Subcellular Localization of \( \text{H}_2\text{O}_2 \) Production in Human Neutrophils Stimulated With Particles and an Effect of Cytochalasin-B on the Cells

By Yoh-Ichiroh Ohno, Kei-Ichi Hirai, Tadashi Kanoh, Haruto Uchino, and Kazuo Ogawa

The ultrastructural localization of \( \text{H}_2\text{O}_2 \) production in suspended polymorphonuclear leukocytes (PMN) stimulated with particles was studied using \( \text{CeCl}_3 \) technique. PMN stimulated with opsonized zymosan or polystyrene latex with or without IgG were incubated in 0.1 \( M \) Tris-maleate buffer with 1 mM \( \text{CeCl}_3 \) and 10 mM aminotriazole. Cells were then fixed and embedded in a resin for electron microscopy. The reaction product of cerium perhydroxide was observed on the phagosomal membranes and on the contact surface of the membrane of adjoining PMN. The plasma membrane was damaged and the electron-dense product was diffused into the cytoplasm. These results clearly show that \( \text{H}_2\text{O}_2 \) production is initiated at the areas of the plasma membrane adherent to the particles and that \( \text{H}_2\text{O}_2 \) is released before the completion of phagocytosis.

ON EXPOSURE to various stimuli, polymorphonuclear leukocytes (PMN) undergo a respiratory burst with the stimulation of \( \text{NAD(P)}\text{H} \) oxidation, \( \text{O}_2 \) consumption, \( \text{O}_2^- \), and \( \text{H}_2\text{O}_2 \) production, and hexose monophosphate shunt (HMPS) activity.\(^1\)\(^2\) The formed \( \text{H}_2\text{O}_2 \) thereafter contributes to the microbicidal activity in cooperation with the myeloperoxidase and halide.\(^3\) \( \text{NAD(P)}\text{H} \) oxidase associated with these phenomena was suggested to be on the plasma membrane.\(^4\)\(^5\) Generally, a cytochemical approach is useful in studying each cell in association with the morphology. Briggs et al. devised a new cytochemical method using \( \text{CeCl}_3 \) for demonstrating the subcellular \( \text{H}_2\text{O}_2 \)-producing site in PMN stimulated with particles.\(^6\) They observed that in PMN adhered to a glass cover slip, \( \text{H}_2\text{O}_2 \) was formed on the whole surface of the plasma membrane and on the phagosomal membrane and that the reaction was NADH dependent. However, no study with PMN in suspension was carried out. Using the \( \text{CeCl}_3 \) technique, we demonstrated the subcellular localization of \( \text{H}_2\text{O}_2 \) production in human PMN stimulated with opsonized zymosan (OPS), polystyrene latex (LA), or IgG-coated latex (LAG) in suspension. An influence of cytochalasin-B (CB) on PMN was also studied.

MATERIALS AND METHODS

Catalase (bovine liver, C-40), ferricytochrome-c (horse heart, type 6; cyto-c), zymosan-A, and cytochalasin-B were from Sigma Chemical Co., St. Louis, Mo. Dextran (mol wt 199,000), \( \text{p-Benzoquinone} \) (\( \text{O}_2^- \) scavenger) inhibited the formation of the deposits, but KCN or \( \text{NaNO}_3 \) enhanced it. Pretreatment with \( \text{p-diazobenzenesulfonic acid} \) inhibited the reaction. In some PMN pretreated with cytochalasin-B, cellular aggregation was observed. The \( \text{H}_2\text{O}_2 \) production in these cells was observed on the membrane adherent to the particles and on the contact surface of the membrane of adjoining PMN. The plasma membrane was damaged and the electron-dense product was diffused into the cytoplasm. These results clearly show that \( \text{H}_2\text{O}_2 \) production is initiated at the areas of the plasma membrane adherent to the particles and that \( \text{H}_2\text{O}_2 \) is released before the completion of phagocytosis.

