H_2O_2 Release from Human Granulocytes during Phagocytosis

RELATIONSHIP TO SUPEROXIDE ANION FORMATION AND CELLULAR CATABOLISM OF H_2O_2: STUDIES WITH NORMAL AND CYTOCHALASIN B-TREATED CELLS

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ABSTRACT Normal and cytochalasin B-treated human granulocytes have been studied to determine some of the interrelationships between phagocytosis-induced respiration and superoxide and hydrogen peroxide formation and release into the extracellular medium by intact cells. By using the scopoletin fluorescent assay to continuously monitor extracellular hydrogen peroxide concentrations during contact of cells with opsonized staphylococci, it was demonstrated that the superoxide scavengers ferricytochrome *c* and nitroblue tetrazolium significantly reduced the amount of H_2O_2 released with time from normal cells but did not abolish it. This inhibitory effect was reversed by the simultaneous addition of superoxide dismutase (SOD), whereas the addition of SOD alone increased the amount of detectable H_2O_2 in the medium. The addition of sodium azide markedly inhibited myeloperoxidase-H_2O_2-dependent protein iodination and more than doubled H_2O_2 release, including the residual amount remaining after exposure of the cells to ferricytochrome *c*, suggesting its origin from an intracellular pool shared by several pathways for H_2O_2 catabolism.

When cells were pretreated with cytochalasin B and opsonized bacteria added, reduced oxygen consumption was observed, but this was in parallel to a reduction in specific binding of organisms to the cells when compared to normal. Under the influence of inhibited phagosome formation by cytochalasin B, the cells released an increased amount of superoxide and peroxide into the extracellular medium relative to oxygen consumption, and all detectable peroxide release could be inhibited by the addition of ferricytochrome *c*. Decreased H_2O_2 production in the presence of this compound could not be ascribed to diminished bacterial binding, decreased oxidase activity, or increased H_2O_2 catabolism and was reversed by the simultaneous addition of SOD. Furthermore, SOD and ferricytochrome *c* had similar effects on another H_2O_2-dependent reaction, protein iodination, in both normal and cytochalasin B cells. When oxygen consumption, $O_2^{\cdot-}$, and H_2O_2 release were compared in the presence of azide under identical incubation conditions, the molar relationships for normal cells were 1.00:0.34:0.51 and for cytochalasin B-treated cells 1.00:0.99:0.40, respectively. Nonopsonized, or opsonized but disrupted, bacteria did not stimulate any of these metabolic functions.

The results indicate that with normal cells approximately 50% of H_2O_2 released during phagocytosis is derived directly from $O_2^{\cdot-}$ by dismutation, the remainder appearing from an (intra)cellular source shared by azide-inhibitable heme enzymes. With cytochalasin B treatment the evidence is consistent with the derivation of all H_2O_2 from an $O_2^{\cdot-}$ precursor which is released from the cell surface. Furthermore, when activated by phagocytic particle

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binding, the neutrophil O_2^- generating system appears to make more of this compound than can be accounted for by dismutation to H_2O_2. This establishes conditions for the direct participation of both compounds in the microbicidal and cytocidal activity of these cells.

INTRODUCTION

The burst in respiration which occurs during phagocytosis or other nonphagocytic perturbations of the granulocyte cell membrane is accompanied by the formation of several reduction products of oxygen including H_2O_2 (1, 2) and the superoxide anion (O_2^-)[1] (3). Evidence for the participation of these and other oxygen reduction radicals in the microbicidal and cytocidal activities of these cells has been recently summarized (4), however, several areas of controversy exist. For example, whereas it can easily be demonstrated in aqueous cell-free media that O_2^- can serve as a precursor to H_2O_2 by the dismutation reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (5–7), studies performed to date have only inferred, but not clearly indicated, that this is the key pathway for H_2O_2 formation in intact cells (8, 9). Enzymatic systems which utilize NADPH (10–13) or NADH (14, 15) as substrate have been proposed to mediate the phagocytic respiratory burst forming H_2O_2 either indirectly through dismutation of O_2^- (7–9) or directly by divalent reduction of oxygen (13, 16). Furthermore, debate exists as to whether H_2O_2 (4, 7, 17, 18) or O_2^- (3, 5, 6, 19–21) is primarily involved in the effector mechanism of oxygen-dependent killing or whether both might play a role through the generation of other active oxygen compounds such as the hydroxyl radical and singlet oxygen (4, 21–25).

In previous investigations we successfully adapted a highly sensitive and specific fluorometric assay for H_2O_2 in aqueous media (26) for studies of the kinetics of its formation and release from intact phagocytizing cells (2). By measuring the effect of scavengers of O_2^- and superoxide dismutase (SOD) on this function and, when necessary, inhibiting H_2O_2 catabolism with sodium azide (4), we thought that it might be possible to determine the precise interrelationships between O_2^- and H_2O_2 formation and utilization in intact cells binding and phagocytizing opsonized particles. Our goals were to determine whether superoxide was in fact the major initial product of oxidase activation during phagocytosis by intact cells. If true, such an observation would exclude enzymatic systems that produced H_2O_2 directly by the divalent reduction of oxygen from playing a primary role in the phagocytic respiratory burst. Second, we wondered whether all superoxide formed could be shown to undergo spontaneous dismutation to H_2O_2. If this were the case, it would imply that the major function of the neutrophil superoxide generating system is to form H_2O_2 rather than to play a more direct role in microbicidal and cytotoxic activity of these cells. Finally, we were concerned with obtaining some information that might identify the primary site for superoxide and H_2O_2 formation. To answer these questions, our studies were greatly aided by the observations of Goldstein et al. (27) that cytochalasin B-treated cells retain the capacity to generate and release O_2^- when stimulated by phagocytic particles and other compounds, despite exhibiting a marked reduction in particle ingestion per se. Their observations of enhanced O_2^- release under these conditions when compared to untreated cells have been confirmed by Curnutte and Babior (28), suggesting that such cells might provide a suitable model for the study of the interrelationships cited above using extracellular detection systems. Our findings support the concept that all H_2O_2 formed during the respiratory burst is indeed derived from an O_2^- precursor; however, O_2^- also appears to be utilized in reactions other than dismutation. Furthermore, the quantitative relationships between oxygen consumed and O_2^- and H_2O_2 formed and released from cytochalasin B-treated cells strongly suggest that these events take place at or near the portion of the cell surface involved in particle binding.

METHODS

Materials. Nitroblue tetrazolium (NBT) was obtained from Sigma Chemical Company (St. Louis, Mo.) and a 2.4-mM solution prepared in bicarbonate-buffered (pH 7.4) Hanks' balanced salt solution containing 5.5 mM glucose (HBSS). This resulted in a supersaturated solution which was clarified before use by centrifugation at 2,200 *g* for 10 min.

Ferricytochrome *c* (horseheart cytochrome *c* Type VI, Sigma Chemical Company) was freshly prepared in HBSS at a concentration of 0.8 mM before use each day.

Scopoletin (Sigma Chemical Company) was dissolved in 50 mM phosphate buffer (pH 7.0) and stored at 4°C until used.

Horseradish peroxidase (HPO; Worthington Biochemical Corp., Freehold, N. J.) was dissolved in 50 mM phosphate buffer at a concentration of 2.4 mg/ml and stored at −20°C until used.

[1-^{14}C]Glucose and carrier-free ^{125}Na were obtained from New England Nuclear (Boston, Mass.).

Sodium iodide and sodium azide were purchased from Fisher Scientific Co. (Pittsburgh, Pa.).

Dimethyl sulfoxide (DMSO) was obtained from Sigma Chemical Company.

[1] *Abbreviations used in this paper*: DMSO, dimethyl sulfoxide; GO, glucose-oxidase; HBSS, Hanks' balanced salt solution; HPO, horseradish peroxidase; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; O_2^-, superoxide anion; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase.

Cytochalasin B was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) dissolved in DMSO to a concentration of 2.5 μg/ml and frozen at −20°C. Fresh aliquots were thawed before use each day.

