# Hydroxyl Radical Generation by Polymorphonuclear Leukocytes Measured by Electron Spin Resonance Spectroscopy

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ABSTRACT Electron spin resonance spectroscopy using the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was employed to detect the formation of hydroxyl radicals (OH·) by phagocytosing polymorphonuclear leukocytes (PMN). An electron spin resonance signal with the identical *g* value and splitting characteristics of the (DMPO/OH)· adduct was detected on incubation of normal PMN with opsonized zymosan. Adduct formation was strongly inhibited by superoxide dismutase and by the OH· scavenger mannitol, but catalase had little or no effect. (DMPO/OH)· was not formed by PMN from a patient with chronic granulomatous disease; in contrast, adduct formation by PMN which lack myeloperoxidase was greater than normal. These findings are discussed in relation to the formation of OH· by PMN.

## INTRODUCTION

The formation of hydroxyl radicals (OH·)[1] by phagocytes and their role in microbicidal activity is the subject of considerable current interest (1). Phagocytes, when stimulated, generate both the superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$). The interaction of these reactants to form OH· was proposed by Haber and Weiss (2) as follows:

$$H_2O_2 + O_2^- \rightarrow O_2 + OH^- + OH\cdot.$$

Recent studies have indicated that the direct interaction of $H_2O_2$ and $O_2^-$ is slow (3), making it unlikely that OH· are generated in biological systems by the above mechanism. Rather, the current view (4) is that a trace metal, such as iron, functions as an oxidation reduction catalyst as follows:

$$\begin{aligned} Fe^{3+} + O_2^- &\rightarrow Fe^{2+} + O_2 \\ Fe^{2+} + H_2O_2 &\rightarrow Fe^{3+} + OH^- + OH\cdot \\ \hline O_2^- + H_2O_2 &\rightarrow O_2 + OH^- + OH\cdot \end{aligned}.$$

The oxygen formed may be initially in the singlet form (5). When a reaction, initiated by a $O_2^-$ (and $H_2O_2$)-generating system, is inhibited by superoxide dismutase (SOD), catalase, and OH· scavengers such as mannitol, ethanol, or benzoate, this has been taken as evidence for the involvement of OH· generated as described above (6). The microbicidal activity of polymorphonuclear leukocytes (PMN) is partially inhibited by SOD, catalase, and the OH· scavengers, and the involvement of OH· was therefore proposed (7).

The direct measurement of OH· by phagocytes has been attempted. Beauchamps and Fridovich (6) noted that the conversion of methional to ethylene by the $O_2^-$-generating system, xanthine plus xanthine oxidase, was inhibited by SOD, catalase, and OH· scavengers thus implicating OH·. Subsequent studies indicated that when stimulated by phagocytosis, PMN convert either methional (8, 9) or a closely related substance, 2-keto-4-methylthiobutyric acid (KMB) (9, 10), to ethylene. However the role of OH· in ethylene formation by PMN has been questioned on the following grounds: (*a*) ethylene formation from methional or KMB can be initiated by oxidants other than OH· (9); (*b*) although ethylene formation by phagocytes is strongly inhibited by SOD, thereby implicating $O_2^-$, inhibition by catalase or OH· scavengers is not striking (8–10); (*c*) ethylene formation by PMN is dependent largely on myeloperoxidase (MPO) (9), suggesting either that MPO is required for OH· formation or that ethylene formation occurs largely by an OH·-independent mechanism.

In this paper, we have employed electron spin resonance (ESR) spectroscopy using a spin trap to seek further evidence for OH· formation by phagocytosing

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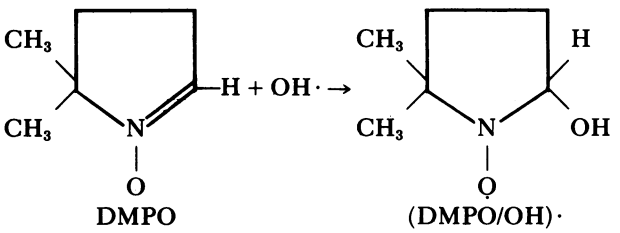
[1] *Abbreviations used in this paper*: CGD, chronic granulomatous disease, DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ESR, electron spin resonance; $HO_2$, perhydroxy radical; KMB, 2-keto-4-methylthiobutyric acid; MPO, myeloperoxidase; $O_2^-$, superoxide anion; OH·, hydroxyl radical; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase.

