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Do Human Neutrophils Make Hydroxyl Radical?

DETERMINATION OF FREE RADICALS GENERATED BY HUMAN NEUTROPHILS ACTIVATED WITH A SOLUBLE OR PARTICULATE STIMULUS USING ELECTRON PARAMAGNETIC RESONANCE SPECTROMETRY*

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Using electron paramagnetic resonance spectrometry and the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), neutrophil free radical production in response to phorbol myristate acetate and opsonized zymosan was investigated. Using phorbol myristate acetate and zymosan (3 mg/ml), the superoxide spin-trapped adduct 2-2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl (DMPO-OOH) and the hydroxyl spin-trapped adduct 2-2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl (DMPO-OH) were detected. Only DMPO-OH was observed with zymosan (0.5 mg/ml). Hydroxyl radical production in the presence of dimethylsulfoxide (Me_2SO) and DMPO yields 2,2,5-trimethyl-1-pyrrolidinyloxyl. The only 2,2-trimethyl-1-pyrrolidinyloxyl detected following neutrophil stimulation was that expected from DMPO-OOH degradation. Superoxide dismutase but not catalase inhibited generation of all three spin-trapped adducts. These data indicate that DMPO-OH arose from DMPO-OOH degradation and does not represent hydroxyl radical production. Under certain conditions DMPO-OH is the predominant spin-trapped adduct resulting from neutrophil superoxide production, perhaps due to cellular bioreduction of DMPO-OOH to DMPO-OH.

Cytochalasin B, which prevents phagosome closure, inhibited zymosan-stimulated neutrophil oxygen consumption and electron paramagnetic resonance superoxide detection. No hydroxyl radical was detected. Spin trapping with DMPO appears to detect intraphagosomal free-radical formation.

Membrane stimulation of human neutrophils results in consumption of a large quantity of oxygen which is ultimately reduced to products important in microbicidal and tumoricidal activity (1), and inflammation (2). Formation of superoxide (O_2^-) (3–6) and hydrogen peroxide (H_2O_2) (7–9) by phagocytic cells has been clearly demonstrated. In cell free systems, superoxide and hydrogen peroxide react in an iron-catalyzed process generating hydroxyl radical ($OH^\cdot$) (10, 11) as outlined below:

$$O_2^- + Fe^{+3} \rightarrow O_2 + Fe^{+2}$$
$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^\cdot$$
$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^\cdot$$

Ethylene detection following stimulation of neutrophils in the presence of a thioether has been offered as evidence of neutrophil production of hydroxyl radical (12, 13). However, other free radicals induce similar results (14–16), leaving in doubt the specificity of this technique.

Spin trapping has been used for detection of free radicals in many cellular systems (17–22). Reaction of unstable free radicals with nitrones or nitroso compounds (spin traps) results in the production of "long-lived" nitroxide free radicals which can be detected using conventional electron paramagnetic resonance (EPR[1]) spectrometry. The hyperfine splitting of a spin-trapped adduct allows identifcation of the original free radical species. Since stable free radicals accumulate, spin trapping is an integrative method of measurement and is inherently more sensitive than procedures measuring instantaneous or steady state generation of free radicals. Reaction of the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) with superoxide and hydroxyl radical produces spin trap adducts with characteristic EPR spectra (23, 24, and Fig. 1). When these free radicals are generated simultaneously in the presence of DMPO, the resulting spectrum is a composite of the individual signals.

However, spin trapping procedures may suffer from technical limitations which make data interpretation difficult (23–26). Interaction of DMPO with hydroxyl radical leads to the formation of DMPO-OH. Detection of DMPO-OH following incubation of DMPO with stimulated neutrophils has been used as proof of hydroxyl radical formation by these cells (20, 21). However, previous work has shown that the superoxide spin trap adduct DMPO-OOH is unstable and rapidly decomposes into three species, including DMPO-OH (25, 26, and Fig. 2). Thus, detection of DMPO-OH does not unequivocally prove the existence of hydroxyl radical.

Because of uncertainty as to the nature of free radicals produced during neutrophil stimulation, we undertook further

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[1] The abbreviations used are: EPR, electron paramagnetic resonance; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO-OH, 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl; DMPO-OOH, 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl; DMPO-CH_3, 2,2,5-trimethyl-1-pyrrolidinyloxyl; DETAPAC, diethylenetriaminepentacetic acid; SOD, superoxide dismutase; PMA, phorbol myristate acetate; HBSS, Hanks' balanced salt solution; PMN, neutrophil.