Lysostaphin was obtained from Schwarz/Mann Div. Becton, Dickinson & Co. (Orangeburg, N.Y.), dissolved in HBSS to a concentration of 100 U/ml, and frozen until used.

Fresh human serum was obtained from the clotted blood of normal AB and Rh positive donors, pooled from several individuals, and stored at −70°C until used for opsonizing *S. aureus* 502A. The latter were grown in overnight culture in trypticase soy broth with or without ^{14}C-mixed amino acids for radioactive labeling and heat-killed as described previously (2). Opsonization was achieved by incubating bacteria in 50% serum for 30 min at 37°C. The opsonized organisms were then twice washed by resuspension and centrifugation in HBSS before being used as phagocytic particles in the assays described below.

Cell separation. Heparinized venous blood taken from normal donors ranging in age from 21 to 37 yr provided granulocyte-rich leukocyte preparations by dextran sedimentation and hypotonic lysis of accompanying erythrocytes as previously described (2). Granulocytes comprised 70–85% of the final mixed cell population and constituted >95% of the phagocytic cells. In all phagocytic and metabolic assays, except as noted below, the final polymorphonuclear leukocyte (PMN) concentration was 2.5×10^6 PMN/ml and the suspending medium was HBSS.

In experiments utilizing cytochalasin B, this compound was added in a final concentration of 5 μg/ml and the cell suspensions incubated at 37°C for 5 min before adding phagocytic particles and making the measurements noted below. Preliminary experiments established that this concentration gave maximal results without demonstrable cytotoxicity. Control suspensions contained 0.1% DMSO, a concentration which did not alter cell function or inhibit any of the assays.

In experiments in which sodium azide was employed, this compound was added in a final concentration of 1 mM to the cell suspensions which had been prewarmed to 37°C. After a 5-min preincubation period, phagocytic particles were added and measurements made as recorded below.

Measurement of H_2O_2 release. In the majority of experiments, the release of H_2O_2 into the extracellular medium during phagocytosis of opsonized *S. aureus* at 37°C was measured and monitored continuously in a 1 × 1-cm light path cuvette using the HPO-mediated extinction of scopoletin fluorescence during its oxidation as described previously (2). The scopoletin concentration was 4 μM and that of HPO 22 nM. The bacteria to cell ratio was 500:1 to insure maximal rates of particle ingestion (2).

The system was modified for the detection of the role of O_2^- in H_2O_2 formation using the principles outlined in Fig. 1. After preliminary experiments established maximum dose-response effects with a minimum of fluorescence quenching at 0.24 mM NBT and 80 μM ferricytochrome *c* concentrations, respectively, these compounds were added to cell suspensions 5 min before the bacteria to serve as O_2^- scavengers. Similarly, 50 μg/ml SOD was added to separate cell preparations or to those containing the above compounds to promote dismutation of O_2^- to H_2O_2. Again, preliminary experiments established that this concentration of SOD produced maximal effects with minimal fluorescence quenching. When required, SOD was inactivated by boiling or autoclaving for 30 min before addition to the suspension. None of the compounds added, including azide, cytochalasin B, or DMSO altered the detection of H_2O_2 by the scopoletin oxidation technique in cell-free systems when H_2O_2 was generated from glucose by glucose-oxidase (GO; 2).

Measurement of O_2^- release during phagocytosis. O_2^- release was measured by the SOD-inhibitable reduction of ferricytochrome *c* by minor modifications of the method of Babior et al. (3). To duplicate the conditions of the fluorometric assay, preparations containing 80 μM ferricytochrome *c*, 2.5×10^6 PMN, and opsonized *S. aureus* (500:1 bacteria/cell ratio) were incubated at 37°C in 12 × 75-mm test tubes with occasional stirring for 10 min. Controls consisted of cell-free preparations in HBSS of ferricytochrome *c* or ferricytochrome *c* plus SOD, as well as cell suspensions that contained no bacteria. All comparisons of absorbance at 550 nm were made to 80 μM cytochrome *c* which was completely reduced with an excess of dithionite. Measurements were made on a rapid scanning dual spectrophotometer (model 635, Varian Associates, Instrument Div., Palo Alto, Calif.) The amount of cytochrome *c* reduction in the cell suspensions that was inhibited by SOD was regarded as an indication of O_2^- release. O_2^- concentrations in nanomoles were calculated using a ΔEmM (ferrocytochrome *c* minus ferricytochrome *c*) at 550 nm of 15.5 (3). In experiments in which the tubes were rotated end over end to increase phagocytosis, the concentration of ferricytochrome *c* was increased to 160 μM to maximize O_2^- trapping (9, 28).

Correlation of oxygen consumption with O_2^- and H_2O_2 release. To measure these parameters of oxidative metabolism under simultaneous incubation conditions, cell suspensions containing 5×10^6 granulocytes/ml in HBSS were incubated in the chamber of a Clark oxygen electrode (oxygen monitor, Yellow Springs Instrument Co., Yellow Springs, Ohio). Sodium azide (1 mM final concentration) was added to inhibit H_2O_2 catabolism, and opsonized staphylococci were added in a 500:1 particle to cell ratio. Some chambers also contained either ferricytochrome *c* (160 μM), SOD (50 μg/ml), or both compounds. Oxygen consumption was measured (2) and compared to control preparations incubated in the absence of either of these compounds. After a 5-min incubation period, H_2O_2 release was quantitated by removing several 10-μl aliquots and adding them serially to a cuvette containing 2 μM scopoletin in 2.5 ml HBSS and an excess of HPO (22 nM) and measuring the extinction of fluorescence. Other aliquots were added to another cuvette containing catalase (400 U) in addition to the HPO and scopoletin to

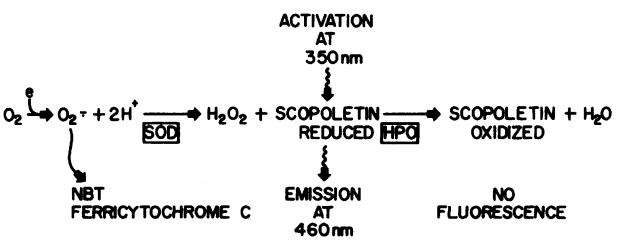

FIGURE 1 Schematic representation of the modification of the scopoletin assay for detecting the interrelationships between superoxide and hydrogen peroxide release into the extracellular medium of appropriately stimulated cells. Hydrogen peroxide concentrations are measured in the presence of the superoxide scavengers nitroblue tetrazolium (NBT) or ferricytochrome *c* or superoxide dismutase (SOD) by the HPO-mediated oxidation of scopoletin as described in the text.

act as a reference blank and indicate fluorescence extinction that was specific for H_2O_2. The mean fluorescence extinction produced by at least three aliquots was used to calculate H_2O_2 concentration. Superoxide release over the same time period was measured by quantitating the SOD inhibitable reduction of 160 μM ferricytochrome *c* in cell-free supernates derived from the suspensions as described above.

Measurement of the recovery of exogenously added H_2O_2. To exclude the possibility that changes in H_2O_2 release measured under the different experimental conditions were the result of alterations in H_2O_2 catabolism or the generation of products (e.g. ferrocytochrome *c*) that might compete with scopoletin for oxidation by HPO using H_2O_2 as substrate (29), known amounts of H_2O_2 were added to the medium of phagocytizing cells and their recovery measured. The experimental design chosen was as follows: (*a*) GO was used to generate H_2O_2 from the glucose in the HBSS. This compound was chosen for its stability and the fact that continuous H_2O_2 generation most closely mimics the release of H_2O_2 by the cells. The activity of the enzyme added was capable of forming 1–1.5 μM H_2O_2/min at 37°C, as determined by the scopoletin assay. (*b*) Cells (normal or cytochalasin B-treated) were preincubated in sodium azide to inhibit intracellular catabolism of H_2O_2. Under these conditions, recoveries of added H_2O_2 from particle-free cell suspensions in the absence of scavengers averaged 90% or better. This recovery was not significantly altered in similar cell suspensions which also contained SOD (50 μg/ml) ferricytochrome *c* (80 μM), NBT (0.24 mm), or combinations of NBT or ferricytochrome *c* with SOD during continuous recording of H_2O_2 concentrations as described above. (*c*) To determine the recovery of exogenously added H_2O_2 from cell suspensions phagocytizing staphylococci in the presence or absence of these O_2^- scavengers, GO was not added until at least 2 min after the particles at which time phagocytosis-induced H_2O_2 release was occurring at maximal rates. H_2O_2 release from phagocytizing cell suspensions to which no GO had been added was subtracted from those containing GO to determine the amount of exogenously added H_2O_2 remaining in the latter. The "recovery" of this added H_2O_2 was then calculated by dividing it by the values obtained with nonphagocytizing cells incubated under the same conditions and converting it to a percentage by multiplication by 100.

