Biological Defense Mechanisms

THE EFFECT OF BACTERIA AND SERUM ON SUPEROXIDE PRODUCTION BY GRANULOCYTES

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ABSTRACT We previously reported that granulocytes are able to produce superoxide (O_2^-), a highly reactive compound formed by the one-electron reduction of oxygen. The demonstration of O_2^- production was based on the observation that the reduction of extracellular cytochrome *c* by granulocytes was greatly diminished by superoxide dismutase, an enzyme catalyzing the conversion of O_2^- to hydrogen peroxide and oxygen. In the present report, studies concerning the effect of bacteria and serum on O_2^--dependent cytochrome *c* reduction by granulocytes are described.

In the absence of bacteria, the O_2^--dependent reduction of extracellular cytochrome *c* by granulocytes under optimal assay conditions amounted to 9.2±2.8 SD nmol/3 × 10^6 cells/20 min. When bacteria (100 organisms/cell) were present, the O_2^--dependent cytochrome *c* reduction under otherwise similar conditions increased by a factor of nearly four (34.5±9.4). There was no effect of albumin or catalase on cytochrome *c* reduction, and boiled dismutase had only a small effect. Omission of granulocytes or substitution of live cells by cells killed by heat abolished O_2^--dependent cytochrome *c* reduction. Bacteria killed by autoclaving were almost as effective as live bacteria in stimulating granulocyte O_2^- production. Measurements of particle uptake and O_2 uptake by granulocytes indicated that superoxide dismutase did not affect granulocyte metabolism nonspecifically, supporting the conclusion that the diminution of cytochrome *c* reduction in the presence of dismutase was due to the destruction of O_2^- by this enzyme.

Stimulation of O_2^- production by bacteria was strongly dependent on the presence of serum in the incubation mixture. Serum heated to 56°C for 45 min was as effective as unheated serum in stimulating O_2^- production in the presence of bacteria, but boiled serum had no effect. Other experiments suggested that incubation of bacteria with serum resulted in the release of a nonparticulate heat-labile substance capable of stimulating O_2^- production in the absence of bacteria.

Certain characteristics of the O_2^--dependent cytochrome *c* reduction by granulocytes were studied, including the dependence of this process on granulocyte, cytochrome *c*, and bacterial concentrations. In addition, O_2^--dependent cytochrome *c* reduction was followed as a function of time. A constant rate was found with resting granulocytes. With bacteria the time course was more complex. A well-defined lag was followed by a fairly brief period of extremely vigorous cytochrome *c* reduction. During this period, the maximum rate of cytochrome *c* reduction exceeded the rate observed in the absence of bacteria by a factor of 12. The rate then decreased until by 40 min, it had slowed to the rate observed in the absence of bacteria.

From the above results, it was concluded that the exposure of the granulocyte to bacteria plus serum initiates a process in which a defined quantity of O_2^- is formed in a rapid burst lasting 20–30 min. It is conceivable that the O_2^- generated by this process may be involved in the killing of bacteria by the granulocytes.

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INTRODUCTION

The polymorphonuclear leukocyte is an important component of the host defense system because of its ability to ingest and destroy invading microorganisms. Although the ingestion of bacteria can take place in an anaerobic environment (1), oxygen is required for the efficient destruction of the microorganisms. It has been postulated that the oxygen-dependent process by which

microorganisms are killed involves the reduction of O_2 to H_2O_2, which participates directly in bacterial killing. The bases for this postulate are the findings that both O_2 uptake and H_2O_2 production by the leukocyte are stimulated by phagocytosis (1, 2) and that leukocytes defective in bacterial killing, i.e., those from patients with chronic granulomatous disease and severe granulocyte glucose-6-phosphate dehydrogenase deficiency, fail to display the increase in O_2 consumption and H_2O_2 production ordinarily associated with the ingestion of bacteria (3, 4). Since the bactericidal effectiveness of hydrogen peroxide itself is relatively low, it has been further proposed that other constituents of the leukocyte act in conjunction with hydrogen peroxide to bring about the destruction of ingested bacteria. The most extensively investigated of these proposals is the one originally outlined by Klebanoff (5), in which the primary bactericidal agent is generated in a myeloperoxidase-catalyzed reaction between a halide anion and hydrogen peroxide. Although there is much evidence documenting the importance of this bactericidal system in leukocytes (5–9), the fact that bacterial killing by cells severely deficient in myeloperoxidase is only moderately impaired (9) implies that other mechanisms for the destruction of bacteria must also exist.

In considering such alternative mechanisms, the possibility occurred to us that O_2^- might participate in bacterial killing by leukocytes. O_2^-, produced by the one-electron reduction of oxygen, is a highly reactive compound formed by several biochemical systems (10). We have recently demonstrated that O_2^- is produced by human granulocytes both in the absence and presence of latex particles (11). The present investigation represents a study of the effect of bacteria and serum on O_2^- production by these cells.

METHODS

Materials. Catalase, human serum albumin, xanthine, and milk xanthine oxidase (grade I) were purchased from Sigma Chemical Co., Inc., St. Louis, Mo. Horse heart ferricytochrome *c* (grade VI, Sigma) was dissolved in water to a concentration of 1.5 mM. Dextran 75 (6%, in 0.9% NaCl) was obtained from Travenol Laboratories, Morton Grove, Ill. Hanks' Balanced Salts Solution (HBSS),[1] concentrated once without phenol red, was purchased from Grand Island Biological Co., Grand Island, N. Y. Before use, HBSS was supplemented with additional glucose to bring the final concentration to 2 g/liter. Pooled human AB serum, purchased from Grand Island Biological Co., was diluted with HBSS (serum: HBSS = 3:7, vol/vol) before use. Gelatin in granular form was obtained from Fisher Scientific Co., Pittsburgh, Pa. Superoxide dismutase (3,000 U/mg) was obtained from Truett Laboratories, Dallas, Tex. Assay of the activity of this enzyme preparation by the xanthine-xanthine oxidase method of McCord and Fridovich (12) showed that at a concentration of 0.3 μM (10 μg/ml) the enzyme completely abolished O_2^--dependent cytochrome *c* reduction in a system generating O_2^- at a rate of 5.5 nmol ml^{-1} min^{-1}. Opsonized paraffin oil emulsion tinted with Oil-Red-O was the gift of Dr. T. P. Stossel. Other reagents were the best grade commercially available and were used without further purification.

