Quantitative and Temporal Characterization of the Extracellular $\text{H}_2\text{O}_2$ Pool Generated by Human Neutrophils*

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The extracellular $\text{H}_2\text{O}_2$ concentration surrounding stimulated human neutrophils was continuously quantitated with a sensitive, $\text{H}_2\text{O}_2$-detecting electrode. Following stimulation of neutrophils with phorbol myristate acetate, opsonized zymosan particles, or N-formyl-Met-Leu-Phe, the extracellular $\text{H}_2\text{O}_2$ concentration rapidly increased and maintained steady state conditions before falling to undetectable levels in a manner that was dependent on the triggering agent used. Total extracellular $\text{H}_2\text{O}_2$ accumulation for each stimulus was quantitated as the integral of the $\text{H}_2\text{O}_2$ concentration with respect to time. $\text{H}_2\text{O}_2$ accumulation in the extracellular milieu was unaffected by the addition of superoxide dismutase, whereas exogenous catalase or myeloperoxidase completely consumed the released $\text{H}_2\text{O}_2$. Analysis of $\text{H}_2\text{O}_2$ metabolism by neutrophils revealed that stimulus-dependent differences in the size of the extracellular $\text{H}_2\text{O}_2$ pool may be partially attributable to differences in hypochlorous acid generation by the $\text{H}_2\text{O}_2$, myeloperoxidase, chloride system. Finally, both the concentration of $\text{H}_2\text{O}_2$ in the extracellular space and its utilization by myeloperoxidase could be diminished in the presence of an extracellular target cell. These data indicate that the ability of a triggering agent to stimulate the neutrophil to generate $\text{H}_2\text{O}_2$ and release myeloperoxidase, coupled with the characteristics of a target cell population, control $\text{H}_2\text{O}_2$ metabolism in effector-target cell interactions.

Increased attention has focused on the ability of phagocytes to generate sufficient $\text{H}_2\text{O}_2$ to mediate extracellular cytotoxicity or modulate cell function in adjacent targets. Recent examples have shown that $\text{H}_2\text{O}_2$ can destroy normal or malignant cells and can alter erythrocyte, platelet, neutrophil, or lymphocyte function (1–15). $\text{H}_2\text{O}_2$ is an unusual oxidant because 1) it reacts slowly with organic substrates and thus can diffuse large distances in biological systems, 2) its small size and lack of charge facilitate its movement across plasma membranes, and 3) its intracellular concentration is controlled by several enzymes (16).

Following contact between a neutrophil and an opsonized particle, a plasma membrane-associated NADPH oxidase is activated, which generates $\text{O}_2^-$ via the univalent reduction of $\text{O}_2$. The $\text{O}_2^-$ then undergoes rapid spontaneous or enzyme-catalyzed dismutation to produce $\text{H}_2\text{O}_2$ (16). As the neutrophil’s plasma membrane begins to encircle the particle, $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ are released directly into the extracellular medium until the particle is completely engulfed within a phagocytic vacuole. Inside this environment, $\text{H}_2\text{O}_2$ may remain in the vacuole or diffuse either into the cytoplasm or outside the cell. Therefore, the $\text{H}_2\text{O}_2$ generated by an intact neutrophil should exist in an equilibrium among interconnected vacuolar, cytosolic, and extracellular “pools.” In turn, this $\text{H}_2\text{O}_2$ equilibrium will be regulated by the relative amounts of $\text{H}_2\text{O}_2$ generated or consumed in each of these individual sites (Fig. 1).

Within the phagocytic vacuole or in the extracellular space, $\text{H}_2\text{O}_2$ may be utilized by the secreted lysosomal enzyme myeloperoxidase or it may react directly with target molecules (16). In contrast, $\text{H}_2\text{O}_2$ that has diffused into the cytoplasm is primarily reduced by either catalase or glutathione peroxidase (16). Thus, there are multiple factors controlling the distribution and concentration of $\text{H}_2\text{O}_2$ in the intracellular and extracellular environment. Nonetheless, it is clear that the ability of a phagocyte to mediate $\text{H}_2\text{O}_2$-dependent damage against an extracellular target is not dictated by the total amount of $\text{H}_2\text{O}_2$ produced but rather the extracellular $\text{H}_2\text{O}_2$ concentration as a function of time.