Measurements of phagocytosis, protein iodination, and [1-^{14}C]glucose oxidation. Cell association of ^{14}C-*S. aureus*, fixation of iodide to a protein-bound form and L-[^{14}C]glucose oxidation during phagocytosis were measured as previously described (2, 30). The ratio of opsonized bacteria to cells was 500:1, and the cell concentrations were 2.5 × 10^6 PMN/ml. When comparisons between the different oxidative functions were made, the values for oxygen consumption in the 5 × 10^6 PMN/ml cell suspensions were corrected to levels expected for 2.5 × 10^6 PMN/ml as described previously (2). Ferricytochrome *c*, SOD, and/or sodium azide in the concentrations noted above were added to different preparations as indicated in different experiments. Incubation periods with phagocytic particles varied from 10 to 20 min as specified below.

Statistics. The statistical analysis employed was the paired-sample *t* test when more than three donors were used. In experiments utilizing cells from three or fewer donors, the data was analyzed by the Pittman-Welch permutation test (31). *P* values of <0.05 for differences between control and experiment preparations were considered to be significant.

RESULTS

Effect of ferricytochrome c and SOD on H_2O_2 release from phagocytizing cells

As shown in Table I, under conditions of constant recording, the maximal rate of H_2O_2 release detected from cells phagocytizing opsonized *S. aureus* was significantly increased in the presence of SOD (147.4 ±6.2% of controls) and reduced in the presence of ferricytochrome *c* (53.0±7.0% of controls). The enhancing effect of SOD was abolished by boiling the enzyme, and the inhibitory effect of ferricytochrome *c* reversed with SOD, consistent with its mediation through O_2^- scavenging. Qualitatively similar results were observed for preparations incubated with NBT (H_2O_2 release, 46.4±3.4% of controls) although complete reversal with SOD to control values did not occur in four out of the five experiments shown and the means averaged 79.2% of control.

The kinetics of phagocytosis-induced H_2O_2 release and the effects of ferricytochrome *c* and SOD on this process are shown in Fig. 2 which represents the mean results of eight experiments. After a 30-s latency following the addition of *S. aureus* free H_2O_2 could be detected in linearly increasing amounts in the medium in control preparations. In the presence of ferricytochrome *c*, no H_2O_2 was found until a mean 59 s later than the controls and the amounts were reduced below control levels by approximately

TABLE I

Effect of Superoxide Scavengers on the Detection of H_2O_2 Released from Granulocytes Phagocytizing Opsonized S. Aureus*

Preparation	H_2O_2 released
	(nmoles/min/2.5 × 10^6PMN)
Control (30)‡	0.545±0.036
+ 50 μg/ml SOD (26)	0.855±0.92§
+ Boiled SOD (4)	0.582±0.05
+ 80 μM cytochrome *c* (11)	0.238±0.031§
+ 80 μM cytochrome *c* + SOD (6)	0.729±0.184
+ 0.24 mM NBT (12)	0.213±0.014§
+ 0.24 mM NBT + SOD (5)	0.432±0.024

Results given are mean±SE.

* All data is expressed as the maximum rate of release of H_2O_2 into the extracellular medium which occurred after varying latent periods. The incubation conditions were those which permitted continuous recording of scopoletin fluorescence with stationary cell suspensions as described in Methods (2).

‡ The numbers in parentheses indicate the number of experiments.

§ $P < 0.01$, Student's *t* test for paired samples in comparison with simultaneously run control preparations.

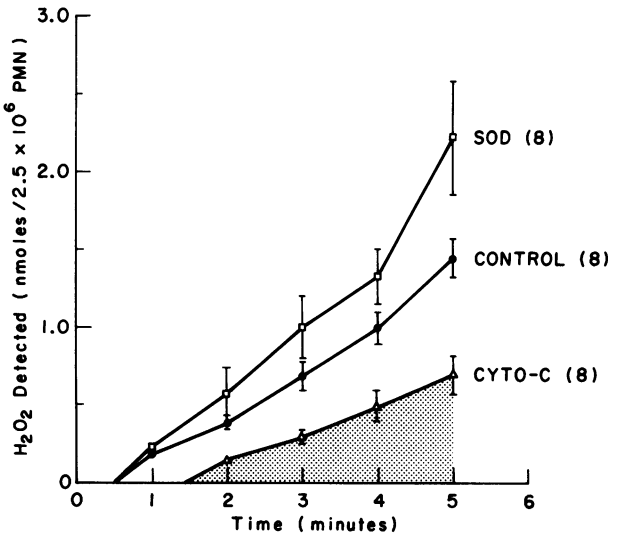

FIGURE 2 Release of H_2O_2 from granulocytes ingesting opsonized *S. aureus* added at time zero in a 500:1 particle to cell ratio. Lines are the means and the brackets the SEM of eight experiments. The control cuvettes contained 2.5 × 10^6/ml PMN in 2.5 ml of HBSS with scopoletin 4 μM and HPO 22 nM to detect released H_2O_2. Ferricytochrome *c* (CYTO-C) cuvettes contained the above plus 80 μM ferricytochrome *c*. SOD cuvettes contained 50 μg/ml SOD. All incubations were performed at 37°C with constant recording of scopoletin fluorescence as described in Methods.

50% at each time through 5 min. The addition of SOD to the cell suspensions did not increase the amount of H_2O_2 detected above control until after 1 min of incubation with bacteria. The latency before H_2O_2 was detected was not significantly changed in the presence of SOD. Thereafter, the pattern maintained through the 5-min incubation period was similar with preparations containing SOD exhibiting approximately 50% higher levels of H_2O_2 than control.

Effect of scavengers on phagocytosis, postphagocytic oxygen consumption, and H_2O_2 recovery

None of the compounds affected H_2O_2 release by changing rates of phagocytosis (Table II). Likewise, SOD did not alter postphagocytic oxygen consumption although ferricytochrome *c* inhibited it by a mean 24% and NBT by 30%, respectively. The inhibitory effect of ferricytochrome *c* was significantly reversed by the simultaneous addition of SOD although the values observed remained slightly below the controls.

Similarly, the observations did not appear to be explained by changes in the rate of catabolism of H_2O_2 or competition for scopoletin oxidation by products of superoxide reduction. As shown in Table III, the recovery of added H_2O_2 was equivalent in all preparations of phagocytizing cells whether scavengers were present or absent, with the exception of suspensions which contained both SOD and ferricytochrome *c*.

TABLE II

Effect of Superoxide Scavengers on Cellular Uptake of S. Aureus *and Postphagocytic Oxygen Consumption*

Additions	Bacterial uptake*	O_2 consumption‡
	% of control	
SOD (50 μg/ml)	98.6±4.1 (3)§	100.8±4.1 (6)
NBT (0.24 mm)	103.2±2.0 (3)	70.2±1.5 (6)‖
Ferricytochrome *c* (80 μM)	106.4±3.7 (3)	76.6±9.3 (3)‖
SOD + Ferricytochrome *c*	—	94.8±1.5 (3)‖
SOD + NBT	—	72.5±7.3 (3)‖

* Values for nine control subjects were 73.0±11.5 bacteria/cell/10 min (mean±SEM).

‡ Values for 12 control subjects were 130.6±7.5 nmol/2.5 × 10^6PMN/10 min (mean±SEM).

§ Numbers in parentheses indicate the number of experiments. Results given are mean±SEM.

