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$_2$O$_2$ [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$_2$O$_2$ 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$_2$O$_2$ + 2 GSH $\xrightarrow{\text{GSH-peroxidase}}$ 2 H$_2$O + GSSG

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

H$_2$O$_2$ is reduced to H$_2$O. 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$_2$O$_2$ by following the kinetics of NADPH oxidation. This oxidation is stoichiometrically in a 1:1 ratio with H$_2$O$_2$ reduction.

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Materials and methods

Reagents

(A) Dulbecco’s buffer (KCl = 2.68 mM; KH₂PO₄ = 1.47 mM; MgCl₂ = 0.49 mM; NaCl = 136.89 mM; Na₂HPO₄ = 8.10 mM; CaCl₂ = 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) 0.7 IJ/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-K₃ and were suspended (10 x 10⁶ cells/ml) in Dulbecco’s buffer containing 5.56 mM of glucose. This preparation yielded at least 8 x 10⁶ PMN (> 95% pure) allowing 16 experiments to be carried out, each with 0.5 x 10⁶ 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 x 10⁶ 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.

H₂O₂ produced was calculated on the basis of 6.21 mmol of NADPH (6.21 mmol⁻¹ cm⁻¹).
Standard \( \text{H}_2\text{O}_2 \) was prepared by photometric measure at 230 nm by using the \( \varepsilon \text{mmol of } \text{H}_2\text{O}_2 \) (0.0622 mmol\(^{-1}\) cm\(^{-1}\)). 30% \( \text{H}_2\text{O}_2 \) was used as a stock and dilutions were made immediately before the assay was performed.

Results

Fig. 1 shows the \( \text{H}_2\text{O}_2 \) 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 \( \text{NaN}_3 \) from the assay (B), the recovery of \( \text{H}_2\text{O}_2 \) was decreased, indicating that part of \( \text{H}_2\text{O}_2 \) was degraded by cellular catalase and myeloperoxidase as previously described [8,9]. The addition of catalase (obviously in absence of \( \text{NaN}_3 \)) inhibited the reaction (C), confirming that it depends on \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \), as detected by consumption of NADPH, was complete and that the presence of PMN did not affect the recovery.
Fig. 2. Rate of NADPH consumption in various assay conditions. A: Standard assay (see methods), PMN stimulated with 1 \( \mu \)M final PMA. B: Omit NaN\(_4\). C: Omit NaN\(_3\), plus 0.2 mg of catalase. D: Omit NaN\(_3\), addition of 2 x 10\(^6\) red cells. E: Omit glutathione peroxidase and glutathione reductase. F: PMN stimulated with 100 \( \mu \)M final arachidonic acid. G: Omit PMA, PMN stimulated with 2 \( \mu \)M 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\(_2\)O\(_2\)/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 different 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\(_2\)O\(_2\). While various fluorimetric methods of H\(_2\)O\(_2\) production by PMN are available, photometric methods that allow kinetic measurement have not been described, with the exception of the methods based

<table>
<thead>
<tr>
<th>H(_2)O(_2) added (nmol)</th>
<th>H(_2)O(_2) measured (nmol)</th>
<th>with PMN (0.5 x 10(^6))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without PMN</td>
<td>with PMN</td>
</tr>
<tr>
<td>10</td>
<td>10.2</td>
<td>10.7–11.7 (CV% = 6.3)</td>
</tr>
<tr>
<td>20</td>
<td>21.8</td>
<td>19.6–21.4 (CV% = 6.2)</td>
</tr>
<tr>
<td>30</td>
<td>31.9</td>
<td>29.3–31.5 (CV% = 5.1)</td>
</tr>
</tbody>
</table>

**TABLE I**

Recovery of H\(_2\)O\(_2\) in the absence and in the presence of 0.5 x 10\(^6\) PMN. In the presence of PMN data were in duplicate between batches.
TABLE II

H₂O₂ 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).

<table>
<thead>
<tr>
<th>Assay runs</th>
<th>Individuals</th>
<th>nmol H₂O₂/min/10⁶ PMN</th>
</tr>
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<tbody>
<tr>
<td>d-1</td>
<td>i-1</td>
<td>7.22</td>
</tr>
<tr>
<td>d-2</td>
<td>i-2</td>
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<td>d-3</td>
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</tr>
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<td>i-6</td>
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</tr>
<tr>
<td>d-7</td>
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</tr>
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<td>8.98</td>
</tr>
<tr>
<td>d-8</td>
<td>i-8rpt</td>
<td>8.41</td>
</tr>
</tbody>
</table>

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% 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 H₂O₂/min/10⁶ 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 H₂O₂.

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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References
