The Role of Superoxide Anion and Hydrogen Peroxide in Phagocytosis-Associated Oxidative Metabolic Reactions

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ABSTRACT The contribution of hydrogen peroxide (H_2O_2) and one of its unstable intermediates, superoxide anion (O_2^-), to the oxidative reactions that occur in phagocytizing leukocytes was explored by depleting these cells of O_2^-. This was accomplished by allowing them to phagocytize latex particles coated with superoxide dismutase (SOD), which catalyzes the generation of H_2O_2 from O_2^-. Although the rate and extent of phagocytosis of latex coated with bovine serum albumin was similar to latex coated with SOD, the rate of oxygen consumption, [^{14}C]formate oxidation, [1-^{14}C]glucose oxidation, and iodination of zymosan particles was significantly enhanced by SOD. In contrast, the rate and extent of reduction of nitroblue tetrazolium (NBT) was diminished by 60%. These studies indicate that the majority of NBT reduction by leukocytes is due to O_2^-, whereas stimulation of the hexose monophosphate shunt and iodination of ingested particles requires H_2O_2 generated from the increased reduction of oxygen by phagocytizing leukocytes.

INTRODUCTION

A series of oxidation-reduction reactions takes place in vitro in phagocytizing leukocytes, including: (*a*) a cyanide-insensitive burst of oxygen consumption; (*b*) production of H_2O_2; (*c*) stimulation of glucose oxidation through the hexose monophosphate shunt (1, 2); (*d*) iodination of ingested particles (3); and (*e*) reduction of nitroblue tetrazolium to insoluble formazan (4). Two cyanide insensitive oxidases, NADH and NADPH oxidase, have been identified in leukocytes and each has received consideration as the primary oxidase mediating these reactions (5, 6). Recent studies confirm that several highly reactive unstable intermediate products of oxygen reduction, including superoxide anion (O_2^-)[1] are generated by human leukocytes during phagocytosis (7–10). In a chemical system, O_2^-, generated by an oxidase-catalyzed reaction, can be assayed by observing the extent of inhibition of the reduction of nitroblue tetrazolium (NBT) or ferricytochrome *c* by superoxide dismutase (SOD) (11). This enzyme catalyzes the dismutation of 2 mol of O_2^- to form 1 mol of oxygen and 1 mol of (H_2O_2).

Johnston et al. have observed diminished phagocytic bacterial killing when SOD, in distinction to bovine serum albumin (BSA) or denatured SOD, was present in the reaction mixture (10). The effect was markedly enhanced if the enzyme was associated with latex particles. It seemed possible that this technique might offer a unique means of depleting phagocytizing leukocytes of O_2^- and simultaneously enhancing the rate of generation of H_2O_2. That is, if dismutation of the O_2^- radical to H_2O_2 occurs within the phagocyte, as it does in chemical systems, it would seem likely that the following sequence of metabolic reactions occurs in leukocytes phagocytizing latex coated with SOD:

$$2O_2 + 2H\cdot \xrightarrow{\text{Oxidase}} O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Assuming that the oxidase-catalyzed reaction is not limiting in this system, as O_2^- is generated it would be

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; NBT, nitroblue tetrazolium; O_2^-, superoxide anion; SOD, superoxide dismutase.

rapidly converted to H_2O_2 and oxygen. Therefore, those metabolic reactions dependent upon superoxide would be depressed by introduction of SOD into the cells, whereas those reactions dependent upon H_2O_2 would be enhanced. We have explored the metabolic effects of introducing latex-associated SOD into phagocytes. Results suggest that the phagocytic reduction of NBT is O_2^- dependent, whereas stimulation of the hexose monophosphate shunt, stimulation of formate oxidation, and iodination of ingested particles are dependent upon H_2O_2 generation.

METHODS

Collection and preparation of leukocyte suspension. Peripheral blood was collected in heparinized plastic syringes from normal young adult volunteers. After mixing with 1–2 ml Plasmagel (HTI Corp., Associated Biomedic Systems, Inc., Buffalo, N. Y.), the syringe was inverted and erythrocytes were allowed to sediment at 25°C for 30 min. The supernatant leukocyte-rich plasma was decanted into 60 ml plastic, conical centrifuge tubes and mixed with 3 vol of 0.87% NH_4Cl for 5 min to remove the red cells. This mixture was centrifuged at 800 *g* for 5 min to obtain a purified white cell pellet, which was washed three times with Krebs-Ringer phosphate buffer (pH 7.4) enriched with 10 mM glucose. Leukocyte and differential counts were performed on these suspensions to obtain the absolute number of granulocytes; the preparations contained 85–90% granulocytes.