At present, little is known about the size or characteristics of the extracellular pool of $\text{H}_2\text{O}_2$ surrounding stimulated neutrophils. In order to continuously monitor the extracellular $\text{H}_2\text{O}_2$ concentration, a detection system must be chosen that ideally does not alter $\text{H}_2\text{O}_2$ production or $\text{H}_2\text{O}_2$ catabolism, or perturb the $\text{H}_2\text{O}_2$ concentration in any of its three pools. To date, no studies have accurately monitored the extracellular pool as a function of time because current techniques either require the scavenging of all the released $\text{H}_2\text{O}_2$ (thus constantly perturbing the normal equilibrium between intra- and extracellular pools) or require the addition of agents that block normal catabolic pathways of $\text{H}_2\text{O}_2$ metabolism. Advances in polarographic techniques have led to the development of $\text{H}_2\text{O}_2$-sensing electrodes that rapidly quantitate $\text{H}_2\text{O}_2$ concentrations while consuming only miniscule amounts for analysis. Utilizing this approach, we have quantitated and examined both the characteristics of the free pool of $\text{H}_2\text{O}_2$ released by human neutrophils and its interaction with an extracellular target cell population.

EXPERIMENTAL PROCEDURES

Cell Preparations—Neutrophils were isolated from the peripheral venous blood of normal volunteers by Ficoll-Hypaque density centrifugation followed by dextran sedimentation as previously described (17). Cells were suspended in Dulbecco’s phosphate-buffered saline (pH 7.4; Grand Island Biological Co., Grand Island, NY) supplemented with 1 mg/ml of glucose.

Erythrocytes were also isolated from the peripheral venous blood of normal volunteers. In order to minimize hemolysis, all cell washes were performed in the presence of 100 µg/ml of human serum albumin.
Extracellular \( \text{H}_2\text{O}_2 \) Pool Generated by Human Neutrophils

**EXTRACELLULAR POOL**

Fig. 1. The distribution and fates of \( \text{H}_2\text{O}_2 \) generated by stimulated phagocytes. The location of and interaction between the different pools of \( \text{H}_2\text{O}_2 \) in the neutrophil and the catabolic pathways present in each pool are shown. Possible routes of catabolism include nonenzymatic (N.E.), myeloperoxidase (MPO)-mediated, catalase-mediated, or glutathione peroxidase (GPO)-mediated using reduced glutathione (GSH) as the hydrogen donor.

\( \text{H}_2\text{O}_2 \)-detecting System—\( \text{H}_2\text{O}_2 \) release was measured continuously with a YSI model 25 oxidase meter (Yellow Springs Instrument Co., Yellow Springs, OH). The electrode was covered with a single layer of polycarbonate membrane (0.015-\( \mu \)m pore size; Nuclepore Corp., Pleasanton, CA). The meter was attached to a Hewlett-Packard 7130A dual channel chart recorder. In order to allow simultaneous recording from 3 probe-meter assemblies, an ATC model 342 flip-flop timer (Automatic Timing and Controls Co., King of Prussia, PA) was interposed between two of the oxidase meters and one of the recorder inputs. Meters were calibrated before each experiment with dilutions of reagent \( \text{H}_2\text{O}_2 \) (30% \( \text{H}_2\text{O}_2 \), Mallinckrodt Inc., Paris, KY) based on an extinction coefficient of 230 nm at 81 \( \text{M}^{-1}\text{cm}^{-1} \) (17). The probe could detect a \( \text{H}_2\text{O}_2 \) concentration as low as 0.25 \( \mu \text{M} \) and the meter response was linear from 0-100 \( \mu \text{M} \) (r = 0.9999). The oxidase probe consumes \( \text{H}_2\text{O}_2 \) at a rate of 1.81 \( \times \) 10\(^{11}\) mol/h at a probe current of 1 namp (42). The electrode did not respond to either reagent HOCl or \( \text{NCl} \)-chlorotaurine. In some experiments, \( \text{H}_2\text{O}_2 \) was also measured spectrophotometrically by the method of Thurman et al. (18) as previously described (1).

**Neutrophil System**—Neutrophils (3 \( \times \) 10\(^{6}\)/ml, except as otherwise noted) were incubated in siliconized cuvettes (final volume of 4 ml) at 37 °C with constant stirring in a YSI model 5301 bath stirrer assembly connected to a Haake Model PE constant temperature water circulator (Haake Instruments, Inc., Rochelle Park, NJ). After a 5-min incubation period, neutrophils were stimulated to generate oxy-

gen metabolites with PMA\(^1\) (Consolidated Midland Corp., Forrester, NY), opsonized zymosan particles (ICN Nutritional Biochemical, Cleveland, OH) prepared as previously described (17) or FMLP, (Sigma). Stock solutions of PMA (1 mg/ml) and FMLP (3 \( \times \) 10\(^{-3}\) M) were prepared in Me\(\text{SO}_2 \) (Sigma) and stored at \(-20°C\) in some experiments, neutrophils were preincubated for 10 min with cytochalasin B (Aldrich Chemical Co.), which was prepared from stock solutions (10 mg/ml in Me\(\text{SO}_2 \)) stored at \(-20°C\).

