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Direct Measurement of Hydrogen Peroxide Release from Rat Alveolar Macrophages: Artifactual Effect of Horseradish Peroxidase

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ABSTRACT: *Investigators disagree on the amount of hydrogen peroxide (H_2O_2) released by resting and stimulated alveolar macrophages. The method commonly used to measure H_2O_2 release involves horseradish peroxidase (HRP)-catalyzed oxidation of scopoletin by H_2O_2. We describe an artifact in this method that may explain the seemingly inconsistent data reported by other investigators. Release of H_2O_2 and luminol-catalyzed chemiluminescence are stimulated in rat alveolar macrophages by type II HRP at concentrations normally used in the HRP–scopoletin method. The amount of H_2O_2 released depends upon the length of time the cells are preincubated at 37.5°C and the time at which type II HRP is added. After stimulation with type II HRP, the cells do not release additional H_2O_2 upon exposure to zymosan particles. Myeloperoxidase, an alternative catalyst to type II HRP, does not stimulate H_2O_2 release and, therefore, can be used to measure H_2O_2 release from rat alveolar macrophages. Using myeloperoxidase, resting H_2O_2 release is negligible; after zymosan stimulation, $6.14\ (\pm\ 0.87) \times 10^{-6}$ nmoles/cell · 10 min is released. In addition, more pure HRP preparations (types VI, VII, VIII, and IX) do not stimulate alveolar macrophages to release H_2O_2 and can be used to monitor zymosan-induced H_2O_2 release. As our data indicate that type II HRP stimulates H_2O_2 release from rat and guinea pig alveolar macrophages, it is not the catalyst of choice for this assay. In conclusion, our data explain the conflicting results found in the literature and indicate that rat alveolar macrophages release minimal amounts of H_2O_2 at rest and can be stimulated by zymosan.*

INTRODUCTION

Alveolar macrophages are mobile phagocytic cells located within the alveoli and small airways of the lung. As phagocytes, they are involved in the clearance of particles deposited deep within the lung and represent a primary line of defense against the adverse effects of particulate inhalation. It has been suggested that these cells also play a role in the killing of microbes that enter the lung [1]. Appropriate stimuli induce alveolar macrophages to produce oxygen metabolites, such as superoxide anion ($\cdot O_2$) and hydroxyl radical ($\cdot OH$), that can kill bacteria. Hydrogen peroxide (H_2O_2) may also be released by alveolar macrophages and participate in killing microbes.

An assay commonly used to investigate H_2O_2 release from alveolar macro-

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phages was designed by Root et al. [2] to determine H_2O_2 release from leukocytes. The assay is based on the oxidation of reduced scopoletin by a horseradish peroxidase (HRP)–H_2O_2 complex [3]. The amount of oxidized scopoletin is directly related to the concentration of H_2O_2 and can be measured spectrofluorometrically. The assay was used successfully to measure H_2O_2 release from polymorphonuclear leukocytes, helping to clarify the events associated with the "respiratory burst" in that cell type [1]. The data generated from alveolar macrophages, however, have not been consistent [4–12]. Rates of H_2O_2 release from resting alveolar macrophages have been reported to be as low as 0.043 nmoles/2.5×10^6 cells · min [11] and as high as 7.0 nmoles/2.5×10^6 cells · min [10]. In addition, some investigators have reported that stimulants, such as zymosan, increase H_2O_2 release from alveolar macrophages [5, 7], whereas others do not observe any additional release upon stimulation [10, 11].

The objective of this investigation was to study the validity of the method designed by Root et al. [2] in measuring H_2O_2 release from alveolar macrophages. A review of the methods used in previous studies reveals slight variations in the length of time that cells were preincubated and the order in which HRP, scopoletin, and particles were added to the assay mixtures. The experiments described herein were performed to determine if the seemingly inconsistent data reported previously could be explained by an artifact within the H_2O_2 assay or by variations within the procedure.

MATERIALS AND METHODS

Isolation of Cells

Alveolar macrophages were obtained by bronchotracheal lavage of male Sprague–Dawley rats (250–300 g) supplied by Charles River Laboratories, Boston, MA [13, 14]. Each animal was anesthetized by intraabdominal injection of 52 mg of sodium pentobarbital and exsanguinated by severing the renal artery and abdominal aorta. The trachea was cannulated with an 18-gauge needle covered with plastic tubing and the lungs were lavaged 12 times with a total of 80 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4). Cell suspensions and buffers were maintained at 4°C to reduce macrophage adherence to the tubes and microbial growth.

