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# Hydroxyl Radical Scavengers Produce Similar Decreases in the Chemiluminescence Responses and Bactericidal Activities of Neutrophils

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The addition of hydroxyl radical (·OH) scavengers caused similar decreases in the chemiluminescence responses and killing of *Staphylococcus aureus* 502A by human neutrophils in vitro.

Ingestion of bacteria by polymorphonuclear leukocytes or neutrophils triggers a burst of oxidative metabolism which is associated with the production of many species of activated oxygen, including superoxide anion ($O_2^- \cdot$) hydrogen peroxide ($H_2O_2$), and hydroxyl radical (·OH) (12). Along with the generation of these activated oxygen species, which are commonly called oxygen radicals, the generation of light or chemiluminescence (CL) occurs (1). Because CL produced by neutrophils and bacteria is decreased by $O_2$ radical scavengers and because $O_2$ radical-deficient neutrophils from patients with chronic granulomatous disease do not produce CL, it is generally believed that $O_2$ radicals are involved in the generation of CL by neutrophils (13). Moreover, neutrophils from patients with decreased CL frequently have abnormalities in $O_2$ radical production or killing of bacteria or both. As a result, CL is often used as a simple screening test to uncover bactericidal defects in neutrophils from patients with recurrent infections. However, which of the aforementioned $O_2$ radicals contribute to CL and the bactericidal function of neutrophils and how they contribute remain the subject of continued investigation (2, 3, 5, 6, 14). In particular, although numerous studies have suggested that superoxide dismutase (SOD) or catalase or both can inhibit neutrophil CL or bactericidal activity, little is known about the parallel effects of ·OH scavengers on neutrophil CL or bactericidal activity.

In the present investigation, we examined the nature of the $O_2$ radicals involved in CL and tested the premise that the measurement of CL reflects changes in bactericidal activity by neutrophils. Our results show that ·OH scavengers decrease CL by neutrophils and that a close relationship exists between ·OH-dependent decreases in neutrophil CL and bactericidal activity.

Dimethyl sulfoxide (DMSO), mannitol, dimethyl urea, SOD, and catalase were obtained from Sigma Chemical Co., St. Louis, Mo. Urea was obtained from Fisher Chemical, Fair Lawn, N.J., thiourea was obtained from Mallinckrodt Chemical, St. Louis, Mo., and dimethyl thiourea was obtained from Alfa Products, Danvers, Mass. Neutrophils were prepared from heparinized venous blood from healthy, drug-free adult donors (11). Neutrophil purification (>95%) involved dextran sedimentation, gradient separation with hypotonic lysis of erythrocytes, and double washing as previously described (11). Serum was obtained and pooled from five nonmedicated, asymptomatic donors and prepared in standard fashion (7). *Staphylococcus aureus* cells 502A were grown overnight in broth and washed twice in phosphate-buffered saline (4). After recovery by centrifugation at 3,000 × *g* for 10 min, *S. aureus* cells were suspended in pooled serum, mixed, and rotated at 37°C for 60 min. These preopsonized *S. aureus* cells were then washed twice and adjusted in a spectrophotometer to a final concentration of $10^9$/ml. Bactericidal activity was evaluated by adding washed *S. aureus* cells ($5 \times 10^6$) to washed neutrophils ($4 \times 10^6$) in a final volume of 1.0 ml of Hanks balanced salt solution with 8% pooled human serum (4). Each siliconized tube was mixed immediately on a Vortex stirrer, sampled by micropipette, capped, and incubated at 37°C. These mixtures were then tumbled end over end for 30 min and subsequently allowed to remain stationary for 30 min. Each tube was similarly resampled after 60 min. Micropipetted samples were appropriately diluted and plated on agar. After 24 h, the number of colonies on each plate was counted (model 620 Automatic Colony Counter, 3M Co., Minneapolis, Minn.) The percentage of *S. aureus* cells killed was calculated using 0- and 60-min bacterial plate counts. Each determination was the average of triplicate samples from duplicate tubes. CL was measured in the absence of luminol in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) which had been kept in darkness for 24 h before use (10). The mixtures tested were the same as those studied in the bactericidal assay. Test vial contents were equilibrated to 37°C, mixed carefully for 30 s, and then counted sequentially. Each sample was maintained in a 37°C water bath between counts.

