Subcellular Localization of Hydrogen Peroxide Production in Human Polymorphonuclear Leukocytes Stimulated With Lectins, Phorbol Myristate Acetate, and Digitonin: An Electron Microscopic Study Using CeCl₃

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The ultrastructural H₂O₂-producing site in human polymorphonuclear leukocytes (PMN) stimulated with soluble stimuli was studied using a CeCl₃-technique. Cellular aggregation and formation of small vacuoles were observed when PMN were stimulated with 100 µg/ml concanavalin-A, 1 mg/ml phytohemagglutinin, or 100 µg/ml wheat germ agglutinin for 10 min at 37°C. Electron-dense deposits formed from the reaction of H₂O₂ and CeCl₃ were observed on the contact surface of the plasma membrane of aggregated PMN stimulated with lectins. Treatment with 5 µg/ml cytochalasin-B before lectin-stimulation induced an enhanced formation of vacuoles, degranulation, rounding of the contour, cellular aggregation, and enhancement of the deposits. Phorbol myristate acetate (PMA; 100 ng/ml) induced strong leukocyte aggregation, the formation of multiple huge vacuoles, degranulation, and H₂O₂ production at almost all of the contact surface between adjoining PMN and between PMN and erythrocytes, mononuclear cells, or thrombocytes. In PMN stimulated with digitonin (8 µg/ml), vacuolar formation, degranulation, multiple projections on the surface, and H₂O₂ production on the whole surface membrane were demonstrated. It is shown that cellular aggregation and cell-to-cell contact have an important role in the induction of O₂⁻ production induced by lectins or PMA and that O₂⁻ production induced by the detergent is not dependent on leukocyte aggregation.

Preparation of the Cells

Venous blood from healthy young adult donors was allowed to sediment at room temperature after mixing 4 parts blood to 1 part 6% dextran (mol wt 177,000) 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 Hanks' balanced salt solution, pH 7.4, with additional glucose (1 mg/ml) (HBSS/G), suspended in conical plastic tubes, and used in the electron microscopic cytochemistry. In the biochemical assay of superoxide production, PMN were separated from the leukocyte-rich plasma by centrifugation at 400 g on Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden). Thereafter, contaminated erythrocytes were hypotonically lysed. The cells were washed 3 times with modified Krebs-Ringer phosphate buffer (KRP/G: 122 mM NaCl, 4.0 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 15.7 mM sodium phosphate buffer, pH 7.4, and 5.5 mM glucose) and suspended in the buffer. In the assay of hydrogen peroxide, the cells were washed and suspended in HBSS/G.

Electron Microscopic Cytochemistry for Subcellular H₂O₂-Producing Site

Stimulants were added to 3 ml of leukocyte-suspended solution containing 2 x 10⁶ PMN at 37°C. The tubes were incubated at 37°C for 10 min in 5% CO₂ atmosphere with occasional stirring. Some...
cells were pretreated with CB in a concentration of 5 µg/ml at 37°C for 10 min before stimulation with lectins in HBSS/G containing CB. The stimulants were as follows; 100 µg/ml Con-A, 1 mg/ml PHA, 100 µg/ml WGA, 100 ng/ml PMA, and 8 µg/ml digitonin. To confirm the specificity of the Con-A reaction, the effect of 60 mM AMM on Con-A stimulation was studied. In the study, the drug was added just before the stimulation of Con-A. After stimulation, the cells were centrifuged at 150 g for 10 min at 4°C and washed once in 0.1 M Tris-maleate buffer, pH 7.4, with 7% sucrose (TMB/S). A standard incubation medium for the cytochemical reaction consisted of TMB/S with 1 mM CeCl₃ and 10 mM aminotriazole. Unfixed cells were sedimented in a conical plastic tube. Ten milliliters of a prewarmed standard incubation medium or the solution with 0.1 mM BQ was added to the sediments. The

![Fig. 1](image)

