H_2O_2 Release from Human Granulocytes during Phagocytosis

I. DOCUMENTATION, QUANTITATION, AND SOME REGULATING FACTORS

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ABSTRACT The extinction of fluorescence of scopoletin during its oxidation by horseradish peroxidase (HPO) provides a highly sensitive and specific assay for small quantities of peroxide in solution. With this assay, the release of free H_2O_2 into the extracellular medium by phagocytizing human granulocytes has been documented and quantitated, and some of the regulating factors have been determined. Under basal conditions granulocytes released less than 0.01 nmol/ml of H_2O_2 (2.5×10^6 polymorphonuclear leukocytes/ml). Upon the addition of phagocyte particles (latex, opsonized yeast, or staphylococci), an abrupt increase in extracellular peroxide concentration was observed (>50-fold above basal levels) after latencies as short as 10 s. Release reflected increased intracellular H_2O_2 production during phagocytosis in that it paralleled the respiratory burst and was absent when phagocytosis was prevented or when cells from patients with chronic granulomatous disease were utilized. Evidence that scopoletin oxidation occurred predominantly in the extracellular medium was obtained by demonstrating a marked inhibition when HPO was omitted from the reaction mixture or when exogenous catalase was added. Similarly, it was found that exogenous serum also inhibited scopoletin oxidation, apparently because of the presence of competing hydrogen donors.

H_2O_2 formation and release were observed at rates which closely paralleled those of phagocytosis. With O_2 consumption as an approximate index of H_2O_2 formation, the fractions released during maximal rates of particle uptake were calculated as follows: for latex, 15.7%; for staphylococci, 10.3%; and for yeast, 4.9%. It is postulated that release is due to diffusion of free H_2O_2 from an expanded intracellular pool of this substance that develops during phagocytosis. This pool represents the net of increased synthesis versus catabolism by various enzymatic pathways for H_2O_2 disposal within the cells.

The close relationship between rates of H_2O_2 formation and rates of phagocytosis by human granulocytes suggests a role for specialized areas of the cell membrane, involved in particle ingestion, in the trigger mechanism for H_2O_2 synthesis. The consequences of H_2O_2 release to other cells or organisms in the immediate environment of phagocytizing granulocytes remain to be determined.

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INTRODUCTION

Phagocytosis by mammalian granulocytes is accompanied by a marked burst in oxygen consumption, which in most cases ultimately results in the production of superoxide anions and H_2O_2 (1). Considerable evidence has been accumulated to indicate that H_2O_2, and perhaps superoxide anions, serve an important role in oxygen-dependent microbicidal activity by these cells (2, 3). In addition, several previous observations suggest that H_2O_2 is released from granulocytes during phagocytosis (4) and that it might affect other cells in the nearby environment (5). Most techniques to measure H_2O_2 production by granulocytes have been indirect, however, and do not provide information on the early stages of synthesis (6, 7). This has hampered a precise examination of the mechanisms of H_2O_2 formation by these cells, as well as a clarification of the importance of different pathways for H_2O_2 catabolism, both within and outside the cell.

Certain fluorescent compounds can act as hydrogen donors in oxidative reactions catalyzed by horseradish

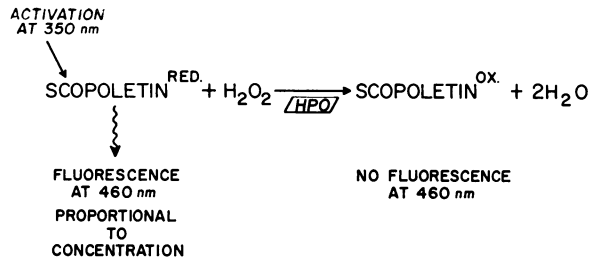

FIGURE 1 Principle of the detection of H_2O_2 by the HPO-mediated oxidation of reduced scopoletin.

peroxidase (HPO)[1] (8), an enzyme that exhibits substrate specificity for hydrogen or monoalkyl peroxide (9). With oxidation, a loss of the fluorescence of these compounds occurs and when HPO is present in excess, this change can be monitored to assay solutions specifically for their content of peroxides. As shown in Fig. 1, scopoletin (7-OH-6-methoxycoumarin) is a compound that fluoresces at 460 nm with an intensity directly proportional to its concentration when activated by light of 350 nm wavelength (10, 11). During its oxidation by HPO, fluorescence is lost with a stoichiometry directly proportional to the peroxide concentration in the medium. The sensitivity of the system is such that 0.01 μM concentrations of H_2O_2 in small volumes of aqueous solutions can be measured with precision. With a constant recording apparatus, small changes in H_2O_2 concentration can thus be closely monitored. This study reports in the utility of this highly sensitive and specific assay to examine the kinetics of H_2O_2 formation by granulocytes and to document and quantitate its release from these cells during phagocytosis. An intimate relationship between phagocytic rates and H_2O_2 formation and release was documented, which suggests a role for specialized portions of the cell membrane involved in phagocytosis in the trigger mechanisms for H_2O_2 synthesis.

METHODS

Scopoletin (7-OH-6 methoxycoumarin) was obtained from Sigma Chemicals (St. Louis, Mo.) and a 2 mM solution was prepared in 0.5 M phosphate buffer (pH 7.0).

HPO was kindly supplied in purified crystalline form by Dr. M. Tamura of the Johnson Research Foundation, University of Pennsylvania, School of Medicine, or obtained from Worthington Biochemical Corp. (Freehold, N. J.) as a purified lyophilized preparation (peroxidase D) with an activity of 400–700 U/mg. Both were prepared for use by dissolving them in 0.05 M phosphate buffer at pH 7.0.

Glucose oxidase (Type II: purified from *Aspergillus niger*) was obtained from Sigma Chemicals and a stock solution at a concentration of 1.6 mg/ml prepared for use by dissolving it in 0.05 M phosphate buffer at pH 7.0. The activity of the glucose oxidase in the stock solution was capable of generating 2 mM H_2O_2/min from 5.5 mM glucose/ml in Krebs-Ringer bicarbonate buffer at pH 7.4 (KRB) and 37°C.

Catalase (2 × crystallized from beef liver) was purchased from Sigma Chemicals as a crystalline suspension in water with 0.1% thymol with an activity of 30,000–40,000 sigma U/mg.