‖ $P < 0.05$ for comparisons with simultaneously run controls (Pittman-Welch permutation test).

Effect of azide on H_2O_2 release in relationship to bacterial cell association and other postphagocytic metabolic events

The data presented in Table I and Fig. 2 are compatible with the concept that at least 50% of the H_2O_2 in the medium was derived directly from the rapid and nonenzymatic dismutation of O_2^- (the difference between control values and those which contained ferricytochrome *c* or NBT). Furthermore, the increase in H_2O_2 release observed with SOD suggested

TABLE III

Recovery of Exogenously Added H_2O_2 from Cell Suspensions Phagocytizing S. Aureus *under Different Conditions*

Additions	% recovery of added H_2O_2*
None (3)‡	93.9±3.4
SOD (50 μg/ml) (3)	99.2±10.1
Ferricytochrome *c* (80 μM) (3)	97.4±2.5
SOD + ferricytochrome *c* (3)	77.4±6.4§
NBT (0.24 mM) (2)	98.5±3.5
NBT + SOD (2)	96.3±0.45

* Recovery of H_2O_2 generated from the glucose in HBSS by GO when compared to nonphagocytizing cells incubated in the presence of the same compounds. See Methods for the details involved in making these calculations. Results given are mean±SEM.

‡ Numbers in parentheses refer to the number of experiments.

§ $P < 0.05$ when compared to preparations containing no additions (Pittman-Welch permutation test).

that not all O_2^- released by the cells underwent spontaneous dismutation to H_2O_2. The source of the residual H_2O_2 left after ferricytochrome *c* or NBT treatment was not defined but possibly came from an intracellular site that might be accessible to other catabolic pathways for H_2O_2. To test this possibility, 1 mM sodium azide was employed to inhibit cellular myeloperoxidase (MPO) and catalase (4), and the effects on different metabolic parameters including H_2O_2 release were measured. As shown in Table IV, azide treatment moderately inhibited bacterial cell association at the high particle to cell ratios employed (500:1) and markedly inhibited MPO-dependent protein oxidation. Oxygen consumption and [1-^{14}C]glucose oxidation were not significantly changed from control although increased relative to phagocytosis; whereas H_2O_2 release was doubled. These data support the concept of a shared common pool for H_2O_2. More importantly, they also indicated that the use of extracellular scavengers of O_2^- might not reach the major site of formation of the compound within the cells, and that another experimental approach was needed.

Use of cytochalasin B-treated cells

Cell association of bacteria and oxygen consumption. We then turned to the use of cytochalasin B-treated cells because treatment with this compound not only inhibits phagocytosis (32, 33) but leads to increased release of O_2^- into the extracellular medium upon exposure of cells to phagocytic stimuli (27, 28).

TABLE IV
Effect of 1 mM Sodium Azide on Cellular Uptake of Opsonized S. Aureus *and Phagocytosis-Induced Oxidative Metabolism*

Function	Incubation time	Control value, Bacteria/cell	Azide treatment
	min		*% of control*
Uptake of bacteria	10 (5)*	45.2±7.0	60.9±13.1‡
	20 (3)	58.0±9.1	69.4±4.8‡
Oxygen consumption (nmoles/2.5 × 10^{6}PMN)	10 (3)	126±8.4	105.2±5.5
	20 (3)	182±18.1	87.1±10.8
[1-^{14}C]glucose oxidation (nmoles/2.5 × 10^{6}PMN)	20 (3)	71.4±1.1	95.1±3.5
Iodination (nmoles/2.5 × 10^{6}PMN)	20 (6)	0.585±0.08	28.8±2.8‡
H_2O_2 release (nmoles/2.5 × 10^{6}PMN)	10 (7)	3.4±0.34	219.6±33.7‡

Results given are mean±SEM.
* Numbers in parentheses refer to the number of experiments. The bacteria/cell ratio was 500:1 in all experiments.
‡ $P < 0.02$ or less for comparisons with nonazide-treated cells, paired sample *t* test.

Furthermore, the fact that cytochalasin B-treated cells also release a considerable fraction of their lysosomal enzymes under similar conditions (27) suggested that measurements made in the extracellular medium surrounding these cells may accurately reflect events that take place in phagocytic vacuoles during particle ingestion. While these experiments were in progress, work from the laboratories of Rossi et al. (34) and Roos et al. (35) was published which used a similar experimental approach to address some of the same issues, although the methodologies employed and information gained were somewhat different.

As shown in Fig. 3, cells preincubated for 5 min in 5 μg/ml of cytochalasin B exhibited reduced cell association of opsonized bacteria when compared to control cells incubated in an equivalent amount of the DMSO solvent used to solubilize the cytochalasin B, as well as reduced consumption of oxygen. The reductions in both were equivalent over a 10-min incubation period, however. This suggested that there was a direct relationship between the extent of particle binding to the cell and oxidase activation. Scanning electron microscope examination revealed that many of the opsonized bacteria were adherent to the cells (Fig. 4). Very few internalized bacteria were observed by light phase microscopy.

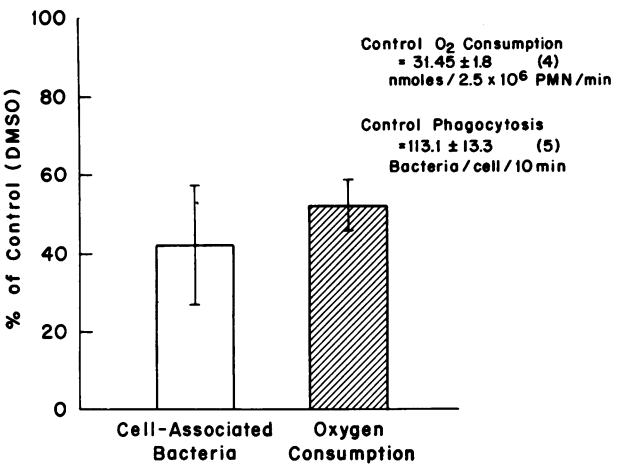

FIGURE 3 Values for phagocytosis-stimulated oxygen consumption and cell association of opsonized *S. aureus* are shown in the upper right corner for cells incubated in HBSS 0.1% DMSO as the mean±SEM of the number of experiments shown in parentheses ("controls"). Oxygen consumption was measured polarographically using a Clark oxygen electrode. Cell association ("phagocytosis") of bacteria was measured by quantitating the uptake of ^{14}C-*S. aureus* after opsonization in 50% normal human serum as described in Methods. The bacteria to cell ratios were 500:1. The results of studies with cells preincubated in the presence of 5 μg/ml cytochalasin B in 0.1% DMSO are given by the bars which depict means and the brackets, the SE of the percent of the simultaneous control values.

