Brief Definitive Report

MONONUCLEAR PHAGOCYTES HAVE THE POTENTIAL FOR SUSTAINED HYDROXYL RADICAL PRODUCTION

Use of Spin-trapping Techniques to Investigate Mononuclear Phagocyte Free Radical Production

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In the presence of Fe^{+3}, O_2^- and H_2O_2 react in vitro to form hydroxyl radical (·OH). Considerable attention has been focused on the role of iron-catalyzed ·OH production in phagocyte microbicidal activity and tissue damage. Early studies implied that both neutrophils (PMN [reviewed in reference 1]) and mononuclear phagocytes (2–9) generated ·OH in the absence of an exogenous catalyst. However, the experimental systems used in these studies probably lacked specificity for ·OH (1).

Using spin trapping, generally considered the most specific technique for ·OH detection, we did not find evidence for ·OH production by human PMN in the absence of an exogenous iron catalyst (10, 11). Even then, ·OH production appears to be inhibited by PMN secretion of lactoferrin and myeloperoxidase (MPO) (11–13). Monocytes do not possess lactoferrin (14) and have less MPO than PMN (15). Differentiation of monocytes to monocyte-derived macrophages (MDM) is associated with the loss of MPO (15) and the acquisition of tartrate-resistant acid phosphatase (TRAP) (16). TRAP is an iron-containing enzyme, similar to uteroferrin, that may act as an ·OH catalyst (17). The current work was undertaken to determine if these factors influenced the potential for ·OH formation by mononuclear phagocytes.

Materials and Methods

Preparation of Phagocytes. Human mononuclear leukocytes and PMN were obtained by dextran and Ficoll-Hypaque separation. Monocytes and lymphocytes were separated by placing them in sterile petri dishes at 37°C, for 2 h. After washing, adherent monocytes were gently scraped into suspension. For MDM, monocytes were incubated in medium 199 (University of Iowa Cancer Center) with 13% autologous serum and gentamicin (50 μg/ml) for 5–7 d. MDM were released with trypsin and EDTA or by gentle scraping. In some cases, MDM were incubated in IFN-γ (100 U/ml) for an additional 4 d.

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TRAP Activity. TRAP activity was assessed using the previously described cytochemical and paranitrophenyl phosphate colorometric assays (16). For TRAP release studies, MDM were exposed to desired stimuli for 30 min, pelleted, and supernatant was removed.

Spin Trapping. Sequential EPR spectra of phagocytes ($0.25–1 \times 10^7$/ml), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO, 0.1 M), DMSO (Me_2SO, 0.14 M), stimulus (PMA, 100 ng/ml; or opsonized zymosan (OZ), 3 mg/ml), and buffer (HBSS containing 0.1 mM diethylenetriaminepentaacetic acid [DTPC]) were recorded at 25°C using an EPR spectrometer (model E-104A; Varian Associates, Inc., Palo Alto, CA). Where desired, ferrous ammonium sulfate (0.1 mM), SOD (10 μg/ml), and catalase (600 U/ml) were included. Exclusion of DTPC did not qualitatively alter the EPR spectra. Spectrometer microwave power was 20 mW, modulation frequency was 100 kHz with an amplitude of 1.0 G, sweep time was 12.5 G/min, and the receiver gain was 3.2×10^4 with a response time of 1 s.

DMPO reacts with O_2^- and ·OH to yield 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl (DMPO-OOH) and 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl (DMPO-OH), respectively (11). However, DMPO-OH is also a decomposition product of DMPO-OOH, making it unreliable as evidence for ·OH production (1, 10, 11). ·OH reacts with Me_2SO to form methyl radical, which can be spin trapped as 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-CH_3) (references 10, 11). When Me_2SO is present in excess of DMPO as in this study, ·OH production is manifested primarily as DMPO-CH_3, providing a more specific detection system for ·OH.

