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Inhibition of Phagocytosis-Associated Chemiluminescence by Superoxide Dismutase

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During the process of phagocytosis, human leukocytes emit a burst of luminescence which can be measured in a liquid scintillation spectrometer. The enzyme superoxide dismutase, which removes superoxide anions ($O_2^-\cdot$), inhibited this chemiluminescence by 70% at a concentration of 100 μg/ml. The enzyme did not inhibit phagocytosis. These results support other studies indicating that $O_2^-\cdot$ is elaborated by phagocytizing leukocytes. They also indicate that $O_2^-\cdot$ plays a major role in phagocytosis-associated chemiluminescence, though not necessarily as the luminescing agent. Catalase and benzoate inhibited the chemiluminescence of phagocytosis to a slight extent, suggesting that hydrogen peroxide and hydroxyl radical, respectively, might also be involved in this phenomenon. The relationship between the mediators of chemiluminescence and those responsible for phagocytic bactericidal activity remains to be defined.

Oxidation reactions involving ground-state molecular oxygen are believed to proceed frequently by one-electron steps that result in production of free radicals, including superoxide anion ($O_2^-\cdot$) (25). The capacity to generate $O_2^-\cdot$ has been demonstrated to exist in plants, bacteria, fungi, and most animal tissues (8). The enzyme superoxide dismutase appears to be responsible for protecting the cell from its own superoxide anion (17) by catalyzing the reaction:

$$O_2^-\cdot + O_2^-\cdot + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

That a product of this disproportionation, hydrogen peroxide, is involved in the killing of bacteria by phagocytes has been well substantiated (15). We have recently demonstrated that the killing of ingested bacteria can be inhibited by superoxide dismutase, suggesting that superoxide anion is also required for this activity (R. B. Johnston, Jr., B. Keele, L. Webb, D. Kessler, and K. V. Rajagopalan. J. Clin. Invest. **52**:44a, 1973; B. B. Keele, Jr., R. B. Johnston, Jr., K. V. Rajagopalan, and D. Kessler. Proc. Ninth Int. Cong. Biochem., 1973).

Allen et al. (1) have reported that polymorphonuclear leukocytes emit flashes of luminescence during phagocytosis of bacteria. This chemiluminescence, which correlates with glucose oxidation through the hexose monophosphate shunt (1), can be quantitated in a liquid scintillation spectrometer. We report here that superoxide dismutase markedly inhibits the chemiluminescence associated with phagocytosis.

MATERIALS AND METHODS

Enzyme and benzoate preparations. Superoxide dismutase was purified from bovine erythrocytes (16). Bovine liver catalase (EC 1.11.1.6) and bovine serum albumin (BSA) were purchased from Sigma Chemical Company, St. Louis, Mo. Catalase was separated from contaminating superoxide dismutase by gel chromatography by using Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, N.J.) in 0.05 M potassium phosphate buffer with 0.1 M KCl, pH 7.8. The catalase was demonstrated to be free of superoxide dismutase activity by using the aerobic xanthine oxidase assay (16). All three proteins were suspended in saline (0.15 M NaCl) to a concentration of 1 mg/ml and sterilized by filtration (Millipore Corp., Bedford, Mass.; 0.22-μm diameter pore size).

Sodium benzoate (Fisher Chemical Co., Fair Lawn, N.J.) was dissolved in water to a concentration of 0.2 M. The conductivity of this solution approximated that of 0.15 M NaCl, which was used as control.

Chemiluminescence assay. Phagocytes (primarily polymorphonuclear neutrophils) were separated from heparinized venous blood, washed, and suspended in Krebs Ringer phosphate buffer, pH 7.4, with 0.2% BSA and 0.2% dextrose (KRP-DA buffer), as previously described (12). Zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared by the method of Wardlaw and Pillemer (26) and suspended in saline to a concentration of 50 mg/ml. One volume of zymosan was incubated with three volumes of fresh normal human serum for 30 min at 37 C. The opso-

nized zymosan was then centrifuged and resuspended in KRP-DA buffer to the total combined volume of zymosan and serum.