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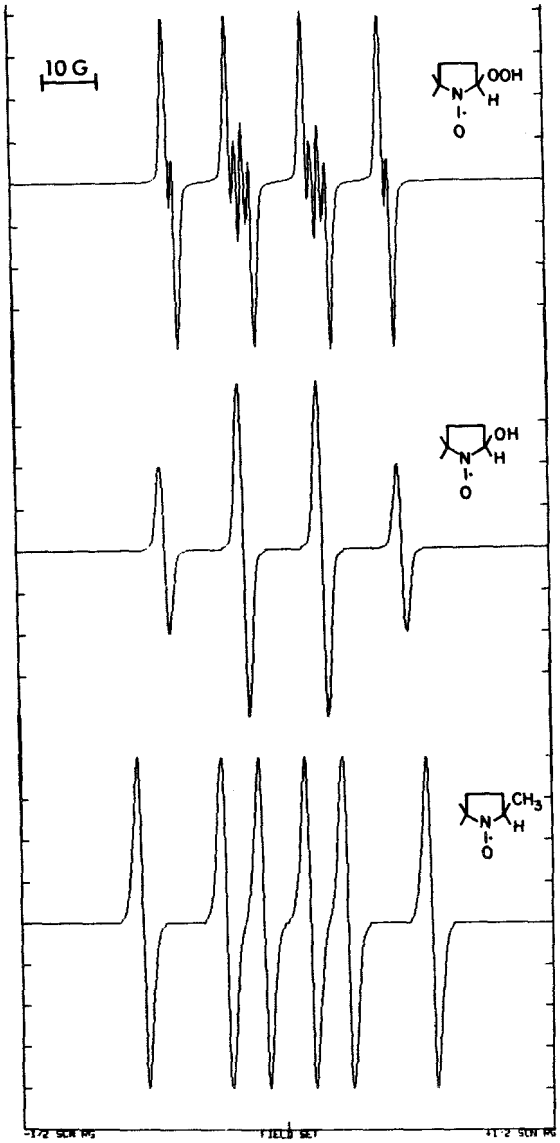

FIG. 1. **Computer simulation of EPR spectra for DMPO-OOH, DMPO-OH, and DMPO-CH_3.** The hyperfine splittings are: for DMPO-OOH, A_N = 14.3 G, A_H^β = 11.7 G, and A_H^γ = 1.25 G; for DMPO-OH, $A_N = A_H$ = 14.87 G; for DMPO-CH_3, A_N = 15.31 and A_H = 22.00 G.

study of this process using EPR spectrometric techniques. Our results show that neutrophil superoxide production leads to a variety of EPR spectra, depending on the stimulus employed and conditions of incubation. These spectral alterations may be misinterpreted as evidence for hydroxyl radical generation unless proper controls are performed. Under the experimental conditions employed in this study, there was no evidence for the formation of hydroxyl radical by human neutrophils.

MATERIALS AND METHODS

Reagents—Diethylenetriaminepentacetic acid (DETAPAC), cytochalasin B, superoxide dismutase (SOD), catalase, ferricytochrome *c*, and zymosan A were purchased from Sigma. Phorbol myristate acetate (PMA) was obtained from Midland Chemical Co., Brewster, NY, dimethyl sulfoxide (Me_2SO), ferric ammonium sulfate, and sodium azide from Fischer Scientific, Fair Lawn, NJ, and ethanol from AAPER Alcohol & Chemical Co., Shelbyville, KY. The spin trap DMPO was synthesized according to the procedure of Bonnett *et al.* (27). Purified human neutrophil myeloperoxidase was kindly provided by Prof. P. Patriarca of the University of Trieste, Italy and was used

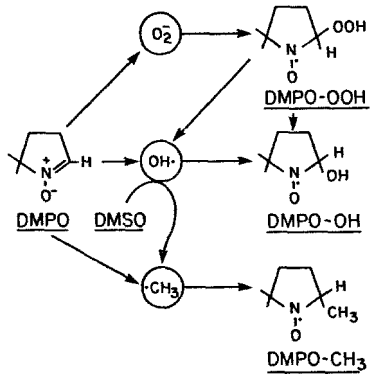

FIG. 2. **Generation of spin trap adducts as a consequence of the interaction between superoxide and hydroxyl radical with DMPO and Me_2SO.** Note the generation of DMPO-OH may occur as a degradation product of DMPO-OOH by two different mechanisms and does not require the generation of hydroxyl radical by the phagocyte.

at a final concentration equivalent to the myeloperoxidase activity of 5×10^6 neutrophils. PMA was suspended in Me_2SO (10 μg/ml) and zymosan in Hanks' balanced salt solution (HBSS). Serum for zymosan opsonization was separated from whole blood of 6–8 healthy donors, pooled and stored at −70 °C until usage. Zymosan was opsonized by incubation in 100% serum at 37 °C for 30 min, followed by 3 washes with final suspension in HBSS (5 or 30 mg/ml).