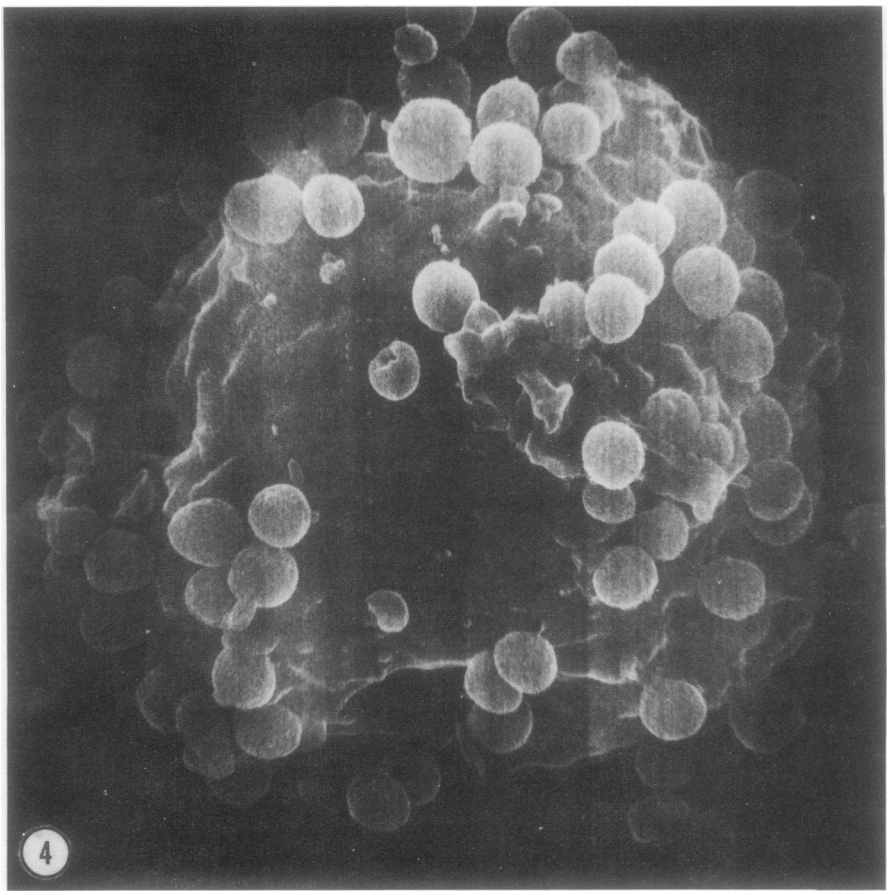

FIGURE 4 Scanning electron micrograph of a cytochalasin B-treated granulocyte from a cell suspension to which had been added opsonized *S. aureus* in a 500:1 particle/cell ratio 15 min previously. The cell surface is studded with intact organisms apparently bound to small pseudopodia projecting from the cell.

Release of H_2O_2 and superoxide from cytochalasin B-treated cells and the effects of superoxide scavengers and azide on H_2O_2 release

As shown in Table V, both H_2O_2 and O_2^- release were significantly increased from cells by cytochalasin B treatment. Values for both almost doubled under equivalent incubation conditions. In the absence of azide to inhibit H_2O_2 catabolism, the molar relationship between O_2^- and H_2O_2 released was approximately 10:1 over the 10-min incubation period.

The effects on H_2O_2 release of the addition of ferricytochrome *c*, SOD, or sodium azide are indicated in Table VI, and some of the findings with cytochalasin B-treated cells are depicted graphically in Fig. 5. H_2O_2 release rates are given as maximums achieved over a 5-min incubation period similar to those in Table I. SOD increased detectable H_2O_2

TABLE V

Effect of Cytochalasin B on Release of H_2O_2 and Superoxide from Cells Exposed to Opsonized S. Aureus*

Cells	H_2O_2 release	O_2^- release
	nmoles/2.5 × 10^6PMN/10 min	
Normal (0.1% DMSO) (3)	2.32±0.45	19.6±0.9
Cytochalasin B-treated (5 μg/ml) (3)	3.77±0.10‡	38.8±2.8‡

* H_2O_2 release was measured continuously by scopoletin assay over a 10-min incubation period. O_2^- release was measured after 10 min under similar incubation conditions (only periodic agitation of stationary suspensions) with 80 μM ferricytochrome *c* and 50 μg/ml SOD as described in Methods. The bacteria/cell ratio was 500:1. Cells from three donors were used as indicated by the numbers in parentheses. Results given are mean±SEM.

‡ $P < 0.05$ for differences when compared to normal cells (Pittman-Welch permutation test).

TABLE VI

Effect of Superoxide Scavengers and Sodium Azide on H_2O_2 Release from Normal and Cytochalasin B-Treated Granulocytes Incubated with Opsonized S. Aureus

Additions	H_2O_2 release*	
	Normal cells (0.1% DMSO) (4)	Cytochalasin B-treated Cells (4)
	nmoles/2.5 × 10⁶PMN/min	
None	0.367±0.02	0.739±0.06‡
+ SOD (50 μg/ml)	0.503±0.03	0.862±0.03‡
+ Ferricytochrome *c* (80 μM)	0.231±0.05	0‡
+ Azide (1 mM)	0.734±0.08	0.792±0.08
+ SOD + Azide	0.825±0.09	0.872±0.21
+ Ferricytochrome *c* + azide	0.694±0.06	0.165±0.06‡

* The results are expressed as the maximal rates of H_2O_2 release observed over a 5-min incubation period using cells from four different donors with assay conditions similar to those in Table I (mean±SEM).

‡ $P < 0.01$ for the differences between normal and cytochalasin B-treated cells (paired sample *t* test).

release from both cytochalasin B and normal cells by 16 and 37%, respectively ($P < 0.05$, paired sample *t* test). In contrast, ferricytochrome *c* totally inhibited H_2O_2 release from the cytochalasin B-treated cells, whereas it was only partially inhibited from the normal cells (37%) as noted previously ($P < 0.05$). The inhibitory effect of ferricytochrome *c* was reversed by the addition of SOD, whereas boiled SOD failed either to enhance H_2O_2 release or reverse this inhibition (Fig. 5).

The addition of sodium azide to the cytochalasin B-treated cells produced no significant enhancement in the rate of H_2O_2 release ($P > 0.5$) in contrast to the effects observed with normal cells ($P < 0.02$). (In fact, when cells from the three donors shown in Table V were permitted to incubate with azide for 10 min, the H_2O_2 released from the normal cells significantly exceeded that from the cytochalasin B-treated cells [11.68±1.9 vs. 6.05±0.68 nmol/2.5 × 10^6 PMN, respectively, $P < 0.02$ paired sample *t* test].)

Like the findings reported in Table III, the recovery of exogenous H_2O_2 from cytochalasin B-treated cells was 90.3±0.4% in the absence of scavengers, 82.5±8.3% in the presence of SOD, 91.9±4.1% in the presence of cytochrome *c*, and 73.4 ±9.0% in the presence of the combination of cytochrome *c* and SOD. Only the last was significantly different from the scavenger-free control preparations ($P < 0.05$ for three experiments). Oxygen consumption by cytochalasin B-treated cells exposed to SOD or ferricytochrome *c* was 87.8±1.5% and 61.5±5.9% of control (nonscavenger exposed) values, respectively ($P < 0.02$ for four experiments).

Effect of SOD and ferricytochrome c *on protein iodination by normal and cytochalasin B-treated cells*

To examine the effect of the scavengers on another H_2O_2-dependent postphagocytic reaction, MPO-mediated protein iodination was measured with both normal and cytochalasin B-treated cells. As shown in Table VII, despite reduced oxygen consumption, bacterial binding, and ingestion, protein iodination by normal and cytochalasin B-treated cells was equivalent. The addition of SOD produced an equivalent increase in iodination (a mean 38–60% for normal and cytochalasin B-treated cells, respectively, $P < 0.05$ for differences between control and SOD-exposed cells in both groups), whereas ferricytochrome *c* addition inhibited iodination to a greater degree in the cytochalasin B-treated cells (87%) than the normal (75%), suggesting more effective scavenging of $O_2^{\cdot-}$. In contrast to the measurements of H_2O_2 release, the inhibition of iodination induced by ferricytochrome *c* was only partially reversed by the simultaneous addition of SOD in three of four experiments.

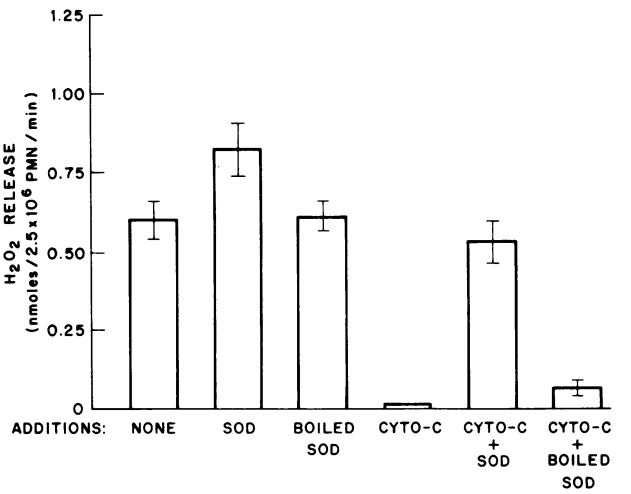

FIGURE 5 The bars depict the maximal rates of H_2O_2 release measured by the scopoletin assay in cell suspensions containing 5 μg/ml cytochalasin B and opsonized *S. aureus* in a 500:1 particle/cell ratio. SOD concentrations were 50 μg/ml. Ferricytochrome *c* (CYTO-C) concentrations were 80 μM. SOD was inactivated by placement in a boiling water bath for 30 min and added to some of the preparations as indicated. H_2O_2 release from cells incubated in the presence of SOD, ferricytochrome *c*, or ferricytochrome *c* plus boiled SOD was significantly different from suspensions containing no additions ($P < 0.01$, paired sample *t* test).