Coating of latex particles. Latex particles measuring 0.81 μm in diameter (Dow Chemical Co., Midland, Mich.) were dialyzed against distilled water at 4°C overnight. Particles were coated with either SOD (Truett Labs., Dallas, Tex. or purified by the method of McCord and Fridovich [12]) or BSA (Sigma Chemical Co., St. Louis, Mo.) by incubation with these proteins at a concentration of 1 mg per 1 ml of undiluted latex particles at 25°C in 25-ml siliconized glass Erlenmeyer flasks for 15 min (12). Coating of latex with apodismutase, hemoglobin, and thrombin was performed in the same way. To determine the extent of protein binding to latex, after incubation the latex particles were centrifuged, the supernate was discarded, and the amount of protein associated with the particles was determined by the method of Lowry, Rosebrough, Farr, and Randall (13). To ensure that SOD associated with latex was enzymatically active, 0.1 ml of coated latex was assayed for SOD activity by the inhibition of cytochrome *c* reduction with xanthine and xanthine oxidase (12). Apodismutase was prepared by the method of McCord and Fridovich (12) and was assayed for SOD activity before incubation with latex.

Rate of uptake of latex by leukocytes. A suspension of 5×10^7 granulocytes in a volume of 1.6 ml was added to 0.4 ml latex-protein suspension and incubated in triplicate in 25-ml siliconized Erlenmeyer flasks at 37°C in a Dubnoff metabolic shaker. Aliquots of 0.5 ml were taken at 0, 5, 15, and 30 min of incubation and placed in siliconized glass test tubes containing 10 ml ice-cold saline. The tubes were then centrifuged at 300 *g* for 5 min at 4°C and washed twice in ice-cold saline. The cell pellets were then placed in a 100°C oven overnight to ensure complete drying. Then 2 ml ρ-dioxane (Matheson Coleman & Bell, Matheson Scientific, Inc., East Rutherford, N. J.) at approximately 60°C was added to each dry button and allowed to stand at 25°C for 2 h until the cell pellet was dissolved. This solution was stirred occasionally to complete solubility of the pellet. Samples were cleared by centrifugation at 300 *g* for 5 min and then read on a Gilford Spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 253 nm against a ρ-dioxane blank. Appropriate blanks containing latex-BSA or latex-SOD without cells, and samples containing cells without latex were run in triplicate at each time point. The combined blank values accounted for less than 10% of the cell values at each time point.

Metabolic reactions of leukocytes

Oxygen consumption. The oxygen consumption by phagocytes ingesting latex coated with BSA or SOD was measured with a Clark membrane oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) using an expanded scale Varian recorder (Varian Associates, Palo Alto, Calif.) calibrated so that a change of 60% oxygen saturation of the incubate produced a full-scale deflection. Additional experiments were performed using latex coated either with apodismutase, hemoglobin, or thrombin. A 1-ml suspension of phagocytes at a concentration of 2.0×10^7 cells/ml was placed in the reaction vessel and 1.9 ml of Krebs-Ringer phosphate glucose buffer was added. After a 5-min incubation at 37°C, 0.1 ml suspension of protein-coated latex was added, and the respiratory burst was followed. After a 1-min period of equilibration, the increased rate of oxygen consumption was linear for at least 10 min. Results were expressed as μl O_2/h per 10^7 phagocytes calculated from the linear slopes produced. Studies in the presence of NBT were performed with 0.1-ml of undiluted plain latex particles or 0.1 ml of a 5% suspension of zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) previously opsonized by incubating with 10% (vol/vol) fresh serum at 25°C for 30 min; 0.1 ml of a 0.2% solution of NBT was added to both preparations. Each experiment was done in triplicate.

[^{14}C]Formate and [1-^{14}C]glucose oxidation. The rates of [^{14}C]formate and [1-^{14}C]glucose oxidation were determined by a previously described method (14). Preliminary studies using 10-min intervals established that the rates were linear for at least 30 min with latex alone or latex coated with either BSA or SOD. To ensure that the metabolic responses observed were due to H_2O_2 produced by SOD and not to nonspecific effects of this enzyme, a series of [1-^{14}C]glucose oxidation experiments were performed using apodismutase, hemoglobin, and thrombin proteins with and without latex particles. To further elucidate the role of H_2O_2, [1-^{14}C]glucose oxidation experiments were performed on leukocytes ingesting latex-SOD to which was added 4.6 U of catalase. Other experiments were performed on leukocytes incubated with a mixture of latex-BSA (90%) and latex-SOD (10%).