The reaction mixture consisted of 0.4 ml opsonized zymosan suspension, the enzymes, benzoate, or their controls, and KRP-DA buffer to a volume of 6.0 ml. To this mixture 10^7 phagocytes in 1.0 ml KRP-DA buffer were added to begin the reaction. The leukocytes were kept at 4 C until addition; all of the other components were at ambient temperature. The phagocyte-to-particle ratio was approximately 1:55.

The reaction took place in siliconized liquid scintillation counting vials (20 ml capacity, Scientific Products, McGaw Park, Ill.). Phagocytes were added at 1-min intervals, the contents of the vial were mixed with a Vortex mixer, and the vial was immediately placed in the chamber for the initial (time zero) 1 min reading. Ater each count, the vial was mixed again and placed in the last position to be counted. The vials were mixed again approximately 20 s before each subsequent count.

Luminescence measurements were made with a Beckman LS-150 ambient-temperature liquid scintillation counter in the tritium region of the spectrum. The coincidence circuit was turned off, and only the front photomultiplier tube was activated (20). Counting vials were wrapped in aluminum foil and stored in the dark for at least 18 h before use. The vials were exposed only to red light during the experiment.

The degree of inhibition was reported as the percentage by which the chemiluminescence at its highest point was reduced by the test material, after the chemiluminescence obtained with resting (noningesting) phagocytes was subtracted from both values.

Phagocytosis assays. The uptake of zymosan by phagocytes was studied in the presence of the enzymes, benzoate, or their controls by two techniques. For both, the reaction mixture and general procedure were the same as those described above for determining chemiluminescence. However, in the first technique, each vial was sampled after incubation at room temperature for 24 min, and smears were made and stained for light microscopy. The stained smears were coded and the number of ingested zymosan particles per 250 phagocytes (polymorphonuclear leukocytes, monocytes, and eosinophils) was determined without knowledge of the contents of the reaction vial from which the slides were made. At least four different smears were read for each vial.

In the second technique the uptake of ^{125}I-labeled zymosan particles was compared in the presence of equal concentrations of superoxide dismutase and BSA. Zymosan was labeled by a modification of the method of Francis et al. (7). To 3 ml of the zymosan suspension in phosphate-buffered saline (PBS, 0.15 M, pH 7.42) were added 1.5 ml of 0.01 M KI in PBS and 0.5 mCi of sodium ^{125}I (New England Nuclear Corp., Boston). Three milliliters of ammonium persulfate, 10 g per 100 ml of PBS, was slowly added to the mixture over a 1-min interval with constant gentle shaking. The mixture was incubated at room temperature overnight and then centrifuged, washed once, resuspended to 3 ml with PBS, and dialyzed against PBS until the dialysate was free of radioactivity.

Phagocytosis was allowed to occur at room temperature for 15 min, and then the vials were placed quickly into an ice bath. To separate uningested [^{125}I]zymosan particles from phagocytized particles, velocity sedimentation in an isokinetic gradient was employed. The separation was kindly performed by T. G. Pretlow II and D. E. Luberoff, University of Albama Medical Center, using previously described techniques (18). The entire contents of a vial were gently layered on top of a linear density gradient of sterile Ficoll (Pharmacia) in Joklik tissue-culture medium, the layers varying in density from 2.7 to 5.5% (wt/wt) Ficoll (18). Gradients were centrifuged at $97 \times g$, at 4 C for 14 min, the speed of the centrifuge being precisely monitored with an electronic stroboscope. After centrifugation, 4-ml fractions were collected by displacement of the gradients with a dense sucrose solution by using a tapping cap described and illustrated previously (19). Cell counts of all fractions were performed by using hemocytometer chambers. Slides for microscope examination were prepared from 0.3 ml of the fraction by cytocentrifugation and stained with Wright stain. The entire remaining fraction was centrifuged at $500 \times g$ for 15 min, and the radioactivity of the centrifugate was determined in a well detector and counter (model 8725, Nuclear-Chicago, Des Plaines, Ill.).