E. coli C, kindly provided by Dr. Andrew Wright, Tufts University School of Medicine, and an unspecified strain of *E. coli* (hereinafter called *E. coli* X), obtained from the bacteriology laboratory of the New England Medical Center Hospital, were grown on nutrient agar and nutrient broth obtained from Difco Laboratories, Detroit, Mich. Experiments were conducted with suspensions of bacteria in HBSS containing 0.1% gelatin (HBSS-gelatin). To prepare these suspensions, bacteria in broth culture were separated from the medium by centrifugation at 1,500 *g* for 15 min at 25°C, washed twice with HBSS-gelatin, and then suspended in HBSS-gelatin at a concentration of 3×10^9 organisms/ml (equivalent to an absorbance at 620 nm of 0.45 [13]).

Preparation of leukocytes. Leukocytes were prepared from whole human blood by a modification of the method of Skoog and Beck (14). 50 ml of blood were mixed with 10 ml of acid citrate dextrose (ACD) solution containing

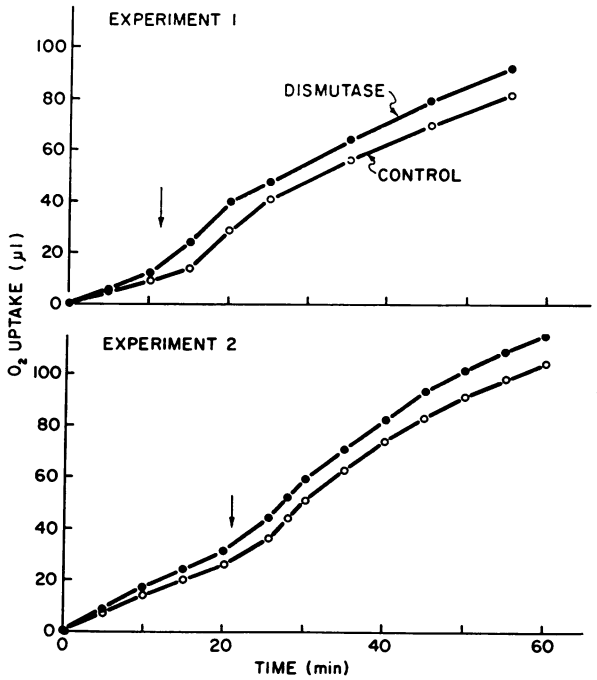

FIGURE 1 Effect of bacteria on granulocyte oxygen uptake in the presence and absence of superoxide dismutase. Granulocyte suspension containing 1.8×10^7 cells in 3.5 ml HBSS-gelatin was placed in a 15-ml Warburg flask along with 2.0 ml diluted serum, 0.48 μmol cytochrome *c*, and (where indicated) 3.6 nmol superoxide dismutase. *E. coli* C suspension (1.8×10^9 bacteria/ml HBSS-gelatin), 0.5 ml, was placed in the sidearm. Oxygen uptake was determined manometrically at 37°C as described in Methods. The arrow indicates the point at which bacteria were added to the mixture in the main compartment of the flask. A different preparation of cells was used for each experiment.

[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; HBSS, Hanks' balanced salt solution.

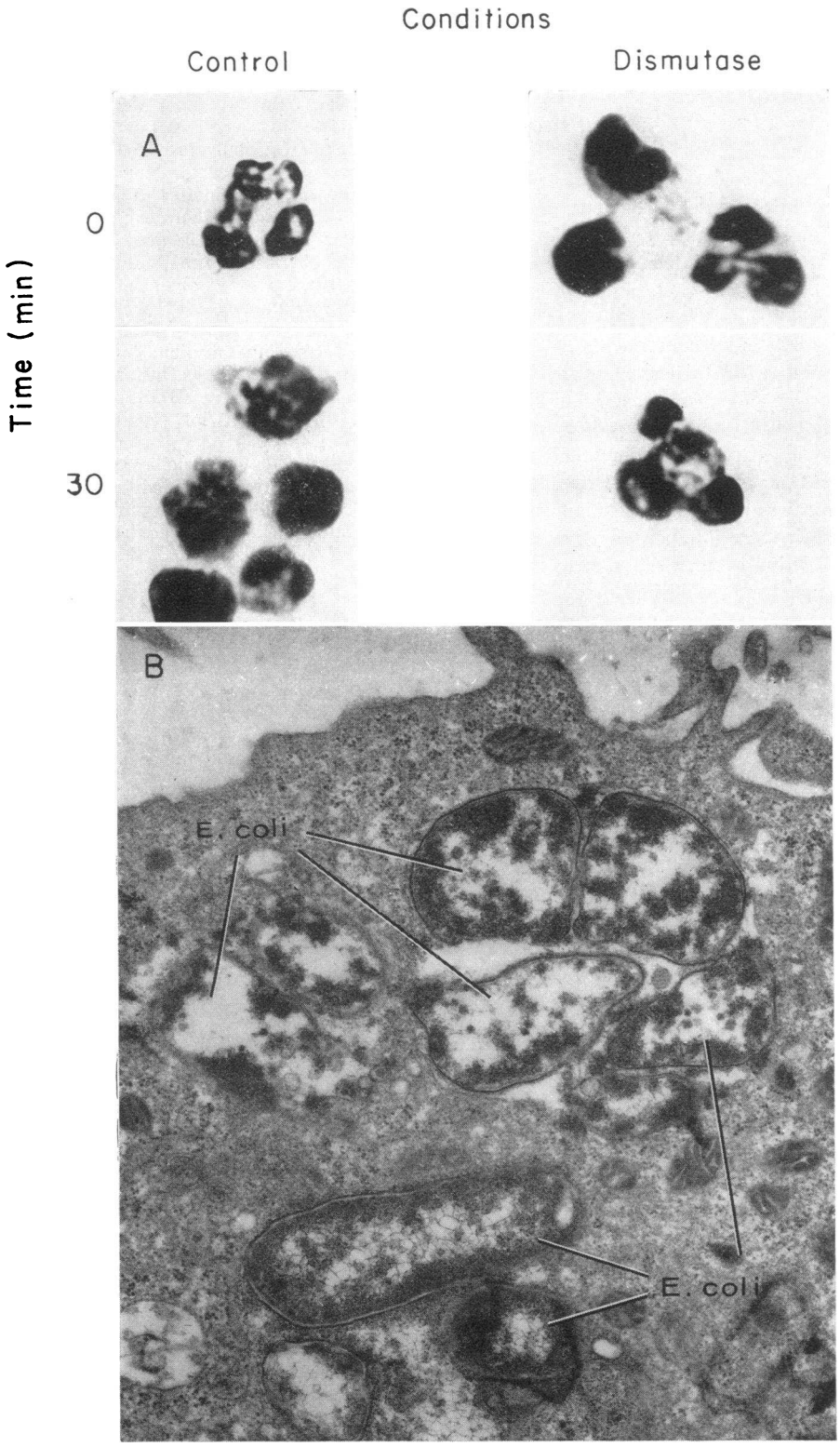

FIGURE 2 Uptake of bacteria by granulocytes in the presence and absence of superoxide dismutase. A. Granulocytes (3×10^6 cells/ml) incubated for the times noted at 37°C with

