

Free Radicals and Inflammation

PROTECTION OF PHAGOCYTOSING LEUKOCYTES BY SUPEROXIDE DISMUTASE

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ABSTRACT Isolated human polymorphonuclear leukocytes engaged in phagocytosis liberate superoxide radical and hydrogen peroxide into the surrounding medium. These two chemical species react to produce the hydroxyl radical, which attacks the leukocyte and leads to premature death of the cell. The hydroxyl radical may be scavenged by mannitol, or its formation can be prevented by the addition of superoxide dismutase or catalase to the medium, thereby eliminating the premature death of the cells. This phenomenon may partially explain the observed anti-inflammatory activity of superoxide dismutase.

INTRODUCTION

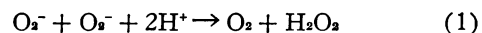
Considerable evidence has accumulated in recent years that the physiological function of superoxide dismutase (SOD)¹ is a protective one (1-6). The reactive free radical substrate O_2^- is generated as a toxic intermediate in a wide variety of biological reactions that reduce molecular oxygen, and several specific mechanisms have been delineated by which its deleterious actions are realized (6, 7). The enzyme scavenges superoxide by catalyzing its dismutation to oxygen and hydrogen peroxide

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¹*Abbreviations used in this paper:* PMN, polymorphonuclear leukocytes; SOD, superoxide dismutase.

(8):



Although it appears that all oxygen-metabolizing cells contain large amounts of SOD, the mammalian extracellular fluids that have been examined contain only traces of the activity (6).

In 1971 a positive identification was made between the enzyme isolated by McCord and Fridovich as superoxide dismutase (8) and a protein isolated several years earlier by Huber and co-workers on the basis of its anti-inflammatory properties (9). This anti-inflammatory protein had been named "orgotein." A relationship between SOD activity and anti-inflammatory activity became obvious with the observation by Babior et al. that phagocytosing polymorphonuclear leukocytes (PMN), effector cells of the acute inflammatory response, release large amounts of superoxide into the medium in which the activated cells are suspended (10). Superoxide production by leukocytes apparently contributes to the killing of ingested bacteria (11). The release of a sizable fraction of the O_2^- into the medium is perhaps an inadvertent and unfortunate side effect of the process (12). It was recently shown that the amount of superoxide radical produced by a concentration of leukocytes such as might be found in an inflamed rheumatoid joint is sufficient to cause extensive and rapid degradation of the joint fluid whereas SOD protected the fluid against degradation (6). The present study illustrates another possible aspect of the anti-inflamma-

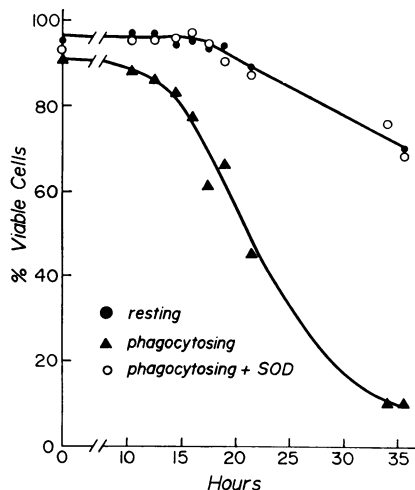


FIGURE 1 Protection of phagocytosing PMN by SOD. Approximately 3×10^6 PMN were incubated at 37°C in 1 ml of Ringer-phosphate buffer, pH 7.4, containing 1% glucose and 5% autologous serum. Those designated "phagocytosing" contained in addition 3×10^7 *E. coli*. Where indicated, SOD was added to a concentration of $300 \mu\text{g/ml}$. At intervals, samples were withdrawn, mixed with 1% trypan blue in 0.9% saline, and the cells examined microscopically. 200 cells were counted and the results expressed as percent cells excluding the dye.

tory action of SOD, namely, the protection of the leukocytes themselves from self-inflicted free radical damage and premature death.