Neutrophil Separation—Whole blood from normal donors was obtained in heparinized syringes. Neutrophils (PMNs) were separated from other cellular components using Plasmagel (Roger Bellon, Neuilly, France) and Ficoll-Hypaque (Pharmacia) sedimentation with osmotic lysis of contaminating erythrocytes (28). PMNs were suspended in HBSS and concentration determined by a model D2N automated blood cell counter (Coulter Electronics, Hialeah, FL). Giemsa stain revealed >98% of cells to be PMNs, and viability was >95% as determined by exclusion of trypan blue dye.

Oxygen Consumption—A 1 ml volume of HBSS with 0.1 mM DETAPAC containing 5×10^6 PMNs was incubated at 37 °C in a Clark oxygen electrode (Oxygen Monitor, Yellow Springs Instrument Co.). Desired stimuli were added and results were expressed as the peak rate of oxygen consumption (nM/min) observed.

Superoxide Detection—Superoxide was mesured as the superoxide dismutase inhibitable reduction of ferricytochrome *c* as previously described (29). The assay was performed in a spectrophotometer (Perkin-Elmer model 557, Mountain View, CA) using an absorbance of 550 nm and a 1 ml volume containing PMNs (5×10^6), ferricytochrome *C* (80 μM), ± cytochalasin *B* (5 μg/ml), ± SOD (150 units/ml) and zymosan (0.5 mg/ml). Control reactions were performed in the absence of stimuli.

Spin Trapping—Reaction mixtures contained 5.0–45 × 10^6 PMNs/ml, spin trap (0.1 M DMPO), neutrophil stimulant (PMA, 100 ng/ml in 0.14 M Me_2SO), or zymosan (0.5 or 3.0 mg/ml), and buffer (HBSS ± 0.1 mM DETAPAC) sufficient to reach final volume of 0.5 ml. Reaction mixtures were transferred to a flat quartz EPR cell, fitted into the cavity of the EPR spectrometer (Varian Associates model E-9 EPR spectrometer, Palo Alto, CA) and spectrum obtained at 25 °C. Control experiments were done in the absence of PMNs and with unstimulated PMNs. Experiments were also performed in which PMNs, spin trap, and stimulus were incubated in a 37 °C water path for defined time periods before transfer to the EPR cell for spectrum determination. Neither 0.1 M DMPO nor 0.1 mM DETAPAC affected neutrophil oxygen consumption. We found that inclusion of DETAPAC (1 μM to 0.1 mM) in the experimental system enhanced EPR signal intensity by a factor of 2 without altering the nature of the EPR spectra.

Statistical Analysis—Paired or unpaired Student's *t* tests were used for all statistical analysis. Results were considered significant if $p < 0.05$. Although, for the purpose of the presentation, data may be expressed as a percentage of appropriate control, only original (raw) data were used for statistical comparisons.

RESULTS

Spin Trapping of Radicals following Neutrophil Stimulation with Phorbol Myristate Acetate—Neutrophils (5×10^6/ml) in

HBSS with 0.1 mM DETAPAC and 0.1 M DMPO were stimulated with PMA and examined by EPR (Fig. 3, *A* and *B*). The resulting spectrum was a composite of three distinct products: the superoxide adduct (DMPO-OOH, *peak 3*), hydroxyl adduct (DMPO-OH, *peak 2*), and the methyl radical adduct (DMPO-CH_3, *peak 1*). The size of the DMPO-OH and DMPO-OOH peaks increased with sequential scans indicating progressive free radical generation (Fig. 3, *A* and *B*). DMPO-OH can arise as a consequence of the degradation of DMPO-OOH (25, 26, and Fig. 2), and its detection does not confirm hydroxyl radical production. DMPO-CH_3 formation should occur with hydroxyl radical formation in the presence of Me_2SO, which was used as the solvent for PMA (30, Fig. 2). As shown in Fig. 3*B*, no increase in DMPO-CH_3 was noted in the sequential scan. No spin trap adducts were seen with unstimulated neutrophils. Catalase had no effect on the EPR spectrum (data not shown). However, PMA stimulation of neutrophils in the presence of SOD resulted in nearly complete inhibiton of all three spin-trapped adducts (Fig. 3*C*).

To assure that our system was appropriate for hydroxyl radical detection, Fe^{+3} (10^{-4} M) was added to the buffer containing 0.1 mM DETAPAC and the EPR spectrum determined (Fig. 4, *A* and *B*). The low field portion of Fig. 4*A*