0.14 M citric acid, 0.20 M trisodium citrate, and 0.22 M dextrose. The blood-ACD mixture was then mixed in a 100-ml graduated cylinder with 30 ml of 6% dextran 75 in 0.9% saline. This mixture was left undisturbed at room temperature for 60 min to allow the red cells to settle out. After the sedimentation period, the straw-colored supernate was removed carefully from the graduate and was centrifuged at 150 *g* for 12 min at 4°C in an International model PR-2 refrigerated centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) to separate the granulocytes from the bulk of the mononuclear cells and platelets. The pellet contained most of the granulocytes and was treated according to the method of Woeber, Doherty, and Ingbar (15) to remove residual red blood cells. After this treatment the leukocytes were suspended in HBSS-gelatin and diluted to a final concentration of 9,000 cells/mm^3 (determined on a Coulter Counter, Model F, Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). Microscopic examination of the leukocyte preparation revealed that there were essentially no red cells present and that there was fewer than 1 platelet per 50 white cells. Very few of the leukocytes were broken (< 2%), and differential counting showed that more than 90% of the cells in the preparation were polymorphonuclear leukocytes.

Determination of cytochrome c *reduction by granulocytes.* To prepare the reaction mixture, 1.0 ml diluted serum, the appropriate volume of leukocyte suspension, and other constituents as indicated in the legends to the figures and tables were placed in siliconized Vacutainer tubes (Becton, Dickinson & Co., Rutherford, N. J.) standing in ice. The volume of the reaction mixture was adjusted with HBSS-gelatin such that after addition of bacteria and cytochrome *c*, the reaction mixture contained 3.2 ml. To begin the assay, the reaction vessel was placed in a 37°C water bath to permit temperature equilibration to take place. 2 min later bacteria and cytochrome *c* were added. Immediately, a 1.5-ml portion of the reaction mixture was reserved in melting ice to serve as reference. The remaining portion was incubated at 37°C for 30 min (unless otherwise noted), after which time the incubation was terminated by placing the reaction vessel in melting ice. Leukocytes and bacteria were removed by centrifugation at 1,500 *g* for 20 min at 4°C. Cytochrome *c* reduction was determined from short-range difference spectra (575–525 nm) of the supernate from the incubated reaction mixture, with the corresponding unincubated supernate as reference. Spectra were obtained on a Cary 118C recording spectrophotometer (Cary Instruments, Monrovia, Calif.). ΔE_{mM} (ferrocytochrome *c* minus ferricytochrome *c*) at 550 nm was taken as 15.5 (16).

Uptake of paraffin oil by granulocytes. Paraffin oil uptake by granulocytes in the presence and absence of superoxide dismutase was determined by the method of Stossel (17). With each preparation of granulocytes, two reaction mixtures were prepared. Each contained 0.4 ml of granulocyte suspension and 0.1 ml of an opsonized emulsion of paraffin oil tinted with Oil-Red-O. Superoxide dismutase (0.6 μM) was present in one of the reaction mixtures. Incubations were conducted at 37°C for 5 min. The reactions were terminated with 3 ml of ice-cold 1 mM *N*-ethylmaleimide in 0.9% saline. Uptake of paraffin oil was then measured as described by Stossel.

Oxygen uptake determinations. Oxygen uptake was determined manometrically, with a Gilson submarine differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.).

Statistics. Statistical significances were determined by means of Student's *t* test, with paired data.

RESULTS

Granulocyte oxygen uptake. The present experiments were performed to determine the effect of bacterial phagocytosis on O_2^- production by granulocytes. Our assay depends on the fact that superoxide dismutase, an enzyme that catalyzes the conversion of O_2^- to hydrogen peroxide and oxygen, interferes with the reduction of added cytochrome *c* by granulocyte suspensions. We have previously demonstrated the inhibition by superoxide dismutase of cytochrome *c* reduction by granulocytes ingesting latex particles. Experiments performed at that time indicated that superoxide dismutase did not interfere with oxygen uptake by such cells. Fig. 1 shows the results of similar experiments, conducted this time with bacteria rather than latex particles. Before the addition of bacteria, oxygen was taken up at an average rate of 37 μl/10^7 cells per h. Upon the addition of bacteria, the rate of oxygen uptake began to rise, reaching a maximum of 100 μl/10^7 cells per h between 5 and 10 min after the microorganisms were added. The rate then began to decline, so that by 30 min after the addition of bacteria, it had returned to the resting level. Augmentation of [1-^{14}C]glucose oxidation by granulocytes incubated with starch particles, as well as phagocytosis of the particles themselves, has been shown by others to follow a similar time-course (18). The presence of superoxide dismutase appeared to increase white cell oxygen uptake slightly both in the absence and presence of bacteria.

Phagocytosis. Microscopic examination of granulocytes exposed to bacteria and serum provided further evidence against a nonspecific effect of superoxide dismutase on white cell function. Photomicrographs of

E. coli C (100 bacteria/cell), 0.24 μmol cytochrome *c*, and (where indicated) 1.8 nmol superoxide dismutase under the conditions used for the determination of cytochrome *c* reduction (see Methods) were smeared on glass slides and stained with Wright's stain. B. A reaction mixture containing 12.0 ml granulocyte suspension (9 × 10^6 cells/ml), 12.0 ml diluted serum, 6.0 ml *E. coli* C suspension (1.8 × 10^9 cells/ml HBSS-gelatin), 6.0 ml HBSS-gelatin, 2.9 μmol cytochrome *c* and 22 nmol superoxide dismutase in a final volume of 38 ml The cells were then isolated by centrifugation at 150 *g* for 20 min at 4°C. The pellet was washed twice with HBSS, then suspended in 2% paraformaldehyde-glutaraldehyde containing 0.2% picric acid buffered with 0.1 M cacodylate to pH 7.3. After fixation at room temperature overnight, the cells were embedded in Epon-A12 (Shell Chemical Co., New York), cut into thin sections, stained with uranyl acetate and lead citrate, and examined by electron microscopy. Intracellular *E. coli* are labeled as such. Magnification × 21,000.