PMN. Spin traps are compounds that readily react with free radicals to produce a relatively long-lived free radical product (spin adduct) which may be identified by its ESR spectrum. The spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) reacts with OH· to form a relatively stable (DMPO/OH)· adduct with readily identifiable ESR characteristics (11, 12).




The formation of the (DMPO/OH)· adduct by PMN has recently been reported (13). This paper describes our observations of (DMPO/OH)· formation by human PMN, the effects of various inhibitors, and the properties of PMN deficient in respiratory burst activity or MPO.

## METHODS

*Special reagents.* DMPO from Aldrich Chemical Co., Milwaukee, Wis., was purified by adsorption with activated charcoal (14). Catalase (bovine liver, 600,000 U/mg) from Worthington Biochemical Corp., Freehold, N. J., was either dialyzed against water or chromatographed on Bio-Gel P-150 (Bio-Rad Laboratories, Richmond, Calif.) to remove trace SOD impurities (15). SOD (bovine erythrocytes, 12,300 U/mg) was obtained from Miles Laboratories, Inc., Elkhart, Ind. Zymosan from ICN Nutritional Biochemicals, Cleveland, Ohio, was opsonized, and MPO prepared and assayed as previously described (16). Human serum albumin, essentially fatty acid free, was obtained from Sigma Chemical Co., St. Louis, Mo., and Hanks' balanced salt solution without calcium, magnesium, or phenol red from Microbiological Associates, Walkersville, Md. Phosphate-buffered saline, pH 7.2, contained 0.145 M NaCl and 0.02 M sodium phosphate buffer, pH 7.2.

*Preparation of leukocytes.* Blood was drawn from normal volunteers, one patient with chronic granulomatous disease (CGD) and one patient (J.F.) with hereditary MPO deficiency (16) using EDTA as anticoagulant. The PMN were separated by dextran sedimentation and hypotonic lysis of contaminating erythrocytes (16). They were washed and suspended at $2.5 \times 10^7$ PMN (85–95% neutrophils)/ml in Hanks' balanced salt solution.

*Measurement of (DMPO/OH)· formation.* $2.5 \times 10^6$ PMN were incubated with 0.1 ml Hanks' balanced salt solution, 0.1 ml phosphate-buffered saline, 250 μg human serum albumin, 250 μg opsonized zymosan, and 25 μmol DMPO in a final volume of 0.5 ml. Resultant salt and glucose concentrations were: sodium 64 mM; potassium 2 mM; chloride 58 mM; bicarbonate 1 mM; phosphate 4 mM; and glucose 1 mM. Tonicity was 180 mosmol. Variations from these conditions are indicated in the legends. Incubation was for 20 min at 37°C with shaking. Samples for ESR spectroscopy were injected into 6-in. glass capillary tubes that were placed in the cavity of a Varian E4 ESR spectrometer (Varian Associates, Palo Alto, Calif.). Measurements were at room temperature at maximum gain, scan speed 6.7 G/min, modulation amplitude 0.5 G, time constant 10 s, X-band frequency 9.5 GHz, field strength ~3,200 G and power ~1 mW.

*Data analysis.* The height of the second peak in the ESR spectrum, which was assumed to be roughly proportional to the amount of (DMPO/OH)· present (14), was employed as a measure of signal intensity. Data were compared using Student's two-tailed *t* test for independent means unless otherwise indicated.

## RESULTS

Hydroxyl radicals are generated by the interaction of $FeSO_4$ and $H_2O_2$ (Fenton's reagent) (17). Incubation of DMPO with Fenton's reagent results in an ESR signal with splitting constants of $a_N = a_H = 15.3 \pm 0.3$ G and 1:2:2:1 intensity distribution (Fig. 1) comparable with those previously reported for the (DMPO/OH)· adduct (11, 12, 17). Incubation of normal PMN with opsonized zymosan and DMPO resulted in an ESR signal identical to that generated by Fenton's reagent (Fig. 1). This signal was not observed when either PMN or zymosan were omitted, when unopsonized zymosan was used, or when normal PMN were replaced by those of a patient with CGD (Fig. 1, Table I). In contrast, PMN which lack MPO formed (DMPO/OH)· in greater than normal amounts (Fig. 1, Table I). Adduct formation by the