Ethyl peroxide was obtained from Ferrosan, Malmö, Sweden. The concentration was determined by assay with cytochrome *c* and yeast cytochrome *c* peroxidase (12).

Mixed ^{14}C amino acids were purchased from the New England Nuclear Company (Boston, Massachusetts) as was Aquasol for liquid scintillation counting.

Serum was obtained from the clotted blood of healthy normal donors of AB, Rh + blood type, pooled, and stored at −70°C until used.

Phagocytic particles. 1.1 μm latex spherules were purchased (Dow Chemicals USA, Membrane Systems Div., Midland, Mich.) and prepared for use as previously described, as were bakers' yeast (Fleischman's Yeast, Standard Brands, Inc., New York) (13). A strain of *Staphylococcus aureus* 502A was cultured in trypticase soy broth (TSB) overnight, heat-killed, washed, and suspended in KRB before use. ^{14}C-labeled *S. aureus* were obtained by incubating the overnight culture in TSB with 50 μCi of [^{14}C]mixed amino acids, after which the organisms were heat-killed and washed repeatedly as reported previously (14). Yeast and staphylococci were opsonized by incubation in 10% normal serum at 37°C for 30 min. After opsonization, the yeast and *S. aureus* were washed free of nonadherent proteins, as previously described (13, 15).

Cell separation. Mixtures of peripheral blood leukocytes containing an average of 75.4±0.2% monocytes, 3.6±0.4% eosinophils, and 20.4±1.1 lymphocytes (mean±SE of 35 preparations) were obtained from heparinized peripheral venous blood of 17 normal healthy donors and two adult men with sex-linked chronic granulomatous disease of childhood (CGD). Dextran sedimentation and hypotonic lysis, as previously described, were used to reduce erythrocyte contamination to less than one per granulocyte (14). After washing by suspension and low-speed centrifugation (150 *g* for 5 min at 4°C) in modified Hanks' solution (MHS) (13), the cells were suspended in KRB and enumerated by hemocytometer or electronic particle counter (Coulter Counter Model Z_{BI}, Coulter Electronics Inc., Hialeah, Fla.).

Measurement of H_2O_2 release from granulocytes. Scopoletin (2–4 μM final concentration) was added to 1 × 1 cm light path cuvettes containing 2.5 ml of KRB and granulocytes in a concentration of 2.5×10^6/ml. Preliminary experiments established that the least screening of fluor signals and the most reproducible results were obtained at this granulocyte concentration, so it was used exclusively for the experiments reported below. The cuvettes were maintained at 37°C by a constant-temperature water bath attached to a jacketed cuvette holder in an Hitachi-Perkin-Elmer spectrophotofluorometer (model MPF-2A) (Perkin-Elmer Corp., Hitachi-Perkin Elmer Instruments, Mountain View, Calif.). The activating light was set at 350 nm wavelength and directed through the cuvettes. The intensity of the emission fluorescence at 460 nm, and 90° from the activating light source, was monitored and recorded continuously on an Hitachi recorder (QPD-33) attached to the spectrophotofluorometer. HPO was added in most experiments at a final concentration of 22 nM. This repre-

[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; HPO, horseradish peroxidase; KRB, Krebs-Ringer bicarbonate buffer; MHS, modified Hanks' solution; MPO, myeloperoxidase; TSB, trypticase soy broth.

sented an amount of enzyme well above that necessary to detect H_2O_2 in concentrations of 10 μM or less. Such concentrations were far above the maximal rates of H_2O_2 production by phagocytizing cells. When necessary, additional HPO was introduced to eliminate any question of inadequate amounts of enzyme necessary to catabolize all free H_2O_2 in the cuvettes. Base-line extinction of the fluorescence by H_2O_2 released from nonphagocytizing cells was recorded for several minutes before adding the phagocytic particles. After particle addition, the suspensions were periodically agitated by stirring. This did not materially alter the rate of H_2O_2 release from the cells.

The system was standardized in the absence of cells with known amounts of peroxide, either generated as H_2O_2 from the glucose in the medium with glucose oxidase, or added directly as ethyl peroxide. The relationship between the extinction of the fluoresence of 2 μM scopoletin and the addition of increasing amounts of ethyl peroxide is shown in Fig. 2. As the amount of scopoletin in the medium decreased to less than 0.9 μM due to its oxidation, the relationship between increasing peroxide concentration and loss of fluorescence became nonlinear. Accordingly, measurements of changes in H_2O_2 concentration were made in the linear portion of the curve and fresh scopoletin was added as necessary to maintain direct proportionality between fluorescence extinction and H_2O_2 concentration. Identical curves were produced when H_2O_2 was generated in the medium from glucose by glucose oxidase of known activity, as calibrated with yeast cytochrome *c* peroxidase and cytochrome *c* (12).

Measurement of the disappearance of reduced scopoletin without oxidation. To document the amount of reduced scopoletin that disappeared from the suspensions under basal and phagocytizing conditions in the absence of its oxidation by H_2O_2 and HPO, latex particles were added before HPO in some preparations and the fluorescence at 460 mM was monitored continuously. In other experiments granulocytes were suspended in a concentration of 2.5×10^6/ml in 2.5 ml volumes of KRB in 12×75 mm glass tubes and kept at 37°C in a water bath. Scopoletin in a final concentration of 2 μM was added to the medium, followed by latex (1,000:1 particle-to-cell ratio) or opsonized staphylococci (500:1 ratio) in different tubes. The high particle-to-cell ratios were chosen after preliminary experiments indicated that they produced maximal H_2O_2 release from the granulocytes (see below). No HPO was added and to inhibit oxidation of scopoletin by cellular myeloperoxidase (MPO), some of the tubes contained 1 mM sodium azide. At various intervals the tubes were removed from the bath and placed on ice, and the cells and particles were removed by centrifugation at 2,500 *g* for 10 min at 4°C. The amount of reduced scopoletin in the cell-free supernates was determined fluorometrically by comparison with known standards and control tubes incubated in the absence of cells.

