Superoxide Dismutase Activity in Leukocytes

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ABSTRACT Superoxide dismutase activity has been identified in both human neutrophils and rabbit alveolar macrophages by two distinct assay procedures. The enzyme is insensitive to both cyanide and azide and is present in the cytosol of the cell. The identification of this enzyme in phagocytic cells is compatible with the theory that superoxide anion might be involved in the bactericidal activity of the cell. It is proposed that the enzyme functions to protect the cell against superoxide generated during the phagocytic process.

INTRODUCTION

During the process of phagocytosis by both neutrophils and macrophages there are profound changes in oxidative metabolism (1). Attention has centered about one such change, the increase in hydrogen peroxide production, as being central to the actual bactericidal activity, either by iodination of the ingested bacterium (2) or by the production of aldehydes from amino acids (3).

Recently Babior, Kipnes, and Curnette (4) have demonstrated that leukocytes can produce the highly reactive superoxide anion (O_2^-) and proposed this species as a likely candidate for a bactericidal agent. This possibility is supported by the demonstration that superoxide production is increased by the phagocytosis of latex particles (4), and by the observation that granulocytes obtained from patients with chronic granulomatous disease do not produce detectable quantities of superoxide (5). Johnston, Keele, Webb, Kessler, and Rajagopalan have reported that purified superoxide dismutase can inhibit leukocytic bactericidal activity (6).

If the leukocyte produces superoxide in sufficient quantity to play a significant role in the bactericidal process, then it seems reasonable that the cell should possess the means to protect itself against the toxic effects of this highly reactive molecule. The metalloenzyme superoxide dismutase, identified in a number of both mammalian and bacterial cells, has been postulated to mediate such protection (7). The present communication describes the identification of superoxide dismutase in sonicated extracts of human peripheral polymorphonuclear leukocytes and BCG-activated rabbit alveolar macrophages.

METHODS

Isolation of cells. Alveolar macrophages were harvested from the lungs of New Zealand white rabbits by the lung lavage technique of Myrvik, Leake, and Fariss (8). The animals were routinely sensitized to BCG 3–4 wk before sacrifice by the intravenous injection into the marginal ear vein of sonic extract of BCG in Bayol F (Humble Oil & Refining Co., Houston, Tex.) (100 μg in 0.10 ml). Human peripheral polymorphonuclear leukocytes (PMNL) [1] were obtained from the venous blood of normal volunteer subjects by a method previously described (9). This routinely resulted in a preparation which was greater than 90% PMNL, as judged by phase contrast microscopy. Lymphocytes were separated from normal human blood in 95% purity by centrifugation in a Ficoll-Hypaque gradient (10).

All cell preparations were washed twice with cold phosphate-buffered saline (PBS). Contaminating red blood cells were removed by lysis for 20 s in deionized water; isotonicity was then restored by the addition of an appropriate volume of 3.5% saline. After centrifugation, the cell pellet was suspended in saline in a ratio of 1 ml of packed cells to 5 ml of 0.9% saline. Cells were ruptured by sonication as previously described (9). For most experiments, the ruptured cell suspension was centrifuged for 15 min at 27,000*g*, and the clear supernate was used in the assay procedures. The protein concentration in the sonic extracts was determined by the biuret procedure of Gornall, Bardawill, and David (11), with bovine serum albumin as a standard.

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[1] *Abbreviations used in this paper:* NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PMNL, polymorphonuclear leukocytes; PMS, phenazine methosulfate.

Assay for superoxide dismutase: epinephrine procedure. Superoxide dismutase activity was detected by its ability to inhibit the autoxidation of epinephrine at pH 10.2 as described by Misra and Fridovich (12). Each cuvette contained in a final volume of 3.0 ml: 0.50 ml of 1.8 mM epinephrine (freshly prepared); 0.50 ml of 0.6 mM EDTA; 0.50 ml of 0.30 M sodium carbonate, pH 10.2, and leukocyte sonicate as indicated. The reaction was initiated by the addition of epinephrine, and the increase in absorbance at 480 nm was followed in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, Ill.) with a Gilford recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The change in absorbance due to the conversion of epinephrine to adrenochrome was markedly inhibited (80%) by the presence of 0.75 μg of purified superoxide dismutase (Truett Labs, Dallas, Tex.) in the reaction vessel.

Control experiments were run with boiled enzyme, bovine serum albumin, catalase, and peroxidase (obtained from Sigma Chemical Co., St. Louis, Mo.). Even when these enzymes were present in 100-fold excess by weight they failed to inhibit the formation of adrenochrome; indeed, peroxidase resulted in rapid increase in adrenochrome formation. Because of this catalytic action of peroxidase, the assay cannot be used with uncentrifuged preparations of PMNL that contain large amounts of myeloperoxidase. The sonication procedure employed was sufficiently gentle to leave the great bulk of the lysosomes intact, as determined by the increase in lysosomal enzymes in the whole sonicate made in 0.1% Triton X-100. Thus, the level of myeloperoxidase in the centrifuged sonicate was reduced to a point where it failed to interfere with the assay.