Other additions to the system included bovine superoxide dismutase (3200 units/mg; Boehringer Mannheim Biochemicals, Indianapolis, IN), bovine catalase (88,000 units/mg; Worthington Biochemical Corp., Freehold, NJ), purified canine myeloperoxidase (gift of Dr. J. Schultz, Paninicolou Cancer Research Institute, Miami, FL), sodium azide (J. T. Baker Chemical Co., Phillipsburgh, NJ), and ferricytochrome c (horseheart cytochrome c, Type III; Sigma). Superoxide dismutase, catalase, and myeloperoxidase were assayed as previously described (17). All additions were incubated with reagent \( \text{H}_2\text{O}_2 \) in cell-free solutions to ascertain that they did not consume \( \text{H}_2\text{O}_2 \) in our assay system.

**N-Chlorotaurine Formation—**N-Chlorotaurine was quantitated spectrophotometrically by the iodide oxidation technique as previously described (17). All results are expressed as the mean ± 1 S.D.

**RESULTS**

\( \text{H}_2\text{O}_2 \) Release by Triggered Neutrophils—Neutrophils are known to generate \( \text{H}_2\text{O}_2 \) in response to a variety of soluble and particulate triggering agents (16). The most powerful soluble stimulus is the croton oil derivative PMA. This agent triggers the oxidative metabolism of neutrophils (19), stimulates lysosomal enzyme release (20), and initiates vacuole formation (21). When 3 \( \times \) 10\(^{4}\) neutrophils/ml (a total of 1.2 \( \times \) 10\(^{10}\) cells in a final volume of 4 ml) were stimulated with PMA (25 ng/ml), \( \text{H}_2\text{O}_2 \) was readily detected in the extracellular space within 40 s (Fig. 2). Continuous monitoring of the extracellular \( \text{H}_2\text{O}_2 \) pool revealed that its concentration peaked at 12.2 ± 1.9 \( \mu \text{M} \) after 26.8 ± 4.0 min (n = 36) and returned to undetectable levels (i.e. < 0.25 \( \mu \text{M} \)) after 97.3 ± 9.9 min. (n = 5). Under identical conditions, resting cells generated a peak \( \text{H}_2\text{O}_2 \) concentration not greater than 0.4 \( \mu \text{M} \) after ~48 min. If aliquots of the supernatant from stimulated cells were removed as the maximal levels were detected and then measured by an independent spectrophotometric assay, the \( \text{H}_2\text{O}_2 \) concentration varied by no more than 15% between the two techniques.

The effect of varying the neutrophil concentration on the extracellular \( \text{H}_2\text{O}_2 \) pool is shown in Fig. 3. Although the extracellular \( \text{H}_2\text{O}_2 \) concentration increased with cell number, the maximal levels of \( \text{H}_2\text{O}_2 \) did not double with a corresponding doubling of the neutrophil concentration. In addition, with increasing numbers of neutrophils, peak levels of \( \text{H}_2\text{O}_2 \) and their subsequent fall to undetectable levels occurred more rapidly (Fig. 3). Apparently, as neutrophil numbers are increased, there is a relatively greater increase in \( \text{H}_2\text{O}_2 \) catabolism than in its generation.

Although PMA-stimulated neutrophils release substantial amounts of \( \text{H}_2\text{O}_2 \) into the extracellular space, PMA is a nonphysiological trigger and may not accurately reflect normal \( \text{H}_2\text{O}_2 \) metabolism. In order to examine the neutrophils' response to stimuli more likely to be encountered in vivo, biologically relevant particulate and soluble triggers were next studied. Serum-treated zymosan particles are coated with the complement fragment C3b which is recognized by specific receptors on the neutrophil membrane (22). Compared to PMA-stimulated cells, the extracellular \( \text{H}_2\text{O}_2 \) concentration peaked more rapidly when cells were triggered with opsonized zymosan but the maximal levels were significantly lower (Fig. 3).

\(^1\)The abbreviations used are: PMA, phorbol myristate acetate; Me\(\text{SO}_2 \), dimethyl sulfoxide; FMLP, N-formyl-Met-Leu-Phe.
2). In 12 experiments, the peak extracellular H$_2$O$_2$ concentration was 3.6 ± 1.0 μM after 8.1 ± 1.7 min and returned to undetectable levels after 66.0 ± 7.9 min (n = 9).