METHODS

Human PMN were isolated from freshly drawn venous blood as previously described (13). The cells were typically greater than 95% viable as judged by their ability to exclude trypan blue (14) and were greater than 98% PMN. 1-ml incubation mixtures contained about 3×10^6 PMN suspended in Ringer-phosphate buffer, pH 7.4, containing 1% glucose and 5% autologous serum. When phagocytosing cells were desired, incubation mixtures contained *Escherichia coli* K-12 that had been washed in deionized water and stored frozen, at a ratio of 10 bacteria per leukocyte. Other additions included, where indicated, bovine liver SOD, prepared as previously described (8), or bovine catalase ($2\times$ crystallized, Sigma Chemical Co., St. Louis, Mo.), bovine serum albumin (Sigma), or mannitol (Sigma). All incubation mixtures were maintained at 37°C in siliconized glass tubes. Longer incubations contained 50 U/ml of penicillin (Sigma) and $50 \mu\text{g/ml}$ streptomycin sulfate (Sigma) to retard extraneous bacterial growth. At the indicated times samples of the mixtures were withdrawn, mixed with a 1% solution of trypan blue, and examined microscopically. The percentage of cells not stained by the dye is represented as "% viable cells." In certain experiments a significant number of dead cells lysed. In these cases, the % viable cells was corrected for the decrease in total cell count.

RESULTS

Fig. 1 shows that "resting" PMN or those incubated in the absence of *E. coli* maintained full ability to exclude

trypan blue for approximately the first 18 h of incubation. Thereafter, the percentage of viable cells slowly decreased, reaching a value of 70% after 35 h. The cells incubated with *E. coli*, designated "phagocytosing," showed a slow loss of viability during the first 15 h of incubation. After that time, loss of viability proceeded rapidly with 90% of the cells dead at 35 h. A third incubation mixture of phagocytosing PMN contained *E. coli* plus SOD at $300 \mu\text{g/ml}$. The addition of the SOD restored the viability of the phagocytosing cells to that of resting cells. Johnston and co-workers (11) found that SOD at comparable concentrations did not interfere with the process of phagocytosis and had little or no effect on bactericidal action by PMN. The concentration of SOD required to produce maximal protection was determined by incubating phagocytosing suspensions of PMN with various concentrations of the enzyme at 37°C for 40 h and by observing the viability of the cells at the end of the incubation. These data are shown in Fig. 2. The maximal protective effect was attained at $200 \mu\text{g}$ of SOD per ml. After prolonged incubation the pH of the mixtures dropped less than 1 U, and this was not affected by the presence of protectants.

The nature of the protection afforded by SOD was further explored by incubation mixtures containing catalase or mannitol rather than SOD. Haber and Weiss proposed in 1934 a reaction between O_2^- and H_2O_2



by which the very powerful oxidizing radical $\text{OH}\cdot$ is produced (15). Several superoxide-dependent phenomena have been shown to result from $\text{OH}\cdot$ produced secondarily from superoxide by this reaction, including bac-

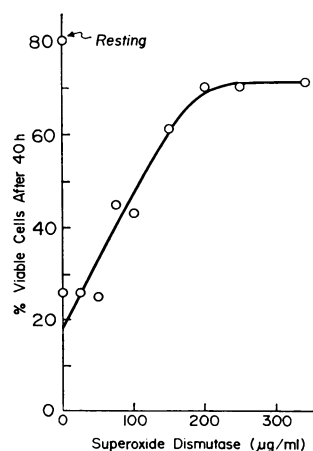


FIGURE 2 Effect of SOD concentration on maintenance of PMN viability. Conditions and procedures were as described in Fig. 1, except that SOD concentration was varied as indicated, and all incubations were examined only after 40 h. All cells were phagocytosing except the point labeled "Resting."

