 °C under atmospheric conditions. After 200 sec, 1.0 ml of 0.01 mM lucigenin (Sigma Co., USA) in PBS was injected into the cell and the CL in the blood sample was continuously measured for a total of 600 sec. The total CL was calculated by integrating the area under the curve (for example see fig. 1) and subtracting it from the background level, which was equivalent to the dark average. The assay was performed in duplicate for each sample and was expressed as CL counts/10 sec. The production of CL per WBC (expressed as CL/WBC) was calculated by dividing the blood level by the WBC count, while the amount of CL per PMNL (CL/PMNL) was calculated by dividing the blood CL level by the PMNL count. The mean ± standard deviation (M ± S.D.) was used to express the values of CL, CL/WBC, and CL/PMNL for each sample. The data were compared between healthy controls and patients with acute pancreatitis using Wilcoxon's rank sum test. A p value of less than 0.05 was considered statistically significant.

The inhibition experiment. To determine the specific type of ROS generated in the blood samples and measured by this system, different concentrations of superoxide dismutase (SOD) (30 to 307.6 U/ml in PBS) or catalase (115 to 460 U/ml in PBS) were added to the blood sample in a stainless steel cell. Both SOD and catalase were purchased from Sigma, USA. Otherwise, the CL level was measured in the same way as described above.

Results

In the absence of blood samples, assessment of CL levels in lucigenin (0.01 mM) or PBS alone gave 11.9 and 13.0 counts/10 sec, respectively, that is, in the same range as that of the dark average (table 1). The CL levels detected in the sera were also within the range of the dark average, whereas minimal to moderate

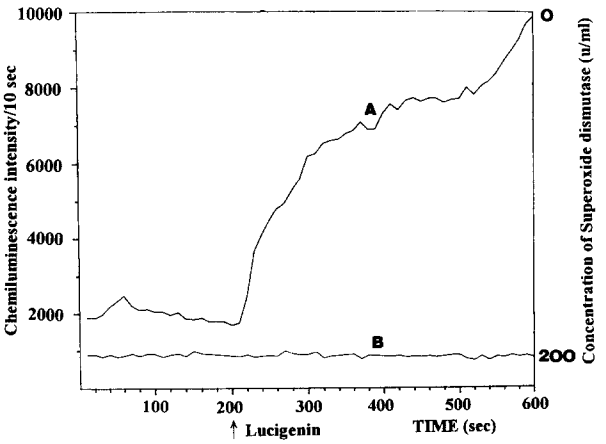

Figure 2. The CL level of a patient with acute pancreatitis. Augmented CL response with a CL level of 6281 counts/10 sec is noted. **A**) actual reading without addition of superoxide dismutase (SOD), **B**) blocking experiment with 200 U/ml of SOD.

Table 2. The relationships of chemiluminescence (CL) levels to peripheral leukocyte counts of healthy subjects and patients with acute pancreatitis.

Unit	CL [counts/10 sec]	CL/WBC [counts/10 sec/cell]	CL/PMNL [counts/10 sec/cell]
Healthy subjects (n = 125)	348.4 ± 324.0[a]	0.056 ± 0.050	0.096 ± 0.085
Acute pancreatitis (n = 12)	2522.0 ± 2014.0[b]	0.194 ± 0.149[c]	0.283 ± 0.243[c]

CL = chemiluminescence, WBC = white blood cells, PMNL = polymorphonuclear leukocytes.
[a]All samples were assayed in duplicate and all results are expressed as mean ± standard deviation.
[b]$p < 0.005$ vs. healthy subjects by Wilcoxon's rank sum test.
[c]$p < 0.05$ vs. healthy subjects by Wilcoxon's rank sum test.

amounts of CL (mean: 348.4 count/10 sec, range: 11.5 to 1484 counts/10 sec) were detected in the heparinized blood of healthy subjects (fig. 1, table 1). The CL blood levels of males (362.8 ± 337.7 counts/10 sec, M ± S.D.) did not differ from those of females (335.4 ± 308.7 counts/10 sec, M ± S.D.) ($p = 0.64$) (table 1). The blood CL levels of patients with acute pancreatitis (mean: 2522 counts/10 sec, range: 416.4 to 6281 counts/10 sec) (fig. 2) were significantly higher than those of healthy controls ($p < 0.005$) (table 1). As shown in table 2, the value of CL/WBC of patients with acute pancreatitis (0.194 ± 0.149 counts/10 sec/cell, M ± S.D.) was significantly higher than that of healthy controls (0.056 ± 0.050 counts/10 sec/cell, M ± S.D.) ($p < 0.05$). The value of CL/PMNL of acute pancreatitis (0.283 ± 0.243 counts/10 sec/cell, M ± S.D.) was also significantly higher than that of healthy controls (0.096 ± 0.085 counts/10 sec/cell, M ± S.D.) ($p < 0.05$). However, the blood CL levels in the pancreatitis patients did not differ significantly between different levels of severity, according to Ranson's criteria[20]. The four patients with fever did not show higher levels of blood CL than those without fever.

The CL levels of whole blood samples, wrapped with aluminium foil and stored in an ice box, could still be measured 48 hours later without a significant change (data not shown). The blood CL was inhibited by superoxide dismutase (SOD) in a dose-dependent fashion and was completely blocked with 100 U/ml of SOD (fig. 3). Conversely, catalase with a concentration from 115 to 460 U/ml did not inhibit the generation of CL in the blood. The generation of blood CL, not inhibited by catalase at a concentration of 115 U/ml, was completely blocked by additional administration of 307.6 U/ml of SOD at the 400 sec time point (fig. 4).