N-Formyl oligopeptides resemble naturally occurring bacterial products and can stimulate neutrophil chemotaxis, lysosomal enzyme release, and oxygen metabolism (23). Although these peptides are considered weak stimuli for oxygen metabolite generation by neutrophils, extracellular H$_2$O$_2$ could readily be detected in our system. In 6 experiments, neutrophils (3 × 10$^6$/ml) triggered with FMLP (10$^{-7}$ M) generated a peak extracellular H$_2$O$_2$ concentration of 1.0 ± 0.3 μM after 2.7 ± 0.3 min which returned to undetectable levels after 24.8 ± 6.1 min (Fig. 4). Pretreatment of neutrophils with cytochalasin B can increase the amounts of O$_2$ released extracellularly by FMLP-triggered cells by inhibiting vacuole formation or closure (24). However, the extracellular H$_2$O$_2$ concentration actually decreased when cytochalasin B-treated neutrophils were studied (Fig. 4) despite a 2–3-fold increase in O$_2$ release (data not shown).

In order to directly compare the total amounts of H$_2$O$_2$ detected in the extracellular space with the three stimuli studied, the values can be expressed quantitatively as the integral of the H$_2$O$_2$ concentration (c) with respect to time (t) or simply c × t. For PMA-, zymosan-, and FMLP-stimulated cells, the c x t values differed markedly and were 614.9 ± 136.8 μM·min (n = 15), 68.2 ± 29.3 μM·min (n = 9), and 8.4 ± 1.8 μM·min (n = 7), respectively.

Effect of Exogenous Catalase, Myeloperoxidase, and Superoxide Dismutase on Extracellular H$_2$O$_2$—As expected, no H$_2$O$_2$ was detected when neutrophils were triggered in the presence of catalase (25 μg/ml; see Fig. 5). If catalase was added once the H$_2$O$_2$ concentration peaked, the H$_2$O$_2$ concentration rapidly fell to undetectable levels (Fig. 5). Identical results were obtained with zymosan-triggered cells and heat-inactivated

**Fig. 2.** The extracellular H$_2$O$_2$ concentration as a function of time surrounding triggered neutrophils. The H$_2$O$_2$ concentration was measured continuously using neutrophils (3 × 10$^6$/ml) stimulated with either serum-opsonized zymosan (1.25 mg/ml) or PMA (25 ng/ml) as described under "Experimental Procedures." H$_2$O$_2$ generation was measured simultaneously from paired cell preparations with or without sodium azide (1 mM). Time is in minutes from addition of stimulus. The curves depicted are from representative experiments using neutrophils from a single donor.

**Fig. 3.** The effect of neutrophil concentration on the extracellular H$_2$O$_2$ pool. The extracellular H$_2$O$_2$ concentration generated by increasing numbers of PMA-stimulated neutrophils was measured continuously in three preparations of cells incubated simultaneously. Numbers shown are the total number of neutrophils present in a 4-ml preparation.

**Fig. 4.** The extracellular H$_2$O$_2$ concentration generated by FMLP-stimulated neutrophils. The H$_2$O$_2$ concentration generated by FMLP-stimulated neutrophils (3 × 10$^6$/ml) was measured continuously in the presence or absence (—) of added azide (---) or cytochalasin-B (......). Azide (1 mM) was added 5 min prior and cytochalasin-B (5 μg/ml) 10 min prior to addition of FMLP. Time is in minutes from addition of stimulus. The curves depicted are from a representative experiment of six performed.

**Fig. 5.** The effect of exogenous catalase and myeloperoxidase on the extracellular H$_2$O$_2$ concentration. The H$_2$O$_2$ concentration generated by PMA-stimulated neutrophils (3 × 10$^6$/ml) was measured continuously in the presence or absence of added catalase or myeloperoxidase. Bovine catalase (25 μg/ml) was added either 5 min prior to addition of PMA (---) or when H$_2$O$_2$ reached peak concentration (——). Purified canine myeloperoxidase (MPO) (2 milliunits/ml) was added at peak H$_2$O$_2$ in a simultaneous assay (-----).
catalase had no effect on the H$_2$O$_2$ concentration with either stimulus (data not shown). Finally, H$_2$O$_2$ and Cl$^-$ can be utilized by myeloperoxidase to generate HOCl (16). If HOCl does not interfere with H$_2$O$_2$ detection by the electrode, then exogenous myeloperoxidase should deplete the extracellular H$_2$O$_2$ pool. Indeed, in the presence of purified myeloperoxidase (8 milliunits), the H$_2$O$_2$ concentration fell to base-line values (Fig. 5). These results underline both the specificity and the rapid response time of the H$_2$O$_2$-sensing electrode in this system.

