In the presence of Fe$^{3+}$, O$_2^-$ and H$_2$O$_2$ 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.
**TRAP Activity.** TRAP activity was assessed using the previously described cytochemical and paraniitrophenyl 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 x 10⁷/ml), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO, 0.1 M), DMSO (Me₂SO, 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 x 10⁷ with a response time of 1 s.

DMPO reacts with O₂− and ·OH to yield 2,2-dimethyl-5-hydroperoxy-l-pyrrolidinyloxyl (DMPO-OOH) and 2,2-dimethyl-5-hydroxy-l-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 Me₂SO to form methyl radical, which can be spin trapped as 2,2,5-trimethyl-l-pyrrolidinyloxyl (DMPO-CH₃) (references 10, 11). When Me₂SO is present in excess of DMPO as in this study, ·OH production is manifested primarily as DMPO-CH₃, 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-CH₃ 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 Fe³⁺ to induce ·OH production yielded EPR spectra dominated by DMPO-CH₃ (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-CH₃ 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 1). Stimulation of IFN-γ-treated MDM with PMA or OZ (Fig. 2) only increased O₂−-derived spin-trapped adducts (DMPO-OOH and DMPO-OH). Minimal DMPO-CH₃ was observed. IFN-γ decreased MDM TRAP (Table 1).

**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 Fe³⁺ (Fig. 3), exhibited sustained ·OH production (stable or increasing DMPO-CH₃). Catalase inhibited DMPO-CH₃ 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-\(\gamma\)-treated and control MDM.
Table I

Acid Phosphatase Activity in Mononuclear Phagocytes

| Phagocytes | Total Acid Phosphatase (mU/mg) | Tartrate-resistant Acid Phosphatase (mU/mg) | Release │ TRAP Stain |
|------------|-------------------------------|---------------------------------------------|---------|------------|
| Monocytes  | 21.5 ± 2.6                    | 18.4 ± 2.4                                  | ND      | ND         |
| MDM        | 43.0 ± 7.5                    | 36.8 ± 6.6                                  | 15      | 19         |
| MDM-γ      | 9.5 ± 2.9                     | 8.3 ± 2.5                                   | 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-CH₃ and all spin-trapped adducts were blocked by SOD but not catalase, these data indicate only O₂⁻ formation. O₂⁻ 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 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 Me2SO, consistent with ·OH production. Unfortunately, no comment was made as to whether DMPO-CH3 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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References

1. Cohen, M. S., B. E. Britigan, D. J. Hassett, and G. M. Rosen. 1988. Do human neutrophils make hydroxyl radical? Evaluation of an unresolved controversy. *Free Radical Biol. Med.* 5:81.

2. Weiss, S. J., G. W. King, and A. F. LoBuglio. 1977. Evidence for hydroxyl radical generation by human monocytes. *J. Clin. Invest.* 60:370.

3. Karnovsky, M. L., and J. A. Badwey. 1983. Determinants of the production of active oxygen species by granulocytes and macrophages. *J. Clin. Chem. Clin. Biochem.* 21:545.

4. Ito, M., R. Karmali, and K. Krim. 1985. Effect of interferon on chemiluminescence and hydroxyl radical production in murine macrophages stimulated by PMA. *Immunology.* 56:533.

5. Niwa, Y., T. Sakane, Y. Miyachi, and M. Ozaki. 1984. Oxygen metabolism in phagocytes of lepromatous patients: enhanced endogenous superoxide dismutase activity and hydroxyl radical generation by clofazamine. *J. Clin. Microbiol.* 20:837.

6. Speer, C. P., D. R. Ambruso, J. Grimsley, and R. B. Johnson, Jr. 1985. Oxidative metabolism in cord blood monocytes and monocyte-derived macrophages. *Infect. Immun.* 50:919.

7. Janco, R. L., and D. English. 1983. Regulation of monocyte oxidative metabolism: chemotactic factor enhancement of superoxide release, hydroxyl radical generation and chemiluminescence. *J. Lab. Clin. Med.* 102:890.

8. Hoidal, J. R., G. D. Beall, and J. R. Repine. 1979. Production of hydroxyl radical by human alveolar macrophages. *Infect. Immun.* 26:1088.

9. Hume, D. A., S. Gordon, P. J. Thornalley, and J. V. Bannister. 1983. The production of oxygen-centered radicals by Bacillus-Calmette-Guerin-activated macrophages: an electron paramagnetic resonance study of the response to phorbol myristate acetate. *Biochim. Biophys. Acta.* 763:245.

10. Britigan, B. E., G. M. Rosen, Y. Chai, and M. S. Cohen. 1986. 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. *J. Biol. Chem.* 261:4246.

11. Britigan, B. E., G. M. Rosen, B. Y. Thompson, Y. Chai, and M. S. Cohen. 1986. Stimulated human neutrophils limit iron-catalyzed hydroxyl radical formation as detected by spin trapping techniques. *J. Biol. Chem.* 261:17026.

12. Winterbourn, C. C. 1986. Myeloperoxidase as an effective inhibitor of hydroxyl radical production: implications for the oxidative reactions of neutrophils. *J. Clin. Invest.* 78:545.

13. Britigan, B. E., D. J. Hassett, G. M. Rosen, and M. S. Cohen. 1987. Comparison of the impact of myeloperoxidase and lactoferrin release on the potential for neutrophil hydroxyl radical formation using spin trapping techniques. *Clin. Res.* 35:857A. (Abstr.)

14. Van Snick, J. L., P. L. Masson, and J. F. Heremans. 1974. The involvement of lactoferrin in the hyposideremia of acute inflammation. *J. Exp. Med.* 140:1068.

15. Johnston, Jr., W. D., B. Mei, and Z. A. Cohn. 1977. The separation, long-term cultivation, and maturation of the human monocyte. *J. Exp. Med.* 146:1613.

16. Snipes, R. G., K. W. Lam, R. C. Dodd, T. K. Gray, and M. S. Cohen. 1986. Acid phosphatase activity in mononuclear phagocytes and the U937 cell line: monocye-derived macrophages express tartrate-resistant acid phosphatase. *Blood.* 67:729.

17. Sibley, J. C., K. Doi, and P. Aisen. 1987. Hydroxyl radical formation and iron-binding proteins: stimulation by the purple acid phosphatases. *J. Biol. Chem.* 262:59.

18. Thompson, B. Y., G. Sivam, B. E. Britigan, and M. S. Cohen. 1988. Oxygen metabolism of the HL-60 cell line: comparison of the effects of monocyteoid and neutrophilic differentiation. *J. Leukocyte Biol.* 43:140.

19. Britigan, B. E., T. J. Coffman, D. R. Adelberg, and M. S. Cohen. 1988 Monocytes and noncyte-derived macrophages lack the endogenous capacity to form hydroxyl radical as assessed by spin trapping. *Clin. Res.* 36:452A. (Abstr.)