RESULTS

Examples of the chemiluminescence elaborated by normal human leukocytes during phagocytosis of opsonized zymosan particles are represented by the top lines of Fig. 1, 2, and 3. In the presence of BSA (200 μg/ml) or 0.15 M saline (as controls), the luminescence rose rapidly from a background level of approximately 8.0×10^3 counts/min at time zero to a maximum of 1.5×10^5 to 2.1×10^5 counts/min at 8 to 18 min, after which it gradually fell during the remainder of the incubation period. The time at which peak chemiluminescence was reached was delayed when fewer zymosan particles and leukocytes were used (in the same phagocyte-to-particle ratio), in agreement with Allen et al. (1), or when less zymosan was used. When zymosan particles were omitted from the reaction mixture, chemiluminescence remained at a baseline level of 3,000 to 10,000 counts/min without significant variation during the 54-min incubation.

When BSA was replaced by an equal concentration of superoxide dismutase (200 μg/ml), peak luminescence was significantly decreased (Fig. 1). The reduction here represents inhibition of about 72% of the chemiluminescence recorded with the control. In a total of 11 experiments, superoxide dismutase in a concentration of 100 μg/ml inhibited peak luminescence by 65 to 76% (mean, 70%). Inhibition by enzyme at this concentration was only slightly

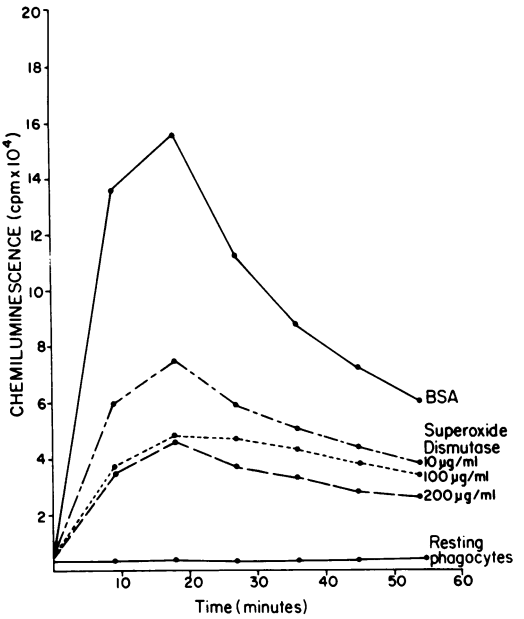

FIG. 1. *Inhibition of phagocytosis-associated chemiluminescence by various concentrations of superoxide dismutase. The extent of chemiluminescence, recorded in counts per minute (cpm), is plotted as a function of time. Protein concentrations were made equal in each vial by the addition of BSA in 0.15 M saline. Inhibition at the peak of luminescence (at 18 min) was 52, 70, and 72% for enzyme concentrations of 10, 100, and 200 µg/ml, respectively.*

less than that obtained at a concentration of 200 µg/ml (Fig. 1); increasing superoxide dismutase to as high as 300 or 400 µg/ml did not significantly increase inhibition over that achieved with 100 to 200 µg/ml, 73 and 78% inhibition being obtained, respectively. As shown in Fig. 1, smaller concentrations of the enzyme inhibited less; however, at concentrations as low as 1 µg/ml, 20 to 35% inhibition was still achieved (data not shown). Heat denaturing the enzyme by autoclaving it for 15 min at 19 lb/in², 124 C, eliminated all but approximately 10% of its inhibitory effect.

Much less inhibition of chemiluminescence was achieved by catalase in concentrations similar to those used for superoxide dismutase (Fig. 2). The inhibition demonstrated here with 200 µg of catalase per ml was 27%. At this concentration, inhibition fell within a range of 16 to 34% of its control in five experiments (mean, 25%). No significant further inhibition was achieved by catalase at 300 µg/ml concentration.