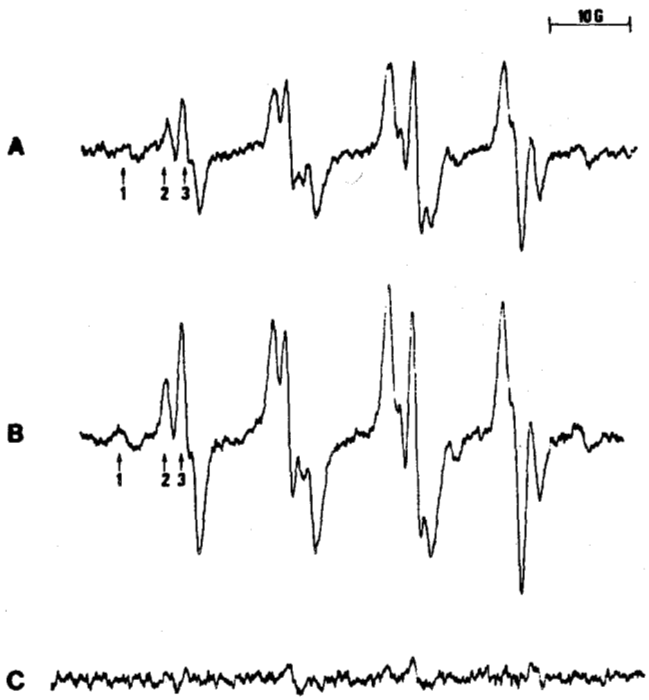

FIG. 3. **EPR spectrum resulting from neutrophil (5.0×10^6 cells/ml) stimulation with PMA (100 ng/ml in Me_2SO) in the presence of DMPO (0.1 M) and DETAPAC (0.1 mM).** *Scan A* was obtained 6 min after the reaction commenced and is the composite of three species: DMPO-CH_3 (*1*), DMPO-OH (*2*), and DMPO-OOH (*3*). DMPO-CH_3 is barely detectable under conditions where DMPO-OOH is present in high concentration. *Scan B* is the continuation of *Scan A*, 12 min after initiation of the reaction. Signal intensity for DMPO-CH_3 (*1*) increased only slightly while DMPO-OOH (*3*) doubled relative to *Scan B*. These data show that DMPO-OH is not generated by neutrophil hydroxyl radical production following stimulation by PMA, but arises from decomposition of DMPO-OOH. *Scan C* was generated under experimental conditions identical to *Scans A* and *B* except that superoxide dismutase (SOD, 5 μg/ml) was added to the reaction mixture. The small quantity of EPR detectable species (DMPO-OH) which persist in the presence of SOD probably results from chemical oxidation of DMPO. Microwave power was 20 milliwatts, modulation frequency was 100 kHz with an amplitude of 0.63 G, sweep time was 12.5 G/min, and the receiver gain was 1.25×10^4 with a response time of 1.0 s.

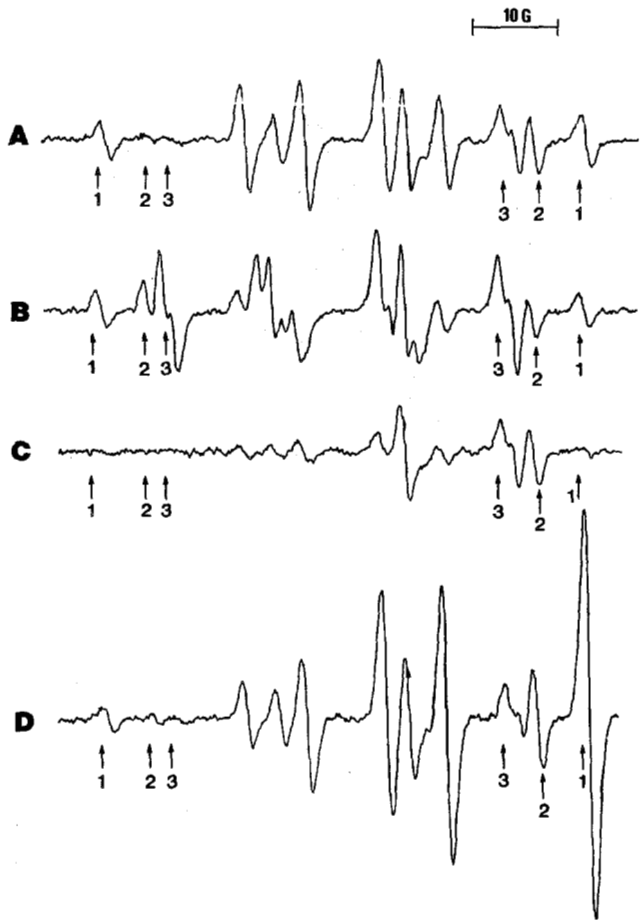

FIG. 4. **EPR spectra resulting from neutrophil (5.0×10^6 cells/ml) stimulation with PMA (100 ng/ml in 0.14 M Me_2SO) in the presence of 0.1 M DMPO, 0.1 mM DETAPAC, and 0.1 mM ferrous ammonium sulfate.** *Scan A* was obtained 3 min into the reaction and is the composite of three species: DMPO-CH_3 (*1*), DMPO-OH (*2*), and DMPO-OOH (*3*). Initially only hydroxyl radical was generated, as suggested by the presence of DMPO-CH_3 and the absence of DMPO-OH and DMPO-OOH. With time, (*Scan B*) superoxide was spin-trapped as DMPO-OOH (*3*). *Scans C* and *D* were performed under the same conditions as *Scan A* except for the addition of catalase (300 units/ml) and SOD (10 μg/ml), respectively, to the reaction mixtures. Microwave power was 20 milliwatts, modulation frequency was 100 kHz with an amplitude of 0.63 G, sweep time was 12.5 G/min, and the receiver gain was 5×10^3 with a response time of 1 s.

