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FULL PAPER

Spin-trapping detection of superoxides in polymorphonuclear leukocytes stimulated with serum-opsonized zymosan

Mikinori Kuwabara[1]*, Tsuneo A. Takahashi[2]
Hajime Nagahata[3], and Osamu Inanami[1]

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Abstract

To clarify where oxygen radicals are generated in polymorphonuclear leukocytes (PMNs) during phagocytosis, superoxides (O_2^-) from opsonized zymosan (OZ) -stimulated human PMNs were detected by the ESR and spin-trapping methods. PMNs were preactivated with OZ for the indicated periods of time at 37℃. Then a spin-trapping agent, 5, 5-dimethyl-1-pyrroline N-oxide (DMPO), was added to them, and they were further incubated for 30sec for ESR observations. The ESR spectra consisted of two components due to the DMPO-OOH and DMPO-OH spin adducts. To clarify where these spin-adducts were present, cells were separated from extracellular fluid by brief centrifugation and resuspended in Hanks' balanced salt solution. ESR examination of two fractions showed that the DMPO-OOH adducts was present in the cell fraction, whereas the DMPO-OH adducts were present in the extracellular fluid. When DMSO was used as a scavenger of hydroxyl radicals (·OH), DMPO-CH_3 adducts were observed in the fluid fraction but not in the cell fraction. Both spin adducts were completely abolished by Cu, Zn-SOD but not catalase. These results indicated that O_2^- were produced inside phagosomes of OZ-stimulated PMNs and ·OH were produced outside them by spontaneous decomposition of the DMPO-OOH adducts.

Key words : ESR-spin-trapping, hydroxyl radical, opsonized zymosan, polymorphonuclear leukocyte (PMN), superoxide anion.

Introduction

When polymorphonuclear leukocytes (PMNs) are stimulated with phagocytic particles, bacteria and phorbol myristate acetate (PMA), they undergo a respiratory burst

[1] Laboratory of Radiation Biology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, JAPAN
[2] Department of Cell Processing, The Institute of Medical Science, The University of Tokyo, Tokyo 108-0071, JAPAN
[3] Department of Animal Health, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069-8501, JAPAN.
* To whom correspondence should be addressed.
M. Kuwabara : Laboratory of Radiation Biology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
Phone : +81-011-706-5235, Fax : +81-011-706-7373, e-mail : kuwabara@vetmed. hokudai. ac. jp

with an uptake of ambient oxygen resulting in the generation of superoxides (O_2^-). This occurs via a single electron transfer mediated by NADPH oxidase[2,3,7,8,25]. It is widely accepted that O_2^- are converted to hydrogen peroxides (H_2O_2) by disproportionation reactions. H_2O_2 are further converted into hydroxyl radicals ($^{\cdot}OH$) by reactions with trace metals via Fenton reaction. $^{\cdot}OH$ are harmfully reactive and result in the disruption of the microorganism and host-tissue injury[1].

When PMNs are activated by phagocytic particles such as opsonized zymosan (OZ) and bacteria, they form phagosomes (phagocytic vacuoles). From histological experiments, Ohno et al. reported that H_2O_2 were accumulated outside the plasma membrane of phagosomes when PMNs were stimulated with OZ or IgG-coated latex particles[21]. The localization of NADPH oxidase components in the phagosome membrane was also reported[5,9,26]. The localization of the cytosolic components of NADPH oxidase and the mechanisms of their assembly in the phagosome membrane have been extensively studied[5,6,9,21,26], but the chemistry of oxygen radicals inside and outside the phagosomes has not been.

Among several techniques for the detection of transient oxygen radicals in cellular systems[16,17], spin trapping with 5, 5-dimethyl-1-pyrroline N-oxide (DMPO) is widely employed to qualitatively and quantitatively analyze oxygen radicals produced in stimulated phagocytic cells [10-12,19,20,22]. However, Rosen et al. demonstrated that DMPO-OOH adducts generated in OZ-stimulated PMNs were extremely unstable. They found that artificial DMPO-OH adducts were induced by the reactions of DMPO with $^{\cdot}OH$ as their decomposition products[13]. This suggested that it is hard to obtain information about the kinetics of O_2^- and $^{\cdot}OH$ generation as well as their localization in cells by this method, though this is advantageous for the accurate determination of radical structures.