The lavage fluid was centrifuged at 500*g* and 2°C for 5 min. The resulting pellets were washed once by resuspending in 3.0 ml of *N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-buffered medium (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM Na-Hepes, and 5.5 mM glucose; pH 7.4) and recentrifuged. The final pellets were resuspended in Hepes-buffered medium and placed in an ice bath (4°C) until needed. Cell concentration and size were determined with a Coulter counter (Model ZB) and Channelyzer II (Coulter Electronics, Inc., Hialeah, FL). For all experiments reported herein, the final suspensions were at least 97% pure alveolar macrophages.

Hydrogen Peroxide Assay

Alveolar macrophages (2×10^6 cells), previously maintained at 4°C, were added to a total of 3.0 ml of Hepes-buffered medium and preincubated for 15 min at 37.5°C in a rotary shaker water bath to warm the cells and restore normal cellular mebatolism. After preincubation, scopoletin and type II HRP were added to achieve final concentrations of 2.4 μM and 6.6 units/ml, respectively. The fluorescence of reduced scopoletin was monitored continuously in a Perkin–

Elmer fluorescence spectrophotometer (MP6-3L; Perkin–Elmer Corporation, Norwalk, CT) equipped with a magnetic stirring unit. The chamber temperature was not controlled, but remained at 32°C for all of the present experiments. The excitation wavelength was 350 nm and the emission at 460 nm was measured. A standard curve was constructed by measuring the fluorescence changes associated with the addition of known amounts of H_2O_2 to a Hepes-buffered medium containing scopoletin and HRP.

The rate of H_2O_2 release from unstimulated alveolar macrophages was measured after the preincubation period as outlined previously. The rate of H_2O_2 release from particle-stimulated macrophages was determined by adding unopsonized zymosan (5.0 mg/ml) to an assay mixture after a stable fluorescence signal was obtained. The zymosan was prepared by boiling 350 mg in 20 ml of phosphate-buffered medium for 15 min. This suspension was centrifuged for 5 min at 500*g* and the pellet resuspended in 1.0 ml of Hepes-buffered medium [13]. At the designated time 43 μl of this zymosan preparation was added to the cell suspension to monitor the effect of particles on H_2O_2 release.

The basic procedure outlined was varied to identify artifacts in the assay.

1. Catalase, an enzyme that acts to degrade H_2O_2, was added to samples before preincubation to ensure that recorded changes of fluorescence were caused by changes in the H_2O_2 concentration of the cell suspension. The final concentration of catalase was 2500 units/ml. In all cases, catalase prevented changes in fluorescence, indicating that the measurements were valid. To ensure that the catalase was not injuring the cells, some catalase-pretreated samples were centrifuged for 15 sec at 15,600*g* in an Eppendorf centrifuge (Model 5412; Brinkmann Instruments, Westbury, NY) after preincubation and before the addition of scopoletin and type II HRP. The pellets were resuspended in catalase-free, Hepes-buffered medium at 37.5°C; scopoletin and type II HRP were added; and the cellular release of H_2O_2 was recorded. These cells produced a normal amount of H_2O_2, indicating that pretreatment with catalase did not alter the production of H_2O_2 by injuring the cells.
2. The effect of preincubating the cells at 37.5°C was investigated by altering the length of preincubation.
3. The time of addition of scopoletin was varied. Scopoletin was added 4 min before, 2 min after, or 4 min after the addition of type II HRP to a preincubated cell suspension to determine if either substance was stimulating the cells to release H_2O_2. Both scopoletin and HRP are required to measure H_2O_2 in solution. If one of these substances stimulated cellular release of H_2O_2, adding that substance alone to a cell suspension would cause an accumulation of H_2O_2 in the medium. This accumulation would be indicated by a decreased initial fluorescence and an altered time course for the change in fluorescence after the addition of the other, nonstimulatory, substance.
4. Release of H_2O_2 from various phacocytes was studied. Human leukocytes from blood and alveolar macrophages from Long–Evans hooded rats, New Zealand white rabbits, and guinea pigs were substituted for the alveolar macrophages from Sprague–Dawley rats.
5. Myeloperoxidase (12 mg/ml) or several other preparations of HRP (types VI, VII, VIII, and IX at 6.6 units/ml) were substituted for type II HRP. In experiments involving myeloperoxidase, the cell concentration of the assay mixture was increased to 1.33×10^6 alveolar macrophages/ml to compensate for the lower activity of this catalyst compared to that of type II HRP. The effect of the peroxidases on the fluorescence of scopoletin was also measured in the absence of cells. None of the peroxidases directly affected scopoletin fluorescence.

The intrinsic rate of H_2O_2 release from unstimulated rat alveolar macrophages

was checked by suspending 1.33×10^8 cells in 40 ml of Hepes-buffered medium and preincubating the suspension for 15 min at 37.5°C. After preincubation, 6.0 ml of the suspension was removed every 3 min and centrifuged in the Eppendorf centrifuge. An aliquot of supernatant was then added to scopoletin (2.4 μM) and type II HRP (6.6 units/ml) and fluorescence monitored. This technique enabled measurement of the H_2O_2 release from cells that were not exposed to HRP or scopoletin.