Measurement of phagocytosis. Ingestion of ^{14}C-labeled *S. aureus* was measured by previously described techniques (14, 15), with the following exceptions. To reproduce conditions occurring in the fluorometry cuvettes, granulocytes were added to 1 ml of KRB in 12×75 mm glass tubes in a concentration of 2.5×10^6/ml. After being warmed to 37°C for 5 min in a water bath, opsonized bacteria in a particle-to-cell ratio of 500:1 were added and the mixtures stirred periodically. The phagocytic reaction was halted by removing the tubes from the water bath and flooding the cells with 4 ml of ice-cold MHS containing 10% fetal calf serum and 0.01 M NaF. The cells were washed free of noningested organisms by repeated low-speed centrifugation at 4°C in cold MHS and 10% fetal calf serum and prepared for counting of radioactivity in Aquasol as previously described (15). To determine cell-associated radioactivity, which occurred as a result of the preparation technique in the absence of phagocytosis, control tubes in which the organisms were added to cell suspensions maintained on ice were treated and counted by similar methods. This "background" radioactivity, representing centrifugation-induced artifact, amounted to less than 0.5% of added counts in all experiments and, when calculated in terms of numbers of bacteria per cell, to a mean of less than one organism per granulocyte. The number of bacteria added to the suspensions was determined by Petroff-Hausser chamber (C. A. Hausser & Son, Philadelphia, Pa.) counts and a "specific activity" (counts per minute/organism) calculated from the radioactivity in the medium. The number of organisms ingested per cell was then calculated by converting cell-associated radioactivity to numbers of organisms and dividing that number by the total granulocyte count in the suspensions.

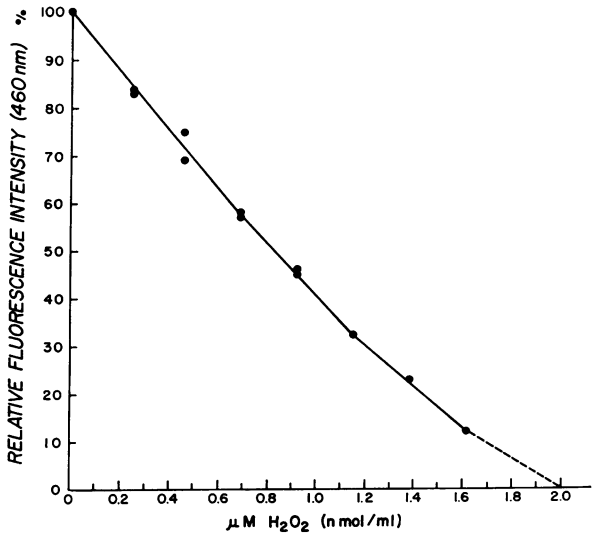

FIGURE 2 Relationship between the intensity of fluorescence of 2 μM scopoletin in the presence of HPO (22 nM) and increasing concentrations of peroxide added to the medium as ethyl peroxide.

Phagocytosis of opsonized yeast was quantitated in similar tubes containing 1.0 ml of KRB, 2 μM scopoletin, and granulocytes in a concentration of 2.5×10^6/ml. Yeast was added to the cells in a particle-to-cell ratio of 40:1 after the tubes had been warmed for 5 min in a 37°C water bath. At intervals after yeast addition, the suspensions were stirred and 10 μl amounts were removed with an Eppendorf pipette (Brinkmann Instruments, Inc., Westbury, N. Y.). They were mixed with an equal amount of phosphate-buffered 3% gluteraldehyde, pH 7.0 (final concentration, 1.5%), in saline on a clean glass slide. The gluteraldehyde provided rapid fixation of the cells to the surface of the slide and immediately halted phagocytosis. The cells were Wright's-stained and scored for phagocytosis by determining the percentage of 100 granulocytes with ingested yeast and the total number of intracellular yeast per 100 granulocytes.

Latex phagocytosis was also examined microscopically with particle-to-cell ratios (1,000:1) that produced maximal

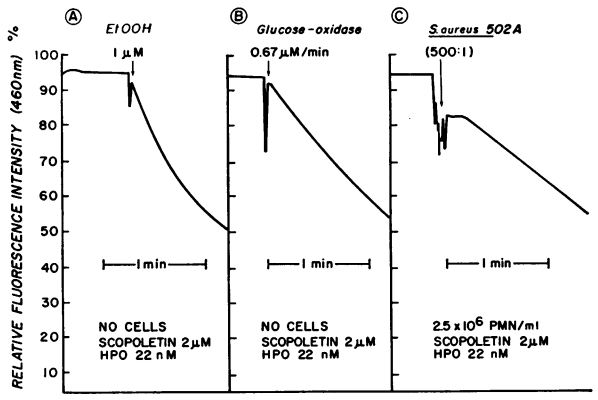

FIGURE 3 Tracings of representative experiments illustrating the loss of scopoletin fluorescence during its oxidation by HPO in the presence of different sources of peroxide.

rates of H_2O_2 release. At these very high particle-to-cell ratios, it was impossible by microscopic techniques to get a reliable quantitative measurement of the number of intracellular latex particles; therefore, results were expressed as the percent of cells engaged in phagocytosis.

It should be noted that all the above ratios of particles to cells produced maximal rates of phagocytosis, both as measured directly, in preliminary experiments in which the ratios were varied, and indirectly by their production of maximal post-phagocytic metabolic activities (13).

To duplicate more accurately the conditions during measurement of oxygen consumption, phagocytic assays were also run with concentrations of particles and cells similar to those above except that the tubes were rotated continuously in a 37°C incubator. Enumeration of phagocytosis was carried out as described.

Measurement of oxygen consumption by granulocytes. Oxygen consumption was measured with a Clark oxygen electrode as described previously (13), with a concentration of granulocytes of 5×10^6/ml suspended in 3 ml of KRB. Precise recordings could not be obtained with granulocyte concentrations of less than 5×10^6/ml, so that conditions in the incubation chamber did not duplicate those in the fluorometer cuvettes exactly. In addition, to insure a continued supply of dissolved O_2, the suspensions were agitated continuously with a magnetic stirrer. The ratios of phagocytic particles to cells were similar to those that produced maximal rates of H_2O_2 release; such ratios produced maximal rates of O_2 consumption at any cell concentration. In preliminary experiments different cell concentrations were employed while the phagocytic particle-to-cell ratio was kept constant to determine the relationship between cell concentration and oxygen consumption. It was found that as the cell concentration was halved, the concentration of O_2 consumed with respect to time fell by a factor of 0.62. By using this factor, it was possible to derive a figure for O_2 consumption at a granulocyte concentration of 2.5×10^6/ml so that the results could be compared directly with those for H_2O_2 release.