As O$_2^-$ is generated by the neutrophil, it can dismutate to H$_2$O$_2$ or be consumed in other redox reactions (16). In an attempt to shuttle all the O$_2^-$ to H$_2$O$_2$, experiments were performed in the presence of exogenous superoxide dismutase (10 ng/ml). For PMA- or zymosan-stimulated neutrophils there was no change in the extracellular H$_2$O$_2$ concentration as a function of time (n = 3). Apparently, the pool of O$_2^-$ available to exogenous superoxide dismutase dissipates spontaneously to H$_2$O$_2$ under the conditions studied.

If the extracellular H$_2$O$_2$ detected in our system arises primarily from the dismutation of O$_2^-$ rather than the direct divalent reduction of O$_2$, we would predict that the O$_2^-$ scavenger cytochrome c would inhibit H$_2$O$_2$ production. Indeed, the extracellular H$_2$O$_2$ fell to <10% of control values when neutrophils were stimulated in the presence of 160 μM cytochrome c. However, the addition of superoxide dismutase in quantities sufficient to inhibit cytochrome c reduction (10 µg/ml) failed to regenerates more than 50% of the expected H$_2$O$_2$. This unexpected result was explained by the finding that ferricytochrome c rapidly consumed H$_2$O$_2$ in cell-free preparations. Thus, it is not possible to accurately interpret data with respect to polarographically detected H$_2$O$_2$ in the presence of cytochrome c.

**Effects of Azide on the Extracellular H$_2$O$_2$ Pool**—The size of the extracellular H$_2$O$_2$ pool at any point in time is regulated by the rate of H$_2$O$_2$ generation versus the rates of enzymatic and nonenzymatic consumption. H$_2$O$_2$ generated by the neutrophil can be enzymatically catabolized via the glutathione peroxidase system, catalase, or myeloperoxidase but the latter two enzymes are heme-containing and their activity can be inhibited by azide (16). If neutrophils were stimulated in the presence of 1 mM azide, the amounts of H$_2$O$_2$ detected extracellularly were dramatically increased (see Fig. 2). The peak extracellular H$_2$O$_2$ concentration with PMA-stimulated cells was increased 5-fold to 61.9 ± 12.3 μM after 79.5 ± 3.9 min (n = 13) and, for zymosan-triggered neutrophils, it increased 12.3-fold to 44.3 ± 9.1 μM after 50.7 ± 5.0 min (n = 3). After the extracellular H$_2$O$_2$ concentration peaked in the presence of azide, the H$_2$O$_2$ slowly disappeared with either population of stimulated cells (Fig. 2). Approximately 75% of the H$_2$O$_2$ could still be detected after a 4-h incubation. In contrast, the addition of azide to FMLP-stimulated neutrophils resulted in an insignificant increase in the H$_2$O$_2$ concentration (Fig. 4). As with PMA andzymosan, there was a slow decay in extracellular H$_2$O$_2$ after the maximal concentration was reached.

**HOCl Generation by Stimulated Neutrophils**—The large increases in the extracellular H$_2$O$_2$ concentration observed in the presence of azide suggest that large amounts of the generated H$_2$O$_2$ are consumed by either catalase or myeloperoxidase. Although the amounts of H$_2$O$_2$ reduced by catalase are difficult to assess, we have recently described a technique to measure H$_2$O$_2$ utilization by myeloperoxidase (17). In the presence of H$_2$O$_2$, myeloperoxidase can oxidize Cl$^-$, Br$^-$, or I$^-$ to their respective hypohalous acids (16). Based on their in vivo concentrations, Cl$^-$ is the most likely halide oxidized and model H$_2$O$_2$-myeloperoxidase-Cl$^-$ systems have been demonstrated to generate free HOCl (25).

### Table I

| Stimulus | Maximum level of extracellular H$_2$O$_2$ | N-Chlorotaurine formed |
|----------|------------------------------------------|------------------------|
|          | −Taurine + Taurine                       | nM                    |
| PMA (n = 5) | 47.5 ± 3.8                             | 36.3 ± 5.1            |
| Zymosan (n = 4) | 16.2 ± 4.2                           | 14.6 ± 3.7            |
| FMLP (n = 4) | 5.9 ± 1.7                          | 5.1 ± 1.5             |

|          | nM | mmol |
|----------|----|------|
| PMA      | 50.8 ± 17.6 |
| Zymosan  | 101.4 ± 20.6 |
| FMLP     | 10.9 ± 2.5  |

Because HOCl is the rate-limiting step in the chlorination reaction (25), exogenous taurine should not markedly alter the size of the extracellular H$_2$O$_2$ pool. Indeed, in the presence of 10 mM taurine, there were only small decreases in the peak H$_2$O$_2$ concentration detected with PMA-, zymosan-, or FMLP-stimulated cells (Table I). If the amounts of N-chlorotaurine generated in these samples were simultaneously quantitated, zymosan-triggered neutrophils generated 2 times more N-chlorotaurine than the PMA-stimulated cells (Table I). Thus, neutrophils triggered with zymosan appear to generate a lower extracellular H$_2$O$_2$ concentration but shuttle larger amounts of H$_2$O$_2$ through the myeloperoxidase system than those treated with PMA.