Chemiluminescence

Chemiluminescence was monitored as an additional indicator of respiratory burst activity. Alveolar macrophages (4×10^6 cells) were suspended in 5.0 ml of Hepes-buffered medium and preincubated for 15 min at 37.5°C in dark-adapted plastic vials. After preincubation, luminol was added to all vials to achieve a final concentration of 1×10^{-8} M and chemiluminescence was measured. The measurements were made as counts per minute (cpm) in the tritium channel of a Packard liquid scintillation counter (Model 3255; Packard Instrument Company, Inc., Downers Grove, IL) operated in the out-of-coincidence mode. At time zero, either type II HRP (3.96 units/ml) or an equal volume of vehicle was added to the vials. Chemiluminescence was measured in all vials at 0.5, 2.0, and 5.0 min. A vial with type II HRP, luminol, and buffer was also counted to ensure that type II HRP and luminol were not reacting to produce light. This procedure was conducted in dim, indirect incandescent lighting.

Materials

Scopoletin (S-2500), HRP (type II; P-8250; type VI, P8375; type VII, P-0889; type VIII, P-1014; type IX, P-1139), zymosan (Z-4250), catalase (C-10), and Hepes (H-3375) were obtained from Sigma Chemical Company, St. Louis, MO. Luminol (12, 307-2) was obtained from Aldrich Chemical Company, Milwaukee, WI. Sodium pentobarbital was supplied by Butler Company, Columbus, OH. Hydrogen peroxide was ordered from Mallinkrodt Chemical Work, St. Louis, MO. Myeloperoxidase was a gift from Denis English, Department of Pathology, University of Vanderbilt, Nashville, TN. The animals and their sources were as follows: Sprague–Dawley rats, Charles River, Boston, MA; Long–Evans hooded rats, Blue Spruce, Altamont, NY; New Zealand white rabbits, Dutchland, Denver, PA; and guinea pigs, Camm, Wayne, NJ.

RESULTS

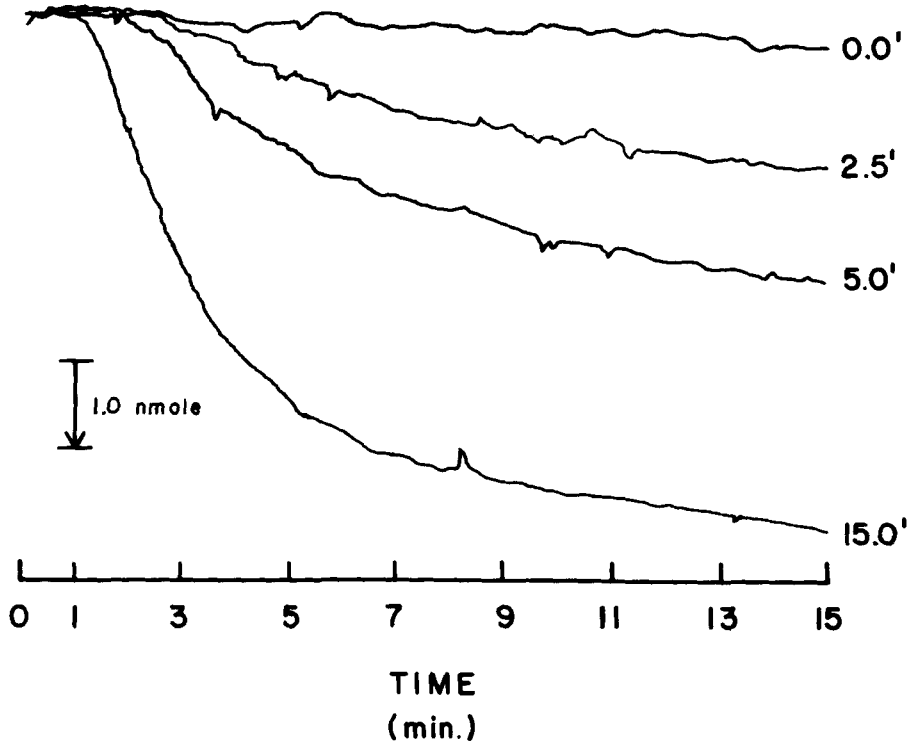
In this investigation we studied H_2O_2 release from rat alveolar macrophages in an effort to resolve conflicting results reported in the literature. Rat alveolar macrophages, previously maintained at 4°C, were preincubated at 37.5°C for 15 min to warm the cells and restore cellular metabolism to its normal rate. Type II HRP and scopoletin were then added to the suspension and changes in fluorescence measured over 10. If one assumes that the change in fluorescence is directly related to the H_2O_2 in solution, $2.73 (\pm 0.5) \times 10^{-7}$ nmoles of H_2O_2 is released per cell during a 10-min period ($n = 7$). This oxidation of scopoletin can be inhibited by the addition of catalase, indicating that the fluorescence change is caused by release of H_2O_2. The inhibition caused by catalase can be reversed by washing the cells and resuspending them in catalase-free medium. These data, which indicate a high rate of H_2O_2 release from resting alveolar macrophages, are similar to those reported by McNulty and Reasor [10] but are

