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Short Communication

A photometric assay for hydrogen peroxide production by polymorphonuclear leucocytes

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Introduction

Activated polymorphonuclear leucocytes (PMN) produce a superoxide anion (O_2^-) that rapidly dismutates into H_2O_2 [1–3]. With the considerable interest in the actions of this reactive oxygen species on cells, we propose a quick reliable photometric method for measurement of H_2O_2 production by activated PMN.

The proposed method for the assay of hydrogen peroxide is based on one of the most important physiologic systems by which cells dispose of toxic oxygen metabolites, i.e. the glutathione cycle [4,5], and by the coupled test procedure for glutathione peroxidase in blood [6].

$$H_2O_2 + 2\,GSH \xrightarrow{\text{GSH-peroxidase}} 2\,H_2O + GSSG$$

$$GSSG + NADPH \xrightarrow{\text{GSSG-reductase}} 2\,GSH + NADP$$

H_2O_2 is reduced to H_2O. Reductive potential is provided by NADPH. By incubating PMN into a medium containing GSH, NADPH, GSH-peroxidase and GSSG-reductase, it is possible to quantify the release of H_2O_2 by following the kinetics of NADPH oxidation. This oxidation is stoichiometrically in a 1:1 ratio with H_2O_2 reduction.

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Materials and methods

Reagents

(A) Dulbecco's buffer (KCl = 2.68 mM; KH_2PO_4 = 1.47 mM; $MgCl_2$ = 0.49 mM; NaCl = 136.89 mM; Na_2HPO_4 = 8.10 mM; $CaCl_2$ = 0.90 mM), containing 5.56 mM of glucose (from Gibco Limited, Paisley, Scotland).
(B) Sodium azide (from Carlo Erba, Milano, Italy) 2 mM final in reagent A.
(C) Reduced glutathione (from Boehringer Co. Mannheim, Germany) 1 mM final in reagent A.
(D) Glutathione reductase EC No. (from Boehringer Co. Mannheim, Germany) 10 μg/ml final in reagent A.
(E) Glutathione peroxidase EC No. (from Sigma Chemical Company, St. Louis, MO, USA) 0.1 U/ml final in reagent A.
(F) NADPH (from Boehringer Co. Mannheim, Germany) 150 μM final in reagent A.
(G) Percoll (from Pharmacia, Uppsala, Sweden).
(H) Stimulants: phorbol myristate acetate (PMA) 2 μM final in reagent A; arachidonic acid 100 μM final in reagent A; *n*-formyl methionyl phenylalanine (fMLP) 2 μM final in reagent A (all from Sigma Chemical Company, St. Louis, MO, USA).

PMN isolation

PMN were isolated over Percoll gradients [7] from 4 ml of blood collected in EDTA-K_3 and were suspended (10×10^6 cells/ml) in Dulbecco's buffer containing 5.56 mM of glucose. This preparation yielded at least 8×10^6 PMN (> 95% pure) allowing 16 experiments to be carried out, each with 0.5×10^6 PMN.

Analytic technique

The assay was carried out at 37 °C in a final volume of 1 ml, by using semi-micro cuvettes. Reagents A, B, C, D, E and F were preincubated in the absence (resting) or in the presence of metabolic stimulants such as PMA, arachidonic acid or fMLP. After addition of 0.5×10^6 PMN, the kinetics of NADPH consumption were recorded at wavelength 340 nm. By using PMA as a stimulant the rate of decrease in absorbance was constant for at least 15 min while, by using arachidonic acid or fMLP, the rate of decrease was constant for the first 1.5–2.5 min only.

For every series, a blank assay, in which the cells were omitted from the assay mixture, was carried out. This blank values was subtracted from each sample value. Under our experimental conditions, values for the blank samples ranged from 0.002 to 0.003 Abs/min.

H_2O_2 produced was calculated on the basis of ϵmmol of NADPH (6.21 $mmol^{-1}\ cm^{-1}$).

Standard H_2O_2 was prepared by photometric measure at 230 nm by using the ϵmmol of H_2O_2 (0.0622 $mmol^{-1}$ cm^{-1}). 30% H_2O_2 was used as a stock and dilutions were made immediately before the assay was performed.