In the present study, we designed an improved spin-trapping method to minimize both the decay of DMPO-OOH adducts and the subsequent formation of DMPO-OH adducts. This was performed by separating PMNs, which were stimulated with serum OZ by incubation at regular intervals followed by the addition of DMPO to trap the oxygen radicals, from the extracellular fluid. Using this method, kinetic analysis of the generation of oxygen radicals and identification of the oxygen radicals inside and outside phagosomes were successfully performed.

Materials and Methods

Reagents

The spin trap, DMPO, zymosan A from Saccharomyces cervisie, phorbol 12-myristate 13-acetate (PMA), luminol and bovine liver Cu, Zn-SOD were purchased from Sigma Chemical Co. Ficol 400 was from Pharmacia P-L Biochemicals (Uppsala). Sodium iothalamate (66.8%w/v) was from Dai-ichi Pharmaceutical Co. Ltd., Japan. The other chemicals were obtained from Wako Pure Chemical Industries, Ltd., Japan. Serum was obtained from whole blood of healthy volunteers and the OZ was prepared by incubating it in the serum (10 mg in 1.5 ml of serum) at 37℃ for 1 h, and then washing it three times with Ca^{2+}- and Mg^{2+}- free Hanks' balanced salt solution, HBSS (-). The final concentration of OZ was adjusted to 10mg/ml.

PMN preparation

Peripheral blood of healthy donors was obtained from Hokkaido Block Red Cross Blood Center and diluted twice by HBSS (-). To collect the leukocyte fraction, 10 ml of 10% dextran was added to 40 ml of diluted blood.

The leukocyte-rich supernatant was ob-

tained after incubation at room temperature for 30min. Thirty milliliters of the leukocyte-rich fraction was placed on 20 ml of a Ficoliotalamate solution adjusted to 1.077 g/ml and centrifuged for 30 min at 2,500 rpm followed by hypotonic lysis of trace erythrocytes. Giemsa stain showed that more than 96% of the cells were PMNs.

The improved spin-trapping method to detect O_2^- from OZ-preactivated PMNs by DMPO.

Preactivation of PMNs was performed by mixing them (2.0 x 10^6 cells/ml) with OZ (4 mg/ml) or PMA (100 ng/ml) in 100 µl of HBSS containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$, HBSS (+), in the presence or absence of the competing reagents (Cu, Zn-SOD 40 mU/ml, catalase 300 U/ml or 0.1 M DMSO). Intraphagosomal free radical generation was detected by designing experiments to minimize the decay of DMPO-OOH. After PMNs were preactivated with serum-OZ for the indicated periods of time at 37℃, 100 µl of 0.5 M DMPO was added to them followed by incubation for 30 sec to allow the spin-trapping reactions. After centrifugation at 6000 rpm for 30 sec, cells were resuspended in a 200 µl of HBSS (+) and examined together with the extracellular fluid by ESR spectrometry. The decay of the spin-adducts was minimized by this technique. Thus, the kinetic analysis of the generation of oxygen radicals as well as their identification inside and outside phagosomes of OZ-stimulated PMNs became possible.

ESR measurements

Cells and the extracellular fluid were separately transferred to a flat quartz ESR cell fitted to the cavity of an X-band ESR spectrometer (RE- 1 X, JEOL Co., Japan). The interval from the end of incubation to the start of ESR measurements was 60 sec. The conditions for recording the ESR spectra were follows : time constant=0.03 sec, scan time=5.0 mT/min, microwave power=15 mW, temperature=23℃, modulation width=0.1 mT, modulation frequency=100 kHz.

O_2^- detection of OZ-stimulated PMNs by the luminol-enhanced chemiluminescence method.

The improved spin-trapping method was compared with another O_2^- detection method, chemiluminescence with luminol[16,17]. A reaction mixture containing 1.5x10^6 cells, 10 µM luminol and 50 µg/ml of horseradish peroxydase in HBSS (+) with a final volume of 315 µl was prepared in a 96-well microplate (F16 Black Polysorp, Nunc, Denmark) for luminometry (Luminescencer-JNR, ATTO, Japan). PMNs were activated by adding 35 µl of 10 mg/ml of OZ. Chemiluminescence for 0.02 sec was measured at 12-sec intervals at 37 ℃.