revealed initial spin trapping of methyl radicals by DMPO (DMPO-CH_3) as well as DMPO-OH, indicating rapid formation of hydroxyl radical. The superoxide spin-trapped adduct (DMPO-OOH) was not detected. In the sequential scan the peak height of DMPO-CH_3 remained constant while DMPO-OOH became the predominant species (Fig. 4*B*). The DMPO-CH_3 peak was markedly inhibited by catalase (Fig. 4*C*). DMPO-OH peak height was only slightly decreased by catalase (Fig. 4*C*), suggesting that DMPO-OH arose at least in part as a degradation product of DMPO-OOH (24–27, and Fig. 2). SOD enhanced hydroxyl radical generation as illustrated by the increase in DMPO-CH_3 peak amplitudes observed in the low and high field portions of the scan (Fig. 4*D*). This most likely reflects the SOD-mediated increase in the rate of H_2O_2 formation and implies that even with the removal of superoxide from the system adequate Fe^{+2} was available to catalyze the reduction of H_2O_2 to hydroxyl radical.

Spin Trapping of Radicals following Neutrophil Stimulation

with Opsonized Zymosan—The EPR spectrum following neutrophil stimulation with opsonized zymosan (with 0.14 M Me_2SO added, Fig. 5*A*) was both quantitatively and qualitatively different from that observed with PMA (Fig. 3*B*). Zymosan (0.5 mg/ml) stimulation resulted solely in DMPO-OH detection; no additional spin-trapped adducts were observed (Fig. 5*A*). As with PMA (Fig. 3*C*), SOD (Fig. 5*B*) but not catalase (Fig. 5*C*) inhibited free radical spin trapping following zymosan stimulation, demonstrating that DMPO-OH arose not from hydroxyl radical formation but as a degradation product of DMPO-OOH.

Our inability to detect DMPO-OOH under conditions in which superoxide is known to be formed (1) suggested that DMPO-OOH could have been bioreduced to DMPO-OH. This has been previously observed with porcine thoracic aorta endothelial cells, possibly as a consequence of glutathione peroxidase (18). Although this enzyme does exist in the neutrophil cytoplasm, it is not felt to enter the phagosome. Myeloperoxidase, a component of the neutrophil azurophilic granules, is deposited in high concentrations into the phagosome during particle ingestion and could also mediate nitroxide reduction. However, inclusion of the myeloperoxidase inhibitor sodium azide (10 mM) in the reaction mixture did not alter the EPR spectrum (data not shown). Moreover, no evidence of increased reduction of DMPO-OOH to DMPO-OH was seen when purified neutrophil myeloperoxidase was added to a cell free superoxide generating system (data not shown).