RESULTS

Measurement of H_2O_2 release from cells. Fig. 3 compares the extinction of fluorescence of scopoletin during its oxidation by peroxide added to the medium as ethyl peroxide (panel A), when generated continuously as H_2O_2 by glucose from glucose oxidase (panel B), or when released from granulocytes as H_2O_2 after the addition of opsonized *S. aureus* 502A (panel C). The first two experiments were run without cells and demonstrate that in the presence of peroxide the scopoletin was oxidized rapidly and linearly without a measurable latency. As shown in panel C, under basal conditions before the staphylococci were added, the cells released almost no detectable H_2O_2 (<0.1 nmol/ml per min.). During phagocytosis of staphylococci, scopoletin fluorescence fell rapidly after a latency of only 10 s. This reflected an increase in H_2O_2 concentration in the medium, at a rate more than 50 times basal conditions. Fig. 4 demonstrates similar results with cells phagocytizing latex. At the higher particle-to-cell ratio the latency was shorter (15 s) and the rate of scopoletin oxidation more rapid, as demonstrated by more rapid loss of fluorescence with time. From the steepest portion of the curves, maximum rates of H_2O_2 release were calculated for each phagocytic particle at different particle-to-cell ratios for latex until a plateau was reached (Fig. 5), corresponding to maximum phagocytic rates. Similar results were found with opsonized staphylococci and yeast, although the plateaus were different, as were the particle-to-cell ratios. Under conditions of maximal phagocytic activity, the latent period before H_2O_2 was

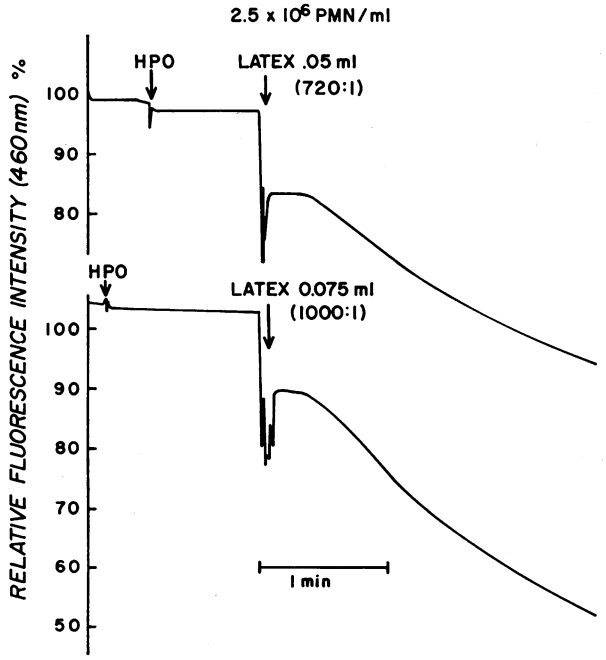

FIGURE 4 Representative tracings depicting the decrease in scopoletin fluorescence by the release of H_2O_2 during phagocytosis of latex by granulocytes. The particle-to-cell ratios are indicated in parentheses.

detected in the medium remained at 10–12 s despite varied concentrations of scopoletin, from 0.5 to 10 μM.

Table I depicts the detection of H_2O_2 under a variety of conditions with these maximal particle-to-cell ratios. The results are expressed as the maximum rates of H_2O_2 release into the medium that developed after the initial latency. Cells ingesting latex released H_2O_2 at greater average maximal rates than those ingesting other particles; however, the range of values was sufficiently broad to overlap with those obtained during yeast and staphylococcal ingestion, and the amounts released over 20 min were similar for all particles (Table III). Release was dependent upon phagocytosis-stimulated H_2O_2 formation, as indicated by its absence when phagocytosis was inhibited by presenting cells with nonopsonized yeast or staphylococci or when cells incapable of H_2O_2 production (CGD leukocytes) were used (16) (Table I). The detection of H_2O_2 in the medium was impaired when catalase was present due to competition by this enzyme with HPO for H_2O_2. It was also completely inhibited for up to 3 min when serum was present in a 10% concentration (Table II).

Table III gives totals for H_2O_2 released over a 20-min-period by cells ingesting latex, yeast, or staphylococci, and compares them to O_2 consumption over a similar period. The amounts of H_2O_2 released after ingestion of the different particles were similar. On the other hand, when O_2 consumption is used as an approximate index of H_2O_2 formation (7), the results suggest that a proportionally greater amount of H_2O_2 formed was released during latex phagocytosis than during ingestion of the other particles. The values given in Table II imply that during ingestion of latex, 5.8% of H_2O_2 formed was released into the medium, while corresponding figures for yeast and staphylococci were 2.0% and 4.4%, respectively. As will be shown below, these are undoubtedly erroneously low proportions due to the different rates of phagocytosis during measurements of O_2 consumption as opposed to H_2O_2 release.

Demonstration that scopoletin oxidation occurs predominantly in the extracellular location. As shown in Fig. 6, scopoletin fluorescence decreased somewhat during phagocyotsis in the absence of HPO in the medium. The extinction rate was immediately increased, however, when HPO was added, which reflected the accumulation of H_2O_2 in the medium during phagocytosis. Likewise, as noted above, when exogenous catalase was added, the decrease in fluorescence was instantaneously inhibited by 80% (Table II).

To evaluate the mechanism of disappearance of reduced scopoletin from the medium when incubated with cells in the absence of exogenous HPO, suspensions con-

TABLE I

Detection of H_2O_2 Released from Human Granulocytes under Different Conditions

Cells	Additions	Number of subjects	H_2O_2 detected
			nmol/2.5 × 10⁶ PMN/min
Normal	None	8	0.012±0.003
	Latex	11	0.445±0.064 (0.276–1.056)
	Opsonized yeast	3	0.283±0.08 (0.093–0.420)
	Opsonized *S. aureus* 502A	3	0.395±0.089 (0.285–0.613)
	Nonopsonized yeast	3	0
	Nonopsonized yeast +10% serum	2	0
	Nonopsonized *S. aureus*	2	0
CGD	Latex	2	0

Results are expressed as the mean ±SE of the maximal rates of H_2O_2 production detected by scopoletin oxidation. The ranges are shown in parentheses. The particle-to-cell ratios were as follows: latex 1,000:1, yeast 40:1, and *S. aureus* 500:1.

