Superoxide-dependent Oxidation of Extracellular Reducing Agents by Isolated Neutrophils

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Incubation of stimulated neutrophils with sulfhydryl (RSH) compounds or ascorbic acid (ascorbate) results in rapid superoxide (O$_2^-$)-dependent oxidation of these reducing agents. Oxidation of RSH compounds to disulfides (RSSR) is faster than the rate of O$_2^-$ production by the neutrophil NADPH-oxidase, whereas about one ascorbate is oxidized per O$_2^-$ Ascorbate is oxidized to dehydroascorbate, which is also oxidized but at a slower rate. Oxidation is accompanied by a large increase in oxygen (O$_2$) uptake that is blocked by superoxide dismutase. Lactoferrin does not inhibit, indicating that ferri (Fe$^{3+}$) ions are not required, and Fe$^{3+}$-lactoferrin does not catalyze RSH or ascorbate oxidation. Two mechanisms contribute to oxidation: 1) O$_2^-$ oxidizes ascorbate or reduced glutathione and is reduced to hydrogen peroxide (H$_2$O$_2$), which also oxidizes the reductants. O$_2$ reacts directly with ascorbate, but reduced glutathione oxidation is mediated by the reaction of O$_2^-$ with manganese (Mn$^{2+}$). The H$_2$O$_2$-dependent portion of oxidation is mediated by myeloperoxidase-catalyzed oxidation of chloride to hypochlorous acid (HOCl) and oxidation of the reductants by HOCl. 2) O$_2^-$ initiates Mn$^{2+}$-dependent auto-oxidation reactions in which RSH compounds are oxidized and O$_2$ is reduced. Part of this oxidation is due to the RSH-oxidase activity of myeloperoxidase. This activity is blocked by superoxide dismutase but does not require O$_2^-$ production by the NADPH-oxidase, indicating that myeloperoxidase produces O$_2^-$ when incubated with RSH compounds.

It is proposed that an important role for O$_2^-$ in the cytotoxic activities of phagocytic leukocytes is to participate in oxidation of reducing agents in phagolysosomes and the extracellular medium. Elimination of these protective agents allows H$_2$O$_2$ and products of peroxidase/H$_2$O$_2$/halide systems to exert cytotoxic effects.

Stimulation of phagocytic leukocytes results in activation of an NADPH-oxidase enzyme in the plasma membrane (1-5). The enzyme is oriented so as to accept electrons from NADPH in the cytosol, transfer one electron at a time to oxygen (O$_2$), and release superoxide free radicals (O$_2^-$) into phagolysosomes or the extracellular medium. The oxidase is specific for O$_2^-$ and does not catalyze the two-electron reduction of O$_2$ to hydrogen peroxide (H$_2$O$_2$), so that the enzyme is committed to producing O$_2^-$.

Nevertheless, the role of O$_2^-$ in leukocyte antimicrobial activity is unclear. O$_2^-$ is short-lived, not highly reactive with most biological materials, and does not readily penetrate bacterial membranes (6-8). Moreover, most microorganisms contain superoxide dismutase enzymes that would prevent the reaction of O$_2^-$ with intracellular components (9, 10). Therefore, O$_2^-$ has little or no antimicrobial activity when microorganisms are incubated with O$_2^-$-generating systems.

Two mechanisms have been described whereby O$_2^-$ contributes to antimicrobial and other cytotoxic activities. First, O$_2^-$ undergoes a rapid spontaneous dismutation reaction that yields H$_2$O$_2$. The H$_2$O$_2$ may react directly with target cells or participate in reactions catalyzed by the hemoprotein peroxidase enzymes of leukocytes. Peroxidase/H$_2$O$_2$/halide systems amplify the toxicity of H$_2$O$_2$ by producing oxidizing and halogenating agents that are more reactive than H$_2$O$_2$ (11-17). In this mechanism, the only role for O$_2^-$ is as an intermediate in H$_2$O$_2$ production.