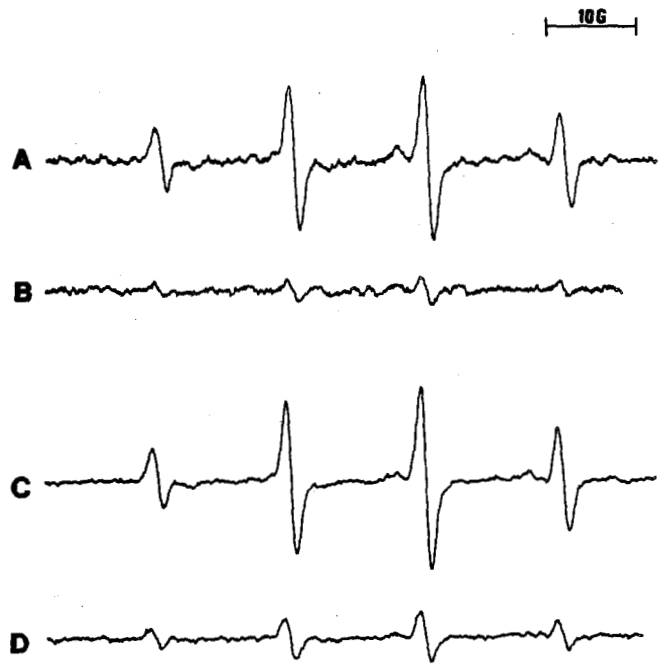

FIG. 5. **EPR spectra obtained with neutrophil (2.0×10^7 cells/ml) stimulation by opsonized zymosan (0.5 mg/ml) in the presence of DMPO (0.1 M), Me_2SO (0.14 M), and DETAPAC (0.1 mM).** Suspensions were incubated at 37 °C in a shaker water bath for 10 min after which contents were transferred to the EPR cell and spectra recorded. EPR spectra in *Scan A* is that of DMPO-OH (note Fig. 2 for computer simulation of this spectrum). The experimental protocol for *Scans B* and *C* was identical to that for *Scan A* except that superoxide dismutase (10 μg/ml) and catalase (300 units/ml) were added to the reaction mixtures in *B* and *C*, respectively. *Scan D* was generated under identical conditions as *Scan A* except that the neutrophils were incubated in the presence of cytochalasin B (5 μg/ml) as well. Inclusion of sodium azide (10 mM) in the reaction mixture of *Scan A* did not alter the spectrum. Microwave power was 20 milliwatts, and the frequency was 100 kHz. EPR spectra were recorded at a rate of 12.5 G/min with a response time of 1 s. The gain wa 6.3×10^3 with a modulation frequency of 100 kHz and an amplitude of 0.63 G, $A_N = A_H = 14.9$ G.

Effect of Cytochalasin B on Zymosan-induced Free Radical Detection—Although zymosan and PMA led to similar rates of neutrophil consumption (Table I), their mechanisms of stimulation are quite different. PMA promotes extracellular secretion of oxygen reduction products (31), while zymosan is phagocytized by neutrophils and most oxygen reduction products are generated within the phagocytic vacuole (32). Experience with other cellular systems (17, 18) suggests that DMPO should penetrate the neutrophil *cytoplasm*, but it was unclear as to whether DMPO achieves significant concentration within the *phagocytic vacuole*. Failure of DMPO to enter the phagosome would only allow spin trapping of products escaping from the neutrophil, possibly preventing detection of intraphagosomal hydroxyl radical formation. Cytochalasin B interferes with closure of the phagocytic vacuole and has been utilized experimentally to increase recovery of vacuolar oxygen reduction products (8, 29). EPR spectra were obtained following zymosan (0.5 mg/ml) stimulation of neutrophils in the presence of cytochalasin B (5 μg/ml, Fig. 5*D*). Relative to results obtained in the absence of cytochalasin B, the size of all spin-trapped adducts was decreased approximately 50% but no qualitative differences in spectral configuration were noted.

Further studies were undertaken to explore the basis for the cytochalasin B-mediated decrease in superoxide detectable by EPR. Cytochalasin B had no effect on the spin trapping of superoxide by DMPO using a xanthine-xanthine oxidase system (data not shown). Consistent with previous reports (8, 29, 33), cytochalasin B increased the rate of ferricytochrome *c* reduction (extracellular superoxide measurement) by zymosan-stimulated neutrophils 267%. However, neutrophil oxygen consumption following cytochalasin B preincubation (Table I) was reduced 45.2% and 30.0% ($n = 4, p < 0.05$) following PMA and zymosan (0.5 mg/ml) stimulation, respectively. A reduction in O_2 consumption would lead to a decrease in the absolute concentration of superoxide (1). It appears that cytochalasin B inhibits intravacuolar neutrophil superoxide production. EPR spectra obtained suggest that DMPO enters the vacuole and accurately reflects intraphagosomal events.

Effect of Zymosan Concentration on Free Radical Detection—Zymosan at the concentration initially used in this study (0.5 mg/ml) does not provide maximal stimulation of neutrophil oxygen reduction (5). In addition it contributed only 10^{-5} M Fe^{+3} to the reaction mixture, as determined by ferrozine method (34), which is 10-fold less Fe^{+3} than was added to PMA-stimulated cells to generate hydroxyl radical (Fig. 4*A*). We, therefore, examined the EPR spectrum resulting when the concentration of zymosan was increased to 3.0

TABLE I

Effect of cytochalasin B on neutrophil oxygen consumption

Mean ± S.E. ($n = 4$) of peak neutrophil oxygen consumption rates (nM/min) in response to phorbol myristate acetate (PMA) or opsonized zymosan after 5 min preincubation in the presence or absence of cytochalasin B.

PMN stimulus	Peak oxygen consumption	
	No cytochalasin B	+ Cytochalasin B
	nM/min	
Resting	2.4 ± 0.3	
PMA	18.3 ± 2.1	10.0 ± 1.8[a]
Zymosan	16.8 ± 1.9	11.4 ± 1.0[a]

[a] Significant decrease relative to rate in the absence of cytochalasin B, $P < 0.05$.

mg/ml to provide maximal neutrophil stimulation and greater Fe^{+3} availability. The result (Fig. 6*A*) was quite different than that seen with 0.5 mg/ml zymosan (Fig. 5*A*): DMPO-OOH was the predominant species. SOD reduced all peaks leaving only a small amount of DMPO-OH detectable (Fig. 6*B*). Catalase unexpectedly increased the size of the DMPO-OH peak, while decreasing DMPO-OOH (Fig. 6*C*). This effect required prolonged (10 min) incubation of catalase with stimulated cells prior to EPR determination (Fig. 6*D*) and was increased in the presence of cytochalasin B (data not shown).

