LETTER TO THE EDITOR

**CYTOCHEMISTRY OF HYDROGEN PEROXIDE PRODUCTION
BY CULTURED ACTIVATED MACROPHAGES
IN PHAGOCYTOSIS**

Our recent cytochemical investigation of oxidative metabolism in human polymorphonuclear leukocytes (PMN) has demonstrated hydrogen peroxide (H_2O_2) production in phagocytosis and in the stimulation with several membrane activators (16, 17). The H_2O_2 production site was on the plasma membrane and contacted with particles and on the phagosome membrane during phagocytosis (16). The H_2O_2 production mechanism is shown by respiratory burst, which is caused by stimulated oxygen consumption, and by the increase in the NAD(P)H oxidation producing superoxide anions (O_2^-) (5). The O_2^- anions are converted into H_2O_2 spontaneously by dismutation (2) or with superoxide dismutase (11). By use of the cerium perhydroxide formation of H_2O_2 with trivalent cerous ions is demonstrated under an electron microscope (3, 15).

As macrophages are also active in the phagocytosis that accompanies H_2O_2 production (9) we tried to demonstrate H_2O_2 production in these cells. Cloned mouse macrophages Mm-1 line, a gift of Dr. Y. Ichikawa of the Department of Cytochemistry, Chest Disease Research Institute, Kyoto University, Kyoto, were cultured in monolayer as reported previously (7, 8), harvested 3 days after inoculation and used. Cells were mixed with IgG-coated polystyrene particles at the ratio of 20 particles per cell (16). The mixture in HBSS with glucose was kept at 37°C under an atmosphere of 5% CO_2 in air for 10 min to allow phagocytosis. Afterward, cells were washed in a cold HBSS and then incubated in a medium consisting of 1.0 mM CeCl_3 and 10 mM NaN_3 in 0.1 M tris-maleate buffer, pH 7.5, with 7% sucrose at 37°C for 15 min (16). NADH and NADPH were not used here as a substrate since they can be supplied endogenously. NAD(P)H does not go through the intact plasma membrane to reach the cytosolic side of the membrane where the NAD(P)H electron accepting site of NAD(P)H oxidase is believed to lie, whereas the H_2O_2 release site appears to be ectocellular (6, 19). Incubated cells were fixed with 2% glutaraldehyde and 1% osmium tetroxide. Ultrathin sections embedded in Spurr's epoxy resin were observed under a JEM-100C or 100CX electron microscope without stain.

The shape of the macrophages in suspension was oval, and the plasma membrane was almost spherical with a few blebs and protrusions. Several particles were randomly engulfed into the cytoplasm, however, no reaction product indicating H_2O_2 production was observed. These macrophages may be included in the category of resting macrophages. Biochemical reports have indicated that the H_2O_2 -producing activity is generally weak in the resident macrophages (resting macrophages) of normal animals; instead, they can be activated *in vivo* and *in vitro* with bacterial endotoxin lipopolysaccharides (LPS) resulting in an increased

microbicidal activity and H₂O₂ production (18). We then demonstrated the H₂O₂ production in LPS-activated macrophages. Activated macrophages were prepared by the culture of Mm-1 cells (1.3×10^6 cells/ml) in the presence of LPS (*E. coli* 0.55 : 135, Difco Lab., Detroit, MI., U.S.A.) at a concentration of 0.1 μ g/ml in a culture flask with a screw cap at 37°C for 20 hr (18). The cells became more irregular in shape with abundant microvilli and cytoplasmic protrusions (Fig. 1). The cell shape reflected the activated macrophages (14). Within the cytoplasm, a marked accumulation of glycogen granules was observed, probably due to an inhibition of mitochondrial oxidative metabolisms by LPS (12). These cells showed highly active phagocytosis. However, the phagocytic site was limited to a particular part of the plasma membrane where extensively protruding cytoplasmic processes had accumulated. The reaction product of cerium perhydroxide was observed only at the area of the plasma membrane in contact with particles (Figs. 1B, C). No reaction product occurred on the remaining free surface of the plasma membrane. After the phagocytosis had progressed, the particles were invaginated into the cytoplasm; the reaction product then became localized on the phagosome membranes (Fig. 1A). The product amount decreased when 0.2 mg/ml cytochrome c (O₂ scavenger; from horse heart, type VI, Sigma Chem. Co., St. Louis, Mo.) or 0.2 mg/ml catalase (H₂O₂ scavenger; from bovine liver, C-100, Sigma) was added to the reaction medium (Fig. 2A). Benzoquinone (1.0 mM; O₂ scavenger) markedly diminished it (Fig. 2B). These results agreed with the physiological evidence (9).

The fact that the initial site of deposit-formation was limited to the particular area where the plasma membrane interacted with the particles may indicate that NAD(P)H oxidase, located in only the intact area, was stimulated through contact with the particles. This is shown strikingly in Fig. 1B. The probable question is: Can all free surface NAD(P)H oxidase be stimulated to produce H₂O₂ and subsequently be internalized into phagosomes? This has been answered through the use of short term phagocytosis (16). This experiment was performed by adding a cold EDTA solution to the cell suspension immediately after the addition of particles, since calcium helps to initiate the activation of NAD(P)H oxidase (4). No difference between short and long term phagocytosis was found in the deposit results. Therefore, the oxidase stimulated only at the membrane which contacted with the particles produced H₂O₂. In the case of guanidine- or saponin-stimulated H₂O₂ production in PMN (17) and macrophages (13), the reaction product was seen on the free surface of the plasma membrane. In the present study, the phagocytizing activated macrophages exhibited a special cell shape like a capped structure with accumulated cytoplasmic protrusions. This shape closely resembles the capped cells induced by concanavalin A and uropods in phytohemagglutinin-stimulated lymphocytes (1). As indicated in Fig. 1, the activated macrophages engulfed particles by protruding the cytoplasmic processes toward the particles.