FMLP-stimulated cells could also chlorinate taurine, thus demonstrating that sufficient amounts of myeloperoxidase were released to catalyze HOCl formation (Table I). We had noted earlier that the extracellular H$_2$O$_2$ pool was smaller and disappeared more rapidly when FMLP-stimulated cells were pretreated with cytochalasin B (see Fig. 4). Because cytochalasin-treated cells, the extracellular H$_2$O$_2$ concentration fell to base-line levels within 5 min (see Fig. 4) by which time 15.8 ± 5.4 nmol (n = 4) of N-chlorotaurine were formed, while control FMLP-stimulated cells had generated only 2.7 ± 0.9 nmol (n = 4). Apparently, differences in the size of the extracellular H$_2$O$_2$ pool as a function of time are at least partially attributable to differences in H$_2$O$_2$ utilization by myeloperoxidase.

**Effect of Erythrocyte Targets on the Extracellular H$_2$O$_2$ Pool**—If neutrophils utilize released H$_2$O$_2$ to attack adjacent cells, then the addition of a target cell population should alter the size of the extracellular H$_2$O$_2$ pool. As shown in Fig. 6,
the extracellular H$_2$O$_2$ concentration was lowered by the erythrocyte target at an effector-to-target cell ratio of 1:10. In four experiments, the peak H$_2$O$_2$ concentration fell from 12.3 ± 2.8 μM at 25.8 ± 2.1 min in the absence of erythrocytes to 2.9 ± 0.7 μM at 15.2 ± 2.3 min in the presence of the targets. In similar experiments with zymosan-stimulated cells, the peak H$_2$O$_2$ concentration fell less markedly from 3.0 ± 0.6 μM at 9.5 ± 2.7 min to 2.1 ± 0.5 μM at 6.1 ± 1.7 min in the presence of erythrocytes (n = 3). Although the extracellular H$_2$O$_2$ concentration might be lowered if O$_2$ crossed the erythrocyte membrane before it dismutated in the extracellular space, the addition of superoxide dismutase did not augment the recovery of H$_2$O$_2$. Finally, H$_2$O$_2$ consumed by small amounts of catalase released from spontaneously hemolyzed erythrocytes (range of lysis 0.8–1.4%; n = 3) accounted for no more than a 10% decrease in the peak H$_2$O$_2$ concentration. Thus, it appears that the extracellular H$_2$O$_2$ concentration is depleted in the presence of an extracellular target.

When neutrophils are triggered to generate oxygen metabolites in the presence of an extracellular target, sufficient quantities of H$_2$O$_2$ may be utilized by myeloperoxidase to mediate cytotoxicity via HOCI (26). If H$_2$O$_2$ that is normally utilized by myeloperoxidase instead interacts directly with the target cell, then the amounts of H$_2$O$_2$ available to myeloperoxidase would be decreased. In order to test the ability of the erythrocytes to "steal" H$_2$O$_2$ from myeloperoxidase, neutrophils (3 × 10$^6$/ml) were triggered with PMA or zymosan in the presence of tauroine (10 mM) with or without erythrocyte targets (3 × 10$^6$/ml) and N-chlorotaurine formation quantitated. Indeed, the quantity of N-chlorotaurine was reduced 61.9 ± 7.2% (n = 4) and 22.5 ± 14.7% (n = 3) for PMA- and zymosan-stimulated cells, respectively (Table II). These reductions were not due to a direct reaction of the generated HOCI with the erythrocyte (rather than the exogenous taurine) because >95% of a bolus of HOCI (100 nmol) added to a mixture of erythrocytes and tauroine could be recovered as N-chlorotaurine. Likewise, >95% of synthesized N-chlorotaurine (100 nmol) could be recovered following a 30-min incubation with 3 × 10$^6$/ml of erythrocytes. It would appear that the erythrocyte targets are capable of reducing both the extracellular H$_2$O$_2$ pool and its subsequent utilization by the neutrophil.