Results

Fig. 1 shows the H_2O_2 produced as a function of PMN number, stimulated with PMA. The reaction was linear up to 10^6 PMN/ml. CV% for PMA-stimulated cells ranged from 1.42 to 2.36.

In Fig. 2 the activity of PMN stimulated with PMA (column A = 100%) is compared with activity obtained in different experimental conditions. By omitting NaN_3 from the assay (B), the recovery of H_2O_2 was decreased, indicating that part of H_2O_2 was degraded by cellular catalase and myeloperoxidase as previously described [8,9]. The addition of catalase (obviously in absence of NaN_3) inhibited the reaction (C), confirming that it depends on H_2O_2 produced. Red cells, which could possible contaminate PMN preparations, did not affect the reaction (D). In the absence of GSH-peroxidase and GSSG-reductase (E), the reaction was practically eliminated, indicating that in this assay system no direct consumption or degradation of extracellular NADPH occurs. The assay was suitable for detecting cell activation by other classical soluble stimulants such as arachidonic acid (F) and fMLP (G).

Table I shows that the measurement of added H_2O_2, as detected by consumption of NADPH, was complete and that the presence of PMN did not affect the recovery.

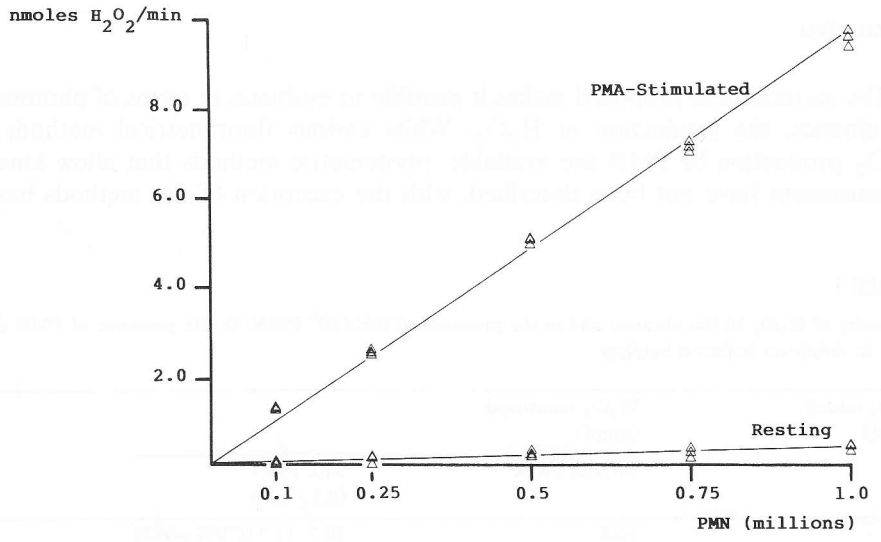

Fig. 1. H_2O_2/min production as a function of PMN number. The cells were incubated in the absence and in the presence of 1 μM PMA. Tests performed in triplicate.

