Biological Defense Mechanisms

THE PRODUCTION BY LEUKOCYTES OF SUPEROXIDE, A POTENTIAL BACTERICIDAL AGENT

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ABSTRACT As a highly reactive substance produced in biological systems by the one-electron reduction of oxygen, superoxide (O_2^-) seemed a likely candidate as a bactericidal agent in leukocytes. The reduction of cytochrome c, a process in which O_2^- may serve as an electron donor, was found to occur when the cytochrome was incubated with leukocytes. O_2^- was identified as the agent responsible for the leukocyte-mediated reduction of cytochrome c by the demonstration that the reaction was abolished by superoxide dismutase, an enzyme that destroys O_2^-, but not by boiled dismutase, albumin, or catalase.

Leukocyte O_2^- production doubled in the presence of latex particles. The average rate of formation of O_2^- in the presence of these particles was 1.03 nmol/10^7 cells per 15 min. This rate, however, is only a lower limit of the true rate of O_2^- production, since any O_2^- which reacted with constituents other than cytochrome c would have gone undetected. Thus, O_2^- is made by leukocytes under circumstances which suggest that it may be involved in bacterial killing.

INTRODUCTION

Polymorphonuclear leukocytes, whose resting metabolism is based largely on anerobic glycolysis, display an increase in oxygen consumption upon phagocytosis of bacteria or other particles (1, 2). Since the ingestion of bacteria by the cells can occur under anerobic conditions (2), it has been postulated that the increase in oxygen uptake associated with phagocytosis is related to the subsequent process whereby the ingested microorganisms are killed. Among the agents thought to be directly responsible for the killing of bacteria are I· (or I⁺) and H_2O_2 (3–5), each presumably produced by the oxygen-consuming reactions that accompany phagocytosis.

Through the work of Fridovich and others, it has recently become apparent that biological systems are able to convert oxygen into a compound of great reactivity (6–8). This compound, the superoxide anion (O_2^-), is an extremely powerful oxidation-reduction reagent, capable of undergoing either oxidation to O_2 or reduction to H_2O_2 with the liberation of large amounts of energy (9). The production of this compound by the one-electron reduction of oxygen in biological systems, together with its reactivity, suggested it as a possible killing agent in leukocytes. In the following communication, we report evidence for the production of O_2^- by phagocytizing leukocytes.
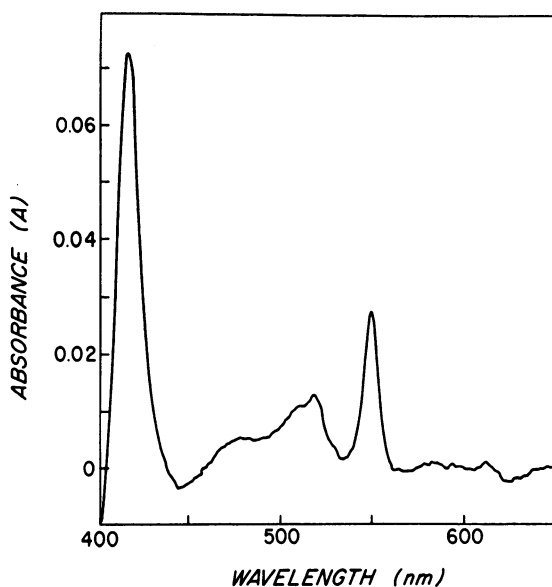
FIGURE 1 Difference spectrum of an incubated vs. an unincubated reaction mixture. For details, see text.

METHODS

Superoxide dismutase (approximately 30,000 U/mg) was purchased from Truett Labs, Dallas, Tex. Its activity was checked with xanthine and xanthine oxidase according to McCord and Fridovich (6). Horse heart ferricytochrome c (type VI), catalase, and human serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. Dextran was obtained from Travenol (Travenol Laboratories, Morton Grove, Ill.). Latex particle suspension (Bacto latex, 0.81 μm, containing 2.8×10^{10} particles/ml) was purchased from Difco Laboratories, Detroit, Mich. Hank's balanced salts solution was obtained from Gibco (Grand Island Biological Co., Grand Island, N. Y.).