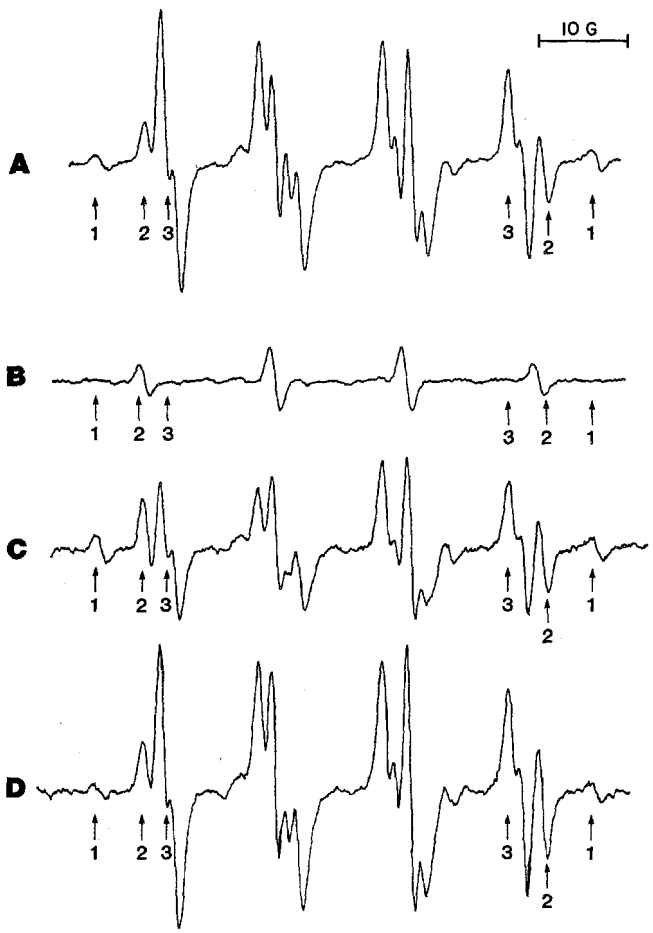

FIG. 6. **EPR spectra obtained with neutrophil (2.0×10^7 cells/ml) stimulation with opsonized zymosan (3.0 mg/ml) in the presence of DMPO (0.1 M), Me_2SO (0.14 M), and DETAPAC (0.1 mM).** *Scan A* was obtained after a 10 min 37 °C incubation of the above contents. The EPR spectra in *Scan A* is the composite of three species: DMPO-CH_3 (*1*), DMPO-OH (*2*), and DMPO-OOH (*3*). *Scans B* and *C* were generated under experimental conditions identical to *Scan A* except that superoxide dismutase (5 μg/ml) and catalase (300 units/ml), respectively, were included in the reaction mixture. SOD resulted in a marked inhibition of all spectra leaving only small DMPO-OH peaks present. This effect was facilitated by cytochalasin B which could reflect incomplete phagosomal penetration of SOD or alternatively cytochalasin B mediated inhibition of neutrophil superoxide production. Catalase produced an increase in DMPO-OH detection while reducing DMPO-OOH amplitude, most likely by increasing the rate of DMPO-OOH bioreduction to DMPO-OH. *Scan D* was obtained under experimental conditions identical to *Scan C* except that the reaction mixture was only allowed to incubate 2 min after the initiation of neutrophil stimulation prior to scanning instead of the usual 10 min. The spectrum is identical to *Scan A*, indicating that the catalase-mediated alteration in the EPR spectrum is related to time of incubation. Microwave power was 20 milliwatts, modulation frequency was 100 kHz with an amplitude of 0.63 G, sweep time was 12.5 G/min, and the receiver gain was 6.3×10^3 with a response time of 1.0 s.

DISCUSSION

Formation of superoxide and hydrogen peroxide has been clearly demonstrated following stimulation of the neutrophil "respiratory burst" (3–9). Since these compounds react in a cell-free system through an Fe^{+3}-catalyzed process to yield hydroxyl radical (10, 11), neutrophil production of hydroxyl radical has been postulated (1, 12, 13).