Preparation for Human Leukocytes and Particles

Venous blood from healthy young adult donors was allowed to sediment at room temperature after mixing 4 parts of the blood with one part of 6% dextran in isotonic saline. The leukocyte-rich plasma was removed, and the cells were sedimented at 150 g for 10 min. The pellets were washed twice in Hank’s balanced salt solution, pH 7.4, with additional glucose (1 mg/ml) (HBSS/G).

OPZ was prepared by incubating zymosan particles in opsonin (5 mg/ml) at 37\(^\circ\)C for 30 min and were washed twice in HBSS/G. LA were washed twice in HBSS/G. LAG were prepared by incubating the mixture of 1.0 ml of latex particles and 1.0 ml of 0.1 \( M \) Tris-glycine buffer, pH 8.5, containing 30 mg of IgG at 37\(^\circ\)C for 30 min.\(^7\) LAG were washed twice in HBSS/G and finally resuspended in the same buffer. The coating of IgG on latex particles was recognized by particulate aggregation using anti-human IgG.

Stimulation of the Leukocytes

Some cells were pretreated with 3 mM DBSA in HBSS/G at 37\(^\circ\)C for 30 min before stimulation with OPZ and were washed twice in HBSS/G.

Leukocytes were suspended in plastic tubes at a concentration of 2 \( \times 10^9 \text{ml}^{-1} \) - HBSS/G. A small volume of concentrated particles was added to the cell suspension such that the numerical ratio of particles to leukocytes was 10 for zymosan and 100 for latex particles. Thereafter, the tubes were incubated in 4% CO\(_2\)/95% air humidified atmosphere at 37\(^\circ\)C with occasional stirring. Ten minutes later, the cells were centrifuged at 150 g for 10 min at 4\(^\circ\)C and washed once in 0.1 \( M \) Trits-maleate buffer, pH 7.4, with 7% sucrose (TMB/S). During the incubation, some cells were stimulated with OPZ in a solution of HBSS/G containing KCN (1 mM) or \( \text{NaNO}_3 \) (10 mM). The short time course studies of phagocytosis were carried out by the addition of 25 ml of cold (O-C) HBSS/G containing 1

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mM EDTA to 1.0 ml of the suspension of cells and OPZ at intervals of 30 sec, 60 sec, or 5 min. The pellet was washed twice in TMB/S. Nonstimulated cells were incubated in HBSS/G without particles.

**Treatment With CB**

Some cells were pretreated with CB (5 μg/ml) in HBSS/G at 37°C for 10 min. Then the solution contained 0.2% DMSO, a solvent of powdered CB. The concentration of DMSO did not influence the cytochemical reaction and the morphology of PMN. CB was not washed out, and the cells were stimulated with particles in HBSS/G containing CB.

**Incubation for Cytochemistry**

The standard incubation medium consisted of 0.1 M Tris-maleate buffer, pH 7.4, with 7% sucrose, 1 mM CeCl₃, and 10 mM AT. Additional drugs were used in the following concentrations; 0.2 mg/ml catalase, 0.1 mM cyto-c, 0.1 mM BQ, 0.71 mM NADH, and 0.71 mM NADPH. The viability of the cells in solutions with these additional drugs was checked with trypan blue dye exclusion. In all cases, the viability was over 98%.

The unfixed cells after phagocytosis were sedimented in a conical plastic tube. Ten milliliters of a standard incubation solution or the solution containing the additional drug was added to the sediment (2 × 10⁶ leukocytes). The suspension was incubated at 37°C for 20 min in 5% CO₂ with occasional stirring.

**Preparation for Electron Microscopy**

Following the cytochemical reaction, leukocytes were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 60 min. After washing with two changes of the buffer, cells were postfixed in 1% OsO₄ in the same buffer at 4°C for 60 min, dehydrated in graded ethanol, and embedded in a Spurr's low viscosity epoxy resin. Ultrathin sections were prepared with a Porter-Blum ultramicrotome and examined by a Hitachi H-700H electron microscope. No counterstaining with uranyl acetate and lead citrate was carried out.