In the second mechanism, some O$_2^-$ ions dismutate to yield H$_2$O$_2$ and others act as reducing agents for metal ions. For example, O$_2^-$ reduces ferric ion (Fe$^{3+}$) to ferrous ion (Fe$^{2+}$), which reacts with H$_2$O$_2$ to yield iron-oxygen complexes (6). These complexes may react directly with target cells, release the highly reactive hydroxyl radical (HO), or mimic the activity of peroxidases by oxidizing the halide ion iodide (18-21). In this mechanism, O$_2^-$ is an intermediate in both H$_2$O$_2$ production and the formation of a catalyst for H$_2$O$_2$-dependent toxicity.

The hypothesis of this study is that O$_2^-$ has an additional role, which is to participate in oxidation of substances in the extracellular phase, consisting of the intraphagolysosomal space and the extracellular medium. When a leukocyte encounters a microorganism in vivo, both cells are bathed in reducing agents such as ascorbate and sulfhydryl (RSH) compounds. Plasma ascorbate is 50 μM in well-nourished individuals (22), and plasma contains 0.6 mM RSH groups, primarily reduced cysteine residues of plasma proteins (23). Reductants may also be found in high amounts at sites of inflammation and infection. For example, damaged cells release intracellular reductants such as ascorbate, GSH, and NAD(P)H, and certain microorganisms create a reducing environment by reducing disulfide (RSSR) compounds and excreting the products (24).

RSH compounds and ascorbate are scavengers for the antimicrobial oxidizing agents produced by leukocytes. These reductants detoxify products of peroxidase/H$_2$O$_2$/halide systems, although they react less rapidly with H$_2$O$_2$. The ability

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1The abbreviations and trivial names used are: GSH, reduced glutathione; cyt, c. cytochrome c; GSSG, glutathione disulfide; Nbs, 5-thio-2-nitrobenzoic acid or TNB; Nbs, 5,5'-dithiobis(2-nitrobenzoic acid) or DTNB; PMA, phorbol 12-myristate 13-acetate.
of leukocytes to use H$_2$O$_2$ and oxidized halides as antimicrobial agents would be facilitated if leukocytes had a mechanism for eliminating protective reductants from the extracellular phase. Without such a mechanism, leukocytes would have to produce enough H$_2$O, or oxidized halides to oxidize both the reductants and the microbial cell components.

O$_2^-$ could participate in two types of reactions that would provide efficient mechanisms for eliminating extracellular reductants. First, free-radicals such as O$_2$ react with readily oxidized substances (RH$_2$) to produce other radicals (RH), and such reactions are self-perpetuating if they produce O$_2$.

$$e^- + O_2 ightarrow O_2^-$
$$H^+ + O_2^- + RH_2 ightarrow H_2O_2 + RH$$
$$RH + O_2^- ightarrow R^- + H^+ + O_2^-$$

net: $e^- + (n + 1)O_2^- + nRH_2 ightarrow nH_2O_2 + nR^- + O_2^-$

The equations show that O$_2^-$ has a catalytic role. O$_2^-$ initiates a series of reactions that repeats n times, but O$_2^-$ is not consumed. O$_2$ rather than O$_2^-$ is the electron-acceptor, so that this is an "auto-oxidation" reaction. The equations also show that production of one O$_2^-$ ion could result in a large amount of O$_2$ uptake, H$_2$O$_2$ production, and RH oxidation. In principle, one O$_2^-$ could initiate oxidation of an unlimited amount of RH$_2$.

In the second mechanism, O$_2^-$ rather than O$_2$ is the electron-acceptor and no free-radical chain reaction occurs. O$_2^-$ oxidizes RH$_2$ to R, and O$_2^-$ is reduced to H$_2$O$_2$, which may also oxidize RH$_2$ to R.

$$2O_2^- + 2H^+ + RH_2 ightarrow 2H_2O_2 + R$$
$$H_2O_2 + RH_2 ightarrow 2H_2O + R$$

net: $2O_2^- + 2H^+ + 3RH_2 ightarrow 4H_2O + 3R$

These equations show that when O$_2^-$ acts as an oxidant (electron-acceptor), up to three RH$_2$ are oxidized per two O$_2^-$.