Quantitative iodination of zymosan particles. The influence on iodination of ingested opsonized zymosan particles by latex coated with BSA or SOD was measured at 0, 15, 30, and 60 min according to the method of Pincus and Klebanoff (15).

Reduction of NBT. NBT reduction was measured as previously described (4). 0.1 mg SOD or 0.1 mg of BSA was added directly to both resting and phagocytic flasks; latex was added to the phagocytizing samples to begin the experiments.

RESULTS

Quantitation of BSA or SOD bound to latex particles. When 1 mg BSA was incubated with 1.0 ml undiluted suspension of latex particles, 33% of the total weight of the protein (i.e., 0.33 mg or 5.50 nmol) was bound. 18% of SOD and apodismutase was absorbed to the latex (0.18 mg or 5.45 nmol). The latex-coated SOD exhibited 20% of the total SOD activity.

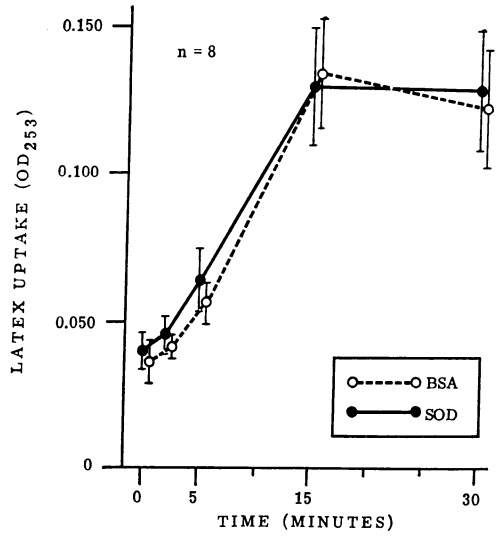

FIGURE 1 Rate and extent of uptake of latex particles by granulocytes. Each point represents the mean of triplicate samples of eight separate studies. The bars represent the SEM. BSA means bovine serum albumin and SOD means superoxide dismutase; either protein was preincubated with undiluted previously dialyzed 0.81-μm latex particles. See text for further details.

Rate of uptake of latex by granulocytes. The rate of uptake of latex by granulocytes determined by the spectrophotometric method was linear from 5 to 15 min during the 30-min incubation period. As indicated in Fig. 1, there was no significant difference in the quantity of either type of latex particle phagocytized or in the rate of this phagocytosis by granulocytes at each time point tested ($P > 0.05$, t test). Maximum uptake of particles was evident by 15 min.

Metabolic studies. The rate of oxygen consumption by leukocytes at rest and during phagocytosis of latex coated with BSA or SOD is given in Table I. There was a significant increase in oxygen consumption observed when latex coated with either protein was ingested. However, the rate of oxygen consumption in the presence of latex coated with SOD was significantly greater than that observed with latex-BSA.

TABLE I

Rate of Oxygen Consumption by Leukocytes at Rest and during Phagocytosis of Latex Particles Coated with SOD and BSA

Protein added	Resting	Phagocytizing
	*μl O_2/h/10^7 phagocytes**	
BSA	2.21±0.71	4.15±1.08
SOD	2.41±0.90	7.01±2.57‡

* Mean±SEM of duplicate values from eight experiments.
‡ Value is significantly different from all other values. ($P < 0.001$, t test).

TABLE II

Rate of Oxygen Consumption by Phagocytizing Leukocytes during Reduction of NBT

Conditions	NBT	O_2 consumption
		*μl/h/10^7 phagocytes**
Resting (6)	Absent	1.92±0.14
Phagocytizing latex (6)	Absent	9.83±1.39
Phagocytizing latex (6)	Present	4.72±0.24‡
Phagocytizing zymosan (5)	Absent	11.08±0.75
Phagocytizing zymosan (6)	Present	6.02±0.94‡

* Mean±SEM. Values in parenthesis indicate number of experiments performed in duplicate.
‡ Values are significantly different than corresponding value obtained without NBT ($P = 0.001$, t test).

The addition of either type latex particle to the system devoid of cells did not evoke an alteration in oxygen consumption. In another series of experiments summarized in Table II, addition of NBT to leukocytes phagocytizing either latex or zymosan particles consistently produced a decrease in oxygen consumption which persisted for more than 15 min at 37°C. This decrease was evident when NBT was added before or after the particles.