Leukocytes were prepared from whole human blood by a modification of the method of Lehrer and Cline (10). 50 ml of whole blood containing 1000 U of heparin was mixed in a 100 ml graduated cylinder with 25 ml of 6% dextran 75 in 0.9% saline containing 0.1% glucose. This mixture was left undisturbed at room temperature to permit red cells to settle out. After 80 min, the straw-colored upper layer was carefully withdrawn from the graduate and centrifuged at 150 g for 12 min at 4°C to separate the granulocytes from the bulk of the mononuclear cells and platelets. The pellet, which contained most of the granulocytes, was treated according to Woeber, Doherty, and Ingbar (11) to remove residual red cells. The final leukocyte preparation was suspended in Hank's balanced salts solution containing two times the usual concentration of glucose, and diluted to a final concentration of 5,000–10,000 cells/mm³ (determined on a Coulter Counter model B, Coulter Electronic, Inc., Hialeah, Fla.). A smear of this preparation showed that it was essentially devoid of red cells, and contained fewer than 1 platelet per 50 white cells. Differential counting showed that more than 90% of the cells in the preparation were polymorphonuclear leukocytes.

The detection of O_2^- was based on its ability to reduce cytochrome c (6). For all experiments except those shown in Fig. 2, reaction mixtures contained 3 ml of leukocyte suspension, latex particle suspension sufficient to provide 10

particles/white cell, and 45 nmol cytochrome c in a final volume of 3.05 ml. For studies of the effects of various proteins (e.g., superoxide dismutase, albumin) on cytochrome c reduction, 33 μg of the protein in question was added to the reaction mixture. Each such reaction mixture was accompanied by a control mixture prepared with the same leukocyte suspension, identical in all respects except for the omission of the protein under study. Once prepared, the reaction mixtures were stored on ice. Before incubation, 1.5 ml of each reaction mixture was reserved at 0°C for use as a blank. The remainder was incubated for 15 min at 37°C. Reactions were terminated by placing the vessels in melting ice. After centrifugation of blanks and incubated mixtures at 30,000 g for 5 min at 4°C to remove leukocytes and latex, cytochrome c reduction was determined by measuring the absorbance of the incubated supernate at 550 nm on a Cary 15 recording spectrophotometer (Cary Instruments, Monrovia, Calif.), using the unincubated blank as reference. ΔE_{mM} (ferrocytochrome c minus ferricytochrome c) at 550 nm was taken as 15.5 (12).

For experiments dealing with the effect of latex particles on O_2^- production (Fig. 2), two pairs of reaction mixtures were prepared from each suspension of leukocytes. Each of the first pair contained 6.0 ml leukocyte suspension and 90 nmol cytochrome c in a final volume of 6.1 ml, while each of the second pair contained 3.0 ml leukocyte suspension, 45 nmol cytochrome c, and 33 μg superoxide dismutase in 3.05 ml. Latex particles (10/cell) were added to one member of each pair of reaction mixtures. The first pair of mixtures was incubated at 37°C as described above, removing 1.5-ml samples to melting ice at 0 (blank), 4, 10, and 20 min. The second pair was incubated similarly, taking samples at 0 (blank) and 20 min. Centrifugation and measurement of cytochrome c reduction were performed as above.

Leukocyte O_2 consumption was determined manometrically using white cell preparations containing 15,000 cells/mm³. From each preparation two reaction mixtures were prepared, each containing 2 ml white cell suspension plus sufficient latex particle suspension to provide 10 particles/cell. Superoxide dismutase (39 μg) was added to one of the reaction mixtures but omitted from the other. O_2 uptake was measured at 35°C with a Gilson differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.).