**TABLE II**

| Stimulus          | Maximum level of extracellular H$_2$O$_2$ | N-Chlorotaurine formed |
|-------------------|------------------------------------------|------------------------|
|                   | − Erythrocytes + Erythrocytes            | − Erythrocytes + Erythrocytes |
| PMA (n = 4)       | 49.3 ± 11.2 11.7 ± 2.7 57.2 ± 9.4        | 22.0 ± 6.8             |
| Zymosan (n = 3)   | 12.0 ± 2.4 8.4 ± 2.0 99.7 ± 28.0         | 69.1 ± 30.1            |

**DISCUSSION**

Recent studies have clearly demonstrated that phagocytes can generate sufficient quantities of H$_2$O$_2$ to destroy cultured endothelial cells (1, 2), fibroblasts (2, 3), or tumor cells (4–6). In addition, H$_2$O$_2$ can mediate a variety of nonlytic effects by altering the function of erythrocytes (7–9), platelets (10), neutrophils (11), T and B lymphocytes (12–14), and natural killer cells (15). Although the biochemical processes underlying these H$_2$O$_2$-dependent effects are unclear, several reports have demonstrated that the rate of H$_2$O$_2$ generation and its concentration as a function of time play a key role in determining target cell damage or destruction (5, 27–29). Triggered neutrophils can generate H$_2$O$_2$, but the extracellular concentration is controlled by both the rates of H$_2$O$_2$ release and H$_2$O$_2$ catabolism at any point in time. Thus, in order to quantitatively measure the extracellular H$_2$O$_2$ concentration surrounding the neutrophil, neither of these parameters can be altered. The polarographic technique used in this report allowed us to continuously monitor the extracellular H$_2$O$_2$ pool while minimally perturbing H$_2$O$_2$ metabolism or distribution. For example, 3 × 10$^6$ neutrophils/ml stimulated with PMA generated a peak extracellular H$_2$O$_2$ concentration of ~12 μM for 2 min. During this time, the electrode would have consumed only 25 × 10$^{-11}$ mol of H$_2$O$_2$ for analysis. Thus, the electrode may be envisioned as an innocent bystander that continuously seases the surrounding pool of H$_2$O$_2$ without altering its size. In contrast, all the other studies that have examined H$_2$O$_2$ release by phagocytes have been limited to cumulative measurements of H$_2$O$_2$ generation (18, 30–38). In the most commonly used technique, phagocytes are stimulated in the presence of exogenous horseradish peroxidase and a hydrogen donor (e.g. scopoletin (35), phenol red (38), diacetyl dichlorofluorescein (32, 34)) whose H$_2$O$_2$-dependent oxidation can be followed spectrophotometrically or fluorometrically. Optimal quantities of the peroxidase and its substrate are added in an attempt to consume all the H$_2$O$_2$ in the extracellular space. Thus, by design, this approach circumvents normal routes of H$_2$O$_2$ catabolism and tends to maximize the amounts of H$_2$O$_2$ released by continuously perturbing the normal H$_2$O$_2$ equilibrium between intracellular and extracellular sites. Although this technique is useful in quantitating the maximal amounts of H$_2$O$_2$ that a phagocyte might release, it does not allow one to assess the instantaneous extracellular H$_2$O$_2$ concentration.

In our system, H$_2$O$_2$ could be detected in the extracellular space within 40 s for all three stimuli. Because the polarographic analyses did not depend on the presence of an oxygen metabolite scavenger or trap (e.g. cytochrome c for O$_2^·$ or peroxidase for H$_2$O$_2$) and consume only trivial amounts for analysis, it appears that H$_2$O$_2$ is released freely into the extracellular milieu under physiologic conditions. The peak extracellular H$_2$O$_2$ concentrations reached by PMA-, zymosan-, and FMLP-triggered cells varied by a factor of 12 and occurred within 26 min, 3 min, and 3 min, respectively. Once attained, the maximal extracellular concentrations remained

**FIG. 6.** The effect of erythrocyte targets on the extracellular H$_2$O$_2$ pool. The H$_2$O$_2$ concentration was measured continuously using PMA-stimulated neutrophils (PMN's, 3 × 10$^6$/ml) in the presence or absence of autologous erythrocytes (RBC's, 3 × 10$^6$/ml). The curves depicted are from a representative experiment of four performed.
constant for various lengths of time (i.e., steady state conditions existed) before the rate of $H_2O_2$ catabolism began to exceed the rate of release and the extracellular $H_2O_2$ concentration fell to undetectable levels. These results indicate that neutrophils would bathe adjacent targets in a constantly changing $H_2O_2$ pool whose concentration and lifetime is regulated by the particular triggering agent used.