Results and Discussion

When PMNs were incubated with DMPO and OZ at the same time for 10 min as described by Rosen and coworkers[10,11,20,21], a strong ESR signal due to DMPO-OH adducts (A_N = $A_{H\beta}$=1.49 mT) was observed, but the ESR signal due to DMPO-OOH adducts (A_N =1.40 mT, $A_{H\beta}$=1.13 mT and $A_{H\gamma}$=0.14 mT) was faint as shown in Fig. 1 A. This suggested that DMPO-OOH adducts were spontaneously transformed to DMPO-OH adducts or ESR silent products by reductive reactions in cytoplasm[13,19,23]. This is why it is hard to detect and to assign oxygen radical species in activated PMNs. Therefore, to minimize the decay of DMPO-OOH adducts, we improved the spin-trapping method by adding DMPO after PMNs were stimulated with serum-OZ and subsequently incubated for the indicated periods of time. Fig. 1 B shows an ESR spectrum of PMNs preactivated with OZ for 10 min and subsequently incubated for 30 sec. The spectrum consisted of two components originating from DMPO-OOH and DMPO-OH

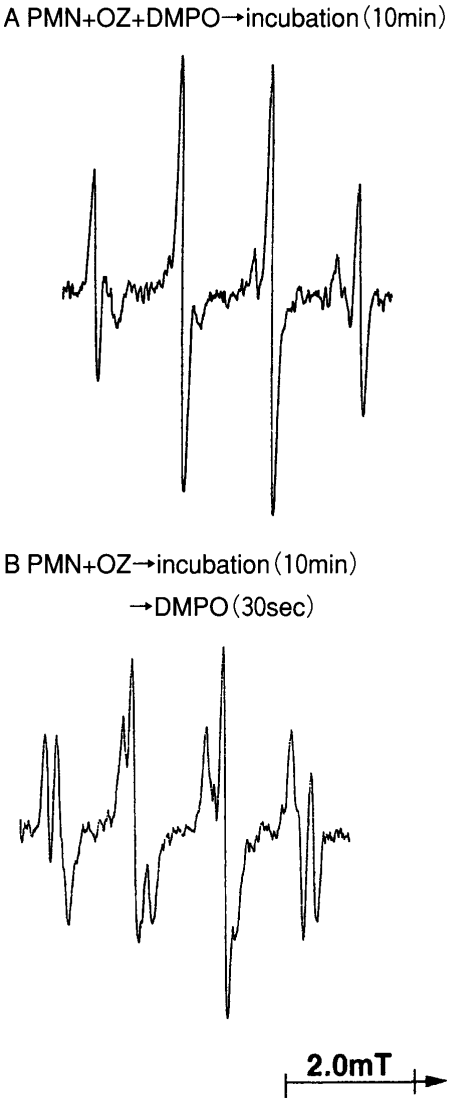

Fig. 1 ESR spectra from polymorphonuclear leukocytes (PMNs) stimulated with serum-opsonized-zymosan (OZ). Scan A was obtained at 5 min after PMNs (2×10^6 cells/ml), OZ (4 mg/ml) and DMPO (0.25 M) were incubated at the same time. Scan B was obtained after DMPO (0.25 M) was added to the preactivated-PMNs, which were then incubated for 30 sec. The preactivation was performed by incubating PMNs and OZ at 37℃ for 5 min. Receiver gain for both ESR spectra was 5×10^2.

adducts. The ESR signal intensity due to DMPO-OOH adducts was significantly stronger than that obtained when using the usual protocol (Fig. 1 A). This meant that the decomposition of DMPO-OOH adducts was minimized by employing this method.

The time course of generation of each spin-adduct at various incubation times used for stimulation is shown in Fig. 2 A. Steep increases in both spin-adducts of DMPO-OOH and DMPO-OH were observed and their production peaked the peak at about 5 min. The shape was very similar to that measured by luminol-enhanced chemiluminescence as shown in Fig. 2 B. The chemiluminescent response of OZ-stimulated PMNs was completely abolished in the presence of Cu, Zn-SOD (data not shown). Therefore, it was thought that the amount of the DMPO-OOH detected by the improved-spin-trapping technique paralleled to the production rate of O_2^- from OZ-stimulated neutrophils detected by chemiluminescence. However, in the case of kinetics obtained by incubating DMPO, OZ and PMN at the same time, a strong ESR signal due to DMPO-OH was observed as shown in Fig. 2 C. The peak of the DMPO-OH was observed at about10min and a gradual decline was observed, whereas the ESR intensity of DMPO-OOH was relatively week and was sustained at the low level. The kinetics was explained by complexity due to the conversion of DMPO-OOH and DMPO-OH to ESR-silent products[18,19], and OH radical generation from DMPO-OOH[13]. Therefore, the analysis of kinetics for O_2^- production in OZ-stimulated PMN was difficult when using the long-standing technique in which the cells, spin-trap and stimulant are incubated at the same time.