RESULTS

Incubation of leukocytes with cytochrome c and latex particles was found to lead to the reduction of the cytochrome. Experiments carried out according to the procedure described above showed that cytochrome c reduction amounted to 1.16 ± 0.84 SD nmol/10^7 leukocytes per 15 min. The fact that the change in absorbance at 550 nm, the parameter from which cytochrome c reduction was determined, actually was attributable to cytochrome c is shown by the spectrum illustrated in Fig. 1. This spectrum, a difference spectrum between an incubated mixture and its corresponding unincubated blank, shows peaks at 418, 519, and 550 nm, in accord with the reported spectral difference between reduced and oxidized cytochrome c (12). Further evidence for the validity of using ΔA_{550} as an indicator of cytochrome c reduction in this system was the observation (not shown) that omis-

sion of cytochrome c abolished the spectral change at this wavelength.

Proof that the reduction of cytochrome c by white blood cells was accomplished by O_2^-, like proof obtained previously for the involvement of O_2^- in other reactions (6–8), depended upon the demonstration that cytochrome c reduction was inhibited by the presence in the reaction mixture of superoxide dismutase. This enzyme, a small copper-containing protein obtained from erythrocytes (6), destroys O_2^- by catalyzing its conversion to oxygen and hydrogen peroxide:

$$2\ O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2$$
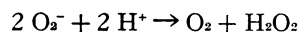
Experiments demonstrating the effect of superoxide dismutase on the reduction of cytochrome c by leukocytes are presented in Table I. In the presence of this enzyme, the reduction of cytochrome c was diminished to 12% of the control level. In contrast, cytochrome c reduction was unaffected by boiled dismutase, albumin, or catalase. Evidence against the possibility that the decrease in cytochrome c reduction represented a nonspecific effect of dismutase on leukocyte metabolism was provided by the observation that the enzyme was without significant effect on leukocyte oxygen uptake. In three experiments, O_2 uptake in the presence of dismutase was 10.9±3.2 SE μl/h per 10^7 cells, compared with a value of 7.8±1.3 SE μl/h per 10^7 cells in the absence of the enzyme. It thus appears that under these conditions, the agent responsible for the reduction of cytochrome c is O_2^-.

An increase in absorbance at 550 nm also took place in the presence of white blood cells which had been heated for 10 min in boiling water. Superoxide dismutase had no effect on the increase in absorbance at 550 nm seen with boiled cells (in three experiments, ΔA_{550} in the presence of dismutase was 0.045±0.019 SE/15 min, compared with a value of 0.040±0.020 SE/15 min in the absence of dismutase; $P > 0.15$). In contrast, the rise in A_{550} associated with cytochrome c reduction by unboiled cells from the same preparations was greatly diminished by dismutase. The lack of effect of superoxide dismutase with boiled cells indicates that O_2^- played no role in the increased A_{550} produced by those cells.

Latex particles had been present in the incubation mixtures for all the foregoing experiments with the idea that the phagocytosis of these particles by granulocytes would stimulate O_2^- production. Such stimulation was confirmed by experiments comparing O_2^- production by leukocytes in the presence and absence of particles. In these experiments, performed as described in Methods, superoxide-dependent cytochrome c reduction was calculated by subtracting the cytochrome c reduction occurring in the presence of dismutase from that occurring in its absence. Cytochrome c reduction at 4 and 10 min in the presence of dismutase was estimated by linear

TABLE I

Inhibition of Cytochrome c Reduction in the Presence of White Blood Cells by Superoxide Dismutase

| Agent | Cytochrome c reduction, nmol/10^7 cells per 15 min | | |
	Agent absent	Agent present	Significance of difference
Superoxide dismutase (20)	1.16±0.84 SD	0.14±0.14 SD	$P < 0.001$
Boiled dismutase (4)	0.48±0.29	0.41±0.30	NS
Albumin (4)	0.48±0.29	0.48±0.21	NS
Catalase (6)	1.11±0.78	1.01±0.52	NS

Incubations were performed as described in the text. The figure in parenthesis indicates the number of experiments with each agent. Statistical significances of the differences were determined by Student's t test, using paired data.

interpolation based on the value determined at 20 min, since the yield of cells was too low to permit a separate determination of superoxide-independent cytochrome c reduction at each time point.