Adrenochrome can be further oxidized in aqueous solutions to compounds that absorb much less light at 480 nm than does adrenochrome itself. The possibility that the sonicate accelerates this process of degradation was examined by incubation of adrenochrome (0.03 mg/ml in 0.05 M sodium carbonate, pH 10.2) with varying quantities of leukocyte sonicate. It was observed that the addition of sonicate actually inhibited the further oxidation of adrenochrome so that the decreased rate of adrenochrome production from epinephrine actually represents a minimum value. Because of this activity, the true superoxide dismutase activity is probably somewhat higher than that observed experimentally.

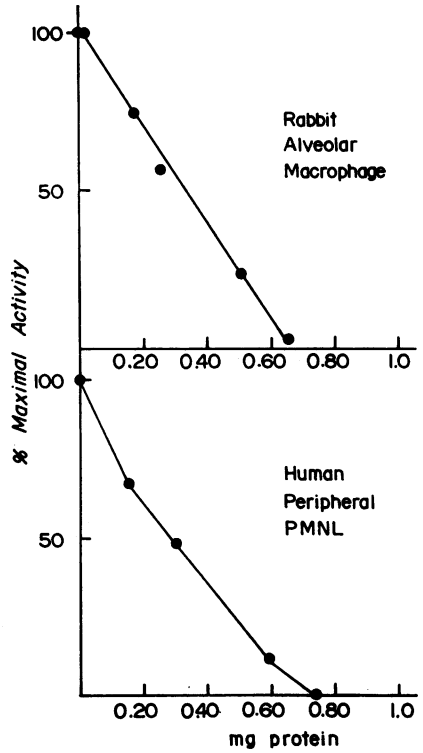

FIGURE 1 Effect of protein concentration on the autoxidation of epinephrine to adrenochrome. Maximal activity seen in the absence of added protein was 0.0186 OD/min. Each point represents the mean of duplicate determinations.

TABLE I
Superoxide Dismutase Activity in Various Leukocytic Extracts

Sample used	% Inhibition
Human PMNL (0.30 mg)	55
Human PMNL (0.75 mg)	99
Human lymphocytes (0.83 mg)	64
Human lymphocytes (2.50 mg)	98
Human lymphocytes (0.79 mg)	38
Human lymphocytes (1.60 mg)	56
Rabbit peritoneal PMNL (0.40 mg)	58

The % inhibition refers to the inhibition of adrenochrome formation in the presence of leukocytic extract. Two different preparations of human lymphocytes were assayed. All values represent the mean of closely agreeing duplicate determinations. The values in parentheses represent the amount of leukocytic protein added in the experiment.

Assay for superoxide dismutase: nitroblue tetrazolium (NBT) procedure. The presence of superoxide dismutase in leukocytes was verified by the procedure of Nishikimi, Rao, and Yagi (13). This assay relies on the ability of the enzyme to inhibit the phenazine methosulfate (PMS)-mediated reduction of NBT dye. Each cuvette contained in a final volume of 3.0 ml: 0.50 ml of 0.3 mM NBT; 0.50 ml of 0.47 mM NADH, 0.50 ml of 0.10 M sodium pyrophosphate buffer, pH 8.3; 0.10 ml of 93 μM PMS (freshly prepared), and varying amounts of leukocytic extract. Reaction was initiated by the addition of PMS, and the increase in absorbance at 560 nm due to the formation of reduced NBT was followed on a recording spectrophotometer as before. Purified superoxide dismutase was shown to inhibit the initial rate of the PMS-induced reduction of NBT (0.50 μg of the purified enzyme giving 80% inhibition). No inhibition of the PMS-mediated reduction of NBT was observed when boiled sonicate or bovine serum albumin was substituted for the sonicate.