TABLE II

Inhibition of the Detection of H_2O_2 Release from Phagocytizing Granulocytes by Exogenous Catalase or Serum

Conditions	No. subjects	H_2O_2 detected*
		nmol/2.5 × 10⁶ PMN/min
Cells + latex	3	1.45 (1.09–1.71)
+ catalase (4 U)	2	0.297 (0.132–0.462)
+ inactivated catalase‡	2	1.32 (0.924–1.72)
+ 10% serum	2	0

* Maximum rate of H_2O_2 release after adding latex particles at a 1,000:1 particle/cell ratio. Data are mean (range).
‡ Catalase was inactivated by heat at 70°C for 2 h and then by treatment with 1 mM azide.

TABLE III

Relationship between Oxygen Consumption and H_2O_2 Release during Ingestion of Different Particles

Phagocytic particle	Number of subjects	Oxygen consumption*	H_2O_2 release*
		nmol	*nmol*
Latex‡	3	68.4±6.6	4.0±0.42
Yeast‡	2	174±26.2	3.4±1.2
S. aureus 502A‡	3	141±15.7	6.2±2.3

* Results are expressed as the mean ±SE/2.5 × 10^6 PMN/20 min.
‡ Particle-to-cell ratios were as follows: latex 1,000:1, yeast 40:1, and *S. aureus* 502A 500:1. Yeast and staphylococci were opsonized before addition to the cells as described in Methods.

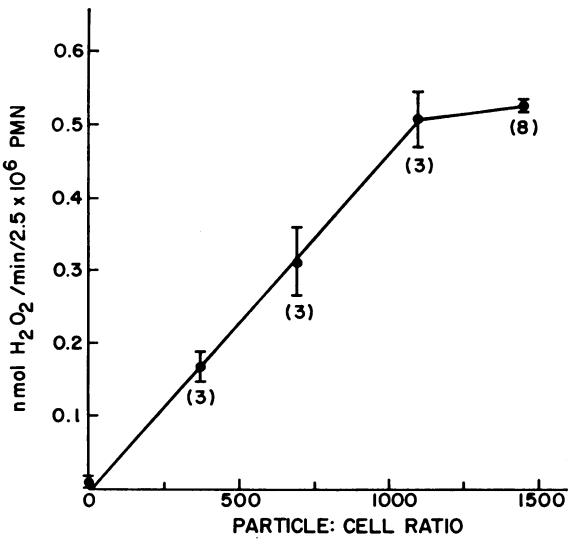

FIGURE 5 Relationship between maximum rates of H_2O_2 release from granulocytes during latex phagocytosis and the particle-to-cell ratio. The line is the mean and the brackets the SE of the number of experiments shown in parentheses.

taining scopoletin and cells were incubated with and without 1 mM sodium azide and the supernates were analyzed for residual reduced scopoletin, as described in Methods. Under basal (nonphagocytizing) conditions, scopoletin fluorescence decreased by 9.6% within 5 min and by 13.2% within 30 min (Table IV). With latex phagocytosis these percentages increased to 25.1% and 41.0%, respectively, within the same time intervals. Addition of sodium azide reduced this loss by 40.6% to 34.1% for basal cells and 54.2% to 54.4% for phagocytizing cells at 5 and 30 min, respectively, presumably due to inhibition of cellular MPO. As noted in Table IV, subtraction of the amount oxidized by MPO left values for scopoletin disappearance of 5.7%–9.8% and 11.5%–17.5% at 5 and 30 min for basal and phagocytizing cells, respectively, that could not be accounted for by its enzymatic oxidation. Expressed in terms of scopoletin concentration, this amounted to a decrease of 0.35 μM for phagocytizing cells at 30 min, only 8.8% of the total amount oxidized at 20 min in the presence of exogenous HPO, as noted in Table III.

Inhibition of scopoletin oxidation by exogenous serum. As shown in Table I, when fresh human serum was added to the medium in a 10% concentration to provide opsonins for the ingestion of nonopsonized yeast, no loss in scopoletin fluorescence was observed over a several-minute incubation period, despite the occurrence of phagocytosis. Furthermore, a similar serum concentration totally and immediately inhibited scopoletin oxidation during latex phagocytosis (Table II), and the inhibition continued for at least 3 min. With glucose oxidase generating H_2O_2 continuously from glucose in the medium, the effect on scopoletin oxidation of various concentrations of serum was examined. The addition of a small concentration of serum totally inhibited scopoletin oxidation for a short period of time; then oxidation resumed at the original rate (Fig. 7). As serum concentrations were increased, the duration of the period of total inhibition also increased and when the concentrations were 0.08% or above (protein concentrations >0.14 mg/ml, Lowry, Rosebrough, Farr, and Randall technique [17]), the subsequent rate of oxidation was also diminished by more than 50% (Table V). The inhibition of scopoletin oxidation by serum at low concentrations (<0.05%) differed then from the inhibition produced by catalase in its complete but temporary nature.

Relationship of H_2O_2 formation and release to phagocytosis. As indicated in Table I, when phagocytosis of staphylococci or yeast was prevented because of a lack of opsonic factors, no formation or release of H_2O_2 occurred. The relationships between phagocytosis, O_2 consumption, and H_2O_2 release are more precisely shown in Figs. 8 and 9. Both metabolic activities lagged slightly behind those for phagocytosis in the first 2 min of incubation. Thereafter they paralleled each other.