much different from those reported by other investigators [5, 6, 8, 11, 12]. Therefore, we studied some of the factors involved in the H_2O_2 assay, such as length of preincubation and order of addition of type II HRP or scopoletin, to determine if they could influence the measurement of H_2O_2 release.

The effects of different preincubation times are shown in Fig. 1. Rat alveolar macrophages preincubated for 15 min at 37.5°C responded to addition of both type II HRP and scopoletin with biphasic release of H_2O_2 after a delay of 1.08 ($\pm$ 0.10) min (n= 8). During the first phase a maximum rate of H_2O_2 release is achieved (5.98 ($\pm$ 0.57) $\times$ 10^{-7} nmoles/min · cell, n = 8). During the second phase a low rate of H_2O_2 release is maintained (4.12 ($\pm$ 0.73) $\times$ 10^{-8} nmoles/min · cell, n= 6). As the length of the preincubation period is decreased, the rate of H_2O_2 release decreases and the division into separate phases becomes obscured. Cells that are not preincubated display a reduced, but constant, resting release of H_2O_2 when type II HRP and scopoletin are added to the suspension. These results indicate that the rate of H_2O_2 release that is measured depends on the time during which the measurement is made. Furthermore, the amount of resting H_2O_2 release that is measured is dependent on the length of the preincubation period.

To determine if H_2O_2 is released and accumulates in the medium during

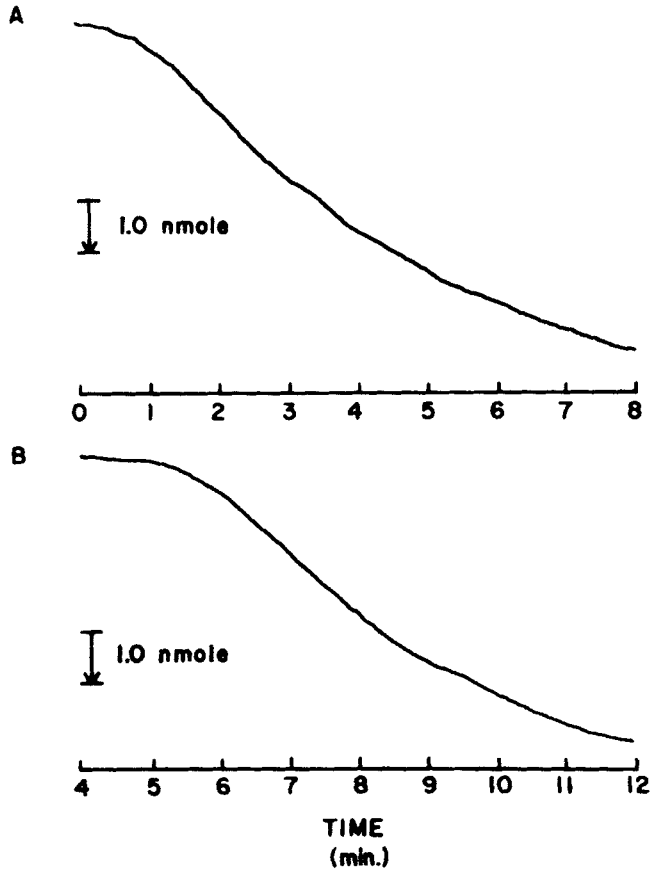
Figure 1 Effects of preincubation at 37.5°C on hydrogen peroxide release by rat alveolar macrophages. Samples containing 2 $\times$ 10^6 alveolar macrophages were removed from an ice bath and preincubated at 37.5°C for 0, 2.5, 5.0, and 15.0 min *(right)*. Type II horseradish peroxidase (6.6 units/ml) and scopoletin (2.4 μM) were added at time zero after preincubation and fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. Each tracing represents three different experiments done for each preincubation time.



preincubation of rat alveolar macrophages before the addition of type II HRP and scopoletin, aliquots of a preincubated cell suspension were drawn at various times and centrifuged. The supernatant was removed and mixed with type II HRP and scopoletin, and the H_2O_2 concentration was measured. No significant H_2O_2 release was detected over a 15-min period after preincubation. When the cell pellet is resuspended in Hepes-buffered medium containing type II HRP and scopoletin, a biphasic decrease in fluorescence, similar to that shown in Fig. 1, is recorded. Therefore, H_2O_2 does not accumulate in the medium during preincubation and before the addition of type II HRP and scopoletin to the suspension. These results suggest that type II HRP and/or scopoletin may be interacting with rat alveolar macrophages to trigger H_2O_2 release.