**X-Ray Energy Spot Pulse Analysis**

X-ray spectra of a point analysis of some ultrathin sections were obtained using a Kevex x-ray microanalyzer attached to a Hitachi H-700H electron microscope (accelerating voltage at 20 kV).

**RESULTS**

The morphology of resting PMN was almost the same as that not incubated in the cytochemical medium, and no electron-dense product was observed.

**PMN Stimulated With OPZ**

In PMN incubated with OPZ for 10 min, the reaction product was visible on the phagosomal mem-

![Fig. 1](image-url)
branes and on the areas of the plasma membrane adherent to OPZ (Fig. 1A). It was absent from the free surface of the membrane, free surface of the particles, and the membrane of some phagosomes. The product was completely lost by pretreatment with DBSA (Fig. 1B). PMN whose phagocytosis was stopped 30 sec after the addition of OPZ apparently phagocytosed fewer particles than the cells stimulated for 10 min (Fig. 1C). In these cells, the pattern of the reaction product was the same as the pattern of cells incubated for 10 min. Cells treated with a cold HBSS/G with EDTA at 60 sec or 5 min after phagocytic stimulation also showed almost the same reaction as the 10-min-stimulated cells (Fig. 1D). By the addition of catalase or cyto-c, the amount of deposits was decreased but was not lost completely (Fig. 2 A and B). In contrast to these scavengers, BQ completely inhibited the formation of the reaction product (Fig. 2C). Addition of NADH or NADPH did not alter the distribution and the amount of the deposits (Figures were not shown.). The presence of KCN or NaN₃ during the phagocytic stimulation apparently enhanced the deposits (Fig. 2D). To quantify the effect of these drugs on the deposits, the deposits associated with 50 zymosan particles involved in cells was classified into four groups: “strong,” “moderate,” “weak,” and “negative.” The results are presented in Table 1.

Phagocytosis of OPZ was apparently inhibited in

Table 1. Effect of Drugs on the Deposits of Cerium Peroxidide Associated With Zymosan particles

<table>
<thead>
<tr>
<th>Addition to Incubation</th>
<th>Deposits Around the Particles Associated With Cells</th>
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<tbody>
<tr>
<td></td>
<td>Strong</td>
</tr>
<tr>
<td>Catalase (0.2 mg/ml)</td>
<td>7</td>
</tr>
<tr>
<td>Cytochrome-c (100 μM)</td>
<td>1</td>
</tr>
<tr>
<td>p-Benzoquinone (100 μM)</td>
<td>0</td>
</tr>
<tr>
<td>KCN (1 mM)</td>
<td>22</td>
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</table>

Number of particles examined: 50 in each study.

The procedures are described in Materials and Methods. The degree of the deposits around the particles associated with cells was classified into four groups: “strong,” “moderate,” “weak,” and “negative.” Selection of the 50 particles on the ultrathin sections was carried out in a blind fashion.
Fig. 3. PMN pretreated with 5 μM cytochalasin B were stimulated with OPZ. Left: Some PMN were aggregated. Deposits were observed on the areas of the membrane adherent to OPZ and on the contact surface of the adjoining PMN (×3400). Right: Higher magnification of the left photograph (×7400).

Fig. 4. PMN stimulated with latex particles (left) and IgG-coated latex (right). The distribution of the dense deposits was the same as that of PMN stimulated with OPZ. Left—×12,800. Right—×10,700.
PMN Stimulated With LA or LAG

In PMN stimulated with LA or LAG, the distribution of the reaction product was the same as the OPZ-stimulated cells: the area of the membrane engulfing the particles and some of the phagosomal membrane (Fig. 4). The difference between PMN with LA and those with LAG was not recognized.