CL produced by mixtures of neutrophils, serum, and *S. aureus* was inhibited by the addition of ·OH scavengers, thiourea, dimethyl thiourea, and DMSO, but not mannitol or the less active analogs urea and dimethyl urea (Table 1). Killing of *S. aureus* by neutrophils was also inhibited by the addition of ·OH scavengers, thiourea, dimethyl thiourea, and DMSO, but not mannitol, urea, or dimethyl urea (Table 1). In contrast, the addition of SOD, a scavenger of $O_2^- \cdot$, or catalase, a scavenger of $H_2O_2$, significantly ($P < 0.05$) inhibited CL by neutrophils and *S. aureus* but did not significantly ($P > 0.05$) decrease the bactericidal activities of neutrophils against *S. aureus* beyond the nonspecific effects caused by the addition of heat-inactived SOD or catalase (Table 1). Furthermore, although the effects of SOD or catalase or neutrophil bactericidal activity or CL were dissimilar, a highly significant correlation ($R = 0.87$; $P < 0.01$) existed between ·OH scavenger-mediated inhibitions in bactericidal activity and CL by neutrophils (Fig. 1).

The results of the present investigation indicate that ·OH

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TABLE 1. Effect of oxygen radical scavengers on the CL responses and bactericidal activity of neutrophils in vitro

| Test conditions (neutrophils, *S. aureus*, and the following addition)[a] | Peak CL by neutrophils (% inhibition) | Bactericidal activity by neutrophils (% inhibition) |
|---|---|---|
| Thiourea (1 mM) | 46 ± 3.7 (15)[b,c] | 31 ± 1.6 (18)[c] |
| Thiourea (5 mM) | 79 ± 3.1 (7)[c] | 65 ± 3.4 (10)[c] |
| Thiourea (10 mM) | 88 ± 2.3 (7)[c] | 89 ± 2.8 (12)[c] |
| Dimethyl thiourea (2.5 mM) | 54 ± 2.5 (20)[c] | 80 ± 2.8 (8)[c] |
| Dimethyl thiourea (10 mM) | 63 ± 2.4 (20)[c] | 91 ± 3.8 (25)[c] |
| DMSO (140 mM) | 15 ± 1.8 (18)[d] | 16 ± 2.6 (16)[d] |
| DMSO (210 mM) | 27 ± 1.7 (18)[d] | 14 ± 2.6 (14)[d] |
| DMSO (280 mM) | 39 ± 2.2 (18)[c] | 38 ± 2.7 (18)[c] |
| Mannitol (50 mM) | 5 ± 3.4 (14)[d] | 5.4 ± 1.3 (14)[d] |
| Dimethyl urea (10 mM) | 2.6 ± 1.4 (10)[d] | 1.0 ± 2.6 (10)[d] |
| Urea (10 mM) | 6.1 ± 3.8 (5)[d] | 5.2 ± 1.4 (5)[d] |
| SOD (200 μg/ml) | 51 ± 2.1 (15)[c] | 12 ± 2.7 (17)[d] |
| SOD (heated) (200 μg/ml) | 3.2 ± 0.8 (4)[d] | 7.3 ± 3.1 (3)[d] |
| Catalase (1 mg/ml) | 24 ± 1.7 (10)[c] | 16 ± 2.4 (10)[d] |
| Catalase (heater) (1 mg/ml) | 2.3 ± 0.9 (5)[d] | 8.1 ± 4.2 (3)[d] |

[a] In the absence of any added inhibitor, neutrophils killed ca. 52 ± 8% of the initial inoculum of *S. aureus* and generated 24,500 ± 2,000 cpm of peak CL at ca. 5 min after mixing.

[b] Mean ± standard error (number of determinations).

[c] Value significantly decreased ($P < 0.05$) from value with no inhibitor added.

[d] Value not significantly ($P > 0.05$) different from value obtained with no inhibitor added.

may be a key component in the CL and bactericidal responses of neutrophils. This impression is based on the observation that the addition of ·OH scavengers similarly decreased CL responses and bactericidal activities of neutrophils, whereas, in contrast, SOD and catalase, scavengers of $O_2^-$· and $H_2O_2$, respectively, inhibited CL responses but not the bactericidal actions or neutrophils in vitro. This discrepancy may reflect the possibility that SOD and catalase are only effective at scavenging $O_2$ radicals that are released from neutrophils and do not enter neutrophils efficiently enough to scavenge $O_2$ radicals generated intracellularly. Indeed, it

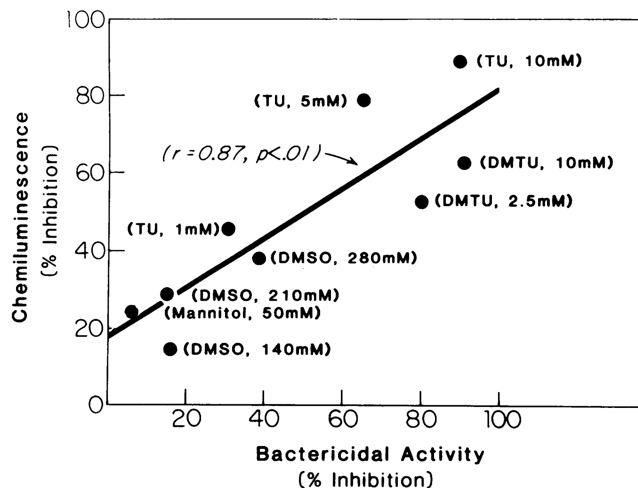


FIG. 1. Significant correlation ($r = 0.87$; $P < 0.01$) occurring between the percent inhibitions seen in CL and bactericidal activity after the addition of hydroxyl radical scavengers (thiourea [TU], dimethyl thiourea [DMTU], DMSO, and mannitol) to mixtures of neutrophils and *S. aureus* 502A.