Both HRP and scopoletin are required to measure the accumulation of H_2O_2 in solution [2, 3]. If one of the substances triggers the biphasic release of H_2O_2 from rat alveolar macrophages, the recorded response would be expected to change according to when the other substance is added. When type II HRP is added at various times to a cell suspension containing scopoletin, the magnitudes and time courses of H_2O_2 release are similar (Fig. 2). These data indicate

Figure 2 Effects of adding type II horseradish peroxidase to a cell suspension containing scopoletin on hydrogen peroxide release by 2.0×10^6 rat alveolar macrophages. Scopoletin was added, after preincubation of cells at 37.5°C for 15 min, at time zero. Type II horseradish peroxidase was added at 0 min (**A**) and 4 min (**B**). Each tracing represents three different experiments.



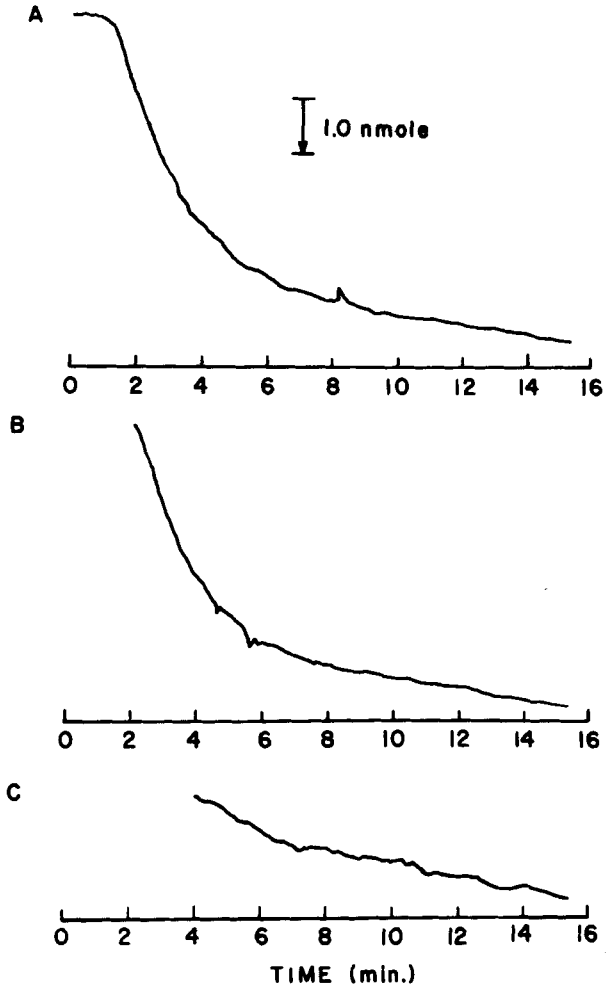


Figure 3 Effects of adding scopoletin to a cell suspension containing type II horseradish peroxidase on hydrogen peroxide release by 2 × 10^6 rat alveolar macrophages. Horseradish peroxidase was added, after preincubation of cells at 37.5°C for 15 min, at time zero. Scopoletin was added at 0 min (A), 2 min (B), and 4 min *(C)*. The hydrogen peroxide scale shown on the right in (A) is applicable to all three tracings. Each tracing represents four different experiments.

that scopoletin does not stimulate H_2O_2 release from rat alveolar macrophages. In contrast, both the magnitudes and the time course of H_2O_2 release change when scopoletin is added to a cell suspension containing type II HRP (Fig. 3). As the time between the addition of type II HRP and scopoletin is lengthened, the initial fluorescence level declines and less of the biphasic response is recorded. These results indicate that type II HRP stimulates rat alveolar macrophages to release H_2O_2, which accumulates in the medium until scopoletin is added.

Chemiluminescence is reported to be a measure of release of active oxygen species [15] and, therefore, was used to verify the ability of type II HRP to stimulate respiratory burst activity in rat alveolar macrophages. Addition of type II HRP to a preincubated cell suspension containing luminol was associated with a rapid increase in chemiluminescence (Fig. 4). Although it is unknown what