Results

Spin Trapping of Oxygen-centered Radicals After Monocyte Stimulation. EPR spectra were obtained after monocyte stimulation with PMA or OZ. PMA spectra comprised mostly DMPO-OOH and DMPO-OH (Fig. 1). With OZ, DMPO-OH dominated with only small DMPO-OOH peaks detected (Fig. 1). Small DMPO-CH_3 peaks observed were not in excess of those expected from the small quantity of ·OH produced as a direct decomposition product of DMPO-OOH (10). SOD markedly inhibited all spin-trapped adducts whereas catalase had no effect (Fig. 1). No qualitative difference was noted in spectra obtained after OZ stimulation of monocytes pretreated with cytochalasin B (data not shown), excluding the possibility that the monocyte phagosome prevented detection of ·OH. Monocytes stimulated with PMA or OZ in the presence of exogenous Fe^{+3} to induce ·OH production yielded EPR spectra dominated by DMPO-CH_3 (Fig. 1).

Detection of Oxygen-centered Free Radical Production by MDM. MDM stimulated with PMA or OZ yielded spectra that were a composite of DMPO-OOH and DMPO-OH (Fig. 2). Minimal DMPO-CH_3 was detected. DMPO-OOH peaks were greater with PMA whereas DMPO-OH peaks were larger with OZ. With either stimulus, SOD inhibited all adducts, whereas catalase had no effect (Fig. 2). Cytochalasin B did not result in qualitative changes in the spectra (data not shown). MDM possessed TRAP that was released by exposure to either stimulus (Table I). Stimulation of IFN-γ-treated MDM with PMA or OZ (Fig. 2) only increased O_2^--derived spin-trapped adducts (DMPO-OOH and DMPO-OH). Minimal DMPO-CH_3 was observed. IFN-γ decreased MDM TRAP (Table I).

Free Radical Production by Monocytes and MDM in the Presence of Exogenous Iron. Next ·OH production by iron-supplemented mononuclear phagocytes and PMN was compared. Monocytes and MDM stimulated with PMA or OZ in the presence of Fe^{+3} (Fig. 3), exhibited sustained ·OH production (stable or increasing DMPO-CH_3). Catalase inhibited DMPO-CH_3 90–100% whereas SOD inhibited 20–40%. Under the same conditions PMN ·OH generation terminated after 10–15 min (Fig. 3).

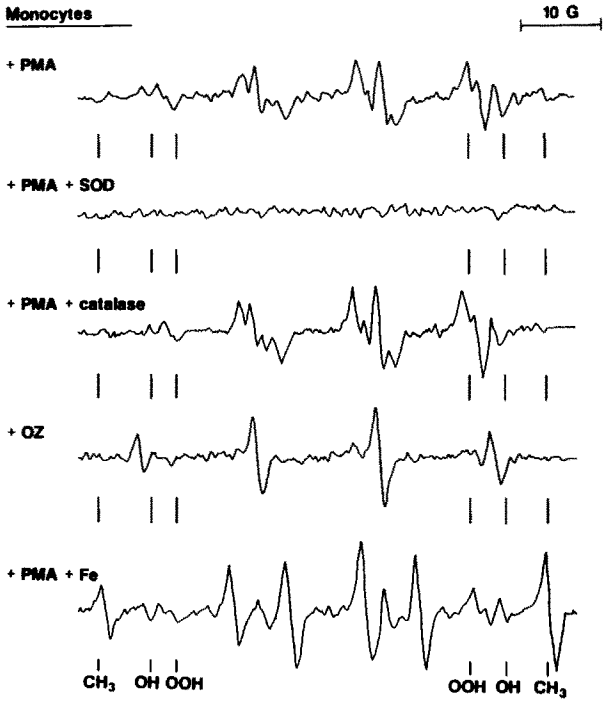

FIGURE 1. Representative (n = 5) EPR spectra obtained immediately after the addition of PMA to monocytes (+PMA) and with the addition of SOD (+SOD) or catalase (+catalase). Also shown is a representative (n = 3) of EPR spectrum obtained 6 min after the addition of OZ to monocytes. SOD and catalase effects were similar to those with PMA. The bottom scan (PMA + Fe) was obtained after PMA stimulation of monocytes in the presence of exogenous Fe^{+3}. Locations of high and low field peaks corresponding to each species are noted.