To discriminate between the intraphagosomal free radical production and the extracellular free radical production, separation of these spin-adducts was performed by brief centrifugation. Fig. 3 A shows an ESR spectrum obtained when PMNs were stimulated with serum-OZ for 10 min and oxygen radical species were subsequently trapped by the added DMPO. Figs.

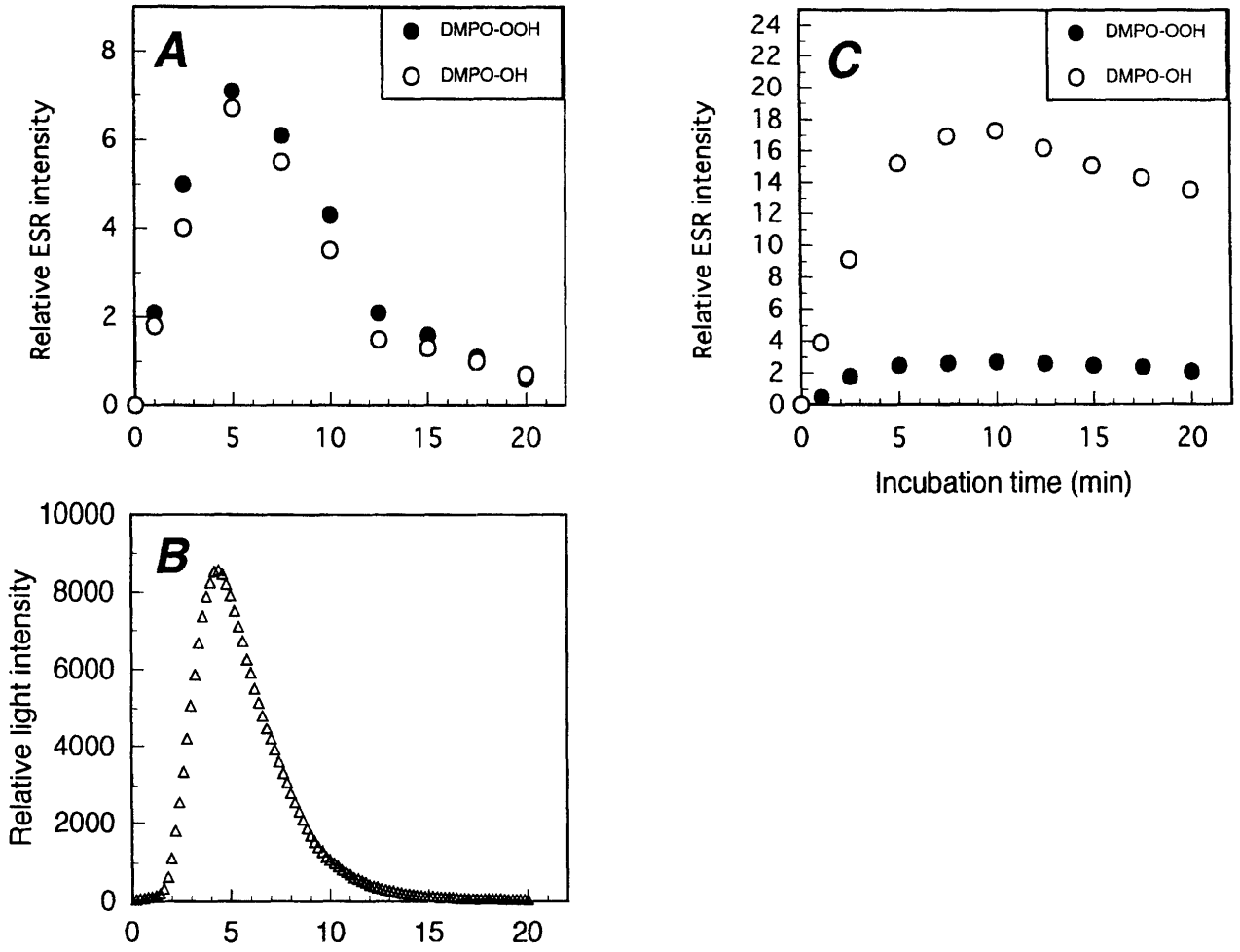

Fig. 2 Time courses of the intensities of DMPO-OOH (closed circles) and DMPO-OH (open circles) in different spin-trapping protocols. The time course in A was obtained with 30-sec incubation after mixing DMPO (0.25M) and OZ-preactivated PMNs (2×10^6 cells/ml). The preactivation was performed by the incubation of PMNs with OZ (4 mg/ml) at 37℃ for various intervals. Receiver gain for both ESR spectra was fixed to 5×10^2. The peak height of low field component of the signals due to DMPO-OH and DMPO-OOH was measured as the relative ESR intensity. The other protocol was similar to that explained in Fig. 1. The time course in B was obtained by chemiluminescence using luminol as described in Materials and Methods. The time course in C was obtained after PMNs, OZ and DMPO (0.25 M) were incubated at the same time for various intervals. Each point indicates the average of three independent experiments.