FIG. 1. H₂O₂ production by LPS-activated macrophages. Reaction product is observed on the plasma membrane in contact with particles. All figures unstained. A. A typical cell with a capped structure (CS) where the particles have been engulfed. Arrows indicate reaction product formation. Gly, glycogen granules; N, nucleus. $\times 7,200$ B. Initial slight stimulation of the activity is indicated by an arrow. $\times 16,000$ C. A heavy reaction product is wrapping the particles on the cytoplasmic protrusions. $\times 17,000$

FIG. 2. Effects of several active-oxygen scavengers on H_2O_2 production. All figures unstained.

A. An addition of cytochrome c to a reaction medium. The amount of the reaction product decreased. $\times 15,000$ B. An addition of benzoquinone. Reaction product was eliminated. $\times 15,000$

This is similar to the receptor-mediated phagocytosis (10). The results indicate that H_2O_2 production by activated macrophages in phagocytosis may occur at the particle-cell contact of the plasma membrane.

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REFERENCES

1. Biberfeld, P.: Uropod formation in phytohaemagglutinin (PHA) stimulated lymphocytes. *Exp. Cell Res.* 66; 433-445, 1971.
2. Bielski, B. H. J.: Reevaluation of the spectral and kinetic properties of HO_2 and O_2^- free radicals. *Photochem. Photobiol.* 28; 645-649, 1978.
3. Briggs, R. T., Drath, D. B., Karnovsky, M. L. and Karnovsky, M. J.: Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical methods. *J. Cell Biol.* 67; 566-586, 1975.
4. Cohen, H. J. and Chovaniec, M. E.: Superoxide generation by digitonin-stimulated guinea pig granulocytes. A basis for a continuous assay for monitoring superoxide production and for the study of the activation of the generating system. *J. Clin. Invest.* 61; 1081-1087, 1978.
5. DeChatelet, L. R.: Initiation of the respiratory bursts in human polymorphonuclear neutrophils: A critical review. *J. Reticuloendothel. Soc.* 24; 73-91, 1978.
6. Green, T. R., Schaefer, R. E. and Makler, M. T.: Orientation of the NADPH dependent superoxide generating oxidoreductase on the outer membrane of human PMN's. *Biochem. Biophys. Res. Commun.* 94; 262-269, 1980.
7. Hirai, K.-I., Maeda, M. and Ichikawa, Y.: Development of annulate lamellae in mouse myeloblastic cell line when differentiated to macrophages. *J. Electron Microsc.* 32; 13-19, 1983.
8. Hirai, K.-I., Nagata, K., Maeda, M. and Ichikawa, Y.: Changes in ultrastructures and enzyme activities during differentiation of myeloid leukemia cells to normal macrophages. *Exp. Cell Res.* 124; 269-283, 1979.

9. Johnston, R. B. Jr., Godzik, C. A. and Cohn, Z. A.: Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J. Exp. Med.* 148; 115-127, 1978.
10. Kaplan, F.: Differences in the mode of phagocytosis with Fc and C₃ receptors in macrophages. *Scand. J. Immunol.* 6; 797-807, 1977.
11. McCord, J. M. and Fridovich, I.: Superoxide dismutase. An enzymic function for erythrocyuprein (hemocuprein). *J. Biol. Chem.* 244; 6049-6055, 1969.
12. McGivney, A. and Bradley, S. G.: Susceptibility of mitochondria from endotoxin-resistant mice to lipopolysaccharide. *Proc. Soc. Exp. Biol. Med.* 163; 56-59, 1980.
13. Moriguchi, K. and Hirai, K.-I.: Cytochemical demonstration of hydrogen peroxide produced by several stimuli in guinea pig resident macrophages. In *International Cell Biology 1984*, ed. by S. Seno and Y. Okada, The Japan Society for Cell Biology, Tokyo, 1984, p. 381.
14. North, R. J.: The concept of the activated macrophages. *J. Immunol.* 121; 806-809, 1978.
15. O'Brien, T. D., Sneed, M. C. and Brasted, R. C.: The lanthanide series. In *Comprehensive Inorganic Chemistry*, ed. by M. C. Sneed and R. C. Brasted, D. Van Nostrand, Princeton, 1955, p. 151.
16. Ohno, Y., Hirai, K.-I., Kanoh, T., Uchino, H. and Ogawa, K.: Subcellular localization of H₂O₂ production in human neutrophils stimulated with particles and an effect of cytochalasin-B on the cells. *Blood* 60; 253-260, 1982.
17. Ohno, Y., Hirai, K.-I., Kanoh, T., Uchino, H. and Ogawa, K.: Subcellular localization of hydrogen peroxide production in human polymorphonuclear leukocytes stimulated with lectins, phorbol myristate, and digitonin: An electron microscopic study using CeCl₃. *Blood* 60; 1195-1202, 1982.
18. Pabst, M. J. and Johnston, R. B. Jr.: Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide. *J. Exp. Med.* 151; 101-114, 1980.
19. Schirmer, R. H., Schulz, G. E. and Untucht-Grau, R.: On the geometry of leukocyte NADPH-oxidase, a membrane flavoenzyme. Inferences from the structure of glutathione reductase. *FEBS Letters* 154; 1-4, 1983.

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