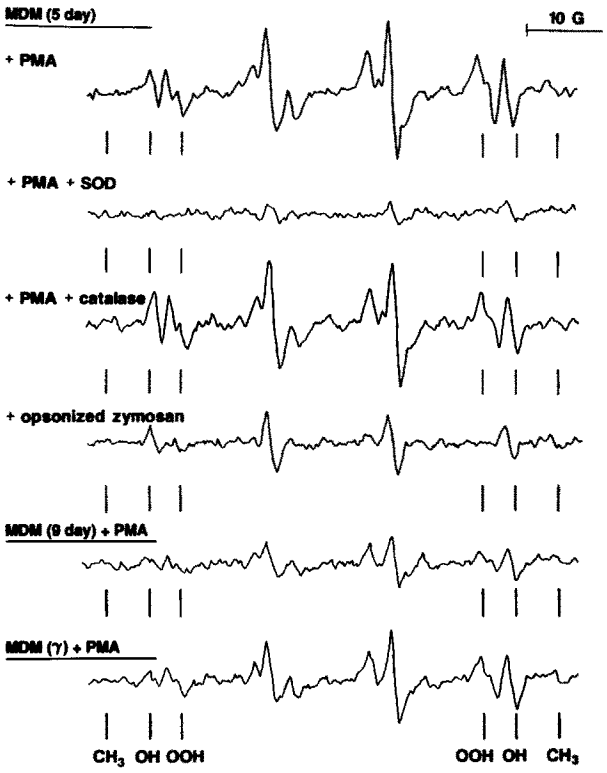

FIGURE 2. Representative (n = 5) EPR scans obtained immediately after the addition of PMA to MDM (+PMA) and under the same conditions except that SOD (+SOD) or catalase (+catalase) were also present. The fourth scan was obtained 6 min after the addition of OZ to the same concentration of MDM (n = 3). Addition of SOD totally ablated this latter spectrum whereas catalase had no effect (not shown). The bottom two tracings were obtained with PMA stimulation of IFN-γ-treated and control MDM.

TABLE I

Acid Phosphatase Activity in Mononuclear Phagocytes

Phagocytes	Acid phosphatase activity					TRAP cytochemical stain
			Release			
	Total	Tartrate resistant	PMA	OZ	OZ/CB	
Monocytes	21.5 ± 2.6	18.4 ± 2.4	ND	ND	ND	-
MDM	43.0 ± 7.5	36.8 ± 6.6	15	25	19	+
MDM-γ	9.5 ± 2.9	8.3 ± 2.5	0	0	0	-

Mean ± SEM (n = 8–18) of total acid phosphatase and TRAP activity expressed as mU acid phosphatase/mg cellular protein of monocytes, MDM, and IFN-γ-treated MDM (MDM-γ). Percentage of TRAP released extracellularly by MDM and MDM-γ after stimulation with PMA, OZ, or OZ after preincubation with cytochalasin B (OZ/CB) is also shown (n = 3), as are results of cytochemical stain for TRAP.

Discussion

PMN do not possess the endogenous capacity for ·OH formation (1, 10, 11) and the lactoferrin and MPO release limits ·OH generation even if the cells are provided with an exogenous iron catalyst (11–13). Their relative lack of MPO (15), absence of lactoferrin (14), and presence of TRAP (16) suggested that mononuclear phagocytes might have a greater propensity than PMN to generate ·OH. However, when monocytes or MDM were stimulated with either PMA or OZ, only DMPO-OH and DMPO-OOH were observed. Since ·OH production should have been manifested as DMPO-CH_3 and all spin-trapped adducts were blocked by SOD but not catalase, these data indicate only O_2^- formation. O_2^- generation manifested as DMPO-OH rather than DMPO-OOH has been noted with PMN and other nonphagocytic cells (1, 10, 11) and appears to result from cell metabolism of DMPO-OOH to DMPO-OH (1). The results observed with cytochalasin B–treated cells suggest that failure of the spin trap to reach intraphagosomal sites was not responsible for lack of ·OH