3 B and 3 C show the ESR spectra obtained from the resuspended cell fraction and extracellular fluid, respectively. The ESR signal originating from DMPO-OOH spin-adducts was predominantly observed in the resuspended cell fraction (Fig. 3 B), whereas the strong ESR signal of DMPO-OH spin-adducts was present in only extracellular fraction (Fig. 3 C). These results suggested that the superoxide anions were produced in the intracellular fraction or phagosomes.

To examine whether the site of production of the superoxide in OZ-stimulated PMN was cytoplasm or phagosomes, we employed a soluble chemical stimulant, PMA, which is a direct activator of protein kinase C and induces NADPH oxidase activation through phosphorylation of $p47^{phox}$ without the formation of phagosomal vesicles[16]. When DMPO was added and incubated for 30 sec after activation of PMNs for 5 min by PMA, the ESR signal originated from DMPO-OOH was

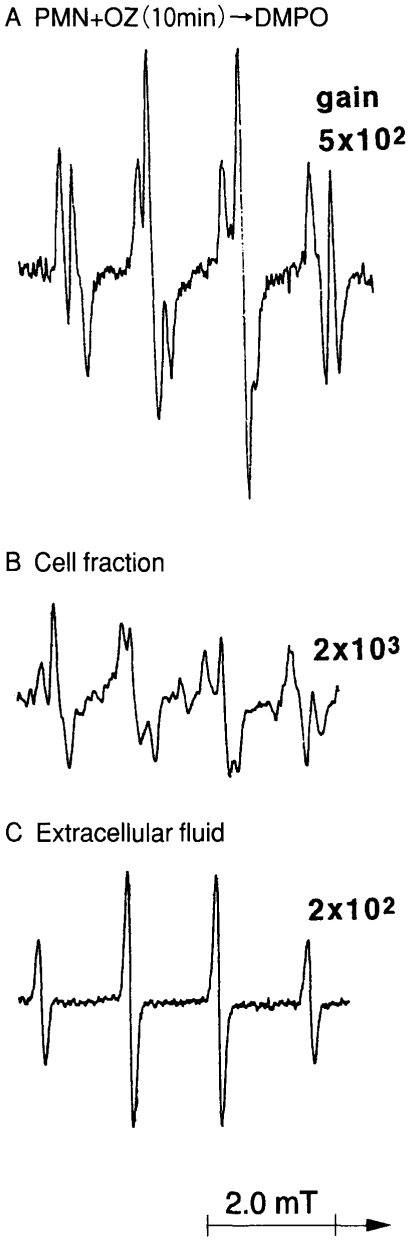

Fig. 3 Localization of DMPO-OOH adducts in phagosomes and DMPO-OH adducts in extracellular fluid. Cells were separated by brief centrifugation at 6000 rpm after 30 sec incubation of OZ-preactivated PMNs with DMPO (0.25 M). The lag-time between centrifugation and ESR measurement was 60 sec. ESR spectrum A was the same as that of Fig. 1 B before separation. ESR spectra B and C were obtained from the cell fraction and the extracellular fluid, respectively.