It should be noted that when the cell suspensions were constantly agitated, phagocytic rates for staphylococci (Fig. 8) were more rapid than under the relatively stationary conditions in which H_2O_2 release was measured (Fig. 9). Over a 30-min incubation, phagocytosis of staphylococci was increased 2.36-fold by agitation and that of yeast 2.55-fold. Similar measurements of the number of intracellular particles could not

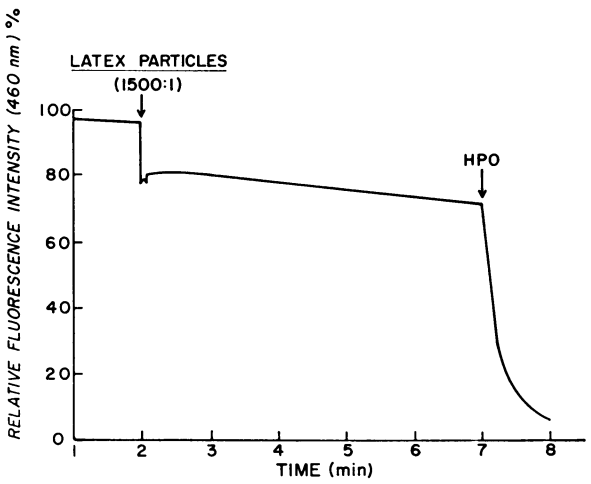

FIGURE 6 Representative tracing depicting the relationship between scopoletin fluorescence and latex phagocytosis (particle-to-cell ratio indicated in parentheses) before and after the addition of HPO to the medium.

be made with the 1,000:1 particle-to-cell ratio of latex, as explained previously, however, under conditions in which H_2O_2 release was measured, the percentages of cells that exhibited ingestion of latex particles were 84, 97.5, and 100% at 1, 2, and 5–20 minutes, respectively. With agitation, 100% of the cells contained intracellular latex by 2 min, as judged microscopically. Under stationary conditions the mean percentages of cells that contained one or more intracellular particles were 35.8, 45.3, 72.8, 84.3, and 92.0%, respectively, at 1, 2, 5, 10, 20, and 30 min of incubation. With agitation 79% of the cells contained intracellular yeast at 2 min, 87% at 5 min, 97% at 10 min, and 99% at 20 min.

Since rates of O_2 consumption and H_2O_2 release were found to be closely correlated with the rates of phagocytosis, the measured increase in phagocytic rates of 2.36-fold for staphylococci and 2.55-fold for yeast, and an approximate correction of 2.5-fold for latex ingestion during agitation were used to estimate more accurately the fraction of H_2O_2 released from that formed by the cells during ingestion of the different particles. With these calculations, the results expressed as a percentage of O_2 consumption were as follows: 15.7% during latex ingestion, 5.1% during yeast ingestion, and 10.3% during staphylococcal ingestion.

TABLE IV

*Disappearance of Reduced Scopoletin from the Medium when Incubated with Human Granulocytes in the Absence of HPO**

Conditions	Percent of control‡	
	5 min	30 min
Basal	90.4 (85.7–95.0)	86.8 (80.0–93.5)
Basal + 1 mM azide	94.3 (91.5–97.0)	91.3 (80.5–103)
% loss due to MPO	3.9 (2.0–5.8)	3.5 (0.5–6.5)
% loss not due to MPO	5.7 (3.0–8.5)	9.8 (0–19.5)
Phagocytizing latex§	74.9 (69.7–80.0)	59.0 (43.0–75.0)
Phagocytizing latex + 1 mM azide	88.5 (80.0–97.0)	81.3 (73.0–90.5)
% loss due to MPO	13.6 (10.3–17.0)	22.3 (15.5–30.0)
% loss not due to MPO	11.5 (3.0–20.0)	17.5 (9.5–25.5)

* Initial reduced scopoletin concentration = 2 μM.

‡ Control preparations were cell-free and the results are expressed as the mean and range of duplicate determinations in two experiments.

§ Ratio of latex particles/cells = 1,000:1.

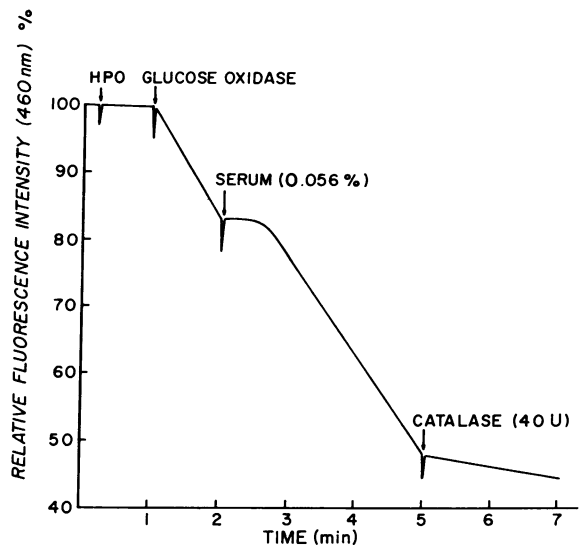

FIGURE 7 Inhibition of scopoletin oxidation by serum (final concentration indicated in volume %) and catalase during the generation of H_2O_2 from glucose by glucose oxidase.

DISCUSSION

The utility of the scopoletin assay to measure small amounts of H_2O_2 in aqueous solution was first reported by Andreae in 1955 (10) and confirmed by Perschke and Broda (11). It has subsequently been employed effectively to measure H_2O_2 formation by subcellular particles (18). Besides its sensitivity, which permits the detection of 0.01 μM concentrations of H_2O_2 in solution, it is highly specific, because hydrogen and monoalkyl hydrogen peroxides are the sole substrates for

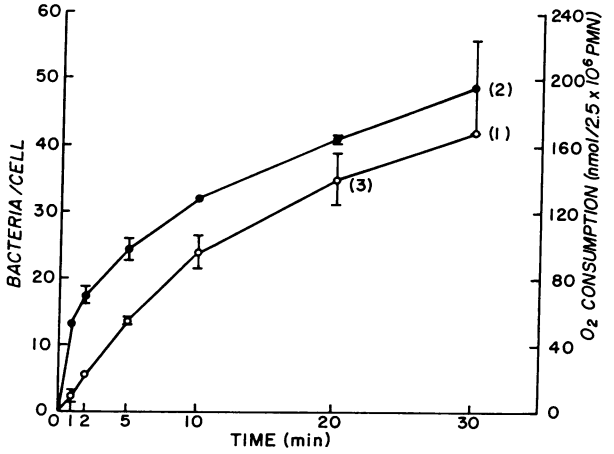

FIGURE 8 Relationship between the number of staphylococci ingested per cell (●—●) and oxygen consumption (○—○) with time. The lines are the mean and the brackets the SE of duplicate determinations on the number of subjects shown in the parentheses.

peroxidase (9), which catalyzes the scopoletin oxidation reaction. The optical geometry used in our experiments provided a system that was relatively unaffected by light-scattering and quenching. This permitted accurate measurements of H_2O_2 concentration in very dense suspensions of cells and phagocytic particles. With constant recording of scopoletin fluorescence, small changes in H_2O_2 concentration could be closely monitored and precise information on the initial phases of H_2O_2 formation and release gained.