PMA and zymosan are both powerful stimuli of oxygen metabolite generation and optimally activate NADPH oxidase activity in human neutrophils (39). Thus, we were surprised to find that PMA-stimulated cells generated peak extracellular $H_2O_2$ concentrations and $c \times t$ values 3.4x and 9x greater, respectively, than those obtained with zymosan-treated cells. However, in the presence of azide, both PMA- and zymosan-triggered neutrophils released $H_2O_2$ extracellularly at comparable rates and the peak $H_2O_2$ concentration detected with zymosan-stimulated cells was 72% of that observed with PMA. Azide can increase the extracellular concentration of $H_2O_2$ by inhibiting catalase and myeloperoxidase and it is tempting to estimate rates of $H_2O_2$ generation and catabolism uncovered the potential of both zymosan- and PMA-stimulated cells to release large amounts of $H_2O_2$ into the extracellular milieu. It is not surprising that heme-enzyme inhibitors have been reported to enhance phagocyte-mediated, $H_2O_2$-dependent effects (1, 5, 41) as the $H_2O_2$ concentrations attained are clearly much higher and maintained longer than those reached under physiologic conditions.

Neutrophils can use $H_2O_2$ directly to mediate cytotoxicity or alter cell function but it is clear that $H_2O_2$ can also be utilized by myeloperoxidase and Cl$^-\$ to generate HOCl (16). Myeloperoxidase, unlike other peroxidases, generates free HOCl and its rate of turnover and $H_2O_2$ utilization should not be altered by the presence of a chlorinateable substrate (25).6 Indeed, the addition of high concentrations of taurine did not markedly alter the characteristics of the extracellular $H_2O_2$ pool in terms of extracellular cytotoxicity, the ratio of $H_2O_2$ to HOCl released will be regulated by the ability of myeloperoxidase to compete with all other routes of $H_2O_2$ utilization. Oposinized zymosan particles are a more potent stimulus for myeloperoxidase release than PMA (16) and we speculated that the low extracellular $H_2O_2$ concentrations and $c \times t$ values obtained with zymosan could be partially related to increased $H_2O_2$ utilization by myeloperoxidase. Indeed, zymosan-triggered cells used at least 2 times more $H_2O_2$ for HOCl generation than the PMA-treated neutrophils. Similar relationships between $H_2O_2$ release and HOCl generation were found when values for FMLP-triggered cells were compared to those for cells preincubated with cytochalasin B before FMLP addition. Thus, there appears to be an inverse relationship between the extracellular $H_2O_2$ concentration and the quantities of HOCl detected. Apparently, stimulated neutrophils can simultaneously release $H_2O_2$ and HOCl in ratios determined by the triggering agent used.

The ability to monitor the extracellular $H_2O_2$ concentration without altering catabolism afforded us the opportunity to examine changes in the $H_2O_2$ pool in a model of a neutrophil-target cell interaction. Continuous monitoring of the extracellular $H_2O_2$ revealed that erythrocytes decreased the peak $H_2O_2$ concentration generated by PMA- or zymosan-stimulated cells by 76 and 30%, respectively. The erythrocytes did not mediate this effect by nonspecifically stimulating the neutrophils' catabolism of $H_2O_2$ because the released $H_2O_2$ can attack the erythrocyte and oxidize intracellular hemoglobin (7-9). Although the processes involved in $H_2O_2$ consumption by the erythrocyte have not been identified, in this report, we have found that increased amounts of $H_2O_2$ are recovered when either catalase activity or the GSH system are inhibited in the erythrocyte targets.4 In addition to a reduction in the extracellular $H_2O_2$ pool, the presence of erythrocytes decreased $H_2O_2$ utilization by myeloperoxidase as reflected in a 61 and 30% inhibition of N-chlorotaurine formation with PMA- and zymosan-triggered cells, respectively. The ability of the erythrocyte to "steal" more $H_2O_2$ from PMA-stimulated cells may be related to the fact that PMA is a weak stimulus for myeloperoxidase release (16) and the erythrocyte can more effectively compete for available $H_2O_2$ when only small amounts of the peroxidase are secreted. Depending on the target cells' ability to catabolize $H_2O_2$, our results suggest that it may be directly damaged by released $H_2O_2$ or the target may protect itself by consuming the released $H_2O_2$ before it is utilized by the myeloperoxidase system to generate a more toxic oxidant. Thus, $H_2O_2$ catabolism by certain target cells may represent an unusual defense mechanism aimed at reducing the extracellular pool of HOCl by stimulated neutrophils.

In conclusion, we have provided the first quantitative and temporal analysis of the extracellular $H_2O_2$ pool generated by intact neutrophils. Our results indicate that the triggering agents' ability to stimulate the neutrophil to generate $H_2O_2$ and release myeloperoxidase, coupled with the characteristics of the target cell, all control $H_2O_2$ metabolism in an effector-target cell interaction. These analyses allow us to directly study the extracellular pool of $H_2O_2$ that is utilized by phagocytes in host defense and the inflammatory response.

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4 Chlorine acceptors could increase $H_2O_2$ utilization indirectly by scavenging HOCl before it inactivates myeloperoxidase.

S. T. Test and S. J. Weiss, unpublished observation.
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