Because neutrophils (and macrophages) contain a diaphorase capable of reacting NBT directly with NADH (14), all experiments had to be corrected for this activity by running the reaction in the presence and absence of PMS. The activity observed in the presence of PMS is a function of both the superoxide-mediated and diaphorase-mediated reactions; it is possible to determine the magnitude of the diaphorase-mediated reaction by eliminating the

TABLE II

Superoxide Dismutase Assayed by the Reduction of NBT

Assay conditions	NBT reduction			% Inhibition‖
	Total activity*	Diaphorase mediated‡	Superoxide mediated§	
		ΔOD/min		%
Human PMNL				
No sonicate	0.114	—	0.114	—
1.49 mg sonicate protein	0.087	0.031	0.056	51
2.98 mg sonicate protein	0.091	0.053	0.038	67
7.44 mg sonicate protein	0.095	0.079	0.016	86
Rabbit alveolar macrophage				
No sonicate	0.101	—	0.101	—
0.12 mg sonicate protein	0.082	0.000	0.082	19
0.24 mg sonicate protein	0.073	0.005	0.068	33
0.48 mg sonicate protein	0.083	0.011	0.072	29
0.73 mg sonicate protein	0.084	0.025	0.059	42

All values represent the mean of closely agreeing triplicate determinations.

* NBT reduction in the presence of PMS.

‡ NBT reduction in the absence of PMS.

§ Determined by subtraction of diaphorase-mediated activity from the total activity.

‖ Inhibition by leukocytic extract of the superoxide-mediated reduction of NBT.

source of O_2^- (PMS) from the reaction mixture. The activity directly attributable to superoxide can then be calculated by substracting the diaphorase activity from the total activity. Control experiments were run in which the NBT was omitted from the reaction cuvette; no change in the absorbance at 560 nm was observed, indicating that the sonicate did not catalyze the oxidation of PMS itself to give a product that might interfere with the assay procedure.

The kinetics of both the epinephrine and NBT assay are complex; rates reported here are derived from the linear portion of the curves. In many experiments, this linearity was observed for only a short interval (2–4 min).

RESULTS

The results in Table I demonstrate the activity of superoxide dismutase in sonicates of various leukocytes as determined by the epinephrine assay procedure. The enzyme activity was observed in all leukocytes examined, but it was substantially lower per milligram of protein in the lymphocyte than in the PMNL, indicating that the observed activity could not be ascribed to lymphocytic contamination. The considerable variation in activity seen with different preparations is apparent in this data. 0.83 mg of one lymphocyte sonicate gave 64% inhibition, while a similar concentration of a second preparation gave only 38% inhibition.

The effect of protein concentration is depicted in Fig. 1. The inhibition of the autoxidation of epinephrine is dependent upon the amount of sonic extract added for both the rabbit alveolar macrophage and the human peripheral neutrophil. Virtually complete inhibition is obtained upon the addition of 0.60–0.80 mg protein in both cases. Control experiments were run in which a comparable amount (i.e., 1.0 mg) of bovine serum albumin was added to the cuvette; no inhibition of adrenochrome formation was observed.

The demonstration of superoxide dismutase activity by the PMS-mediated reduction of NBT is documented in Table II. The addition of sonicate results in only a slight inhibition of total NBT reduction due to the presence of a diaphorase in the cell. If a correction is applied for the diaphorase, as described under Methods, it becomes apparent that there is a marked inhibition of the superoxide-mediated reduction of NBT, as illustrated in the last column. Control experiments employing bovine serum albumin demonstrated that the effect was not due to nonspecific interactions with protein.

This estimation of NBT reduction due to superoxide by subtraction of the diaphorase activity obtained in the absence of PMS assumes that the PMS has no effect on the diaphorase reaction itself. Experiments with purified superoxide dismutase indicate that this assumption might not be true and that there might be an interaction between the PMS and sonicate which results in increased diaphorase activity. The essential findings are these:

(*a*) In the presence of 0.01 mg of authentic superoxide dismutase, the PMS-mediated reduction of NBT is virtually completely inhibited.

(*b*) If leukocytic sonicate is added along with the purified superoxide dismutase, the inhibition is relieved to a significant extent (about 70% inhibition is now observed).

(*c*) The diaphorase activity observed in the absence of PMS is not sufficient to explain this degree of relief of the inhibition.

These data are consistent with the interpretation that PMS acts to stimulate the diaphorase activity, although other interpretations are certainly possible. If, indeed, PMS stimulates the diaphorase activity, then the correction factor we are applying is too low, and the actual superoxide dismutase activity is higher than calculated. This is consistent with the fact that we generally observe a lower activity of superoxide dismutase by the NBT procedure than by the epinephrine assay.

The demonstration of superoxide dismutase in rabbit alveolar macrophages by this assay is complex. The maximum inhibition observed by the addition of sonicate is about 40%; the addition of larger quantities of sonicate results in an increase in the initial velocity. Obviously, there are competing reactions which can obscure the assay; the most likely candidate is the diaphorase enzyme, which has a higher specific activity in the alveolar macrophage than in the PMNL.