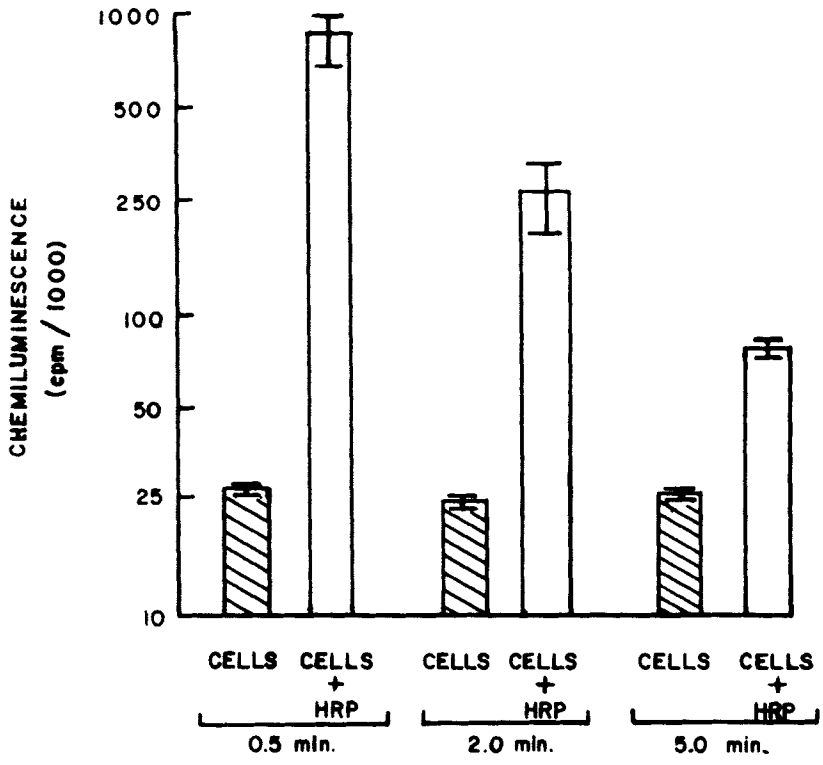


Figure 4 Luminal-catalyzed chemiluminescence of rat alveolar macrophages. Chemiluminescence of 4 × 10^6 cells in the absence *(hatched bars)* and presence *(open bars)* of type II horseradish peroxidase (HRP; 6.6 units/ml) is represented on a log scale. Type II HRP was added, after preincubation of cells at 37°C for 15 min, at time zero. Chemiluminescence was measured as counts per minute (cpm) for 30 sec at 0.5, 2.0, and 5.0 min. Values are expressed as mean ± standard error of mean for three trials.

factors are responsible for the phenomenon of chemiluminescence, these data do indicate that type II HRP stimulates rat alveolar macrophages.

Myeloperoxidase can catalyze the reaction between scopoletin and H_2O_2 and, therefore, was tested as a possible substitute for type II HRP. Stepwise addition of H_2O_2 to a solution containing myeloperoxidase (12 mg/ml), scopoletin (2.4 μM), and Hepes-buffered medium caused a rapid stepwise decrease in fluorescence, indicating that myeloperoxidase can be used as a catalyst in this assay. When myeloperoxidase and scopoletin are added to a suspension of rat alveolar macrophages, no resting H_2O_2 release is observed (Fig. 5). These results suggest that, unlike type II HRP, myeloperoxidase does not stimulate H_2O_2 release. Therefore, myeloperoxidase may be an acceptable substitute for HRP in this assay.

We compared the effectiveness of the catalysts, type II HRP and myeloperoxidase, in measuring cellular release of H_2O_2 after particulate stimulation by exposing rat alveolar macrophages to zymosan and measuring the resultant change in fluorescence. Zymosan does not cause an increase in H_2O_2 release from rat alveolar macrophages when type II HRP is used as a catalyst (Fig. 5*A*, Table 1). It should be noted that zymosan was added after the initial rapid increase in H_2O_2 release caused by type II HRP stimulation. When myeloperoxidase is substituted for type II HRP, an increase in the rate of H_2O_2 release is measured upon stimulation of rat alveolar macrophages with zymosan (Figs. 5*B* and 5*C*,