As expected, the oxidation reaction was found to be significantly inhibited in the presence of enzymes such as catalase, which compete with HPO for H_2O_2 as substrate, or materials that compete with scopoletin as hydrogen donors for oxidation by the HPO-H_2O_2 enzyme-substrate complex (9). The latter point provides an explanation for the complete but temporary inhibition of the detection of H_2O_2 by oxidation of scopoletin in the presence of small amounts of serum. As the serum concentrations were increased, the subsequent rates of scopoletin oxidation were permanently diminished, perhaps due to the presence of catalase released from erythrocytes and other cells during the clotting process. Care must be taken then to exclude such materials from the medium when possible, or take their presence into consideration to provide reliable measurements of H_2O_2 concentration by this method.

Peroxidase-mediated oxidation of scopoletin did not account completely for the disappearance of the material from the extracellular medium. As shown by the experiments in which HPO was eliminated and cellular

TABLE V

Effect of Serum on Scopoletin Oxidation by H_2O_2 Produced from Glucose by Glucose Oxidase

Serum volume	Protein concentration	Duration of total inhibition	Scopoletin oxidation rate
%	*mg/ml*	*s*	*% of control**
0.008	0.014	0	113.2
0.016	0.028	0	115.7
0.024	0.042	6	104.9
0.032	0.050	12	123.6
0.040	0.070	17	75.7
0.048	0.084	24	107.2
0.056	0.098	36	90.5
0.080	0.140	36	52.6
0.20	0.350	60	45.1
0.40	0.700	96	32.8

* Rate of extinction of scopoletin fluorescence after the period of total inhibition compared to control preparations containing glucose oxidase without serum. Control H_2O_2 generation from glucose by glucose oxidase varied between 0.288 and 0.385 nmol/ml/min. Scopoletin oxidation in the presence of 4 U of catalase was 2% of control.

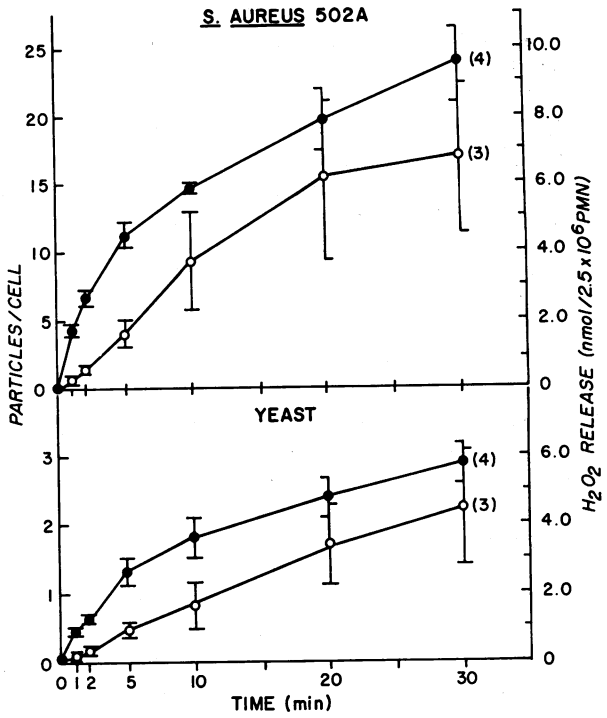

FIGURE 9 Relationship between the number of staphylococci or yeast ingested per cell (●—●) and H_2O_2 release (○—○) with time. The lines are the mean and the brackets the SE of duplicate determinations on the number of subjects shown in parentheses.

MPO inhibited with sodium azide, almost 10% of reduced scopoletin could not be recovered from the medium of cells phagocytizing latex. Part of this loss may be due to entry of scopoletin into cellular water, either by passage through the plasma membrane or by being swept into phagocytic vacuoles along with particles. If scopoletin distributes equally in intra- and extracellular water, then such equilibration alone would lead to an approximate decrease of only 0.1% in the extracellular concentration of the compound.[2] This suggests that other mechanisms are operative in diminishing scopoletin concentrations in the medium, such as its accumulation or catabolism within the cells or perhaps some oxidation by nonenzymatic means during H_2O_2 forma-

[2] Based on data accumulated earlier, that 2.0×10^7 granulocytes are equivalent to approximately 1 mg of cellular protein (13) and that protein represents 10% of cell weight, 70% of which is water, a calculation of 2.6 μl of intracellular water can be derived for the 7.5×10^6 granulocytes in the suspension. During phagocytosis Chang's data (19) indicates that this figure would be increased by 80 nl. This amounts to only 0.108% of the total water in the 2.5-ml suspensions. With a starting concentration of 2.0 μM scopolet in the medium, such an equilibration would decrease the extracellular concentration to 1.998 μM, i.e., only 0.1% from the initial concentration.

tion. The use of another marker of scopoletin concentration and location (e.g., radiolabeled material) would aid in the analysis of this question, which remains unanswered by the present studies.

Regardless of the mechanisms, however, non-peroxidase-mediated disappearance of scopoletin and MPO-mediated oxidation represent only small fractions (10 and 30%, respectively) of the total oxidized in the presence of exogenous HPO. Dramatic evidence of the predominant extracellular location of the reaction was provided by demonstration of an immediate drop in fluorescence when HPO was added to phagocytizing cells, due to the accumulation of H_2O_2 in the medium and the immediate inhibition of the HPO-mediated reactions when exogenous catalase or serum was added. The rapidity of these events and the documentation that exogenous enzymes such as catalase (20) can enter cells only in miniscule amounts during phagocytosis[3] support the concept that HPO-mediated scopoletin oxidation in our system measures extracellular H_2O_2 almost exclusively.