The stimulation of [1-^{14}C]glucose oxidation to $^{14}CO_2$ was enhanced by latex coated with SOD when added to resting leukocytes or to leukocytes ingesting latex-BSA. As noted in Table III, leukocytes ingesting latex BSA

TABLE III

Rate of [1-^{14}C]Glucose Oxidation by Leukocytes at Rest and during Phagocytosis of Latex Particles Coated with SOD and BSA

	[1-^{14}C]Glucose → $^{14}CO_2$/30 min/10^7 phagocytes*
	nmol
Resting	2.52±1.65
Phagocytizing latex-BSA	7.08±2.11
Phagocytizing latex-SOD	20.43±1.39
‡Phagocytizing latex-SOD with added catalase	7.61±2.03
‡Phagocytizing latex-BSA +latex-SOD	19.03±3.50

* Mean±SEM of duplicate values from eight experiments. The values for latex-BSA and latex-SOD + catalase are significantly different from the resting value and from the values for latex-SOD and latex-BSA + latex-SOD ($P < 0.001$, t test).
‡ Mean±SEM of 12 determinations from three experiments.

TABLE IV

Rate of [^{14}C]Formate Oxidation by Leukocytes at Rest and during Phagocytosis of Latex Particles Coated with SOD and BSA

	[^{14}C] Formate → $^{14}CO_2$/30 min/10^7 phagocytes*
	nmol
Resting	0.89±0.03
Phagocytizing latex-BSA	1.36±0.04
Phagocytizing latex-SOD	1.87±0.04

* Mean±SEM of duplicate values from eight experiments. Each value is significantly different from the other two. ($P < 0.001$, t test).

exhibited an almost threefold increase in hexose monophosphate shunt activity, whereas ingestion of latex-SOD or a 10% mixture of latex-SOD to latex-BSA produced an eightfold increase. The addition of apodismutase, hemoglobin, or thrombin did not alter the hexose monophosphate shunt activity of resting leukocytes. Similar to BSA, a two- to threefold increase occurred when the leukocytes ingested latex coated with either of the three proteins. Also the stimulation of [1-^{14}C] glucose oxidation was not observed in leukocytes to which catalase was added less than 1 min after latex-SOD (Table III). There was a less striking yet significant increase in [^{14}C]formate oxidation when leukocytes ingested latex-SOD compared to latex-BSA (Table IV).

Results of the quantitation of the rate of iodination of zymosan particles by phagocytizing leukocytes simultaneously ingesting latex particles are given in Table V. At each time interval during the 60-min incubation, significantly geater iodination was observed in the presence of latex-SOD compared to latex-BSA. No iodination occurred when zymosan was omitted from the system. In contrast to the above responses, the presence of SOD with leukocytes at rest or during

TABLE V

Rate of Iodination of Zymosan Particles by Leukocytes Phagocytizing Latex Particles Coated with SOD or BSA

Time	BSA	SOD
min	*cpm/10^7 phagocytes**	
0	81±20	102±12‡
15	706±106	1,059±129
30	1,423±136	2,886±379
60	2,077±237	4,063±404

* Mean±SEM of triplicate values from eight experiments.
‡ At each time point the values obtained in the presence of SOD were significantly higher than those with BSA ($P < 0.001$, t test).

TABLE VI

Rate of NBT Reduction by Leukocytes Suspended in Either 0.1 mg SOD or BSA at Rest and during Phagocytosis of Latex Particles

Protein added	Rest	Phagocytizing
	*OD_{515}/15 min/10^7 cells**	
BSA	0.139±0.005	0.337±0.018
SOD	0.055±0.006	0.129±0.012

* Mean±SEM of duplicate tubes from five experiments. Both resting and phagocytizing values were significantly less than corresponding values with BSA ($P < 0.001$, t test).

phagocytosis of latex inhibited the reduction of NBT to formazan. This is evident in Table VI which indicates that NBT reduction in the presence of SOD was decreased by approximately 62% in comparison to reduction of NBT in the presence of BSA.

DISCUSSION

These studies were designed to determine the contribution of H_2O_2 and its unstable intermediate O_2^-, to some well-known oxidative metabolic reactions of phagocytizing leukocytes. It is apparent from the studies of Babior, Kipnes, and Curnutte (7) and of Johnston et al. (10) that O_2^- is generated as a by-product of phagocytosis. The enzyme, SOD, catalyzes the conversion of O_2^- to H_2O_2 and oxygen. The stoichiometry of these two related reactions is likely as follows:

$$2O_2 + 2H\cdot \xrightarrow{\text{Oxidase}} O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

These reactions would generate either equimolar O_2^- for each mole of O_2 consumed, or 1 mol each of H_2O_2 and O_2 for every 2 mol of O_2 consumed if the reaction goes to completion. In previous experiments with phagocytizing leukocytes, we and others could account for only 3–15% of the consumed oxygen as H_2O_2 available for oxidation of [^{14}C]formate or reduced glutathione (16, 17).