**Fig. 1.** Electron microscopic cytochemistry for subcellular H₂O₂-producing site in PMN stimulated with concanavalin-A (Con-A). Cells separated from leukocyte-rich plasma were stimulated with 100 µg/ml Con-A for 10 min at 37°C. Cells were then incubated in 0.1 M Tris-maleate buffer, pH 7.4, with 7% sucrose, 1 mM CeCl₃, and 10 mM aminotriazole for 20 min at 37°C and prepared for an electron microscopic examination. (A) Cells stimulated with Con-A. Leukocyte aggregation and formation of small vacuoles are observed (×2800). (B) Cells treated as in A. Electron-dense deposits are localized on the contact surface of the plasma membrane and on the vesicles of adjoining PMN (×8400). (C) Cells pretreated with 5 µg/ml cytochalasin-B (CB) for 10 min at 37°C before stimulation with Con-A. Decrease of the number of granules, rounding, and cellular aggregation are observed. Electron-dense deposits on the contact surface of aggregated PMN are enhanced (×2800). (D) Cells treated as in C. Formation of multiple vacuoles and the electron-dense deposits on them are observed. Dense deposits are also visible on the contact surface between a neutrophil and an erythrocyte (×5000). (E) CB-pretreated cells stimulated with Con-A in the presence of 60 mM α-methyl-α-mannoside. Cellular aggregation, degranulation, and vacuolar formation are not observed. No dense product is formed (×4800). (F) Cells treated as in C were incubated in the cytochemical medium plus 0.1 mM p-benzoquinone. Formation of the dense deposits is severely reduced (×3200).
suspension was incubated at 37°C for 20 min in 5% CO₂ with occasional stirring. Following the cytochemical reaction, the pellets were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 60 min. After washing, the 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 resin. Ultrathin sections were prepared with an LKB ultramicrotome and examined under an electron microscope (type JEM-100CX: Nihon Denshi Co., Tokyo, Japan). Counterstaining with uranyl acetate and lead citrate was used on the sections.

**Assay of Hydrogen Peroxide Production**

This study was planned to demonstrate whether H₂O₂ production could be continued in TMB/S after the stimulant was washed out. The assay of H₂O₂ production was studied following the method by Root et al. PMN were suspended in HBSS/G in a concentration of 10⁶/ml at 37°C in plastic tubes. The stimulants used in the cytochemical study were added to the tubes in the same concentration and the tubes were incubated at 37°C for 10 min in 5% CO₂ with occasional stirring. Some PMN were pretreated with 5 μg/ml CB for 10 min at 37°C before stimulation with lectins. Thereafter, cells were centrifuged at 150 g for 10 min at 4°C, washed once in TMB/S, suspended in the prewarmed TMB/S at 37°C, and assayed for H₂O₂ production. Three milliliters of TMB/S with 3 x 10⁶ PMN were transferred to a 1-cm lightpath cuvette. The cuvette was set on a spectrofluorometer (type 500, Shimadzu Seisakusho, Kyoto, Japan) equipped with a thermoregulatory apparatus. Scopoletin (2 μM) and HPO (22 nM) were added to the cells and the fluorescence intensity was recorded with excitation wave length at 350 nm and the emission at 460 nm. The maximal rate of H₂O₂ production was determined from the initial slope of the scopoletin extinction curve and expressed as nanomoles of H₂O₂ production per minute. The amount of H₂O₂ production during the first 3 min was also calculated. The relationship between the extinction of the fluorescence and known amounts of H₂O₂ was previously determined in the TMB/S.

**Superoxide Production Assay Using Ferricytochrome-c**

Superoxide production was measured by ferricytochrome-c reduction following the method of Cohen and Chovaniec. One milliliter of KRP/G containing 70 μM ferricytochrome-c and 2.5 x 10⁶ PMN was put in a 1-cm pathlength cuvette, which was set on a spectrophotometer (type 140, Shimadzu Seisakusho, Kyoto, Japan) equipped with a thermoregulatory apparatus. Thereafter, stimulants were added in a volume of 20 μl. The concentration of the stimuli was the same as used in the cytochemistry. The effect of CB (5 μg/ml) treatment on the stimulation with lectins was studied. The drug was added 5 min before or after the stimulation with lectins. The absorbance at 550 nm was continuously monitored for 6 min after stimulation at 37°C. To see O₂⁻-independent change, the same assay was carried out in the presence of superoxide dismutase (10 μg/ml). O₂⁻ production was expressed as nanomoles of ferricytochrome-c.
reduced per 10^6 cells using an extinction coefficient 21 /mM/cm at 550 nm. The amount of O_2 released within 5 min was calculated. The maximal rate per minute was determined from the slope of the linear portion of the initial curve to zero absorbance change.