TABLE VII

The Effect of Superoxide Scavengers on Protein Iodination by Normal and Cytochalasin B-Treated Granulocytes during Contact with Opsonized S. Aureus*

Additions	Normal cells (0.1% DMSO) (4)	Cytochalasin B-treated cells (4)
	nmoles iodide fixed/2.5 × 10^6PMN/10 min	
None	1.93±0.35	1.98±0.43
+ SOD (50 μg/ml)	2.64±0.45	2.92±0.43
+ Ferricytochrome *c* (80 μM)	0.478±0.85	0.259±0.03‡
+ SOD + ferricytochrome *c*	0.878±0.18	1.18±0.21

Results given are mean±SEM.

* Data obtained from four donors after a 10-min incubation period using a bacteria/cell ratio of 500:1.

‡ $P < 0.05$ in comparison with normal cells (paired sample *t* test).

Molar relationships between O_2 consumption, O_{2^-}, and H_2O_2 release by normal and cytochalasin B-treated cells

Next we sought to determine the relationships between oxygen consumed and superoxide and H_2O_2 released under identical incubation conditions using cell suspensions that were phagocytizing staphylococci in the chamber of an oxygen electrode as described in Methods. To inhibit catabolism of the H_2O_2 by neutrophil heme enzymes and permit its measurement at the end of the incubation period it was necessary to add sodium azide (1 mM) to the suspensions. As noted in Table IV, this concentration of azide caused some suppression of cell association of bacteria at the high particle/cell ratios used (500:1). Furthermore, azide also had a more pronounced effect on enhancing H_2O_2 release from normal as opposed to cytochalasin B-treated cells as noted above. Finally, phagocytosis and therefore H_2O_2 release is markedly increased by continuous agitation (2) so that the figures given in Table VIII must be interpreted with these points in mind.

As shown in Table VIII, oxygen consumption by the cytochalasin B-treated cells averaged 44% of that of the normal cells in the presence of 1 mM sodium azide. This finding is almost identical to the results with nonazide-treated cells shown in Fig. 4. Measurements of H_2O_2 release under the same incubation conditions were likewise reduced to a mean 34% of the DMSO control cells, whereas measurable superoxide release was equivalent in the two types of cell preparations, despite significantly reduced oxygen consumption by the cytochalasin B-treated cells. In experiments (three each) in which all three functions were measured on the same cell suspensions, the molar relationships between O_2 consumption, O_{2^-}, and H_2O_2 release were 1.00:0.34:0.51 for normal cells vs. 1.00:0.99:0.40 for cytochalasin B-treated cells, respectively.

Requirement for intact opsonized bacteria to stimulate H_2O_2 and O_{2^-} release

Finally, to determine whether a specific interaction between the cell surface and the phagocytic particles was required to activate the cells for O_{2^-} and H_2O_2 release, experiments were run with normal and cytochalasin B-treated cells comparing opsonized staphylococci, nonopsonized staphylococci, and opsonized, then lysostaphin-treated staphylococci as stimuli. To achieve disruption of the opsonized bacteria, lysostaphin was added in a 10 U/ml concentration and incubated with the organisms for 10 min at 37°C before adding them to the cells. Examination of the organisms by phase microscopy and gram staining revealed the uniform presence of poorly staining organisms that had the appearance of protoplasts. As shown in Fig. 6, only the intact opsonized organisms were capable of inducing the metabolic response by either normal or cytochalasin B-treated cells.

DISCUSSION

The importance of oxygen to the normal antimicrobial activity of human granulocytes has been well documented and, as discussed above, several reduction products of oxygen, including O_{2^-} and H_2O_2, have

TABLE VIII

Relationship between Oxygen Consumption, O_{2^-}, and H_2O_2 Release during Contact with Opsonized S. Aureus *by Normal and Cytochalasin B-Treated Granulocytes**

Cells	O_2 consumption	O_{2^-} release	H_2O_2 release
	nmoles/2.5 × 10^6PMN/5 min		*mean±SEM*
Normal	(5) 128.3±5.2	(3) 43.9±2.4	(5) 65.6±3.1
Cytochalasin B-treated	(4) 56.4±8.0‡	(3) 47.9±0.44	(4) 22.4±3.0‡

* Measurements of all three functions were made on granulocyte suspensions from the same donors incubated with 1 mM sodium azide and opsonized *S. aureus* in a 500:1 bacteria/PMN ratio for a 5-min period in the chamber of a Clark oxygen electrode. Granulocyte concentrations were 5 × 10^6/ml in 3 ml of HBSS. Superoxide release was measured by adding 160 μM ferricytochrome *c* to some suspensions and 50 μg/ml SOD to others as described in Methods. At the end of the incubation period the concentration of accumulated H_2O_2 was measured by placing an aliquot of the cell suspensions in a cuvette containing 2.5 ml HBSS with 2 μM scopoletin and 22 nm HPO and recording the resulting extinction in fluorescence as described in Methods. The numbers in parentheses refer to the number of experiments.

‡ The difference between cytochalasin B-treated and normal cells is statistically significant ($P < 0.01$, paired sample *t* test).

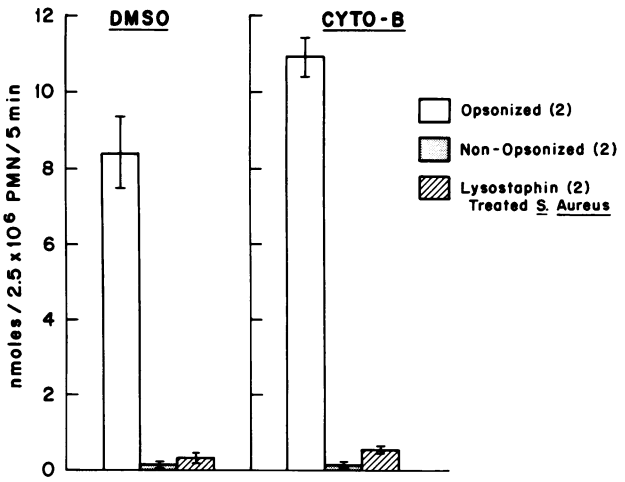

FIGURE 6 Release of H_2O_2 from normal and cytochalasin B-treated (CYTO-B) cells in the presence of SOD when exposed to opsonized *S. aureus* (clear bars), nonopsonized *S. aureus* (cross-hatched bars), and opsonized but lysostaphin-disrupted *S. aureus* (solid bars). The height of the bars depicts the means and the brackets, the SEM of the number of experiments shown in parentheses.

been implicated as playing key roles in their effector mechanisms (4). The experiments reported in this paper were conducted to measure the interrelationships between O_2^- and H_2O_2 produced and utilized during the respiratory burst in order to determine (a) whether the neutrophil superoxide generating system is the sole source for H_2O_2 formation in intact cells; (*b*) whether all superoxide could be quantitatively accounted for by dismutation to H_2O_2; and (*c*) to gain some information concerning the cellular site and stimulus to production of superoxide and H_2O_2. As discussed in the Introduction, resolution of these issues should aid in identifying the nature of the key oxidase(s) involved in promoting the phagocytic respiratory burst in granulocytes as well as defining mechanisms utilized in oxygen-dependent killing and inflammation by these cells. Although designed to provide answers to some of these questions, the experimental methods employed in the present investigations permitted a careful examination only of those fractions of O_2^- and H_2O_2 formed during the phagocytic respiratory burst which escaped into the extracellular medium. Thus, it became necessary to utilize a compound, cytochalasin B, which inhibits phagosome formation (32, 33) to improve the access of superoxide scavengers to the presumed site of formation or release of the compound.