Discussion

An enhanced single photoelectron counting technique has been introduced to estimate ROS as CL on the basis of the degeneration of singlet oxygen to triplet oxygen[21]. In biological systems, the source of ROS can be superoxide radicals, hydrogen peroxide, or hydroxyl radicals[22]. In theory, the detection of ROS can be amplified by measuring the CL emitted by luminescence-generating substrates such as luminol and lucigenin. Luminol-

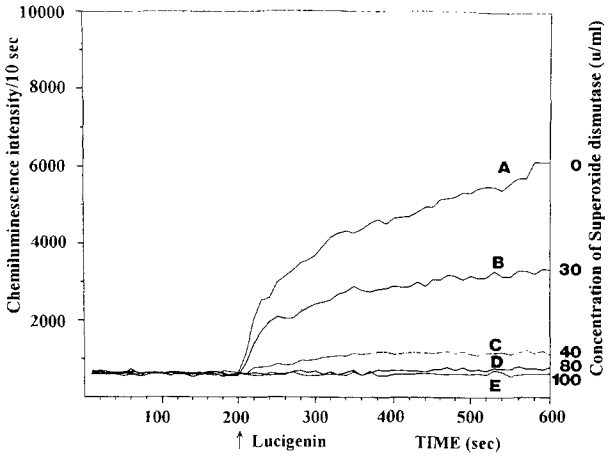

Figure 3. Inhibition experiment with different concentrations of superoxide dismutase (SOD). **A**) actual reading without SOD, **B**) with 30 U/ml of SOD, **C**) with 40 U/ml of SOD, **D**) with 80 U/ml of SOD, **E**) with 100 U/ml of SOD.

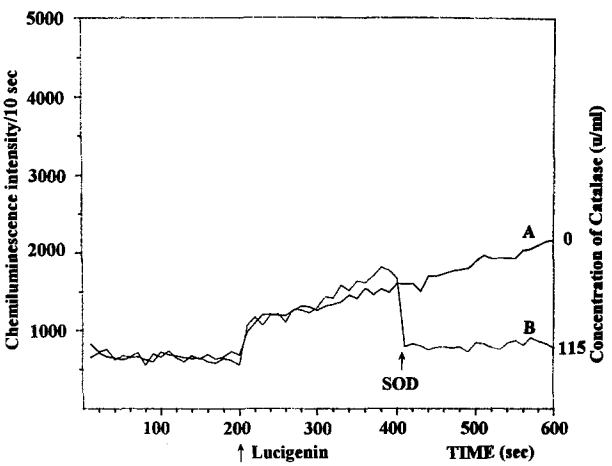

Figure 4. Inhibition experiment with SOD and catalase. **A**) actual reading without SOD and catalase, **B**) 115 U/ml of catalase incubated with whole blood at the zero sec time point, and 307.6 U/ml of SOD is added to the system at 400 sec time point. Lucigenin is injected into the system at 200 sec time point in both A and B curves.

enhanced CL primarily reflects myeloperoxidase activity, while lucigenin-dependent CL measures oxidase-associated oxygenation[23,24]. In the current study, CL was detected only in heparinized blood but not in serum, indicating that leukocytes might be the major source of the CL released. Another cellular source of superoxide other than leukocytes is mitochondria[25]. Further experiments are needed to clarify the actual source of CL in whole blood. The CL amplified by lucigenin was specifically inhibited by SOD but not by catalase, which suggests that superoxide anion is the major component of ROS involved in this assay system.

Detection of luminol-dependent CL in isolated leukocytes has been used to screen for chronic granulomatous disease[9,10]. Nakagawara et al. also described an assay to measure the superoxide radicals from a small amount of whole blood[11]. However, all these measurements required isolation of leukocytes. In the current study, a simple method has been adopted using whole blood samples as small as 0.2 ml without prior isolation of leukocytes.

In earlier methods used to detect the generation of ROS by leukocytes various stimulants, such as Bacto latex particles, opsonized zymosan, PMA, and N-formyl-methionyl-leucyl-phenylalanine, were used to activate the phagocyte respiratory burst[12]. Because the leukocytes were stimulated, the measured ROS might not have represented the natural state. In the current assay, no stimulant is required because the Model CLD-110 detector is sensitive enough to detect the ultra-weak CL related to superoxide anion. The method was able to detect the low amount of superoxide anion present in the whole blood of healthy subjects. The spontaneous release of superoxide anion by non-stimulated leukocytes in whole blood implies that normal individuals produce superoxide anion under conditions of baseline physiological status. Therefore, the high sensitivity of this method may be useful to detect minute changes in superoxide anion generation.

It has been reported that inflammatory processes such as acute pancreatitis are associated with excessive production of ROS by leukocytes[2]. Using this new assay, we have confirmed an augmented release of superoxide anion in acute pancreatitis. For further clarification of the possible mechanisms responsible for the enhanced CL in acute pancreatitis, the measured CL per peripheral WBC or per PMNL was calculated. These results also showed higher values for CL/WBC and CL/PMNL in acute pancreatitis than in healthy controls. It was in line with previous speculation that excessive ROS is released from the activated WBC, especially from the PMNL[2]. However, there was no association between the blood CL level and the cytokine-associated fever in acute pancreatitis in this study. The discrepancy may be due to the small number of patients in the pancreatitis group. With the accumulation of more records of cases with fever symptoms and varied counts of blood leukocytes and CL levels, the relationship between PMNL, CL and fever may be elucidated. Because of its simple and sensitive nature, it may be possible to use the release of superoxide anion as an index to correlate with the prognosis of such a disease as acute pancreatitis, which is characterized by enhanced leukocyte response. Furthermore, this assay may also be potentially valuable to measure superoxide anion in ageing and in disorders such as atherosclerosis, diabetes, inflammatory processes, and neoplasms. However, whether or not this assay can be utilized in early diagnosis or in predicting the outcome of acute pancreatitis awaits further investigation.

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