The results (Fig. 2) show that at each time interval, O_2^- production in the presence of latex particles exceeded that observed in their absence. (The lack of direct determinations of dismutase-inhibited cytochrome c reduction at 4 and 10 min renders the statistics at these times perhaps somewhat less reliable than the 20-min values.) These results show that O_2^- production was stimulated by the presence of latex particles in the reaction mixture.

DISCUSSION

It is likely that at least some of the O_2^- formed in these incubations was the product of the granulocytes contained in the cell preparations. Since the preparations were 90% pure, it could be argued that the contaminating lymphocytes were responsible for the production of O_2^-. However, the increase in O_2^- production with latex particles, an increase presumably associated with the phagocytosis of these particles by the granulocytes, provided strong circumstantial evidence for the production of O_2^- by these cells.

From the above data, phagocytizing white cells produce O_2^- at an average rate of 1.03 nmol/10^7 leukocytes per 15 min. This rate, however, only refers to O_2^- which has reacted with cytochrome c. It is likely that a significant fraction of the highly reactive O_2^- produced by the leukocytes failed to be detected because it was consumed by reaction with other constituents of the incubation mixture before it could reduce cytochrome c. The rate of O_2^- production calculated from these experiments therefore represents a lower limit; it is possible that the
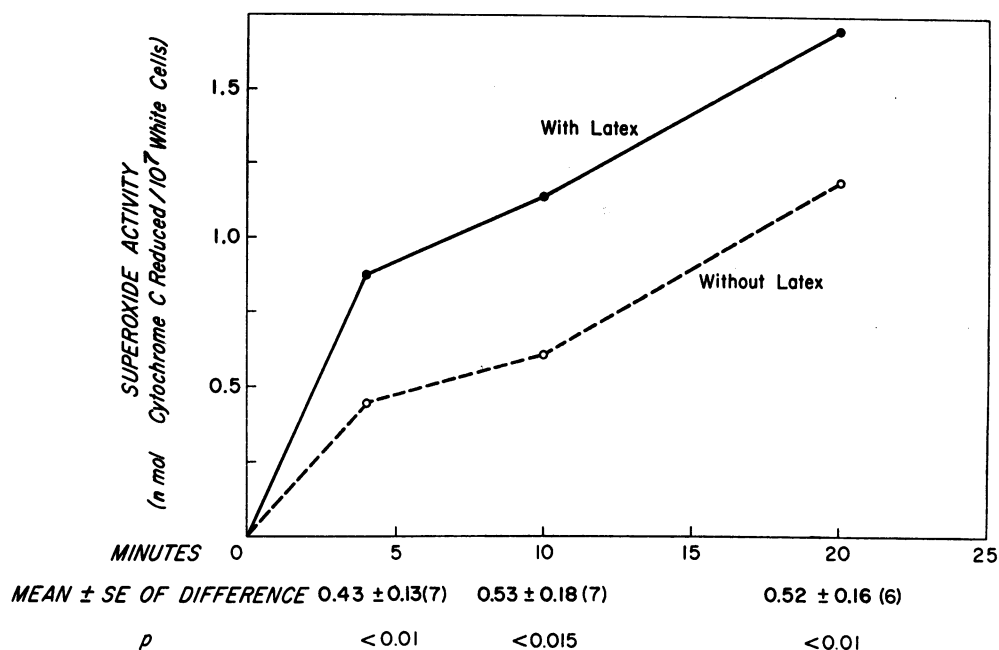
FIGURE 2 Effect of latex particles on superoxide production by white cells. For details, see text. The figure in parenthesis represents the number of determinations at each time point. Statistical significances were determined by Student's t test, using paired data.

true rate of O_2^- production is much greater than that observed here.

The circumstances under which O_2^- is produced suggest that superoxide as well as H_2O_2 may participate in bacterial killing. The possibility is thus raised that the destruction of microorganisms by the leukocyte may be accomplished by a number of alternative oxygen-dependent mechanisms.

ACKNOWLEDGMENTS

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