The techniques utilized in these studies require that there is an equal rate of uptake of latex coated with SOD or BSA. Variable rates of uptake will influence the rates of oxidation-reduction reactions in leukocytes. In previous studies, we bound glucose oxidase to latex and restored defective oxidase activity in chronic granulomatous disease leukocytes which resulted in improved [1-^{14}C]glucose oxidation and [^{14}C]formate oxidation as well as improved intracellular bactericidal activity (14, 18). However, the rate of uptake of latex coated with enzyme by phagocytes was diminished compared to uncoated latex (14). As can be observed in this study, comparison of Table I with rows 1 and 2 of Table II

confirmed that protein bound to latex attenuated the normal particle-induced stimulation of oxygen consumption. Since the extent of these metabolic perturbations depends in part on the rate of particle uptake, proper interpretation of our studies demands that the rate and extent of uptake by leukocytes of latex coated with SOD must equal that of latex coated with BSA. We found no differences in the initial rate of uptake of latex coated with either SOD or BSA or in the extent of ingestion of latex by leukocytes during a 30-min incubation period. Thus, we believe the technique to be a reliable method of studying the influence of SOD on the oxidative metabolic reactions of phagocytizing leukocytes.

These studies focus on the altered rate of several oxidative reactions in phagocytizing leukocytes produced by introduction of SOD into the cell. The fact that the rate of oxygen consumption was enhanced threefold by SOD suggestes that the oxidase catalyzed reaction was not limiting in this system. Iyer, Islam, and Quastel (19) previously have shown that [^{14}C] formate oxidation is dependent upon H_2O_2. Our finding of increased [^{14}C]formate oxidation suggests that the rate of H_2O_2 production was increased by SOD. The addition of catalase, which rapidly converts H_2O_2 to O_2 and H_2O, inhibited the increase in [1-^{14}C]glucose oxidation observed in these leukocytes ingesting latex-SOD. The increase in hexose monophosphate shunt activity and rate of iodination confirms that these two metabolic reactions are stimulated primarily by H_2O_2 rather than superoxide in phagocytizing leukocytes. In addition, the increase in H_2O_2 generated from O_2^- dismutation is linked with activation of the shunt, perhaps through the glutathione peroxidase pathway (20). which favors this mechanism as an explanation for shunt activation during phagocytosis (21).

The observation by Johnston and co-workers (10) that, under experimental conditions similar to those described here, intracellular microbicidal activity was consistently diminished suggests that O_2^- or its by-product does play a role in bacterial killing and lends support to the concept that killing is not necessarily dependent upon iodination, as previously recognized (22).

The metabolic explanation for NBT reduction by phagocytizing leukocytes based upon our studies suggests two possible mechanisms. Since NBT is a redox dye with an oxidation-reduction potential of close to zero, it competes with oxygen for electrons and protons. This is apparent in those studies which showed a consistent decrease in oxygen consumption by phagocytizing leukocytes during NBT reduction to insoluble formazan. An alternative explanation for the diminished oxygen consumption observed during NBT reduction could involve the reaction of O_2^-, formed in the univalent reduction of oxygen with NBT. O_2^- can reduce NBT to formazan and oxygen is generated. In this way, NBT could also decrease the net consumption of oxygen in the system. Since NBT reduction was diminished 62% by SOD, this indicates that 38% of the reduction of NBT is independent of oxygen, and perhaps involves membrane-associated reductases (23, 24). Since the redox potential of the H_2O_2-O_2^- half-cell at pH 7.4 is -0.98, the reduction of NBT by H_2O_2 is not possible. Our studies, as well as those of Johnston et al. (10), show that SOD does decrease NBT reduction by about two-thirds, compared to the result observed during leukocyte ingestion of BSA-coated latex. Thus, it is likely that two metabolic reactions control NBT reduction in phagocytizing leukocytes: (*a*) a reductase which catalyzes the direct reduction of NBT independent of the univalent reduction of oxygen to superoxide anion; and (*b*) an oxidase which endows oxygen with an extra electron that can be displaced for the reduction of ferricytochrome *c* or NBT. These quantitative studies support the idea that the majority of NBT reduction by phagocytizing leukocytes is mediated by the latter reaction through the generation of O_2^-.

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