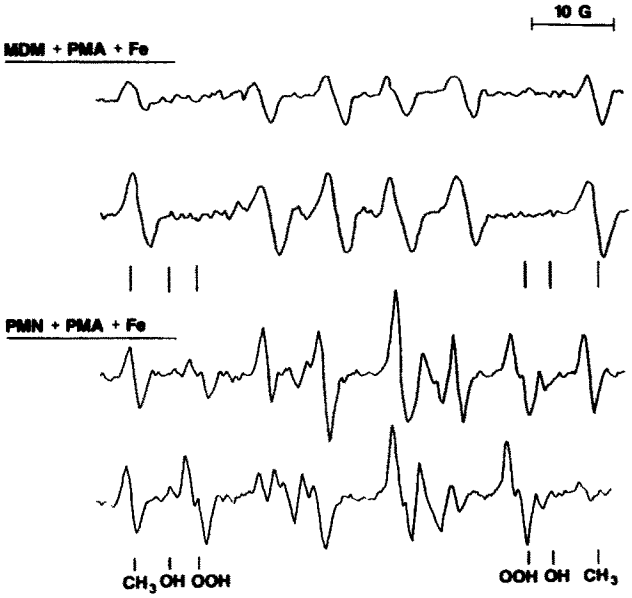

FIGURE 3. Representative (n = 3) EPR scans obtained beginning immediately after (scan 1) and ~35 min after (scan 2) stimulation of MDM with PMA in the presence of Fe^{+3}. DMPO-CH_3 remained the only detectable adduct, indicating ongoing ·OH formation. The bottom two scans are sequential 6-min scans obtained immediately after PMA stimulation of PMN in the presence of exogenous Fe^{+3}. By the end of the second scan DMPO-OOH was the dominant species with only a small DMPO-CH_3 peak detectable. This indicates termination of ·OH production in spite of continued O_2^- formation.

detection. Thus, neither monocytes nor MDM appear to possess the endogenous capacity to generate ·OH, presumably because they do not possess and/or mobilize an appropriate catalyst. MDM possessed TRAP and appeared to release it when stimulated. It is not clear why TRAP release did not allow MDM ·OH production.

Previous studies (2–9) reported ·OH production by monocytes and/or macrophages. However, the specificity of the assay systems for ·OH used in these reports has been questioned (1). In addition, the possibility that iron contaminating the buffers allowed ·OH to be produced was not addressed. To our knowledge no spin-trapping studies of monocytes have been reported. Using spin trapping we showed that monocytic HL-60 cells lack the endogenous capacity for ·OH production (18). Mouse macrophages have been studied by EPR (9). DMPO-OH was detected and inhibited by Me_2SO, consistent with ·OH production. Unfortunately, no comment was made as to whether DMPO-CH_3 was detected. Endogenous capacity to form ·OH may differ among macrophages of different species or anatomical sites.

Although IFN-γ increases MDM microbicidal and tumoricidal activity, we found no evidence that IFN-γ induces human MDM to generate ·OH. IFN-γ reportedly increased murine peritoneal macrophage ·OH (ethylene) production 19-fold (4). This may again reflect species differences but more likely relates to differences in the specificity of the two ·OH detection systems (1).

Monocytes and MDM, but not PMN, stimulated in the presence of exogenous iron produced sustained ·OH. This would be anticipated since monocytes and MDM lack lactoferrin, which terminates PMN ·OH production (11, 13).

Although we find no evidence for the endogenous capacity for ·OH production by human phagocytes (PMN or mononuclear), their remains the potential for phagocytes to induce formation of ·OH in vivo under conditions where an appropriate catalyst is present. The sustained ·OH production observed with iron-supplemented mononuclear phagocytes suggests ·OH might play a significant role in the cytotoxicity of these cells. However, the contribution of mononuclear phagocyte-derived ·OH to inflammatory mechanisms clearly requires additional study.

Summary

Monocytes lack lactoferrin and have much less myeloperoxidase than neutrophils. They also acquire a potential catalyst for ·OH production (tartrate-resistant acid phosphatase) as they differentiate into macrophages. Consequently, the nature of free radicals produced by these cells was examined using the previously developed spin-trapping system. When stimulated with either PMA or OZ neither monocytes nor monocyte-derived macrophages (MDM) exhibited spin trap evidence of ·OH formation. Pretreatment with IFN-γ failed to induce MDM ·OH production. When provided with an exogenous Fe^{+3} catalyst, both stimulated monocytes and MDM, but not PMN, exhibited sustained ·OH production, presumably due to the absence of lactoferrin in mononuclear phagocytes. Sustained production of ·OH could contribute to the microbicidal activity of mononuclear phagocytes as well as inflammatory tissue damage under in vivo conditions where catalytic Fe^{+3} may be present.

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