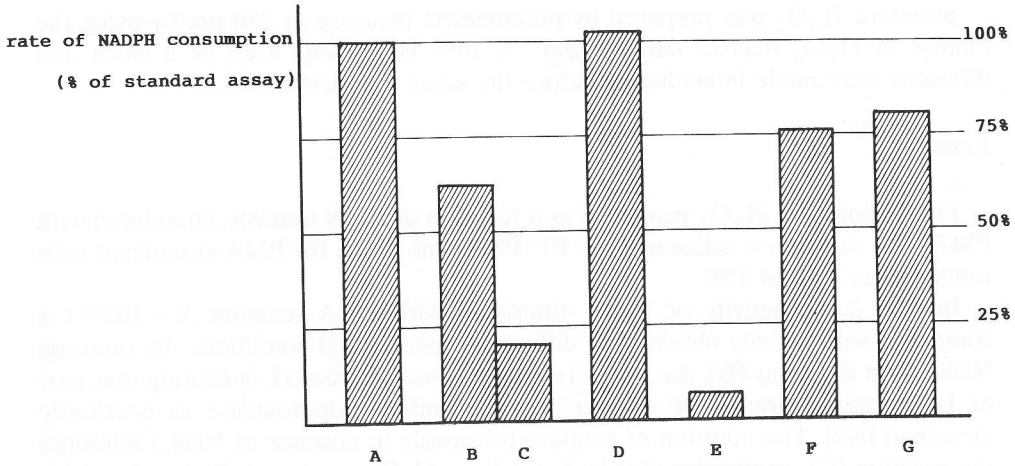

Fig. 2. Rate of NADPH consumption in various assay conditions. A: Standard assay (see methods), PMN stimulated with 1 μM final PMA. B: Omit NaN_3. C: Omit NaN_3, plus 0.2 mg of catalase. D: Omit NaN_3, addition of 2×10^6 red cells. E: Omit glutathione peroxidase and glutathione reductase. F: PMN stimulated with 100 μM final arachidonic acid. G: Omit PMA, PMN stimulated with 2 μM final fMLP.

As reported in Table II, the activity of PMN of 12 healthy individuals, measured in separate experiments, ranged from 7.22 to 10.31 nmol H_2O_2/min/10^6 PMN, with a mean of 8.91 and a CV% of 10.86. Comparing the values of two individuals that were submitted to two tests in two differents days gave a CV% of 2.32 for i-5/i-5rpt and 8.24 for i-8/i-8rpt.

Discussion

The method here proposed makes it possible to evaluate, in terms of photometric kinetics, the production of H_2O_2. While various fluorimetrical methods of H_2O_2 production by PMN are available, photometric methods that allow kinetic measurement have not been described, with the exception of the methods based

TABLE I

Recovery of H_2O_2 in the absence and in the presence of 0.5×10^6 PMN. In the presence of PMN data were in duplicate between batches

H_2O_2 added (nmol)	H_2O_2 measured (nmol)	
	without PMN	with PMN (0.5×10^6)
10	10.2	10.7–11.7 (CV% = 6.3)
20	21.8	19.6–21.4 (CV% = 6.2)
30	31.9	29.3–31.5 (CV% = 5.1)

TABLE II

H_2O_2 production by PMA-stimulated PMN in a sample of 12 healthy individuals. Assay runs were carried out on 9 different days (d-1–d-9). Individuals i-5 and i-8 were submitted to two tests on different days (i-5 on d-5 and d-8, i-8 on d-7 and d-8)

Assay runs	Individuals	nmol H_2O_2 /min/10^6 PMN
d-1	i-1	7.22
d-2	i-2	9.57
d-3	i-3	9.84
d-4	i-4	7.42
d-5	i-5	9.28
d-6	i-6	8.77
d-7	i-7	10.31
d-7	i-8	9.45
d-7	i-9	8.00
d-8	i-10	8.56
d-8	i-11	9.66
d-9	i-12	8.90
d-8	i-5rpt	8.98
d-8	i-8rpt	8.41

on the peroxidase spectral changes [10,11], which have the disadvantage of requiring special equipment (double beam plus double wavelength measurement) and reagents which are not commercially available (cytochrome C peroxidase).

Technical execution is easy and has proved fast and sensitive so as to allow multiple tests in a short time with few milliliters of blood.

The recovery data indicate that accuracy is fairly good (Table I).

The within-batch precision is high; in fact in the dose-response curve (Fig. 1), for PMA-stimulated cells, the CV% ranged from 0.42 to 2.36.

A reasonable between-batch precision can be deduced from these data: (a) the duplicate recovery experiments performed in two different occasions (Table I) showed a CV% ranged from 5.1 to 6.3. (b) The CV%s of individuals that were submitted to tests in different days (Table II) were 2.32 and 8.24. (c) The activity of 12 healthy individuals ranged from 7.22 to 10.31 nmol H_2O_2/min/10^6 PMN with a CV% of 10.9 (Table II).

Compared to other procedures, the method here proposed has the advantage of not requiring the standard curve for H_2O_2.

This method could be adapted to automation and could possibly be applied on less purified PMN preparation, thus enabling rapid performance of multiple tests.

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