H_2O_2 release into the medium was observed to parallel the respiratory burst of normal cells and to be absent from CGD cells, which are incapable of H_2O_2 formation (16). This indicates that the phenomenon is dependent upon the synthesis of H_2O_2 in increased amounts during phagocytosis. Considerable evidence indicates that several metabolic pathways exist within granulocytes, which catabolize free H_2O_2 in different subcellular locations, including catalase and glutathione peroxidase within the cytosol (21, 22) and MPO within phagocytic vacuoles (2, 13, 22). Furthermore, H_2O_2, supplied in an extracellular location, readily diffuses into granulocytes to stimulate intracellular metabolic events (21, 23), indicating that cellular membranes provide no barrier to diffusion of this substance. Thus it seems evident that the release of H_2O_2 from granulocytes during phagocytosis represents diffusion from an expanded intracellular pool of free H_2O_2 that develops during particle ingestion (Fig. 10). The amount released should depend upon and reflect the concentration of free intracellular H_2O_2. This in turn represents that fraction of H_2O_2 synthesized not utilized by other catabolic pathways. In support of this concept, evidence has been obtained in our laboratory to indicate that H_2O_2 release is increased

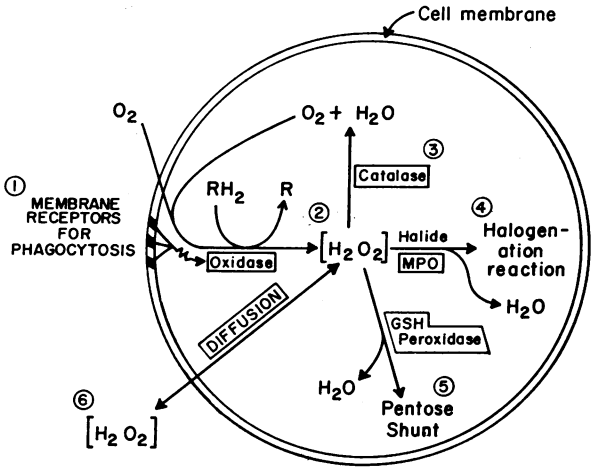

FIGURE 10 Postulated events involved in H_2O_2 formation, catabolism and release by human granulocytes. (1) Initiation of phagocytosis by the interaction between particles and membrane phagocytic receptors triggers the activation of an oxidase, which generates free intracellular H_2O_2 (2) from substrate oxygen and appropriate hydrogen donor(s). Portions of this free H_2O_2 are catabolized by catalase (3), MPO (4), and glutathione peroxidase with a subsequent link to the pentose shunt (5). The resultant concentration of free H_2O_2 that diffuses from the cells (6) is then a direct reflection of the remaining concentration of free intracellular H_2O_2.

when one or more of the other pathways are blocked by metabolic inhibitors.[4] When oxygen consumption is used as an approximate index of H_2O_2 formation (7), the fraction which is released and detected by scopoletin oxidation varies from 4.9 to 15.7%, depending upon the phagocytic particle employed. Such calculations, however, do not take into consideration that oxygen produced during the catalatic action of catalase on H_2O_2 might be used for further H_2O_2 synthesis, as indicated in Fig. 10; thus they can be considered only as approximations. An important point to be addressed by future work, then, is to accurately determine the relationship between extracellular and intracellular free H_2O_2 concentrations under different conditions. With this type of information it should be possible to calculate the total amounts of H_2O_2 formed by phagocytizing cells with more precision.

An outstanding advantage of the technique employed in this investigation is that it provides direct information on the initial rates of H_2O_2 formation within granulocytes during phagocytosis (as reflected by diffusion of H_2O_2 out of the cells), which heretofore has been lacking. It was observed that H_2O_2 synthesis and release were closely related to the rates of particle ingestion.

[3] In our experiments, where catalase concentrations in the medium were 4 U/ml, the amounts of this enzyme that entered phagocytic vacuoles were calculated to be only 4×10^{-12} U/vacuole, from data provided by Chang (19) and Mandell (20). Under conditions of optical activity, this amount of enzyme would be capable of catabolizing only 4×10^{-9} nmol of H_2O_2/min (9). From the extracellular concentrations of H_2O_2 that develop during phagocytosis (about 1 nmol/ml per min) and the number of cells in the suspensions, each cell can be estimated to contribute 4×10^{-7} nmol H_2O_2/min to the medium.

[4] Root, R. K., and J. Metcalf. 1974. H_2O_2 release from human granulocytes during phagocytosis. II. Relationship to intracellular enzymatic pathways for H_2O_2 formation and catabolism. Manuscript in preparation.

When the existence of multiple pathways to dispose of H_2O_2 within cells is considered together with the requirement for diffusion of H_2O_2 to an extracellular location before it can be detected, as well as the presence of potential hydrogen donors in the cell suspensions that might compete with scopoletin for oxidation (e.g., proteins and lipids in cell membranes), the latency of 10 s observed for the detection of extracellular H_2O_2 as an index of formation might be artifactually prolonged. On the other hand, that the latent period was fixed, despite the 20-fold variation in the concentration of scopoletin, suggests that the role of competing hydrogen donors in the suspensions in prolonging the detection of scopoletin oxidation is relatively minor, and that a fixed period of time exists between contact of particles with critical portions of the granulocyte cell membrane and stimulated H_2O_2 formation.

In support of the concept that perturbations in the plasma membrane are responsible for triggering H_2O_2 formation, it has been shown that membrane-active agents such as endotoxin, deoxycholate, digitonin, and chemotactic factors (24, 25) all produce a stimulation of the leukocyte pentose shunt without inducing phagocytosis. Stimulated pentose shunt activity indirectly indicates increased intracellular H_2O_2 levels (1, 21). Granulocytes have been documented to contain receptors for IgG and C3 that appear to be responsible for binding opsonized particles to cells to initiate phagocytosis (26, 27). In view of the failure of nonoponsized staphylococci and yeast to induce H_2O_2 production, and the close relationship between phagocytic and H_2O_2 formation rates observed in our experiments, it seems quite conceivable that opsonic and other membrane receptors involved in phagocytosis might also contain the trigger mechanisms or even the enzymes for H_2O_2 synthesis (Fig. 10). Such an arrangement would insure the delivery of high concentrations of H_2O_2, and perhaps superoxide anions, to within phagocytic vacuoles for oxygen-dependent microbicidal reactions, as suggested by our previous studies (13). Furthermore, if the plasma membrane is the site of H_2O_2 synthesis, part of release of this material into the medium during phagocytosis may be through partially closed phagocytic vacuoles, rather than by diffusion through intact cell membranes. Whether such an arrangement actually exists remains to be determined, as does the impact of H_2O_2 release on microorganisms or other cells within the immediate environment of phagocytizing granulocytes.

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