TABLE I

Stimulation by Bacteria of O_2^--dependent Cytochrome c Reduction by Granulocytes

Conditions	Cytochrome *c* reduction	
	Bacteria absent	Bacteria present
	nmol/3 × 10^6 cells/30 min	
Dismutase absent (17)	13.2±3.9 SD	36.4±8.6
Dismutase present (17)	4.2±2.2*	2.0±1.8*
Average difference (= O_2^--dependent cytochrome reduction)	9.2±2.8	34.5±9.4‡

Reaction mixtures contained 9×10^6 granulocytes, 80 μM cytochrome *c*, *E. coli* C (9×10^8 organisms), and superoxide dismutase (0.6 μM) as indicated. Cytochrome *c* reduction was determined as described in Methods. The figure in parentheses indicates the number of experiments performed. A different preparation of cells was used for each experiment.

* $P < 0.001$ for the difference between cytochrome *c* reduction in the presence and absence of superoxide dismutase.

‡ $P < 0.001$ for an effect of bacteria on O_2^--dependent cytochrome *c* reduction.

representative cells, shown in Fig. 2A, indicate that bacteria were efficiently taken up by granulocytes in the presence of 0.6 μM superoxide dismutase. This finding was confirmed by electron microscopy, which revealed the presence of microorganisms within the cytoplasm of granulocytes incubated with bacteria in the presence of superoxide dismutase (Fig. 2B).

The lack of effect of superoxide dismutase on phagocytosis was confirmed by experiments quantitating the uptake of opsonized emulsified paraffin oil by granulocytes in the presence and absence of the enzyme. Paraffin oil uptake by three granulocyte preparations incubated in the absence of dismutase was 0.162±0.020 SE mg/10^7 cells per min, compared with 0.177±0.012 mg/10^7 cells per min for the same preparations incubated in the presence of dismutase. Similar values for paraffin oil uptake have been reported elsewhere (17). These results indicate that superoxide dismutase has little if any effect on phagocytosis by granulocytes.

Stimulation of O_2^- production by bacteria. It has been shown previously that granulocytes are able to generate O_2^-. Table I presents evidence that O_2^- production is stimulated when granulocytes are exposed to microorganisms. The data show that the reduction of cytochrome *c* by granulocytes incubated in the presence of *E. coli* was substantially greater than the reduction that occurred in the absence of the bacteria. Cytochrome *c* reduction by resting leukocytes was diminished to 30% of the control value in the presence of superoxide dismutase, while for leukocytes incubated with bacteria, the reduction of cytochrome *c* in the presence of dismutase was less than 10% that observed in the absence of the enzyme. O_2^--mediated cytochrome *c* reduction was calculated by subtracting the value obtained in the presence of dismutase from the corresponding control value. This calculation showed that granulocytes incubated with bacteria exhibited a nearly four-fold increase in the production of O_2^- when compared with their companion resting cells during a 30-min incubation.

To establish that the difference between the control and the dismutase-containing reaction mixture actually represented O_2^--mediated cytochrome *c* reduction, it was necessary to demonstrate that this difference was the result of the specific enzymatic property of the dismutase. This was accomplished by comparing the effects of active superoxide dismutase, boiled dismutase, albumin, and catalase on the amount of cytochrome reduced by a leukocyte-bacteria mixture. The results of these experiments are presented in Table II. Superoxide dismutase was found to diminish the extent of cytochrome *c* reduction by 95%. In contrast, similar amounts of albumin and catalase caused no change in observed cytochrome *c* reduction. Dismutase inactivated by boiling for 10 min caused a slight but statistically significant ($0.025 < P < 0.05$) decrease in cytochrome *c* reduction. However, the inhibition of cytochrome *c* reduction by boiled dismutase was much less than that seen with active dismutase ($P < 0.001$). The small but significant inhibition by boiled dismutase may be due to the quenching of O_2^- by the copper released upon denaturation of the enzyme (10). These data show that substantial inhibition of granulocyte-mediated cytochrome *c* reduction requires the presence of active superoxide dismutase.

TABLE II

Specificity of Inhibition of Granulocyte Cytochrome c Reduction by Superoxide Dismutase

Agent	Cytochrome *c* reduction	Significance of difference (vs no agent)	Significance of difference (vs superoxide dismutase)
	Cytochrome *c* reduction		
	nmol/3 × 10^6 cells/30 min		
No agent	22.1±0.7 SD	—	$P < 0.001$
Superoxide dismutase	1.0±0.1	$P < 0.001$	—
Boiled* superoxide dismutase	18.1±1.8	$P < 0.05$	$P < 0.001$
Albumin	22.8±1.4	NS	$P < 0.001$
Catalase	21.8±0.7	NS	$P < 0.001$

With each preparation of cells, reaction mixtures containing granulocytes (9×10^6 cells), cytochrome *c* (40 μM), *E. coli* X (9×10^8 organisms), and 30 μg of the agent under investigation (equivalent to 0.3 μM superoxide dismutase). Cytochrome *c* reduction was determined as described in Methods. The data represent the results from three cell preparations.

* Inactivated by heating for 10 min in boiling water.

In Table III are presented results that establish that granulocytes are the source of O_2^- generated in these incubations. O_2^- was not generated in significant quantities when granulocytes were omitted from the incubation mixture. Similarly, little O_2^- production was seen in reaction mixtures containing leukocytes heated for 10 min in boiling water before incubation. By contrast, granulocyte O_2^- production was stimulated by killed bacteria almost as effectively as by live bacteria.

The effect of serum on bacterial stimulation of O_2^- production. For all of the above experiments, serum was present in the incubation mixture, since opsonization by serum is ordinarily required for the optimal uptake of bacteria by granulocytes (3). Table IV shows that when serum was omitted from a leukocyte-bacteria mixture, O_2^- production was greatly curtailed. In addition, these results show that O_2^- production by granulocytes appears to be affected by serum even in the absence of bacteria. Elimination of serum from a reaction mixture containing resting leukocytes diminished O_2^--dependent cytochrome *c* reduction to less than 50% of its control value. The effect of serum on the leukocyte-bacteria mixture represents a true synergism, since the stimulation of O_2^- production with the combination of bacteria plus serum exceeds by a factor of three the sum of the effects produced by each of these constituents alone.