tericidal action by leukocytes (6, 11, 16). Thus, it was a possibility that the chemical species causing the loss of PMN viability was the hydroxyl radical, $\text{OH}\cdot$. The production of $\text{OH}\cdot$ by reaction 2 can be prevented by the action of SOD or of catalase, either of which halts the reaction by scavenging a reactant. Alternatively, the $\text{OH}\cdot$ may be scavenged as it is formed by such agents as mannitol (17). Fig. 3 shows that SOD, catalase, or mannitol maintained the viability of phagocytosing PMN equal to that of resting cells. The failure of bovine serum albumin to protect the cells rules out any nonspecific protein effect, as does the fact that the incubation mixtures contained 5% autologous serum. We therefore conclude that the agent causing the death of the cells is the hydroxyl radical produced secondarily from the superoxide radical. Superoxide radical, per se, which was not scavenged in those incubation mixtures containing mannitol or catalase, did not cause the premature death of the cell. Likewise, hydrogen peroxide, per se, which was not scavenged in those incubation mixtures containing mannitol or SOD, did not cause the premature death of the cell. Only when O_2^- and H_2O_2 are simultaneously present is hydroxyl radical formation possible, and only under these conditions was premature death following phagocytosis observed.

The time-course of cell death varied considerably for cells obtained from different donors, but the maximal rate of cell death was always seen to take place between 12 and 30 h after the phagocytic challenge. This delayed death was not anticipated since the metabolic burst of O_2^- production was shown to occur in the 30 min after phagocytosis. After 30 min the O_2^- production of the cells returns to the level of resting cells (10, 12, 18). Thus, it appeared that the cells were "mortally wounded" by the initial burst of radical production but that death did not result from this damage until sometime later. To test this hypothesis, PMN were induced to phagocytose

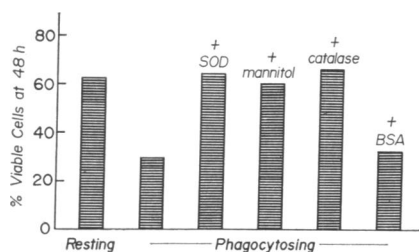


FIGURE 3 Protection of phagocytosing cells by SOD, mannitol, and catalase. Incubation mixtures were as described in Fig. 1 with the following additions where indicated: SOD, 250 $\mu\text{g}/\text{ml}$; mannitol, 1 mM; catalase, 250 $\mu\text{g}/\text{ml}$; bovine serum albumin (BSA), 250 $\mu\text{g}/\text{ml}$. Viability was determined after 48 h. These results represent the average of three separate experiments. Percent viability for unprotected cells was $32 \pm 8.5\%$ ($n = 6$), and for protected phagocytosing cells was $65 \pm 5\%$ ($n = 9$).

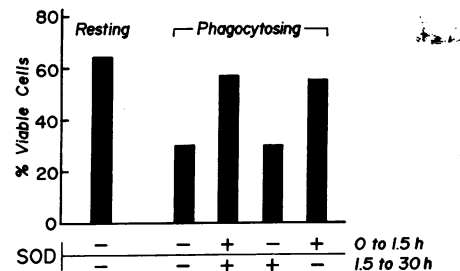


FIGURE 4 Time-course of protection of phagocytosing PMN by SOD. Conditions were as described in Fig. 1. After allowing 1.5 h for the completion of phagocytic activity, the cells were centrifuged at 750 g for 5 min and resuspended in fresh medium, then incubated an additional 28.5 h. SOD was present, when and where indicated (+), at 300 $\mu\text{g}/\text{ml}$. The percentage of viable cells at the end of the incubation was based on the number of viable cells present at $t = 0$. These data represent the results of a single experiment. The phenomenon was also observed with a 2 h initial incubation period.

in the presence and absence of SOD. After 1.5 h, when superoxide production would have ceased, samples of each incubation mixture were gently centrifuged down and resuspended in media with or without SOD for the remainder of the observation period. The results of this experiment, seen in Fig. 4, clearly show that cell death can be prevented only if SOD is present during that part of the incubation in which the O_2^- production takes place and that SOD cannot prevent cell death if added after the exposure to superoxide has occurred.