Aims of this study were to determine whether O$_2^-$ participates in oxidation of extracellular reductants by stimulated neutrophils and to determine whether O$_2^-$ initiates auto-oxidation or acts as an oxidant. As described below and in another study on ascorbate oxidation (25), O$_2^-$ contributes to oxidation. O$_2^-$-dependent auto-oxidation is observed with certain RSH compounds, requires Mn$^{++}$, and is due in part to the RSH-oxidase activity of the neutrophil enzyme myeloperoxidase. On the other hand, O$_2^-$ acts as an oxidant for GSH and ascorbate. Mn$^{++}$ is required for oxidation of GSH but not ascorbate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Acetaldehyde was from Eastman Kodak; L-ascorbate from Gallard-Schlesinger, dehydroascorbate and dimethyl sulfoxide (spectrophotometric grade) from Aldrich, PMA from Pharmacia LKB Biotecnologies Inc., and NaCl ("ultra") from Atomergic Chemetals Corp. Bovine serum albumin, chelators, cyt c (Type V) from E. L. Thomas, manuscript in preparation. Potassium superoxide (KO$_2$, Pfaltz and Bauer) solutions (27) were prepared by adding 0.5 g of KO$_2$ to 4 ml of dimethyl sulfoxide and incubating 1 h at 37 °C in a glass-stoppered tube. Catalase crystals (Boehringer Mannheim) were washed in water and dissolved in 0.14 M NaCl with 15 mM potassium phosphate, pH 7.4 (Cl$^-$-medium) or in 67 mM Na$_2$SO$_4$, with 32 mM potassium phosphate, pH 7.4 (Cl$^-$-free medium). PMA (5 mg) was dissolved in 0.1 ml of dimethyl sulfoxide and diluted 100-fold into 48 ml of bovine serum albumin in water. This solution was diluted 100-
Oxidation of extracellular reductants

The complete system consisted of neutrophils (2 x 10^6/ml) with PMA or 5 mg/ml opsonized zymosan, incubated 1 h with 1 mM reductant: CYS, L-cysteine; DTT, dithiothreitol (1 mM RSH); PEN, D-penicillamine; 2ME, 2-mercaptoethanol, ASC, ascorbate. When added, catalase was 5 µg/ml, and superoxide dismutase was 15 µg/ml.

| Complete (– Cl⁻) | CYS | DTT | PEN | Nbs | 2ME | GSH | ASC |
|------------------|-----|-----|-----|-----|-----|-----|-----|
| + Catalase       | 590 | 620 | 520 | 440 | 380 | 260 | 290 |
| + Super oxide dismutase | 530 | 610 | 370 | 330 | 360 | 250 | 220 |
| − PMA            | 50  | 80  | 100 | 50  | 60  | 30  |     |
| − Cells          | 40  | 20  | 30  | 20  | 50  | 60  | 20  |

The complete system consisted of neutrophils (2 x 10^6/ml) and PMA incubated 1 h with 1 mM reductant. When added, catalase was 5 µg/ml, myeloperoxidase (MPO) was 0.1 µM, superoxide dismutase was 15 µg/ml, lactoferrin (LF) or Fe³⁺-lactoferrin (Fe-LF) was 10 µM, EDTA or diethylenetriaminepentaacetate (DETAPAC) was 0.1 mM, and Ca²⁺ was 1 mM.

| Complete (+ Cl⁻) | Nbs | GSH | Ascorbate |
|------------------|-----|-----|-----------|
| + Catalase       | 590 | 650 | 360 430   |
| + Super oxide dismutase | 470 | 400 | 430 370   |
| − PMA            | 20  | 290 | 170 300   |
| − Cells          | 30  | 30  | 30 40     |

Role of H₂O₂—Oxidation in Cl⁻-free medium was stimulated by adding azide to inhibit endogenous catalase (Table II). Oxidation was also stimulated by adding Cl⁻ to permit H₂O₂-dependent oxidation of Cl⁻ to HOCl. The results indicate that part of the H₂O₂ was scavenged by intracellular GSH-peroxidase and catalase, and the remainder reacted with extracellular reductants. Azide or Cl⁻ increased the participation of H₂O₂ in extracellular oxidation.

The effect of Cl⁻ was blocked by adding catalase, confirming that H₂O₂ was involved in the stimulation by Cl⁻. Similar stimulation was obtained with other halide substrates for myeloperoxidase (Br⁻, I⁻) or a pseudohalide substrate (SCN⁻). The highest rates of oxidation were obtained with 80 mM Cl⁻, 10 mM Br⁻, or 1 mM I⁻ or SCN⁻, consistent with the specificity of myeloperoxidase.