The extinction of the fluorescence of scopoletin during oxidation by HPO provides a highly sensitive and specific assay for H_2O_2 in solution (26). We have previously used this assay to document and quantitate H_2O_2 released from phagocytizing normal cells as well as to define the kinetics of the reaction, because continuous recording of fluorescence is easily performed (2). The addition of the superoxide scavengers ferricytochrome *c* or NBT (5) to the medium of cells ingesting opsonized staphylococci reduced the amount of H_2O_2 detected by approximately 50%, but did not abolish it. The evidence that these findings were the result of scavenging of O_2^- in competitive reduction reactions involving O_2^- rather than some other mechanism is provided by the demonstration that neither one altered the detection of H_2O_2 itself under cell-free conditions, and finally, that the recovery of exogenously added H_2O_2 was over 90%, a value essentially equivalent to that seen with cell phagocytizing in the absence of these scavengers.[2] Whereas both compounds produced a 25–38% inhibition of postphagocytic oxygen consumption, the inhibitory effect of ferricytochrome *c*, but not NBT, was reversed almost completely with SOD. The most likely explanation for these findings is the fact that O_2 is regenerated mole for mole during reduction of ferricytochrome *c* by O_2^-, whereas only 1 mol of O_2 is formed for every 2 mol of O_2^- consumed during dismutation to H_2O_2 (5). Oxygen regenerated in the medium by these reactions is presumably available to the cells for reutilization during continued phagocytosis and O_2^- and H_2O_2 formation. Thus, no convincing evidence that oxidase activity per se was impaired by ferricytochrome *c* could be obtained. Furthermore, it appears unlikely that ferricytochrome *c* serves as a significant electron acceptor in place of oxygen during the oxidase-mediated reduction of oxygen, thus inhibiting the formation of superoxide itself, inasmuch as ferricytochrome *c* reduction during phagocytosis is inhibited by over 95% by SOD (3, 9), a finding which we confirmed in the present investigations.[3] Despite the similarity of the findings with NBT to those with ferricytochrome *c*, the inhibition of the reduction of NBT by SOD is reportedly less pronounced, and data has been presented to indicate that NBT reduction by phagocytizing cells occurs only in part through O_2^- production (8, 21, 38).

[2] Ferricytochrome *c* (36) and SOD (37) may catabolize H_2O_2 under the right incubation conditions, and ferrocytochrome *c* can be oxidized by HPO using H_2O_2 as substrate (29). In a separate series of experiments, we confirmed a 30–40% loss of recoverable H_2O_2 generated from glucose by GO in both cell-free and phagocytizing cell suspensions when HPO and scopoletin were omitted from the incubation mixtures. The accumulated H_2O_2 in the mixture was measured at the conclusion of a 10-min period by addition of 10-μl aliquots to cuvettes containing scopoletin and HPO as described in Methods. Thus, continuous trapping and utilization of H_2O_2 during its formation in scopoletin oxidation by HPO is necessary to avoid this potential source of error.

[3] Root, R. K., and J. Metcalf. Unpublished data.

Thus, NBT might inhibit O_2^- formation directly thereby accounting for the failure of SOD to completely reverse the inhibitory effect of NBT on H_2O_2 release in the majority of experiments. For these reasons, in our studies with cytochalasin B-treated cells only ferricytochrome *c* was employed as an O_2^- scavenger.

As reported by others, cytochalasin B-treated cells exhibited significant reductions in oxygen consumption and cell association of bacteria (32–35). These reductions occurred in parallel to each other, and by scanning electron microscopy the surfaces of the cytochalasin B-treated cells were studded with adherent opsonized bacteria suggesting that most of the organisms were bound to the cell surface rather than inside the cells. Small pseudopodial extensions of the cell membrane appeared to be attached to the bacteria. Despite this reduced cell association of phagocytic particles and a resulting decrease in oxidase activation, the release of both O_2^- and H_2O_2 were enhanced from the cytochalasin B-treated cells when compared to normal cells incubated under the same circumstances and in the absence of azide. These observations are compatible with the formation and release of these products of oxidase activation directly into the extracellular medium, an event which may take place at or near the site of particle binding as others have suggested (16, 27, 39). In this regard it was of interest that a second H_2O_2-dependent reaction, MPO-mediated protein iodination, was unaffected by cytochalasin B treatment indicating that this event may also take place on the cell surface or in the medium once H_2O_2 formation has been triggered (40, 41). After cytochalasin B treatment, access of the ferricytochrome *c* to the site of O_2^- formation must have been significantly improved because virtually no H_2O_2 release was observed in its presence, and iodination was markedly inhibited—more so than with normal cells. Again, the inhibitory effects were reversed with SOD and could not be explained by altered catabolism of added H_2O_2 or the generation of products (ferrocytochrome *c*) which competed significantly with scopoletin for oxidation by HPO using H_2O_2 as substrate. The effects of ferricytochrome *c* on oxygen consumption were similar to those described for normal cells. These observations strongly support the concept that virtually all H_2O_2 formation during the respiratory burst must proceed through an O_2^- precursor and that other pathways involving the direct divalent reduction of oxygen to H_2O_2 are of no quantitative importance. An hypothesis of O_2^- and H_2O_2 formation and catabolism in intact cells which incorporates our findings is shown in Fig. 7.

The studies employing sodium azide provided some information concerning the role that endogenous heme enzymes play in regulating H_2O_2 release into the medium surrounding phagocytizing cells. In 1-mM concentrations this compound inhibited the uptake of bacteria moderately by normal cells (30–40%) after 10–20 min of incubation at the high particle cell ratios employed (500:1). (In most experiments only 10–20% of the opsonized bacteria in the medium became associated with the cells.) Others have reported that azide can inhibit bacterial ingestion under these "saturating" conditions (42). Whether this is due to a reduction in energy supplies necessary to establish maximum phagocytic rates because of inhibition of cytochrome oxidase by azide or to some other mechanism remains to be established. Whatever the mechanism, neither oxygen consumption nor pentose shunt activity were inhibited in parallel. In fact, they were increased by almost 30–40% relative to the inhibition

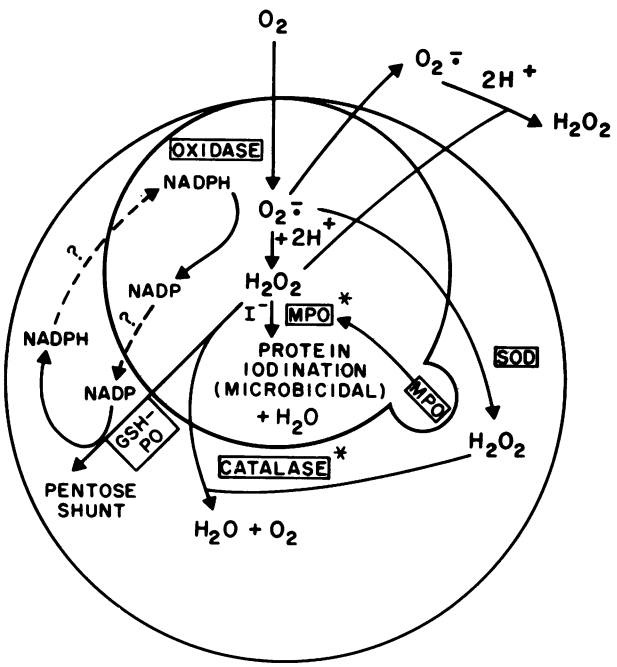

FIGURE 7 Postulated pathways for superoxide and peroxide formation and utilization during phagocytosis. Through the activity of an oxidase, superoxide is produced as the first product of oxygen reduction in forming phagocytic vacuoles, illustrated schematically by the smaller circle within the larger circle, which denotes the cell membrane. H_2O_2 is then formed from superoxide by the dismutation reaction and utilized in the vacuole by myeloperoxidase (MPO); protein iodination serves as a marker of this interaction. Free H_2O_2 and perhaps superoxide leave the forming phagocytic vacuole directly or by diffusion through intact membranes and can be detected in the extracellular medium by scopoletin oxidation and ferricytochrome *c* reduction, respectively. They also enter the cytosol to be catabolized by SOD, catalase, and glutathione peroxidase-linked (GSH-PO) pentose shunt activity. Whether the immediate substrate involved in the initial formation of superoxide is NADPH and can enter the phagocytic vacuole is conjectural as indicated by the question marks. Stars denote enzymes that are inhibited by azide (catalase and MPO).