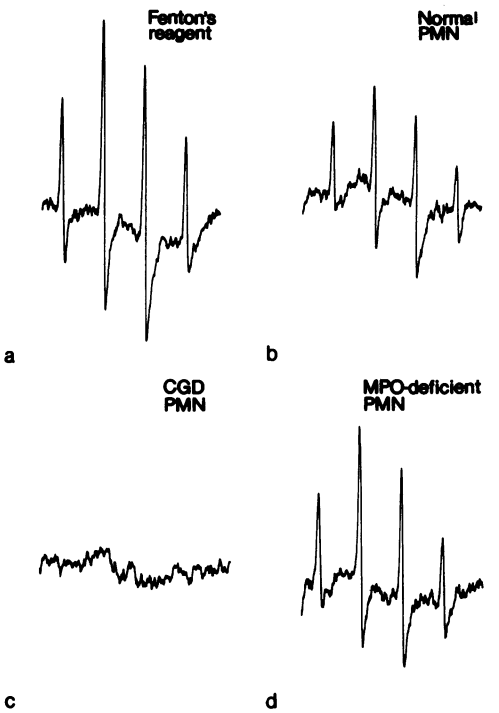



FIGURE 1 (DMPO/OH)· adduct formation by Fenton's reagent and by phagocytosing normal, CGD, and MPO-deficient PMN. The reaction mixture in *a* consisted of 0.05 M DMPO, 5 μM $FeSO_4$, and 10 μM $H_2O_2$. The remaining reaction mixtures contained DMPO and zymosan-activated PMN from normal (*b*), CGD (*c*), and MPO-deficient (*d*) donors as described in Methods.

TABLE I
*(DMPO/OH)·Formation by PMN*

| Additions | Peak height | |
|---|---|---|
| | *mm* | |
| Normal PMN + opsonized zymosan | 83±3 (10)* | |
| PMN omitted | 6±6 (6) | <0.001‡ |
| Zymosan omitted | 0±0 (7) | <0.001 |
| Zymosan unopsonized | 0±0 (3) | <0.001 |
| CGD PMN + opsonized zymosan | 0 (1) | <0.001 |
| MPO-deficient PMN + opsonized zymosan | 104±14 (3) | <0.05 |
| MPO (16 mU/ml) added | 83±15 (3) | |

* Mean±SE of experiments (number of experiments in parentheses).
‡ *P* value for the difference from normal PMN plus opsonized zymosan where significant ($P < 0.05$).

MPO-deficient PMN fell to a normal level when purified MPO was added (±MPO, $P < 0.05$ by paired analysis). MPO at a comparable concentration did not affect the ESR signal of normal PMN.

SOD at a concentration of 50 μg/ml strongly inhibited (DMPO/OH)· formation by normal PMN (95%), an effect that was abolished by heat treatment (Table II). In contrast, catalase at 600 μg/ml had a modest (20%) but significant inhibitory effect which, however, was unaffected by heat treatment. This enzyme was without effect at 60 μg/ml. The results were comparable when either dialyzed or chromatographed catalase was employed. The OH· scavenger mannitol at 0.06 M inhibited signal formation by 68%. Comparable results were observed with MPO-deficient PMN except that no inhibition by catalase was observed (Table II).

## DISCUSSION

Earlier studies are consistent with the following mechanism for (DMPO/OH)· formation by phagocytosing PMN: PMN stimulated by phagocytosis generate $O_2^-$ and by dismutation $H_2O_2$; $O_2^-$ and $H_2O_2$ interact in a trace metal-catalyzed reaction to generate OH· (Haber-Weiss reaction); OH· is trapped by DMPO to form the (DMPO/OH)· adduct. Our findings and those of Green et al. (13) generally support this mechanism, although modifications are necessary.