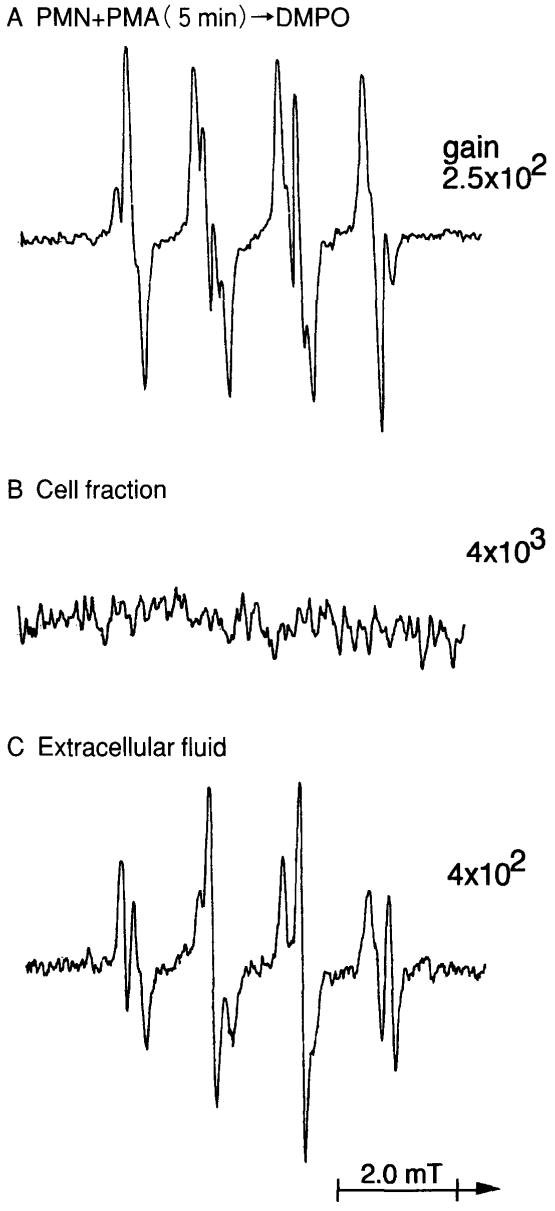

Fig. 4 Localization of both DMPO-OOH and DMPO -OH in extracellular fraction. Cells and extracellular fluid were separated by brief centrifugation at 6000 rpm for 30 sec after PMA-preactivated cells were incubated with DMPO (0.25 M) for 30 sec. The preactivation was performed by the incubation of PMNs with PMA (100 ng/ml) for 5 min. Receiver gain is indicated in each spectrum. ESR spectrum A was obtained before separation. ESR spectra B and C were obtained from the cell fraction and the extracellular fluid, respectively.

mainly produced as shown in Fig. 4 A. Figs. 4 B and 4 C show the ESR spectra obtained from the resuspended cell fraction and extracellular fluid after separation by brief centrifugation, respectively. The results obtained

from OZ-stimulated PMNs with phagocytosis and PMA-stimulated PMNs without phagocytosis suggested that the ESR signal of DMPO-OOH adducts shown in Figs. 1 B and 3 B was primarily produced in the phagosomes of OZ-stimulated PMNs.

In the present experiments using OZ-stimulated PMNs, we detected no DMPO-OOH adducts in the extracellular fluid but detected them in phagosomes. This suggested that DMPO-OOH adducts were generated close to site of O_2^- release in phagosomes, since it was reported that the O_2^- -forming enzyme, NADPH oxidase, was located on plasma membrane after stimulation[6,26] and H_2O_2 production in human PMNs treated by particles was observed at the membrane adherent to the particles inside the phagosome[5,6,9,22]. Using the spin-label technique, Fujii and Kakinuma demonstrated that the O_2^- -releasing sites were not located at the outer surface of the plasma membrane but in the inner hydrophobic environment a short distance (0.4～0.5 nm) from its outer surface[14]. Therefore, it is likely that O_2^- is transformed to H_2O_2 at the outer surface of the phagosomal membrane as soon as O_2^- emerges from the hydrophobic membrane to hydrophilic intraphagosomal space but not to cytoplasm.

Figs. 4 A and 4 C show that DMPO-OOH adducts easily decomposed after brief centrifugation (within 30 sec) of PMA-prestimulated PMNs to undesired DMPO-OH adducts, although these were a minor component just after stimulation. This phenomenon may lead us to misunderstand hydroxyl radical generation from NADPH oxidase. Therefore, to clarify the generation mechanism of ·OH in activated PMNs, DMSO was used as an ·OH scavenger. The reaction of OH radicals with DMSO is known to produce a methyl radical (·CH_3) and the methyl radical is trapped by DMPO and converted to ESR-detectable DMPO-CH_3 adducts (A_N=1.53 mT and $A_{H\beta}$=2.2 mT)[10,11,22]. Figs. 5 A and 5 B show the ESR spectra obtained from the resuspended cell fraction and extracellular fraction in OZ-stimulated PMNs in the presence of 0.1 M DMSO, respectively. The ESR spectrum of DMPO-OOH was observed in the resuspended cell fraction as in the absence of DMSO. However, in the extracellular fluid, the ESR spectrum of DMPO-CH_3 adducts was newly detected as indicated by triangles in the spectrum traced at high receiver gain (Fig.