Experiments with heated serum showed that the effect of serum was probably not mediated by complement. Table V shows that serum that had been heated at 56°C for 45 min, a procedure that abolished both the classical (19) and alternate (20) pathways for complement activation, is as effective as unheated serum in the stimulation of O_2^- production by granulocytes in the presence of bacteria. The ability of the serum to stimulate O_2^- production was destroyed, however, by boiling for 5 min. These findings suggest that the activity responsible for the stimulation of O_2^- production is associated with a protein which interacts with bacteria by a pathway independent of that for complement activation.

The O_2^--stimulating activity produced by exposure of serum to bacteria appears largely to be present in the serum rather than associated with the microorganism. The experiments of Table VI show that when serum preincubated with bacteria was added to granulocytes after removal of the bacteria by centrifugation, O_2^- production was almost as great as when uncentrifuged serum was used. Boiling the preincubated serum abolished stimulation. In contrast, preincubated bacteria had a substantially smaller effect. Control experiments (not shown) indicated that the process of preincubation alone was not sufficient to generate the O_2^--stimulating activity, since neither serum preincubated by itself nor bacteria preincubated in HBSS-gelatin were able to stimulate granulocyte O_2^- production more than constituents that had not been preincubated. Moreover, HBSS-gelatin in which bacteria had been preincubated had no effect on granulocyte O_2^- production after removal of the bacteria.

TABLE III

Dependence of O_2^--Dependent Cytochrome c *Reduction on the Presence of Granulocytes*

Conditions	O_2^--dependent cytochrome *c* reduction
	nmol/3 × 10⁶ cells/30 min
Set 1 (4)	
Complete mixture	24.1±6.0 SD
Omit bacteria	6.2±1.3
Omit granulocytes	0.6±0.4
Boiled* granulocytes	0.8±0.4
Set 2 (3)	
Live bacteria	29.6±6.2
Killed‡ bacteria	24.6±5.5
Omit bacteria	12.7±3.4

For each assay, duplicate reaction mixtures were prepared, each containing granulocytes (9×10^6 cells), cytochrome *c* (for set 1, 40 μM; for set 2, 80 μM), and bacteria (for set 1, 9×10^8 *E. coli* X; for set 2, 9×10^8 *E. coli* C), with omissions as noted. Superoxide dismutase (0.3 μM) was determined as described in Methods. The difference in cytochrome *c* reduction between the duplicate reaction mixtures was taken to represent O_2^--dependent cytochrome *c* reduction. The figure in parentheses indicates the number of experiments performed. A different preparation of cells was used for each experiment.
* Cells were heated for 10 min in boiling water before incubation.
‡ Bacteria in nutrient broth were autoclaved for 20 min at 121° before centrifugation and resuspension in HBSS-gelatin.

TABLE IV

Effect of Serum on O_2^--Dependent Cytochrome c *Reduction in the Presence and Absence of Bacteria*

Conditions	O_2^--dependent cytochrome *c* reduction		
	Serum present	No serum	Significance of difference
	nmol/3 × 10⁶ cells/30 min		
Bacteria absent (5)	9.2±2.8 SD	3.7±3.1	<0.02
Bacteria present (5)	30.3±4.1	6.7±3.3	<0.001

For each assay, duplicate reaction mixtures were prepared, each containing granulocytes (9×10^6 cells), cytochrome *c* (80 μM), and either diluted serum or HBSS. Where indicated, *E. coli* C were present at 9×10^8 organisms/reaction mixture. Superoxide dismutase (0.6 μM) was present in one of the two reaction mixtures. Cytochrome *c* reduction was determined as described in Methods. The difference in cytochrome *c* reduction between the duplicate reaction mixtures was taken to represent O_2^--dependent cytochrome *c* reduction. The figure in parentheses indicates the number of experiments performed. A different preparation of cells was used for each experiment.

TABLE V

Effect of Heated Serum on O_2^--Dependent Cytochrome c Reduction in the Presence of Bacteria

Serum*	O_2^--dependent cytochrome *c* reduction	
	Bacteria present	Bacteria absent
	nmol/3 × 10^6 cells/30 min	
Diluted normal serum	17.2±2.1 SD	8.4±1.8
56°C serum	19.8±4.7	6.2±3.5
Boiled serum	9.3±3.5	4.8±1.7
None	8.8±1.4	4.6±1.8

To prepare 56°C serum, 3-ml portions of pooled human AB serum in siliconized 15 × 100 mm Vacutainer tubes were heated at 56°C in a water bath for 45 min. The serum was then diluted with HBSS as described in Methods. Boiled serum was prepared by mixing 3 ml of pooled human AB serum with 7 ml HBSS, heating the diluted serum for 5 min in boiling water, centrifuging at 2000 *g* for 15 min at room temperature to remove precipitated protein, and recentrifuging the supernate at 2000 *g* for 10 min at room temperature to remove the last traces of precipitate. For each assay, duplicate reaction mixtures were prepared, each containing granulocytes (9 × 10^6 cells), cytochrome *c* (80 μM), *E. coli* C (9 × 10^8 organisms), and serum as indicated. Superoxide dismutase (0.6 μM) was present in one of the two reaction mixtures. Cytochrome *c* reduction was determined as described in Methods. The difference in cytochrome *c* reduction between the duplicate reaction mixtures was taken to represent O_2^--dependent cytochrome *c* reduction. The data represent the results from four cell preparations.

* "Diluted normal serum" refers to untreated pooled human AB serum diluted with HBSS as described in Methods. "None" refers to reaction mixtures containing HBSS instead of serum.

Further information concerning the properties of the O_2^--stimulating activity of preincubated serum was obtained by ultracentrifugation experiments. The results of Table VII show that most of the activity remains in the supernate after centrifugation of preincubated serum at 105,000 *g* for 1 h. It thus appears that incubation of bacteria with serum causes the release of a soluble heat-labile substance capable of stimulating O_2^- production by granulocytes.