RESULTS

Cells Stimulated with Lectins

Cells stimulated with Con-A showed aggregation and the formation of a small number of vacuoles (Fig. 1A). Electron-dense deposits were visualized on the contact surface of the plasma membrane in a small number of aggregated PMN (Fig. 1B). They were absent from the free surface. In the cells pretreated with CB, an enhanced formation of vacuoles, rounding of the contour, a decrease in the number of cellular granules, and cellular aggregation were observed following the Con-A stimulation (Fig. 1C and D). Deposits were observed on the contact surface of aggregated PMN and between PMN and erythrocytes and on the membrane of the vacuoles. The formation of the dense deposits was enhanced.

When CB-pretreated cells were stimulated with Con-A in the presence of AMM, no cellular aggregation, degranulation, or enhanced vacuolar formation were found. Rounding of the contour was observed, as in cells treated with only CB (Fig. 1E). No electron-dense product was formed. The presence of BQ, an electron acceptor that has been reported to have a strong affinity for O_2 under aerobic conditions, in the cytochemical reaction severely reduced the formation of the product (Fig. 1F).

Cells stimulated with P1-IA also showed aggregation and formation of vacuoles (Fig. 2A). The reaction product was visible on the contact surface of the plasma membrane in a small number of PMN. When pretreated with CB, multiple vacuolar formation, degranulation, and rounding were observed (Fig. 2B) and deposits were visualized on the contact surface of the plasma membrane of many cells (Fig. 2C). Deposits were also observed on the membrane of vacuoles and on the contact surface between PMN and thrombocytes (Fig. 2D). They were absent on the contact surface of aggregated thrombocytes.

WGA induced strong cellular aggregation and the formation of vacuoles. Weak deposits were recognized on the contact surface (Fig. 3A). When pretreated with CB, increased numbers of vacuoles, rounding, and degranulation were observed and the deposits on the contact surface between aggregated PMN were enhanced (Fig. 3B). Deposits were also observed in some vacuoles.

Cells Stimulated With PMA (Fig. 4)

PMA induced strong leukocyte aggregation and formation of multiple vacuoles. Electron-dense deposits were visible on the contact surface of the plasma membrane of aggregated PMN. Contrary to the study of lectins, the very strong deposition was formed on almost all of the contact surface of the plasma membrane of granulocytes. Some of the vacuoles were positive. It was noted that deposits were clearly formed on the contact surface between granulocytes and other cells: thrombocytes, erythrocytes, and mononuclear cells. No reaction was observed on the contact surface of aggregated thrombocytes.

Cells Stimulated with Digitonin

No cellular aggregation was observed (Fig. 5A). Multiple projections on the cell surface were observed in some cells as reported by Graham et al. (Fig. 5B). The formation of the vacuoles and a marked decrease in the number of cellular granules were observed. Electron-dense deposits were observed on the whole surface of the plasma membrane and on some vacuoles that originated from the free surface by enhanced pinocytosis. From this finding, it was demonstrated that the retention of cerium deposits visualized on the cellular contact in lectin- or PMA-stimulated cells...
could not arise from the restriction in the diffusion of H$_2$O$_2$ in the region of contact.

**Morphometric Quantitation of the Cerium Deposits**

To quantify the effect of drugs on the deposits, the depositions associated with 30 PMN selected on the ultrathin sections in a blind fashion were classified into three groups: "strong," "weak," and "negative." The results are presented in Table 1.

**Biochemical Demonstration of H$_2$O$_2$ Production in the Cytochemical Reaction**

The results are summarized in Table 2. It was demonstrated that enhanced H$_2$O$_2$ production was
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ultrathin sections was carried out in a blind fashion. Production was not observed during 6 min according to long time was, the greater the amounts of $O_2^-$ production. The shorter the lag time was, the greater the amounts of $O_2^-$ production. When stimulated with only WGA or PHA, $O_2^-$ production was not observed during 6 min according to this assay system.