When superoxide dismutase and catalase were both present in the reaction mixture, the extent of inhibition achieved was only slightly greater than that achieved by superoxide dismutase alone and considerably less than the sum of the inhibition obtained by either enzyme alone (Table 1). This held true even at the optimal inhibitory concentrations of both proteins (100 to 200 µg/ml).

Chemiluminescence observed in the presence of concentrations of 0.01 M and 0.02 M sodium benzoate is illustrated in Fig. 3. Inhibition of chemiluminescence by the higher concentration of benzoate varied from 20 to 38% in four experiments, the mean value being 29% inhibition.

To determine whether the decreased chemiluminescence found with superoxide dismutase, catalase, or benzoate could be due to decreased ingestion of zymosan, smears were made of the chemiluminescence assay mixture for visual determination of ingested particles (Table 2). By this technique there was no significant suppression of phagocytosis of zymosan particles by concentrations of superoxide dismutase, catalase, or benzoate which were maximally inhibitory in the chemiluminescence assay.

Ingestion in the presence of superoxide dismutase and BSA was also compared by using ^{125}I-labeled zymosan. Phagocytized particles were separated from uningested ones by velocity sedimentation in an isokinetic gradient of Ficoll (18). When the radioactivity found in phago-

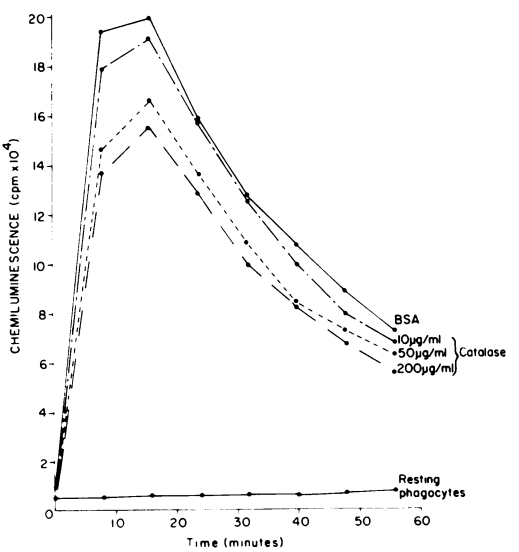

FIG. 2. *Inhibition of phagocytic chemiluminescence by catalase. Protein concentrations were made equal by the addition of BSA in saline. Inhibition by catalase was 5, 17, and 27% at concentrations of 10, 50, and 200 µg/ml, respectively.*

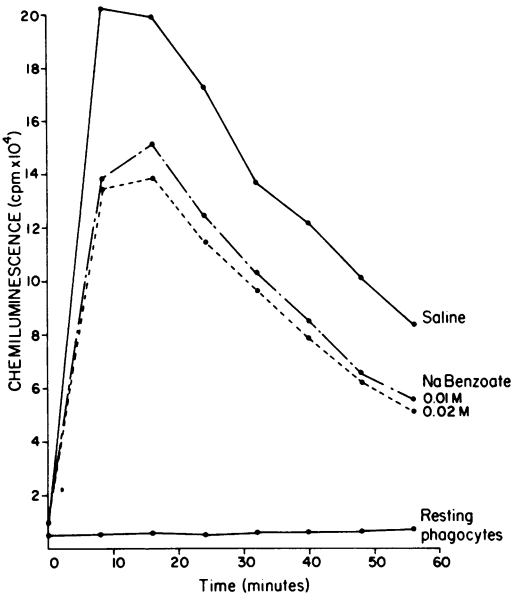

FIG. 3. *Inhibition of phagocytic chemiluminescence by sodium benzoate, a scavenger of hydroxyl radical (·OH). The conductivity of the benzoate solutions was made equal to that of the saline control by the addition of NaCl. Inhibition at 16 min by 0.01 M benzoate was 25%, and by 0.02 M benzoate, 31%.*