Characteristics of O_2^--mediated cytochrome c reduction by granulocytes in the presence and absence of bacteria. As discussed previously, dismutase-inhibitable cytochrome *c* reduction was the reaction used to detect the production of O_2^- by the leukocyte. Since O_2^- is an extremely reactive compound, it would be expected that other O_2^--consuming compounds would compete with cytochrome *c* for the O_2^- produced by the white cells. According to this scheme, when the initial cytochrome concentration is low enough so that most of the O_2^- is consumed by competing reactions, an increase in cytochrome *c* concentration should be accompanied by a proportional increase in the rate of reduction of cytochrome *c*; at cytochrome concentrations high enough so that cytochrome *c* reduction becomes the major O_2^--consuming reaction, the rate of reduction should be independent of cytochrome concentration. Experiments in which O_2^--dependent cytochrome *c* reduction was determined as a function of cytochrome *c* concentration (Fig. 3) are consistent with this formulation. Cytochrome *c* reduction was roughly proportional to cytochrome *c* concentration below about 15 μM. Above this concentration, the proportionality fell progressively until at 60–80 μM cytochrome *c*, the rate of reduction was

TABLE VI

Stimulation of O_2^--Dependent Cytochrome c Reduction by Serum Pretreated with Bacteria

Reaction conditions		O_2^--dependent cytochrome *c* reduction	
Serum	Bacteria	Experiment 1	Experiment 2
		nmol/3 × 10^6 cells/30 min	
None	None	4.0	3.2
Not preincubated	None	11.9	11.2
None	Not preincubated	5.8	7.6
Not preincubated	Not preincubated	20.2	22.4
Preincubated serum plus bacteria		19.6	26.1
Preincubated	None	19.2	19.8
Boiled preincubated	None	2.7	5.1
None	Preincubated	11.7	16.9

To prepare pretreated serum, 20 ml of a suspension of *E. coli* C in HBSS-gelatin (1.8 × 10^9 bacteria/ml) was added to 40 ml of 30% pooled human AB serum, prepared by mixing pooled human AB serum with HBSS in a ratio of 3:7 (vol/vol). The bacteria and serum were incubated together for 30 min at 37°C. A 5-ml portion of the preincubation mixture, designated "preincubated serum plus bacteria" in the table, was reserved at 0°C. The remainder was centrifuged at 1,500 *g* for 15 min at 4°C to separate the bacteria from the serum. The serum was decanted, and a 10-ml portion was heated for 5 min in boiling water. The protein precipitated by boiling was removed by centrifugation at 1,500 *g* for 15 min at 4°C, and the supernate, designated "boiled preincubated" serum, was reserved at 0°C. The rest of the decanted serum was centrifuged again at 1,500 *g* for 15 min at 4°C to remove any bacteria not eliminated by the first centrifugation, and the supernate from this centrifugation, designated preincubated serum, was reserved at 0°C. The bacteria spun down by centrifugation of the original preincubation mixture were washed twice with HBSS-gelatin, then were suspended in HBSS-gelatin to a concentration of 1.8 × 10^9 bacteria/ml. This suspension is designated preincubated bacteria.

Constituents not preincubated before addition to the O_2^- assay mixture are so designated in the table.

For each assay, duplicate reaction mixtures were prepared, each containing granulocytes (9 × 10^6 cells), cytochrome *c* (80 μM), serum (final concentration 10% vol/vol), and bacteria (final concentration 3 × 10^8 cells/ml) as indicated. Where serum or bacteria were omitted, they were replaced by equal volumes of HBSS or HBSS-gelatin, respectively. Superoxide dismutase (0.6 μM) was present in one of the two reaction mixtures. Cytochrome *c* reduction was determined as described in Methods. The difference in cytochrome *c* reduction between the duplicate reaction mixtures was taken to represent O_2^--dependent cytochrome *c* reduction.

Different preparations of serum, bacteria, and granulocytes were used for each experiment. In every case, "not preincubated" serum was prepared from the same sample of pooled human AB serum used to prepare preincubated serum.

TABLE VII

Sedimentation Behavior of Granulocyte O_2^--Stimulating Activity Produced by the Incubation of Serum with Bacteria

Pretreatment of serum		O_2^--dependent cytochrome *c* reduction		
Preincubation	Centrifugation	Experiment 1	Experiment 2	Experiment 3
		nmol/3 × 10⁶ cells/30 min		
With bacteria	None	26.1	31.9	40.2
With bacteria	Centrifuged to remove bacteria	19.8	28.1	32.5
With bacteria	Centrifuged at 105,000 *g*	18.9	22.4	26.0
With HBSS-gelatin	None	12.4	12.9	18.6

The preincubation of serum with bacteria was accomplished as described in Table VI. A 5-ml portion of preincubated serum was set aside and stored at 0°C, while the remainder was centrifuged twice at 1,500 *g* for 15 min at 4°C to remove bacteria. Part of the bacteria-free supernate was stored at 0°C without further treatment, while the remainder was centrifuged at 105,000 *g* for 1 h at 4°C in a Spinco Model L2-65B ultracentrifuge with a Model SW-65K swinging bucket rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

While the preincubation with bacteria was being carried out, an additional 10-ml portion of 30% pooled AB serum (prepared as described in Table VI) was incubated for 30 min at 37°C with 5 ml of HBSS-gelatin. After incubation, this serum was stored at 0°C without further treatment.

For each assay, duplicate reaction mixtures were prepared, each containing 1.5 ml of serum pretreated as indicated, together with HBSS-gelatin (0.5 ml), granulocytes (9 × 10^6 cells), and cytochrome *c* (80 μM), in a total volume of 3.2 ml. Superoxide dismutase (0.6 μM) was present in one of the two reaction mixtures. Cytochrome *c* reduction was determined as described in Methods. The difference in cytochrome *c* reduction between the duplicate reaction mixtures was taken to represent O_2^--dependent cytochrome *c* reduction.

Different preparations of serum, bacteria, and granulocytes were used for each experiment.

independent of cytochrome concentration. In this concentration range, it is likely that cytochrome *c* reduction was the major extracellular pathway for superoxide consumption. In general, the cytochrome *c* concentrations used for the experiments in this paper were in the range where reduction is independent of concentration.

The dependence of O_2^--mediated cytochrome *c* reduction on granulocyte concentration is shown in Fig. 4. For the experiments presented in the upper and middle panels of Fig. 4, the cytochrome *c* concentration was 80 μM. The upper panel shows data obtained with incubations containing a constant number of bacteria—that is, incubations in which the bacteria/leukocyte ratio varied inversely with the cell concentration—while the middle panel shows data obtained when the concentration of bacteria was adjusted so that each incubation contained 100 microorganism/leukocyte. The results were qualitatively similar in each case, although O_2^- production at high concentrations of leukocytes was greater when the bacteria/granulocyte ratio was constant than when the bacterial concentration was constant, a result in accord with the effect of bacterial concentration on granulocyte O_2^- production (see below). Both in the presence and absence of bacteria, the proportionality between cell concentration and cytochrome *c* reduction began to fail at granulocyte levels of about 2,000/mm³. The failure of proportionality at higher cell concentrations suggests the existence of a granulocyte-dependent process that destroys extracellular O_2^- at a rate that becomes large enough to compete with cytochrome *c* reduction at cell concentrations of the order of 2,000/mm³. This suggestion is supported by the observation that when the cytochrome *c* concentration was reduced from 80 to 40 μM, the proportionality began to fail at a cell concentration of only 1,000/mm³ (Fig. 4, bottom panel).