in phagocytosis. Similar findings have been previously reported (43) and can be explained in part by the inhibition of heme enzymes (MPO and catalase) normally involved in H_2O_2 catabolism (Fig. 7). For example, when H_2O_2 is catabolized by catalase, the products are H_2O and O_2. As discussed above, regenerated O_2 can presumably be reutilized by the cells during phagocytosis. Thus, when O_2 regeneration is impaired by azide treatment, increased consumption of O_2 from the medium should be observed. The inhibition of MPO activity by azide was demonstrated by the marked reduction in protein iodination (30, 40, 41). With inhibition of two major pathways for H_2O_2, catabolism increased levels of free cellular H_2O_2 and catabolism of the compound by pathways not involving heme enzymes should occur. In keeping with this, [1-^{14}C]glucose oxidation was increased, presumably reflecting H_2O_2 catabolism by a glutathione recycling mechanism linked to the pentose shunt as proposed by Reed (44) and H_2O_2 release from normal cells was more than doubled by treatment with azide. Because an acidic pH is required for effective binding of azide to HPO (45), no apparent inhibition of the activity of this enzyme in the slightly alkaline medium (pH 7.4) surrounding the cells was observed, and the increased H_2O_2 release was readily detected by the scopoletin assay.

When normal cells were exposed to both azide and ferricytochrome *c*, the residual H_2O_2 found in the presence of these scavengers was more than doubled, indicative of its origin from an intracellular pool shared by the catabolic pathways noted above. In contrast, similar incubations with cytochalasin B-treated cells led to a much smaller rise in residual H_2O_2. Furthermore, prolonged measurement of H_2O_2 release in the presence of azide from normal and cytochalasin B-treated cells documented a reversal of the initial observation of increased H_2O_2 release seen with the latter in the absence of azide. Both observations are consistent with the more effective access of O_2^- scavengers to the site of its production in cytochalasin B-treated cells as well as an overall reduction in O_2^- and H_2O_2 formation by these cells when total bacterial uptake (i.e. phagocytosis and phagosome formation) is inhibited by the compound.

The increase in H_2O_2 release and iodination observed when both normal and cytochalasin B-treated cells were exposed to SOD was of interest regarding the fate of O_2^- produced during phagocytosis. Whereas it is possible that the enzyme was merely speeding the rate of dismutation of "free" O_2^- in the medium to H_2O_2, the rapid spontaneous dismutation rates in aqueous solution make this highly unlikely (5). Rather, it is far more consistent with the likelihood that a portion of the O_2^- produced during phagocytosis is not normally involved in spontaneous dismutation but is diverted into competitive oxidation or reduction reactions. The addition of exogenous SOD presumably inhibited some of these competing reactions and increased cellular H_2O_2 and extracellular H_2O_2 recovery, accordingly. Besides the detection of more H_2O_2 released by the scopoletin assay, MPO-dependent protein iodination was also increased in the presence of SOD. This emphasizes the point that the extent of this reaction is in part a function of H_2O_2 availability (8, 30, 41).

The stoichiometric relationships found between O_2 consumed and O_2^- + H_2O_2 released from cytochalasin B-treated cells lend weight to the concepts stated above. The complete reduction of oxygen to peroxide through an O_2^- intermediate pursues the following path:

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

thus 2 mol of oxygen and O_2^- are required to generate 1 mol of H_2O_2, with the regeneration of 1 mol of O_2 in the process. The exact relationship found for measurement of all compounds in phagocytizing cell suspensions (assuming optimal detection systems) would be a function of how much O_2 is utilized in O_2^- formation, how much O_2^- undergoes dismutation relative to diversion in other reactions, and finally, how much oxygen generated during dismutation is reutilized. In the presence of sodium azide, used to inhibit H_2O_2 catabolism, rates of oxygen consumption and O_2^- release over a 10-min incubation period by cytochalasin B-treated cells exposed to opsonized *S. aureus* were virtually identical (O_2^- detected = 99±14% of O_2 consumed). In contrast, with normal cells O_2^- recovery averaged only 34±2% of oxygen consumed, indicative of the formation of a considerable portion of O_2^- in a location which was presumably intracellular and inaccessible to free ferricytochrome *c* in the medium. H_2O_2 recoveries as a percentage of oxygen consumption averaged 51±1.3% for normal cells and 40±2% for cytochalasin B-treated cells, respectively. Similar results have been obtained by Roos et al. with serum-treated zymosan as a stimulus and a different technique to measure H_2O_2 (35). Inasmuch as 1 mM azide was noted to have disparate effects on phagocytosis and oxygen consumption under the conditions employed, inhibiting the former while increasing the latter over a 10-min incubation period, it is likely that the values for O_2^- and H_2O_2 produced relative to O_2 consumed are artifactually low.[4] Allowing for recycling of O_2 as noted in the formula above, it would not be surprising to detect levels of O_2^- formation in excess of measurable O_2 consumption, if the techniques employed were optimal. Nevertheless, the results are compatible with

[4] In preliminary experiments, we have confirmed this possibility as have Babior et al. (personal communication). O_2^- formation and release induced from cells by nonparticulate activators were not inhibited by azide.

the obligation of most or all of stimulated O_2 consumption into O_2^- production by the cytochalasin B-treated cells. The molar relationships between O_2^- and H_2O_2 released by the cytochalasin B-treated cells averaged 2.48:1. Even considering the fact that some H_2O_2 may have been catabolized by the glutathione-linked pathway to the pentose shunt (44), this is greater than the 2:1 ratio predicted by the formula above. Taken together with the enhanced recovery of H_2O_2 from cells exposed to SOD this observation strongly suggests that phagocytizing granulocytes generate more O_2^- than can be accounted for by spontaneous dismutation to H_2O_2. Potential pathways for O_2^- utilization other than dismutation include oxygenation reactions involving unsaturated lipids either in the bacteria or the leukocytes themselves (5), competing reduction reactions, and in the presence of H_2O_2, its consumption in the generation of singlet oxygen and hydroxyl radicals (21–25). The relative importance of these other reactions to the microbicidal and inflammatory capabilities of the granulocyte remain to be determined, but our studies provide quantitative support for their existence.

Finally, the actual site of O_2^- and therefore H_2O_2 formation in intact phagocytizing granulocytes remains to be defined. Our studies indicate that when staphylococci are used as a stimulus they must be intact and coated with serum opsonins, indicating that their interaction with specific regions on the cell membrane is a prerequisite to trigger oxidase activation as well as phagocytosis. Whether or not these regions are actually "receptors" on the cell surface which are specific for immunoglobulins and complement coating the organisms (46–49) must be clarified. The nonspecific nature of many activators of the granulocyte respiratory burst (see 34 for review) suggests that the common property of all these materials is their ability to produce certain conformational changes in the plasma membrane (34, 50). These changes are then responsible for stimulating O_2^- production through activation of a specific oxidase. The effectiveness with which scavengers such as ferricytochrome *c* remove O_2^- from the medium surrounding cytochalasin B-treated cells and block H_2O_2 formation suggests that O_2^- production must occur at or near the surface engaged in particle binding. Both chemical (27, 39) and morphological (16) evidence supporting this point of view has been recently presented. If this is not the case, then a mechanism must be defined for the highly efficient delivery of O_2^- from or through the cell surface and into phagocytic vacuoles before it has undergone significant dismutation to H_2O_2. A major challenge of future investigations in neutrophil physiology, then, is to precisely define the locus of operation of the critical enzymes involved in O_2^- generation as well as the mechanisms involved in their activation.

ACKNOWLEDGMENTS

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