is likely that ·OH is probably formed in some part from the reaction of $O_2^-$ and $H_2O_2$. However, the inability of SOD or catalase or both to penetrate intracellularly may be an important consideration in studies which use SOD or catalase to elucidate the contributions of various oxygen radicals to CL or bactericidal activity by neutrophils.

The results of the present study also provide additional evidence to support the use of assay to CL as an indicator of the bactericidal activity of neutrophils. The close correlation between the inhibitory effects of ·OH scavengers on neutrophil bactericidal activity and CL observed in the present investigation can be added to previous observations made with normal and chronic granulomatous disease neutrophils to support the belief that CL is a good indicator of neutrophil bactericidal capability. It is also important to note that the degree of inhibition produced by these ·OH scavengers closely paralleled their reported ability to scavenge ·OH in other systems (8, 9).

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## LITERATURE CITED


1. **Allen, R. C., R. L. Stjernholm, and R. H. Steele.** 1972. Evidence for generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. **47**:679–684.
2. **Cheson, B. D., R. L. Christensen, R. Sperling, B. E. Kohler, and B. M. Babior.** 1976. The origin of the chemiluminescence of phagocytosing granulocytes. J. Clin. Invest. **58**:789–796.
3. **Cheung, K., A. C. Archibald, and M. F. Robinson.** 1983. The origin of chemiluminescence produced by neutrophils stimulated by opsonized zymosan. J. Immunol. **130**:2324–2329.
4. **Clawson, C. C., and J. E. Repine.** 1976. Quantitation of the maximal bactericidal capability in human neutrophils. J. Lab. Clin. Med. **88**:316–327.
5. **DeChatelet, L. R., G. D. Long, P. S. Shirley, D. A. Bass, M. J. Thomas, F. W. Henderson, and M. S. Cohen.** 1982. Mechanism of the luminol-dependent chemiluminescence of human neutrophils. J. Immunol. **129**:1589–1593.
6. **McPhail, L. C., L. R. DeChatelet, and R. B. Johnston, Jr.** 1979. Generation of chemiluminescence by a particulate fraction isolated from human neutrophils. J. Clin. Invest. **63**:648–655.
7. **Repine, J. E., C. C. Clawson, and P. S. Friend.** 1977. Influence of a deficiency of the second complement on the bactericidal activity of neutrophils *in vitro*. J. Clin. Invest. **59**:802–809.
8. **Repine, J. E., J. W. Eaton, M. W. Anders, J. E. Hoidal, and R. B. Fox.** 1979. Generation of hydroxyl radical by enzymes, chemicals and human phagocytes *in vitro*: detection using the anti-inflammatory agent—dimethyl sulfoxide. J. Clin. Invest. **64**:1642–1651.
9. **Repine, J. E., R. B. Fox, and E. M. Berger.** 1981. Hydrogen peroxide kills *Staphylococcus aureus* by reacting with staphylococcal iron to form hydroxyl radical. J. Biol. Chem. **256**:7094–7097.
10. **Repine, J. E., J. R. Hoidal, G. D. Beall, F. L. Rasp, D. P. Clifford, J. W. Eaton, S. Davies, C. C. Clawson, J. G. White, and R. B. Fox.** 1979. The effect of temperature on chemiluminescence by polymorphonuclear leukocytes *in vitro*, p. 725–736. *In* W. S. Caughy (ed.), Biochemical and clinical aspects of oxygen—1977. Academic Press, Inc., New York.
11. **Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes.** 1974. Effects of phorbol myristate acetate on the metabolism and ultrastructure of neutrophils in chronic granulomatous

disease. J. Clin. Invest. **54**:83–90.

12. **Root, R. K., and M. S. Cohen.** 1981. The microbicidal mechanisms of human neutrophils and eosinophils. Rev. Infect. Dis. **3**:565–598.
13. **Rosen, H., and S. J. Klebanoff.** 1976. Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. J. Clin. Invest. **58**:50–60.
14. **Stevens, P., D. J. Winston, and K. Van Dyke.** 1978. In vitro evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutropenia and cellular deficiency states. Infect. Immun. **22**:41–51.