Fig. 5 shows the dependence of O_2^--mediated cytochrome *c* reduction on bacterial concentration. No significant change in cytochrome *c* reduction was seen until the bacteria/granulocyte ratio reached a value of 4. At higher concentrations of bacteria, O_2^- production rose, reaching a maximum at a bacteria/granulocyte ratio of 100, and then falling again at higher concentrations. The fall at the highest concentrations of bacteria may have represented either quenching of O_2^- by the

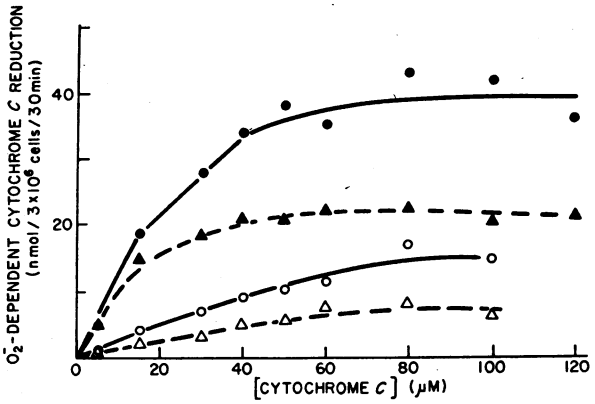

FIGURE 3 The effect of cytochrome *c* concentration on O_2^--dependent cytochrome *c* reduction. Experimental points were determined by measuring total cytochrome *c* reduction both in the absence and in the presence of superoxide dismutase, then subtracting the latter value from the former to obtain O_2^--dependent cytochrome *c* reduction. For each experimental point, two reaction mixtures were prepared. One contained 9 × 10^6 granulocytes, cytochrome *c* at the concentrations shown, and *E. coli* C (9 × 10^8) as indicated. The other was identical except for the addition of 1.8 nmol of superoxide dismutase. Cytochrome *c* reduction was determined as described in Methods. The difference in cytochrome *c* reduction between the two reaction mixtures was taken to represent O_2^--dependent cytochrome *c* reduction. The results of two separate experiments are represented by different symbols (○, △). Closed symbols indicate incubations containing bacteria; open symbols represent incubations from which bacteria were omitted. Each experiment was conducted with a different cell preparation.

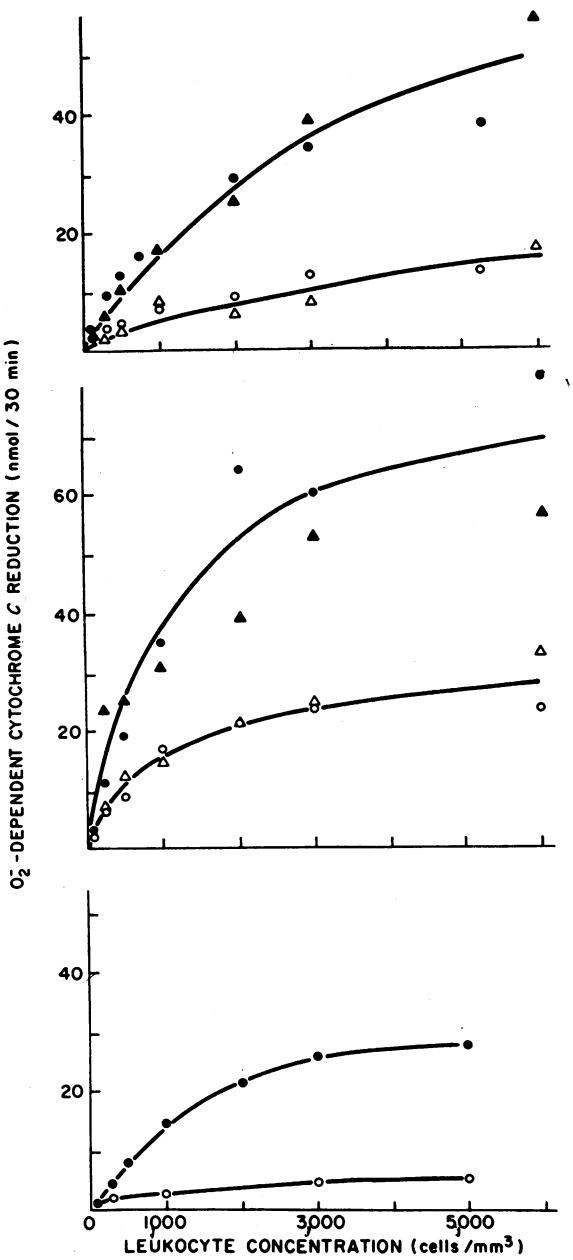

FIGURE 4 The effect of granulocyte concentration on O_2^--dependent cytochrome *c* reduction. Experiments were performed as described in Fig. 3. Concentrations of cytochrome *c* and bacteria were as follows: top panel, 80 μM and 3×10^8 organisms/ml; middle panel, 80 μM and 100 organisms/*granulocyte*; bottom panel, 40 μM and 3×10^8 organisms/ml. Granulocyte concentrations were as indicated.

In each panel, the results of separate experiments are represented by different symbols (○, △). Closed symbols indicate incubations containing bacteria; open symbols represent incubations from which bacteria were omitted. Each experiment was conducted with a different cell preparation.

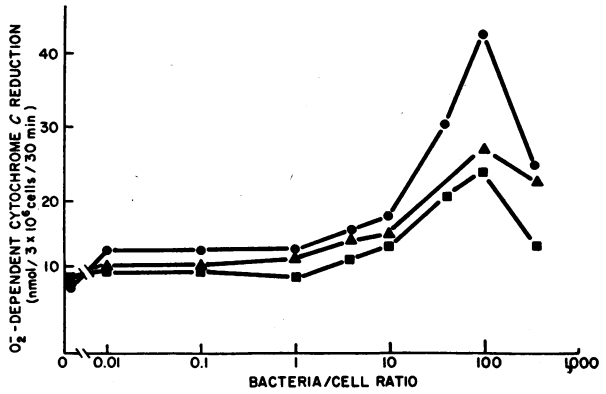

FIGURE 5 The effect of bacterial concentration on O_2^--dependent cytochrome *c* reduction. Experiments were performed as described in Fig. 3. Reaction mixtures contained 9×10^6 granulocytes, 80 μM cytochrome *c*, and *E. coli* C as shown. The results of three separate experiments are represented by different symbols (▲, ●, ■). Each experiment was conducted with a different cell preparation.

microorganisms or a toxic effect of the bacteria on the leukocyte.