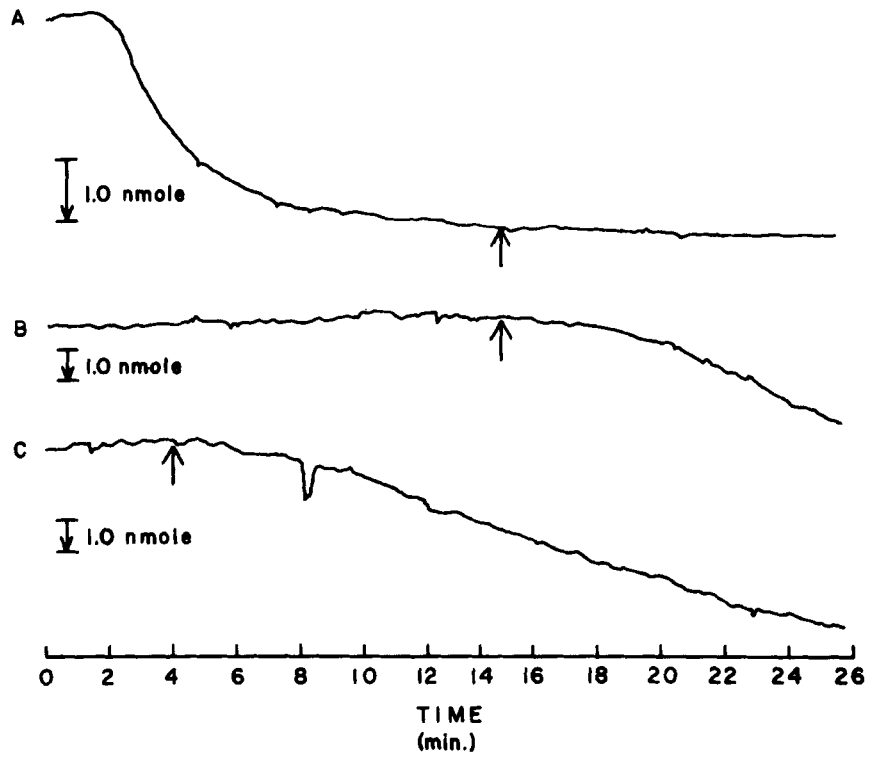


Figure 5 Effects of zymosan particles on hydrogen peroxide release by rat alveolar macrophages. Type II horseradish peroxidase (A) and myeloperoxidase (B and C) were used as catalysts at 6.6 units/ml and 12 mg/ml, respectively. Addition of zymosan (5 mg/ml) is indicated by the arrows. The fluorescence of scopoletin (2.4 μM) was measured at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. For tracing A, 2.0×10^6 alveolar macrophages were used; for tracings B and C, 4.0×10^6 alveolar macrophages. Each tracing represents at least seven different experiments.

Table 1). Zymosan stimulation causes a release of 6.14 (± 0.87) nmoles of H_2O_2/cell · 10 min. These results indicate that rat alveolar macrophages that have not been previously stimulated by type II HRP do release H_2O_2 upon stimulation with zymosan and that this release can be measured when myeloperoxidase is used as the catalyst. Previous stimulation with type II HRP eliminates the zymosan-induced response.

Four other HRP preparations (types VI, VII, VIII, and IX) were studied to determine if a factor unique to the type II HRP preparations was stimulating the cells. Rat alveolar macrophages in the presence of scopoletin and type VI, VIII, or IX HRP release H_2O_2 at the very slow rate of approximately 1×10^{-8} nmoles/min · cell (Table 1). A significant increase in H_2O_2 release occurs when zymosan is added. These results indicate that HRP types VI, VII, VIII, and IX do not stimulate H_2O_2 release from rat alveolar macrophages and that the ability to stimulate these cells may be unique to the type II preparation. Therefore, these peroxidase preparations are acceptable substitutes for type II HRP in this assay.

We also screened human leukocytes from blood and alveolar macrophages from Long–Evans hooded rats, New Zealand white rabbits, and guinea pigs to ensure that sensitivity to type II HRP is not unique to alveolar macrophages from Sprague–Dawley rats. At least two separate experiments were conducted

Table 1 Release of hydrogen peroxide by rat alveolar macrophages[a]

Catalyst	Release of H_2O_2 (10^{-7} nmoles/cell · min)	
	Catalyst induced	Zymosan induced
Horseradish peroxidase		
Type II	5.98 ± 0.57	Negligible
Type VI	0.08 ± 0.08	2.55 ± 0.49
Type VII	0.08 ± 0.02	3.02 ± 0.70
Type VIII	0.09 ± 0.07	6.11 ± 0.69
Type IX	0.07 ± 0.05	4.54 ± 0.98
Myeloperoxidase	Negligible	9.2 ± 1.07

[a]Alveolar macrophages were preincubated in Hepes-buffered medium for 15 min at 37.5°C before the addition of catalyst (either 6.6 units/ml horseradish peroxidase (HRP) or 12 mg/ml myeloperoxidase) and scopoletin (2.4 μM). Catalyst-induced H_2O_2 release was measured in the absence of zymosan. Zymosan was added (5 mg/ml) after stable H_2O_2 release was established following the addition of the catalyst. Values are expressed as mean ± standard error of mean. There were eight trials for type II HRP and myeloperoxidase and three trials for type VI, VII, VIII, and IX HRP.

with each cell type. Alveolar macrophages from Long–Evans hooded rats and guinea pigs release H_2O_2 at high rates (11.8 (± 5.1) × 10^{-7} and 39 (± 0.9) × 10^{-7} nmoles/min · cell, respectively) at rest after exposure to type II HRP. Resting alveolar macrophages from rabbits and leukocytes from humans release negligible amounts of H_2O_2 after exposure to type II HRP. These results indicate that sensitivity to type II HRP is not universal among phagocytes, as it is not found in leukocytes or rabbit alveolar macrophages. It is not unique to alveolar macrophages from Sprague–Dawley rats, however, as stimulation by type II HRP also occurs with alveolar macrophages from Long–Evans hooded rats and guinea pigs.