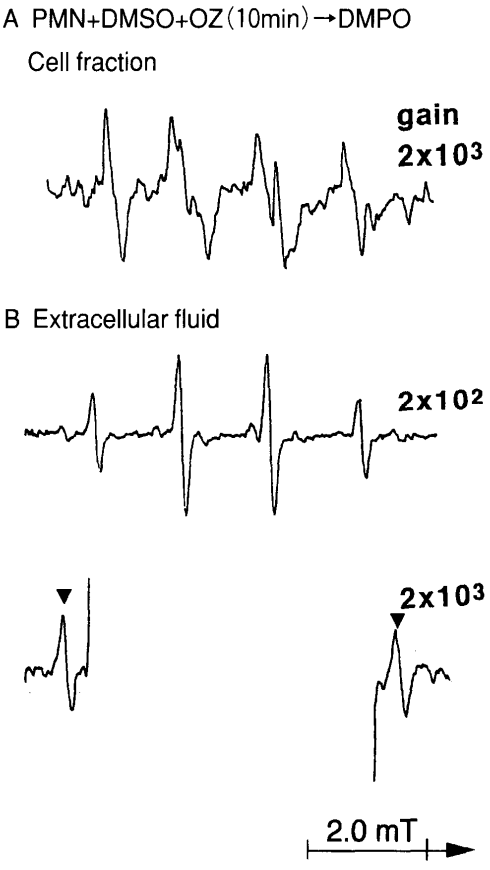

Fig. 5 Hydroxyl-radical formation of OZ-stimulated PMNs. Cells were separated by brief centrifugation at 6000 rpm for 30 sec after OZ-preactivated-PMNs were incubated with DMPO (0.25 M) in the presence of DMSO (0.1 M). ESR spectra A and B were obtained from the cell fraction and the extracellular fluid, respectively. The lower spectrum in B was recorded at high gain (2×10^3) to compare it with the upper spectrum in B (2×10^2).