DISCUSSION

Lectins have a cell-aggregating activity due to a binding activity for specific saccharides. However, reports of leukocyte aggregation by lectins are rare. Ryan et al. reported that when PMN adhering to glass cover slips were treated with Con-A in a concentration of 100 $\mu$g/ml at 4°C and were warmed to 37°C, capping of the Con-A-receptor complex and the internalization of it were observed. We demonstrated in this study, using electron microscopic examination, that PMN in suspension were aggregated by 100 $\mu$g/ml Con-A at 37°C and that the formation of a small number of vacuoles, probably from active pinocytosis, was also observed. These phenomena were inhibited by the presence of AMM and were dependent on the specific receptor binding of Con-A. It has been reported, using aggregometric study, that PHA (50 $\mu$g/ml) has neutrophil-agglutinating activity. It was further demonstrated that WGA also could aggregate human PMN. The effect of CB pretreatment and subsequent lectin stimulation on the morphology of PMN has not been fully studied. Only one report by Hoffstein et al. showed the enhanced assembly of the microtubules and degranulation of the specific granules in CB-Con-A-stimulated PMN. We found markedly enhanced vacuolar formation in CB–lectin-stimulated PMN. When Con-A stimulation was carried out in the presence of AMM in cells pretreated with CB, this vacuolar formation was obliterated. Based on these results, it is suggested that the formation of these vacuoles was dependent on the binding of lectins on the specific receptor sites and that vacuoles originated from active pinocytotic process. It has been known that CB causes a disappearance of microfilament and inhibition of phagocytosis by PMN. As to pinocytosis, the effect of CB on PMN is not fully studied. Pinocytosis of the protein by mouse peritoneal macrophage was not influenced by CB. In mouse peritoneal macrophage, Con-A-induced vacuolization was diminished by pretreatment with CB under light microscopic study. Our study suggests that active pinocytosis induced by various lectins is enhanced by CB pretreatment. The ultrastructural change of PMN stimulated with PMA has been studied by White and Estensen and Repine et al. Multiple vacuolar formation, degranulation of specific granules, and cell aggregation were reported. The effect of various drugs, extracellular calcium ion, and temperature on PMA-induced leukocyte aggregation has been also studied using platelet aggregometer.

Con-A, PHA, WGA, PMA, and digitonin (0.1%) are known to induce respiratory burst in PMN. It has been reported that $O_2^-$ production in PMN stimulated with lectins is enhanced by the pretreatment with CB. We studied the subcellular localiza-
The formation of which was promoted by CB.

vacuoles, observed on the contact surface of the plasma membrane ofaggregated PMN. When cells were pretreated with CB before stimulation with lectins, the amount of deposition was enhanced on the cell-to-cell contact, with CB. In the case of neutrophil-aggregating agents (lectins and PMA), H2O2 production was observed on the contact surface of the plasma membrane of aggregated PMN. When cells were pretreated with CB before stimulation with lectins, the amount of the deposits was enhanced on the cell-to-cell contact, and that the enhancement of active pinocytotic process is important in the induction of O2 production.

Further investigation that the quality of the membrane of PMN stimulated with lectins or PMA plays an important role in the induction of O2 production in PMN. In our previous report, similar findings were presented in CB-pretreated human PMN stimulated with opsonized zymosan or latex. 

In this system, H2O2 may be produced on the cell-to-cell contact. In a recent report by Badwey et al., subcellular H2O2-producing site in PMN stimulated with PMA was demonstrated. They observed the cerium deposits on the whole surface of the membrane and within small vesicles. The difference in the reaction pattern between their work and our study may have resulted from the difference in the cellular condition, i.e., adhered versus free. They used the cells adhering to cover slips, and a cellular aggregation was not seen under this condition. In the case of digitonin, a nonaggregating stimulant, H2O2 production was induced on the whole surface of the membrane and on the vesicles. The findings show that H2O2 is produced by the action of detergent on the external surface and that the vacuoles are formed from the free surface membrane by a pinocytotic process. An increase in pinocytosis in PMN stimulated with digitonin has been previously reported by Graham et al.

It has been reported that the change in the quality of the plasma membrane is associated with phagocytosis: a content of membrane-bound calcium ion and the composition of the lipid. It is suggested from our investigation that the quality of the membrane of cell-to-cell contact is different from the membrane of the free surface after PMN are stimulated with neutrophil-aggregating agents. Further study of the quality of the membrane of cell-to-cell contact would be important in elucidating the activation mechanism of O2-producing enzyme.

REFERENCES
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