In some PMN treated with CB before stimulation with LAG, cellular aggregation was also observed. Phagosome-like pictures were found in some sections but these small particles may communicate to the outer environment by a narrow slit. The deposits in these cells were visible on the particle-adhering site of the membrane and on the contact surface of the membrane of adjoining PMN (Fig. 5). It was also visualized that the electron-dense deposits were diffused into the cytoplasm (Fig. 5).

X-Ray Spectra of the Electron-Dense Product

The energy spectra of a point on the electron-dense deposits and a point on the cytoplasm were obtained (Fig. 6). On the deposits, Ce peak (Lα: 4.839 kV, Lβ1 5.261 kV, Lβ2 5.162 kV) and Cu peak (Kα: 8.040 kV, Kα 8.904 kV) were obvious. On the cytoplasm, the Cu peak was only detected because of the use of copper-grid meshes. It was demonstrated that the amount of cerium was greater on the reaction product than on the cytoplasm.

DISCUSSION

The mechanism of respiratory burst in PMN is as follows: \( \text{O}_2 \) is reduced to \( \text{O}_2^- \) with the oxidation of NAD(P)H. Next, the dismutation of \( \text{O}_2^- \) results in \( \text{H}_2\text{O}_2 \) formation. The reaction is catalized by superoxide dismutase, but spontaneous dismutation can take
Fig. 6. X-ray spectra of a point of the electron-dense deposits (DEP) and the cytoplasm (CP). (Left) Ce peak and Cu peak were obvious on DEP. (Right) Only the Cu peak was visible on CP. The amount of cerium was greater on the reaction product than on the cytoplasm.

A cytochemical approach using CeCl₃ was reported by Briggs et al. for demonstrating the subcellular H₂O₂ production in stimulated PMN. H₂O₂ production was observed on the whole surface of the plasma membrane and on the phagosomal membrane. They used PMN adhered to glass cover slips. However, biochemical O₂ assay was carried out in PMN in suspension. Free phagocytic cells like PMN can respond to various inflammatory stimuli and migrate to the focus. Therefore, we studied the cells in suspension. Electron-dense deposits were demonstrated on the areas of the plasma membrane adherent to the particles and on the phagosomal membrane. These were absent from the free surface. The dependency on NADH reported by Briggs et al. was not found. The reactions were different from Briggs et al.’s observations. Then the effects of catalase, cyto-c, BQ, KCN, and NaN₃ were studied and the reliability of our modified method for the H₂O₂ producing site was evaluated. By the addition of catalase (H₂O₂ scavenger) or cyto-c (O₂ scavenger), the amount of the reaction product was decreased but was not lost completely. The size of catalase (mol wt 251,000) and cyto-c (mol wt 12,400) are much larger than Ce³⁺. Therefore, these exogenous scavengers may not diffuse through the surface membrane and into the space between the particles and the membrane. The suggestion was reported by Hafeman and Lucas. Dependency on active oxygen species was shown in PMN-mediated and antibody-dependent cellular cytotoxicity. But extracellular catalase or superoxide dismutase did not inhibit the target cell damage. They considered that these enzymes were unable to diffuse into the intermembranous space between PMN and target cells. In contrast to these drugs, BQ (mol wt 108), an electron acceptor that has been reported to have a strong affinity for O₂ under aerobic conditions, completely inhibited the formation of electron-dense deposits. The enhancement of the deposit formation by addition of cyanide or azide can take place because these agents inhibit heme enzymes, catalase, or myeloperoxidase (MPO), which transform H₂O₂ to other oxygen species. Also, it was suggested by Jandl et al. that MPO terminated the activity of O₂ - forming enzymes. We also examined the effect of DBSA. The deposits were not formed by pretreatment with DBSA before phagocytic stimuli. DBSA has been considered to be a nonpermeable reagent that blocks several membrane-associated enzymes. Effects of DBSA on the O₂ - forming enzyme were previously reported by some authors. Briggs et al. showed that DBSA treatment after phagocytic stimuli decreased the surface cerium deposition, not the phagosomal reaction. Goldstein et al. demonstrated that O₂ - production in PMN stimulated with concanavalin-A was inhibited by DBSA. However, it was reported recently that treatment of resting cells with DBSA did not diminish the O₂ - production by human neutrophils stimulated with phorbol myristate acetate. The different effect of the drug on superoxide production of PMN may result from the difference of the stimuli. From these studies, we demonstrated that the cerium deposition in our method was really formed by the reaction of H₂O₂ and that it was related to O₂ - H₂O₂ reacts with Ce³⁺ to form an electron-dense precipitate, cerium perhydroxide [Ce(OH)₃OH]. The stoichiometry is as follows:

\[ 2Ce^{3+} + 6OH^- + 3H_2O_2 \rightarrow 2Ce(OH)_3OOH + 2H_2O \]

The subcellular localization of the O₂ - forming enzyme has been under discussion. Recently it has been reported, that O₂ - forming enzyme is localized on the plasma membrane. However, whether the H₂O₂ production arises at the whole surface or the restricted area of the membrane has not been determined. Our study directly demonstrated that H₂O₂...
production in human PMN in suspension stimulated with particles arose at the areas of the membrane adherent to the particles and that the H2O2-producing site was carried in the phagosome. Other investigators also indirectly suggested the theory. Green et al. showed that the ratio of O2− released to O2 consumed in human PMN stimulated with concanavalin-A was 0.95 and that it decreased to 0.20 when stimulated with OPZ.24 The O2−-forming system, localized on the extracellular side of the outer membrane, was suspected of being carried inside the vacuoles during phagocytosis and to disappear from the outer surface. Nabi et al. showed that the formation of H2O2 induced by cytochalasin-D was almost completely inhibited by p-chloromercuribenzenesulfonic acid, a nonpermeable drug that inhibits surface sulfhydryl groups, whereas H2O2 production induced by bacteria was not affected by the inhibitor.25

There were several reports on the change of quality of the plasma membrane of stimulated PMN. Takeda et al. analyzed the localized calcium in stimulated PMN.23 Using a fluorescent probe to monitor the mobilization of intracellular divalent cations, they showed that the translocation of membrane-bound calcium to the cytoplasm was closely associated with the induction of the O2− production. Hoffstein studied the membrane-bound calcium using an electromagnetic cytochemistry.24 Preincubation of PMN with OPZ or other particles resulted in the loss of pyroantimonate-precipitable calcium from the area of the membrane adherent to the particles but not from the remainder of the membrane. These studies strongly suggest that Ca2+ mobilization from the particle-binding site of the membrane towards the cytoplasm is essential for the induction of metabolic burst.

Studies with the CB-pretreated cells clearly showed that the H2O2 production was induced before the completion of phagocytosis. O2− production in CB-treated cells has been reported by several investigators. Goldstein et al. pointed out that O2− was produced in the PMN pretreated with CB when these cells were stimulated with OPZ, aggregated IgG, or C5a.25 Root and Metcalf reported that PMN pretreated with CB showed a decrease in O2 release, and an increase in H2O2 release when stimulated with opsonized bacteria.5 Electron microscopically, cellular aggregation was observed in these cells and H2O2 was also produced on the contact surface of the plasma membrane of adjoining PMN. It is another interesting finding that the electron-dense product was diffused over the damaged membrane into the cytoplasm. Recently, PMN stimulated with particles,26,27 lectins,28,29 or phorbol myristate acetate30,31 have been thought to have cytotoxic activity with the formation of activated oxygens. In these experiments, cellular contact between PMN and the target cells was shown to be essential. Stimulated PMN may injure the plasma membrane of their own and that of the target cells with the formation of H2O2 at the contact surface. The mechanisms involved in the enhancement of the cellular aggregation and the membrane damage by the treatment of CB should be further studied.

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Subcellular localization of H2O2 production in human neutrophils stimulated with particles and an effect of cytochalasin-B on the cells

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