DISCUSSION

The data generated from these experiments suggest that the duration of the preincubation period and the catalyst employed to mediate the oxidation of scopoletin have significant effects on the direct measurement of H_2O_2 release from rat alveolar macrophages. Type II HRP stimulates these cells to release H_2O_2 and, therefore, is not a suitable catalyst for this assay. Using catalysts other than type II HRP, we have shown that rat alveolar macrophages release a small amount of H_2O_2 at rest but a large amount when exposed to zymosan. The results of these experiments may explain the inconsistencies recorded in the literature. McNulty and Reasor [10] reported a high rate of H_2O_2 release (2.8 × 10^{-6} nmoles/cell · 10 min) from resting alveolar macrophages of Long–Evans hooded rats. No significant change in H_2O_2 release occurred upon exposure to zymosan. Their procedure included 1) incubating the cells in the presence of type II HRP, scopoletin, and, when appropriate, unopsonized zymosan; 2) removing aliquots of the incubation mixture after 5, 10, and 15 min; 3) centrifuging the aliquots; and 4) measuring the H_2O_2 concentration in the supernatant. We have verified that type II HRP can stimulate H_2O_2 from alveolar macrophages from Long–Evans hooded rats. As their cells were incubated with type II HRP before measurement of scopoletin fluorescence, H_2O_2 release and scopoletin oxidation would have occurred during incubation and no true resting release of H_2O_2 would have been measured. Furthermore, zymosan would cause no significant increase in H_2O_2 release, because the cells would already have been stimulated by HRP (Fig. 3).

Holian and Daniele [8] reported that guinea pig alveolar macrophages release negligible amounts of H_2O_2 at rest. Upon stimulation with *N*-formylmethionylphenylalanine (FMP), the rate of H_2O_2 release was reported to have increased to 1.59×10^{-6} nmoles/cell · min. In their study, it appears as though the resting rate was determined during a 40-sec period after the addition of HRP and 2,7-dichlorofluorescein diacetate (a substitute for scopoletin). At this 40-sec point, FMP was added (Fig. 1 of [7]). Our data indicate a 1-min delay before H_2O_2 is released after addition of type II HRP to a suspension of rat alveolar macrophages. Holian and Daniele did not state the type of HRP used. If type II HRP was used, the low resting rate they reported may actually have been the rate during the delay period before the H_2O_2 release stimulated by type II HRP began. The stimulated rate, therefore, would have been a combination of both FMP and type II HRP stimulation. We have verified that type II HRP stimulates release of H_2O_2 from guinea pig alveolar macrophages.

Boxer et al. [5], Gee et al. [7], and Tsan [12], working with rabbit alveolar macrophages, reported low resting rates of H_2O_2 release regardless of the method employed. This finding is consistent with our observation that rabbit alveolar macrophages are not sensitive to type II HRP.

Biggar and Sturgess [11] reported low rates of H_2O_2 release by rat alveolar macrophages at rest (0.017×10^{-6} nmoles/cell · min) and after stimulation with unopsonized zymosan (0.02×10^{-6} nmoles/cell · min). The investigators do not state whether these cells had been preincubated. If the cells were maintained at 4°C and not preincubated at 37.5°C before measurement, the rate of cellular metabolism would be lower and low rates of H_2O_2 release would be expected (Fig. 1).

We report that type II HRP does not stimulate H_2O_2 release from human blood leukocytes and that H_2O_2 release is activated when human leukocytes are exposed to zymosan. These results agree with data reported by other investigators [2, 11].

We have found that alveolar macrophages are not stimulated by HRP types VI, VII, VIII, and IX (Table 1). Type II HRP contains at least five isoenzymes. Type VI HRP contains two and type IX only one of the basic isoenzymes. Types VII and VIII each contain one acidic isoenzyme. It is possible that the additional purity of these preparations may account for their inability to stimulate alveolar macrophages.

In conclusion, our data suggest that type II HRP stimulates H_2O_2 release from rat and guinea pig alveolar macrophages. The response to type II HRP is complex and, therefore, leads to inconsistent data if the assay procedures are varied. Myeloperoxidase and pure HRP preparations (types VI, VII, VIII, and IX) appear to be appropriate catalysts for the assay designed by Root et al. [2]. Myeloperoxidase is not commercially available and must be isolated from large quantities of granulocytes. The pure HRP preparations are available commercially. By using appropriate catalysts, we have determined that resting rat alveolar macrophages release very small amounts of H_2O_2 and that the cells can be induced to release large quantities of H_2O_2 by stimulating them with zymosan particles.

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