Our data suggest that adduct formation is dependent on $O_2^-$ and is mediated by OH·; however, the inhibition by catalase is low, requires high concentrations, and is not reversed by heat treatment, suggesting little or no requirement for free $H_2O_2$. A possible mechanism consistent with these findings is as follows. The superoxide anion in its protonated form ($HO_2^·$) reacts directly with ferrous ions to form a complex:

$$Fe^{2+} + HO_2^· \rightarrow Fe^{3+}O_2H^- \quad k = 1.2 \times 10^6\ M^{-1}S^{-1} \quad (18).$$

The further reduction of this product either by $O_2^-$ or directly by the NAD(P)H oxidase of the PMN could theoretically yield OH· for reaction with DMPO as

TABLE II
*Effect of Inhibitors*

| Inhibitor | Percentage activity* | | | |
|---|---|---|---|---|
| | Normal PMN | | MPO-deficient PMN | |
| SOD (50 μg/ml) | 5±3 (9)‡ | <0.001§ | 20±11 (3) | <0.025 |
| Heated 120°C, 15 min | 111±14 (6) | | 114±7 (2) | |
| Catalase (600 μg/ml) | 80±7 (20) | <0.01 | 108±13 (3) | |
| Heated 100°C, 10 min | 81±5 (10) | <0.01 | 112±12 (3) | |
| Catalase (60 μg/ml) | 91±9 (6) | | | |
| Heated 100°C, 10 min | 89±6 (6) | | | |
| Mannitol (0.06 M)‖ | 32±14 (4) | <0.01 | 19±13 (3) | <0.025 |

* Percentage of the activity without inhibitor.
‡ Mean±SE of experiments (number of experiments in parentheses).
§ *P* value for the difference from no inhibitor where significant ($P < 0.05$).
‖ When mannitol was employed, an isosmotic amount of sodium chloride was omitted.

follows:

$$Fe^{3+}O_2H^- \xrightarrow[H^+]{e^-} Fe^{3+} + OH^- + OH\cdot.$$

Although iron was used here as the example, oxygen may bind coordinately to other transition metals, either free or protein bound to form superoxo- and peroxo-complexes (19) which may yield OH· on reduction. Such a mechanism would be sensitive to SOD and mannitol but insensitive to catalase.

It is also possible that (DMPO/OH)· can be formed without the involvement of OH·. The $HO_2^\cdot$ radical adduct (DMPO/OOH)· is formed when DMPO reacts with $O_2^\cdot$ (or $HO_2^\cdot$) (11). The subsequent reduction of this adduct to (DMPO/OH)· may occur. This mechanism for (DMPO/OH)· formation would be dependent on $O_2^-$ but not $H_2O_2$ or OH·; it thus does not account for the inhibition by mannitol. It has been suggested, on theoretical grounds, that the spin trap could react with singlet oxygen (or other one electron oxidants) to form a radical cation which on the addition of water would yield the same spin adduct as that formed with OH· (20).[2]

Previous efforts to chemically identify OH· formation by PMN have relied on the conversion of methional or KMB to ethylene. Both ethylene and (DMPO/OH)· formation are dependent on phagocytosis, normal respiratory burst activity, and $O_2^-$ formation (8–10), and the susceptibility of both to inhibition by catalase is either absent or very slight (8–10) (Table II). However (DMPO/OH)· formation by PMN is more sensitive to mannitol inhibition (Table II) than is ethylene formation (8). Further, ethylene formation by PMN is strongly dependent on MPO, whereas (DMPO/OH)· formation is not. This difference is most clearly demonstrated when PMN that lack MPO are employed. Ethylene formation by MPO-deficient PMN is <10% of normal (9), whereas (DMPO/OH)· formation is greater than normal, a finding compatible with the generally increased respiratory burst by MPO-deficient PMN (1). When purified MPO is added to MPO-deficient PMN, (DMPO/OH)· formation falls, whereas ethylene formation increases (9). A cell-free, MPO-dependent ethylene-forming model system was found with many of the properties of intact PMN (9); we could not detect (DMPO/OH)· formation by this system (data not shown). These findings suggest that the mechanisms for ethylene and (DMPO/OH)· formation by PMN, although similar in some respects, are fundamentally different. Because (DMPO/OH)· formation is more sensitive to mannitol inhibition and in the absence of persuasive evidence implicating MPO in OH· formation, we are inclined to look on the spin trapping assay as a more reliable indicator of OH· formation by PMN.

[2] Foote, C. S. Personal communication.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