Studying neutrophils stimulated by PMA or a high concentration (3.0 mg/ml) of zymosan, we observed an EPR spectrum consistent with the superoxide adduct (DMPO-OOH) and the hydroxyl adduct (DMPO-OH). Low concentration (0.5 mg/ml) zymosan stimulation led to DMPO-OH detection only. Formation of DMPO-OH (Fig. 3*A*) would be expected following neutrophil production of hydroxyl radical. However, several lines of evidence suggest that a different mechanism, independent of hydroxyl radical production, was responsible for the formation of DMPO-OH in our system. First, DMPO-OH arising from hydroxyl radical generation via the Fenton reaction should have been partially inhibited by catalase and increased by SOD, as occurred following PMA stimulation of neutrophils in the presence of 10^{-4} M Fe^{+3}. Instead, SOD inhibited DMPO-OH production while catalase had either no effect or increased it. Second, since hydroxyl radical reacts rapidly with either Me_2SO or DMPO (23, 30) (and the concentration of Me_2SO in the reaction mixture was 40% higher than DMPO) hydroxyl radical production should have promoted formation of DMPO-CH_3 at the expense of DMPO-OH. However, in the absence of exogenous Fe^{+3}, only negligible amounts of DMPO-CH_3 were detected in response to PMA (note Fig. 3, *A* and *B*, low field peaks) and zymosan (Fig. 5*A*). These results are most consistent with the formation of DMPO-OH from degradation of DMPO-OOH and not neutrophil hydroxyl radical production (25, 26). Since a small amount of hydroxyl radical formation results from DMPO-OOH degradation (25, 26, see Fig. 2), this mechanism would also account for the small amount of SOD (but not catalase) inhibitable DMPO-CH_3 observed. These results explain similar spectra observed in earlier EPR studies (20, 21) reporting neutrophil hydroxyl radical formation by a mechanism other than the Haber-Weiss reaction.

The lack of EPR evidence for neutrophil hydroxyl radical generation is contrary to reports using other detection systems. Neutrophil stimulation in the presence of methional or 2-keto-4-thiomethylbutyric acid has resulted in the production of ethylene (12, 13) which may result from the interaction of hydroxyl radical with either of these thioethers (10). However, free radicals other than hydroxyl radical have been implicated in similar reactions (14–16) leaving in doubt the specificity of the observation. A single report has appeared in which methane detection following neutrophil stimulation in the presence of Me_2SO has been offered as evidence of neutrophil hydroxyl radical formation (36). In this study azide-inhibited methane production suggesting this reaction may not be specific for hydroxyl radical formation either.

The ability of DMPO to diffuse to all potential sites of neutrophil free radical production is critical to assure detection of all radicals generated. The observation that DMPO-mediated spin trapping of superoxide decreased in proportion to O_2 consumption (in contradistinction to ferricytochrome *c* reduction) suggests that the nitrone is able to detect intraphagosomal superoxide formation. In addition, since SOD markedly inhibited the detection of superoxide by DMPO after zymosan stimulation, it seems likely that SOD has some ability to penetrate the phagosome, consistent with earlier work (3).

DMPO spin trapping of superoxide generated by neutrophils may yield a different EPR spectrum than occurs in a cell-free system (24). The most likely explanation is bioreduction of DMPO-OOH to DMPO-OH, as has been postulated to occur as a consequence of cytoplasmic glutathione peroxidase in another cellular system (18). Because of its lipid solubility, DMPO-OOH formed in the phagosome might freely diffuse into the cytoplasm where it could interact with glutathione peroxidase, alleviating the need for the enzyme's transport to the phagosome. Myeloperoxidase does not appear to be involved in this bioreduction. Catalase (in the presence of cytochalasin B) induced an increase in DMPO-OH detection when neutrophils were stimulated with zymosan. Several explanations for this phenomena are currently being investigated. An increase in phagosomal oxygen from H_2O_2 catabolism could lead to greater superoxide formation and EPR signal amplification. However, this would not be expected to selectively increase DMPO-OH detection. Alternatively, H_2O_2 may be an additional substrate for the system responsible for the bioreduction of DMPO-OOH to DMPO-OH. Catabolism of H_2O_2 could increase the rate of DMPO-OOH bioreduction by removing a competitive substrate (H_2O_2) from the system.

In contrast to cell-free systems (24), DMPO-CH_3 failed to accumulate with time following PMA-induced neutrophil hydroxyl radical formation observed in the presence of 10^{-4} M Fe^{+3}. This could also be the result of selective nitroxide bioreduction. Alternatively, neutrophil hydroxyl radical generation may slow with time. Lactoferrin, which is secreted extracellularly following PMA stimulation (37), could bind free iron making it unavailable as a Haber-Weiss catalyst (38), and we have recently generated additional evidence to support this hypothesis.[2]

EPR appears to be a promising technique for the study of neutrophil free radical formation. Extracellular events may be markedly different from those occurring inside the phagosome. The apparent ability of spin trapping technology to measure intraphagosomal events makes this method superior in many respects to other commonly employed systems. However, recognition of the chemistry and kinetics of oxygen radical spin trapping, as well as potential secondary reactions mediated by cellular enzymes, are critical for accurate data interpretation. Our results confirm generation of superoxide by human neutrophils. Formation of hydroxyl radical was not observed under the experimental conditions employed, unless a large quantity of exogenous (free) iron was added. However, it is possible that neutrophils could use iron ingested from another biological source to catalyze formation of hydroxyl radical (39), and experiments to test this hypothesis are in progress.

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