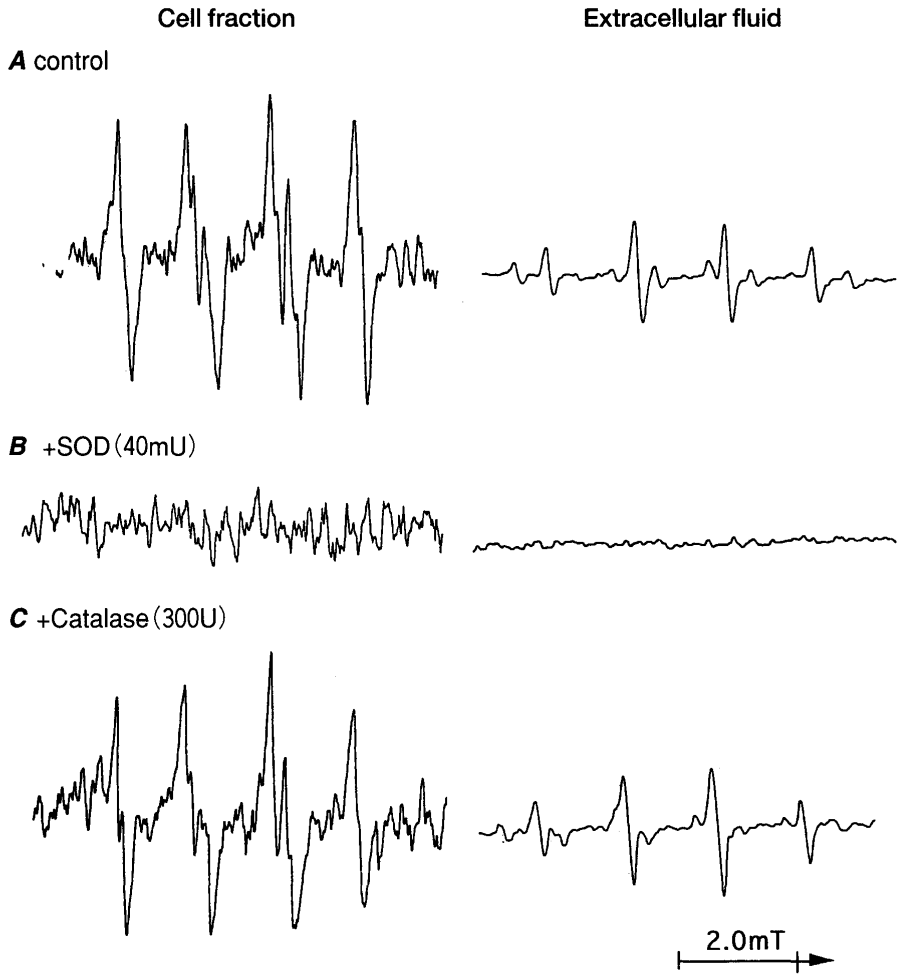

Fig. 6. Effects of Cu, Zn-SOD (40 mU/ml) and catalase (300 U/ml) on DMPO -OOH, DMPO-OH and DMPO-CH_3 adducts formed in the OZ-preactivated PMNs in the presence of DMSO. The preactivation and separation were similar to those of Fig. 4. ESR spectra A, B and C were obtained without enzymes and with Cu, Zn-SOD (40 mU/ml) and catalase (300 U/ml), respectively.

5 B). This could be explained by a leak of DMPO-CH_3 from inside phagosomes or cells since no ESR components due to DMPO-CH_3 and DMPO-OH were observed in the resuspended cell fraction, indicating that hydroxyl radicals were extracellularly generated from OZ-stimulated PMNs.

It has been believed that OH radicals are generated from H_2O_2 by a metal-catalyzed Harber-Weiss reaction (Fenton reaction) in various biological systems. On the other hand, Finkelstein et al. reported OH radical production due to spontaneous decomposition of DMPO-OOH adducts[13]. To examine whether hydroxyl-radical generation was associated with not only OH-radical release from DMPO-OOH but also the metal-catalyzed Harber-Weiss reaction, a spin-trapping experiment using OZ-stimulated PMNs was performed in the presence of Cu, Zn-SOD or catalase. Cu, Zn-SOD is well-known to catalyze the disproportionation reaction of O_2^- to produce H_2O_2. Therefore, if hydroxyl-radical production in OZ-stimulated PMNs was associated with the metal-catalyzed Haber-Weiss reaction, Cu, Zn -SOD would induce high OH-radical generation from excess H_2O_2 and then enhance the intensity of the ESR signal due to DMPO-CH_3.

However, it was shown that not only DMPO-OOH but also DMPO-OH and DMPO-CH_3 were completely diminished with Cu, Zn-SOD (Fig. 6 A and Fig. 6 B). Moreover, catalase, which can eliminate H_2O_2, did not affect the intensities of DMPO-OOH, DMPO-OH and DMPO-CH_3 adducts (Fig. 6 C). This indicated that OH radicals producing DMPO-CH_3 in OZ-stimulated PMNs did not originate from H_2O_2. Thus, it was concluded that the extracellular OH radicals detected in this experiment were O_2^- -dependent but did not associated with H_2O_2. Rosen and coworkers also observed similar phenomena[10-12]. Therefore, it was concluded that the production of OH mainly arose from decomposition of DMPO-OOH adducts.

Since it was reported that 2', 7'-dichlorofluorescin diacetate (DCFH-DA) -detectable hydrogen peroxide was intracellularly generated during phagocytosis in activated PMNs[4] and DMPO could penetrate the cytoplasm of the cell[15,24], DMPO-OH accompanied by OH production in cytoplasm might be detected if metal ions are intracellularly present. However, we had no evidence for the presence of DMPO-spin-adduct species inside cytosols of PMNs activated by OZ and PMA. This phenomenon could be explained by quick conversion of intracellular DMPO-OOH spin-adducts to an ESR-silent compound by intracellular reducible substances. This is because Samuni et al. showed that DMPO-OH in γ-irradiated reticulocytes and V79 cells in the presence of DMPO are not intracellular species but extracellular species, and suggested that DMPO-OH adducts formed inside cells could not survive as ESR-detectable spin-adducts[24]. Thus, it was concluded that spin-trapping using DMPO was not useful for detection of intracellular OH radicals even if H_2O_2 produced inside cells were converted to OH radicals by trace metals because of the instability of the spin-adducts.

In summary, using an improved-spin-trapping technique, it was clearly demonstrated that O_2^- was produced just inside phagosomes of OZ-stimulated PMNs. However, OH radical generation from NADPH oxidase was not observed outside or inside the cells, although the OH radicals produced by decomposition of DMPO-OOH were detected as a byproduct.

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