TABLE 1. *Inhibition of phagocytosis-associated chemiluminesence by superoxide dismutase and catalase present together in the reaction mixture*

Protein concn (μg/ml)		Inhibition (%)
SOD[a]	Catalase	
1		29
	20	10
	50	15
1	20	31
1	50	32
4		46
	20	18
	50	20
4	20	48
4	50	54
100		70
	200	34
100	200	78

[a] SOD, Superoxide dismutase.

cyte-containing fractions (13 to 21) was plotted for the two reaction mixtures, the distribution of counts was almost identical (Fig. 4), and the total radioactivity of the peak fractions (20 and 21) was equal in the presence of superoxide dismutase or BSA (2.01×10^4 counts/min). On microscope examination of smears of each fraction, uningested zymosan appeared only in the first 12 fractions. The total radioactivity of these fractions was also very similar for the two assay mixtures: 2.09×10^5 counts/min for the enzyme preparation and 2.03×10^5 counts/min for the BSA preparation.

DISCUSSION

The involvement of superoxide anion in oxidation-reduction reactions of biological significance has been widely supported (8). Babior et al. have reported the extracellular increase of superoxide anion during the process of phagocytosis and have suggested that this free radical might be involved in the phagocytic killing of bacteria (3). In agreement with this concept, we have demonstrated inhibition of the bactericidal activity of neutrophils by superoxide dismutase (R. B. Johnston, Jr. et al., J. Clin. Invest. **52**:44a, 1973). The experiments reported here offer additional evidence that $O_2^-\cdot$ is elaborated by the phagocytizing leukocyte.

The association of chemiluminescence with phagocytosis was reported by Allen et al. (1). It has been shown that leukocytes from patients with chronic granulomatous disease, which can ingest but cannot kill bacteria normally, fail to emit chemiluminescence (24) or elaborate $O_2\cdot$ (5) (R. B. Johnston, Jr., B. B. Keele, Jr., and R. L. Baehner, unpublished data). The inhibition of chemiluminescence by superoxide dismutase further supports an interrelationship between superoxide anion, chemiluminescence, and bactericidal activity.

The inhibition by superoxide dismutase of 70 to 75% of the chemiluminescence associated with phagocytosis suggests that $O_2^-\cdot$ generation is essential to the development of most, if not all, of the luminescence. However, we have no evidence to indicate that $O_2^-\cdot$ is the actual luminescing agent. Allen et al. believe singlet oxygen ($^1O_2$) to be that agent (1). The presence

TABLE 2. *Uptake of zymosan particles in the presence of superoxide dismutase, catalase, benzoate, or their controls*

Chemiluminescence inhibitor or its control	No. of particles per phagocyte
Superoxide dismutase[a]	2.49
BSA[a]	2.44
Catalase[a]	3.29
BSA[a]	3.34
Sodium benzoate	
0.02 M	3.08
0.01 M	2.98
Saline	3.07

[a] At a concentration of 200 μg/ml.