An attempt was made to localize the superoxide dismutase in the human PMNL. A heavy suspension of human PMNL in PBS was homogenized for 15 strokes in a Potter-Elvehjem homogenizer. Unbroken cells were sedimented at 70*g* for 10 min in an IEC refrigerated centrifuge (Damon/IEC Division, Needham Heights, Mass.). A fraction of the supernate was saved for assay, and the remainder was centrifuged at 10,000*g* for 10 min to obtain a lysosomal rich pellet. The 10,000*g* supernate was then centrifuged at 105,000*g* for 60 min to obtain a microsomal pellet and a final supernatant solution. The various pellets were suspended in a known amount of PBS, and all fractions were assayed for superoxide dismutase activity by the epinephrine procedure. The only fraction in which activity could be measured was the final supernate, suggesting that the enzyme is found in the soluble fraction (cytosol) of the cell. It is possible that high peroxidase activity observed in other fractions masks the existence of superoxide dismutase in those fractions.

Table III illustrates the effect of cyanide and azide on the superoxide dismutase activity of human PMNL as determined by the epinephrine assay procedure. The enzyme was observed to be completely insensitive to both inhibitors in concentrations as high as 1.65 mM. The possibility that cyanide or azide might be directly inhibiting adrenochrome formation was examined. The same rate of reaction was observed in the presence or absence of either compound at a concentration of 1.65 mM.

TABLE III

Effect of Cyanide and Azide on Superoxide Dismutase Activity in Human PMNL

Conditions	Activity
	ΔOD/min
Control	0.0263
+ sonicate	0.0028
+ sonicate + CN^- (0.33 mM)	0.0000
+ sonicate + CN^- (1.65 mM)	0.0000
+ sonicate + azide (0.33 mM)	0.0000
+ sonicate + azide (1.65 mM)	0.0009

Superoxide dismutase activity was assayed by the epinephrine procedure described under Methods. The data are taken from a single experiment, which is representative of four different experiments, each employing a different cell donor. Reaction was initiated by the addition of 0.30 mg of leukocytic protein in each case.

DISCUSSION

We have identified superoxide dismutase in sonic extracts of both PMNL and alveolar macrophages by two distinct assay procedures. Although both assays are indirect procedures, the weight of the combined data suggests that the activity is due to superoxide dismutase in the cells. The activity measured is proportional to protein concentration and is not observed with nonspecific protein. The insensitivity of the enzyme to both cyanide and azide demonstrates that the activity cannot be ascribed to either catalase or peroxidase, both of which are markedly inhibited by the concentrations of the compounds used in the present experiments.[2] The enzyme is present in the cytosol of the cell, although its possible existence in the granule fraction could not be ascertained because of the interference by the high levels of peroxidase in that fraction.

This situation is somewhat different from that reported by Weisiger and Fridovich (15) for the superoxide dismutases from chicken liver, which contains both a cytoplasmic and a mitochondrial enzyme with differing metal ion composition. The cytoplasmic enzyme described by Fridovich differs from the leukocytic enzyme in that it is strongly inhibited by 1 mM cyanide. The reason for this difference in the properties of the soluble enzyme from the different sources is unknown.

Superoxide dismutase catalyzes the dismutation of the highly reactive superoxide anions according to the following reactions (16):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The function of the enzyme within the leukocyte is probably protective, preventing the highly reactive O_2^-

[2] De Chatelet, L. R. Unpublished data.

from damaging the cell itself. This is analogous to the proposed function of catalase in the cytoplasm of the leukocyte; namely, the elimination of excess H_2O_2 and protection of the cell against oxidant damage by this compound (17). An alternative role for the enzyme might involve the generation of H_2O_2, which has been implicated in the bactericidal activity of both neutrophils (18) and macrophages (19).

Although it has been demonstrated that the neutrophil is capable of generating superoxide (4), the exact mechanism of its formation is unknown. Odajima (20) has suggested that the aerobic oxidation of NADH (or possibly NADPH) might provide the source of superoxide ions according to the following reactions:

$$NADH + O_2 \rightarrow NAD\cdot + HO_2,$$

$$HO_2 \rightarrow O_2^- + H^+,$$

$$NAD\cdot + O_2 \rightarrow NAD^+ + O_2^-$$

He has further demonstrated that leukocytic myeloperoxidase can catalyze the aerobic oxidation of NADH and that the reaction is inhibited by superoxide dismutase (21).

Whatever the source of superoxide ion, its importance to the cell is indicated by the following observations. (*a*) During phagocytosis, the production of O_2^- by human PMNL doubles (4). (*b*) Cells from patients with chronic granulomatous disease, a syndrome characterized by defective bactericidal activity (22), do not produce detectable quantities of O_2^- (5), and (*c*). The inclusion of superoxide dismutase within the phagocytic vacuole results in marked impairment in the bactericidal activity of normal cells (6).

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