O_2^--dependent cytochrome *c* reduction by granulocytes followed a time course remarkably similar to that

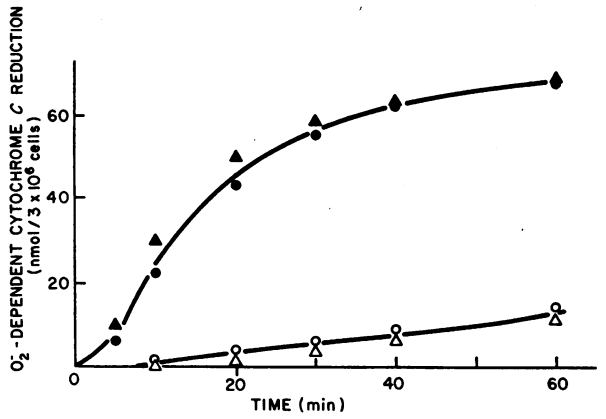

FIGURE 6 Time-course of O_2^--dependent cytochrome *c* reduction. For each curve, two reaction mixtures were prepared. For these reaction mixtures, all quantities were scaled up by a factor of 2.3, compared to the conditions described in Methods. One mixture contained 2.1×10^7 granulocytes, 80 μM cytochrome *c*, and *E. coli* C (2.1×10^9) as indicated. The other was identical except for the addition of 4.2 nmol of superoxide dismutase. Incubations were conducted at 37°C. At the times shown, 1.0-ml portions of each reaction mixture were withdrawn and placed in melting ice. Cytochrome *c* reduction was determined as described in Methods. O_2^--dependent cytochrome *c* reduction at each time point was calculated as described in Fig. 3. The results of separate experiments are represented by different symbols (○, △). Closed symbols indicate incubations containing bacteria; open symbols indicate incubations from which bacteria were omitted. Each experiment was conducted with a different cell preparation.

of leukocyte oxygen uptake (Fig. 6). In the absence of bacteria, the rate of reduction appeared to be constant with time for at least an hour. With bacteria, the time-course was more complex. A well-defined lag was followed by a fairly brief period of extremely vigorous cytochrome *c* reduction. During this period, the maximum rate of cytochrome *c* reduction exceeded the rate observed in the absence of bacteria by a factor of 12. The rate then decreased, until by 40 min it had slowed to the rate observed in the absence of bacteria. It appears from these data that the ingestion of bacteria by the leukocyte initiates a process in which a defined quantity of O_2^- is formed in a rapid burst lasting 20–30 min.

DISCUSSION

Oxygen consumption in the presence of superoxide dismutase appeared to be somewhat greater than in its absence (Fig. 1). While this may to some extent represent an effect of superoxide dismutase on leukocyte metabolism, it is at least in part attributable to the differing fates of O_2^- in the two incubations. In the reaction mixture containing dismutase, extracellular O_2^- was converted to hydrogen peroxide and oxygen according to the following stoichiometry: $2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$. Production of two molecules of extracellular O_2^- thus led to the net consumption of one molecule of oxygen. In the other reaction mixture, however, extracellular O_2^- was quantitatively converted back to oxygen with the reduction of cytochrome *c*. In these experiments, then, net oxygen uptake would be expected to be greater in the presence of dismutase than in its absence, other things being equal.

The increase in leukocyte oxygen consumption associated with phagocytosis has been known for some time (1). The data presented here suggest that a substantial fraction of this oxygen is used to generate O_2^-, a compound whose chemical properties raise the possibility that it may be involved in bacterial killing. There is a striking similarity between the time-course of O_2^- production and that of oxygen uptake. Upon exposure of the leukocyte to bacteria, the rates of both processes increased. These rates reached a maximum between 5 and 10 min after the addition of the microorganisms, and then gradually subsided to the original level over the next 20–30 min. In terms of stoichiometry, the amount of O_2^- detected under optimal assay conditions in the presence of bacteria corresponded to the one-electron reduction of 25% of all the oxygen taken up by the leukocyte in excess of its resting oxygen consumption. Since the assay probably detects only that portion of O_2^- that appears in the extracellular medium, it seems likely that the amount of O_2^- actually produced by the cell is substantially larger than the quantity detected in the present experiments.

Optimal O_2^- production appears to require an interaction between the microorganism, the leukocyte, and the serum in which they are both suspended. The nature of this interaction was clarified by experiments showing that preincubation of serum with bacteria generated a heat-labile activity capable of stimulating granulocyte O_2^- production in the absence of bacteria. Most of this activity remained in the supernate after centrifugation of the preincubated serum at 105,000 *g* for 1 h. Some of the constituents of serum shown by previous work to be capable of stimulating granulocyte function can be excluded as the agents responsible for the increase in O_2^- production observed in these experiments. The fact that serum heated to 56° for 45 min was capable of generating this activity on incubation with bacteria indicates that neither the classical nor the alternate complement activation pathway is involved in its production (19, 20). Generation of the activity in the absence of granulocytes suggests that "tuftsin" is not involved in the stimulation of granulocyte O_2^- production, at least under the present experimental conditions (21). An effect of opsonization of bacteria by serum proteins (3) can be excluded because the stimulation of granulocyte O_2^- production occurred in the absence as well as in the presence of bacteria. Whatever the nature of the stimulating activity, its nonparticulate nature suggests that its effect may be mediated by contact with cell membrane rather than by actual phagocytosis. A mechanism of this sort is seen, for example, in the stimulation of lysosomal enzyme release by exposure of granulocytes to immune complexes attached to Millipore filters (22).

Many previous studies have indicated that the rise in leukocyte oxygen consumption that accompanies bacterial phagocytosis is related to the process of bacterial killing by these cells (2–8). Evidence presented here and elsewhere raises the possibility that O_2^- may be involved in this process. The similarity between the time-courses of O_2^- production and of oxygen uptake and the fact that O_2^- production accounts for a significant fraction of the oxygen consumption of leukocytes engaged in phagocytosis are both consistent with this possibility, as is the interference with the bactericidal activity of granulocytes by superoxide dismutase reported recently by Johnston, Keele, Webb, Kessler, and Rajagopalan (23). The evidence to date, however, is only suggestive. The true importance of O_2^- in bacterial killing by granulocytes remains to be elucidated.

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