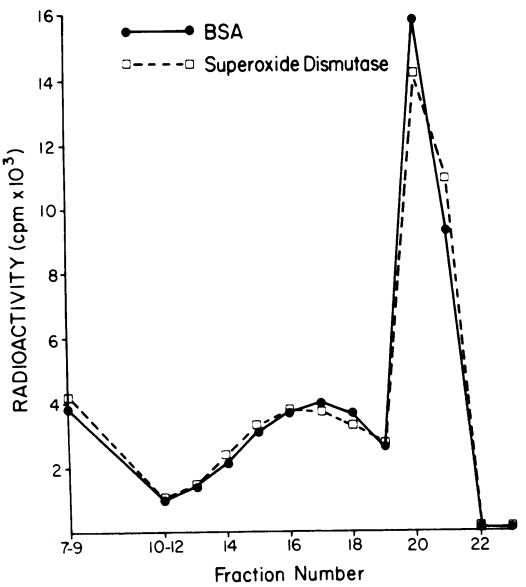

FIG. 4. *Comparison of the distrubition of ^{125}I-labeled zymosan particles in fractions of chemiluminescence assay mixtures containing superoxide dismutase or BSA. Phagocytes were allowed to ingest radiolabeled zymosan in the presence of the enzyme or an equal concentration of BSA (100 μg/ml). The reaction mixtures were then fractionated by sedimentation at 97 × g through a Ficoll gradient. Radioactivity present in each fraction is plotted. Mean values are given for fractions 7 to 9 and 10 to 12, which were pooled. In fractions 20 and 21, 98 to 100% of the cells were phagocytes; 94 to 97% of these contained at least one zymosan particle.*

of this excited form of oxygen has not been clearly demonstrated in biological systems, but it appears reasonably well established that this species participates in ordinary chemical reactions involving molecular oxygen (14).

Khan has shown that potassium superoxide evolves $^1O_2$ in dimethylsulfoxide solution (13). Several laboratories have shown that the xanthine oxidase-catalyzed oxidation of xanthine by molecular oxygen, which generates $O_2^-\cdot$, can generate chemiluminescence (2, 6, 10, 23). Inhibition of this luminescence by superoxide dismutase has also been shown (2, 6, 9). Thus two $O_2^-\cdot$ radicals might produce a molecule of oxygen in the excited singlet state according to the reaction:

$$O_2^-\cdot + O_2^-\cdot \rightarrow O_2^{2-} + {}^1O_2 \qquad (2)$$

$$\downarrow 2H^+$$

$$H_2O_2$$

as proposed by Stauff (21–23). The enzyme-catalyzed dismutation does not give rise to $^1O_2$ (6, 9), which could explain the inhibition of chemiluminescence by superoxide dismutase.

A second possible explanation of the relationship between $O_2^-\cdot$ and chemiluminescence has been offered by Arneson. Using the xanthine-xanthine oxidase system to generate $O_2^-\cdot$, he noted that the addition of H_2O_2 greatly enhanced chemiluminescence (2). He suggested that some of the $O_2^-\cdot$ produced by this enzyme system is converted to hydroxyl radical ($\cdot OH$) by the cycle of Haber and Weiss (11):

$$O_2^-\cdot + H_2O_2 + H^+ \rightarrow \cdot OH + H_2O + O_2 \qquad (3)$$

$$\cdot OH + H_2O_2 \rightarrow O_2^-\cdot + H_2O + H^+ \qquad (4)$$

and that the reaction responsible for luminescence in this system would be:

$$O_2^-\cdot + \cdot OH \rightarrow OH^- + {}^1O_2 \qquad (5)$$

The relatively slight but consistent inhibition achieved with catalase in our experiments is compatible with the concept that H_2O_2 is involved in the luminescence emitted by leukocytes, perhaps through the generation of $\cdot OH$ according to reactions 3 and 4. That $\cdot OH$ is involved in the generation of chemiluminescence is suggested by the consistent inhibition achieved with sodium benzoate, an $\cdot OH$ scavenger (4). Also compatible with this possibility is the finding that even high concentrations of catalase in combination with superoxide dismutase inhibited phagocytosis-associated chemiluminescence only slightly more than did superoxide dismutase alone (Table 1), as if, in this system, the two enzymes act primarily to interfere with the same pathway of luminescence generation. On the other hand, inhibition by benzoate was considerably less than that achieved by superoxide dismutase, suggesting that most phagocytic chemiluminescence is generated independently of $\cdot OH$. Whether reaction 2, reaction 5, or another reaction is responsible for the chemiluminescence of phagocytosis remains to be proven.

Allen and co-workers showed that emission of luminescence by phagocytizing cells correlated with activity of the hexose monophosphate shunt in these cells (1). The precise relationships between chemiluminescence and particle ingestion must be defined. However, this technique offers promise of being a relatively simple, inexpensive, rapid, and reproducible assay of phagocytosis.

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