DISCUSSION

Results identical with those described above were obtained by stimulating the PMN with serum that had been incubated with bacteria, then centrifuged to remove the bacteria themselves before being added to the PMN. That is, the presence of particulate matter for inclusion into phagocytic vacuoles was not necessary for the observance of the premature death phenomenon or for its prevention by SOD. This finding rules out the possibility that the leukocyte death might be related to bacterial metabolic events taking place within the phagocytic vacuoles. It has been shown that "activated" serum (18) and other soluble activating factors such as endotoxin or lymphokine can induce superoxide production in PMN in the absence of particulate matter (12).

Fig. 2 indicates that a concentration of SOD of 200 $\mu\text{g}/\text{ml}$ was required to produce a maximal protective effect. Other phenomena thought to proceed by essentially the same mechanism (i.e., hydroxyl radical production via reaction 2) have been approximately 100-fold more sensitive to inhibition by SOD (6, 16). This difference may be rationalized by realizing that the events taking place in the present study are not occurring in free solu-

tion, as in the previous studies, but are occurring in a thin shell of solvent in contact with the outer surface of the plasma membrane of the PMN. Thus, a small fraction of the total SOD may have to compete for superoxide with very high local concentrations of H_2O_2 . The inhibition of nitroblue tetrazolium reduction by PMN, a process brought about by the superoxide generated at the cell surface, requires a concentration of SOD in excess of 100 $\mu\text{g}/\text{ml}$ for maximal inhibition (12). The high concentration of SOD required to inhibit events taking place at the cell surface may also be related to the fact that both the SOD and the cell surface are negatively charged at the pH of these studies.

These studies have demonstrated a protective effect of SOD, catalase, and mannitol on the viability and integrity of phagocytosing PMN in vitro. This phenomenon of protection of cells against free radical death may contribute to the clinically observable anti-inflammatory activity of SOD (19, 20). It is clear that the death of leukocytes and the concomitant or subsequent release of hydrolytic enzymes and chemotactic factors play major roles in the perpetuation of the inflammatory cycle (21). The cells of surrounding healthy tissue would, of course, also be subject to serious insult leading to death and lysis, either from primary attack by the radicals in the interstitial fluid or from secondary attack by hydrolytic enzymes released from lysed PMN. By intravenous injection, it may not be possible to maintain the necessary concentration of SOD in the extracellular fluids to protect PMN from their own free radical-induced destruction, since the protein is cleared quite rapidly from the circulation.² By local injection, however, such a concentration may be realistically achieved at the site of the inflammation (19, 20). The preparation of derivatives of SOD with longer circulating half-lives is currently under investigation.

The data reported herein and elsewhere (6) support the notion that the superoxide radical per se may not be so detrimental a species in a physiological context. Through its reaction with H_2O_2 (reaction 2), however, it is capable of generating another radical species, $OH\cdot$, which is apparently capable of causing damage of grave consequence to physiological systems. Because of the nearly complete absence of SOD and catalase in extracellular fluids, the generation of $OH\cdot$ in vivo seems a real possibility. In the present case, the chemical nature of the damage to the PMN is unknown. The site of the lethal damage appears to have been the outer surface of the plasma membrane, since extracellular SOD and catalase prevented it.

² Dr. Wolfgang Huber. Personal communication.

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REFERENCES

- McCord, J. M., B. B. Keele, Jr., and I. Fridovich. 1971. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 1024-1027.
- McCord, J. M., C. O. Beauchamp, S. Goscin, H. P. Misra, and I. Fridovich. 1973. Superoxide and superoxide dismutase. In *Oxidases and Related Redox Systems*. T. E. King, H. S. Mason, and M. Morrison, editors. University Park Press, Baltimore, Md. **1**: 51-76.
- Lavelle, F., A. M. Michelson, and L. Dimitrijevic. 1973. Biological protection by superoxide dismutase. *Biochem. Biophys. Res. Commun.* **55**: 350-357.
- Michelson, A. M., and M. E. Buckingham. 1974. Effects of superoxide radicals on myoblast growth and differentiation. *Biochem. Biophys. Res. Commun.* **58**: 1079-1086.
- Petkau, A., and W. S. Chelack. 1974. Protection of *Acholeplasma laidlawii* B by superoxide dismutase. *Int. J. Radiat. Biol.* **26**: 421-426.
- McCord, J. M. 1974. Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science (Wash. D. C.)*. **185**: 529-531.
- Fridovich, I. 1974. Superoxide dismutases. *Adv. Enzymol.* **41**: 35-97.
- McCord, J. M., and I. Fridovich. 1969. Superoxide dismutase. An enzymic function for erythrocyte (hemocuprein). *J. Biol. Chem.* **244**: 6049-6055.
- Carson, S., E. E. Vogin, W. Huber, and T. L. Schulte. 1973. Safety tests of orgotein, an antiinflammatory protein. *Toxicol. Appl. Pharmacol.* **26**: 184-202.
- Babior, B. M., R. S. Kipnes, and J. T. Curnutte. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **52**: 741-744.
- Johnston, R. B., Jr., B. B. Keele, Jr., H. P. Misra, L. S. Webb, J. E. Lehmeyer, and K. V. Rajagopalan. 1975. Superoxide anion generation and phagocytic bactericidal activity. In *The Phagocytic Cell in Host Resistance*. J. A. Bellanti and D. H. Dayton, editors. Raven Press, New York. 61-75.
- McCord, J. M., and M. L. Salin. 1975. Free radicals and inflammation: studies on superoxide-mediated NBT reduction by leukocytes. In *Erythrocyte Structure and Function*. G. J. Brewer, editor. Alan R. Liss, Inc., New York. 731-746.
- Salin, M. L., and J. M. McCord. 1974. Superoxide dismutase in polymorphonuclear leukocytes. *J. Clin. Invest.* **54**: 1005-1009.
- Fallon, H. J., E. Frei, III, J. D. Davidson, J. S. Trier, and D. Burk. 1962. Leukocyte preparations from human blood: evaluation of their morphologic and metabolic state. *J. Lab. Clin. Med.* **59**: 779-791.
- Haber, F., and J. Weiss. 1934. The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. R. Soc. Ser. A*. **147**: 332-351.
- Beauchamp, C., and I. Fridovich. 1970. A mechanism for the production of ethylene from methional. The generation of the hydroxy radical by xanthine oxidase. *J. Biol. Chem.* **245**: 4641-4646.

17. McCord, J. M., and I. Fridovich. 1973. Production of O_2^- in photolyzed water demonstrated through the use of superoxide dismutase. *Photochem. Photobiol.* 17: 115-121.
18. Curnutte, J. T., and B. M. Babior. 1974. Biological defense mechanisms. The effect of bacteria and serum on superoxide production by granulocytes. *J. Clin. Invest.* 53: 1662-1672.
19. Lund-Olesen, K., and K. B. Menander. 1974. Orgotein: a new anti-inflammatory metalloprotein drug: preliminary evaluation of clinical efficacy and safety in degenerative joint disease. *Curr. Ther. Res. Clin. Exp.* 16: 706-717.
20. Marberger, H., W. Huber, G. Bartsch, T. Schulte, and P. Swoboda. 1974. Orgotein: a new antiinflammatory metalloprotein drug evaluation of clinical efficacy and safety in inflammatory conditions of the urinary tract. *Int. Urol. Nephrol.* 6: 61-74.
21. Hirsch, J. G. 1974. Neutrophil leukocytes. In *The Inflammatory Process*. B. W. Zweifach, L. Grant, and R. T. McClusky, editors. Academic Press, Inc., New York. 2nd edition 1: 411-447.