As described below, catalase and other hemoproteins such as myeloperoxidase had RSH-oxidase activity. Therefore, the weak inhibition by catalase (Tables I and II) underestimates the contribution of H₂O₂ to RSH oxidation. Fig. 2 (left) shows oxidation of Nbs by neutrophils in the presence of catalase and oxidation of Nbs by catalase without neutrophils. The difference between the two curves indicates that about 50% of the neutrophil Nbs oxidation was H₂O₂-dependent. H₂O₂ accounted for about 60% of GSH oxidation and 30% of higher ratios were obtained when Mn²⁺ was added or NADPH-oxidase activity was suppressed by lowering the pH. Nevertheless, superoxide dismutase inhibited strongly under all conditions.

Table II

Comparison of RSH and ascorbate oxidation

The complete system consisted of neutrophils (2 x 10^6/ml) and PMA incubated 1 h with 1 mM reductant. When added, catalase was 5 µg/ml, myeloperoxidase (MPO) was 0.1 µM, superoxide dismutase was 15 µg/ml, lactoferrin (LF) or Fe³⁺-lactoferrin (Fe-LF) was 10 µM, EDTA or diethylenetriaminepentaacetate (DETAPAC) was 0.1 mM, and Ca²⁺ was 1 mM.

| Complete (– Cl⁻) | Nbs | GSH | Ascorbate |
|------------------|-----|-----|-----------|
| + Azide (1 mM)   | 158 | 313 | 113       |
| + Cl⁻ (80 mM)    | 146 | 184 | 137       |
| + Cl⁻, + catalase| 80  | 102 | 111       |
| + Catalase       | 75  | 85  | 94        |
| + Br⁻ (10 mM)    | 142 | 254 | 136       |
| + I⁻ (1 mM)      | 139 | 238 | 126       |
| + SCN⁻ (1 mM)    | 153 | 277 | 87        |
| + MPO            | 225 | 134 | 140       |
| + MPO, + super oxide dismutase | 88  | 70  | 93        |
| + MPO, + Cl⁻     | 203 | 190 | 181       |
| + MPO, – PMA     | 61  | 15  | 7         |
| + MPO, – cells   | 75  | 20  | 7         |
| + MPO, – cells, + super oxide dismutase | 0  | 14  | 6         |
| + LF             | 100 | 96  | 78        |
| + Fe-LF          | 100 | 97  | 106       |
| − Mg²⁺           | 105 | 99  | 93        |
| − Mg²⁺, + Ca²⁺   | 100 | 101 | 97        |
| − Mg²⁺, + EDTA   | 33  | 92  | 93        |
| − Mg²⁺, + DETAPAC | 58 | 98  | 80        |
| − Mn²⁺ (10 µM)   | 204 | 253 | 98        |
| − Mn²⁺, – PMA    | 70  | 8   | 10        |
| − Mn²⁺, – cells  | 5   | 8   | 7         |
ascorbate oxidation (Fig. 2, right). Fig. 2 also shows that at pH 7.4 the oxidase activity of catalase was nearly undetectable at concentrations up to 5 μg/ml. This catalase concentration was used in all other experiments.

Role of Lysosomal Components—To determine whether stimulated neutrophils secrete an enzyme or factor that catalyzes oxidation, neutrophils were incubated in Cl\(^-\)-free medium with PMA or opsonized zymosan and centrifuged, and portions of the supernatants were incubated with Nbs, GSH, or ascorbate. There was no oxidation, indicating that lysosomal components did not catalyze oxidation in the absence of Cl\(^-\) production by the NADPH-oxidase. However, these results do not rule out a role for myeloperoxidase because most of the purified enzyme is associated with the cell membrane rather than free in the supernatant (15).

Adding purified myeloperoxidase resulted in a large increase in Nbs oxidation, although no Cl\(^-\) or other halide was added (Table II). In fact, myeloperoxidase was more effective without Cl\(^-\), indicating that the effect was unrelated to halide oxidation. Myeloperoxidase also promoted Nbs oxidation in the absence of PMA. This oxidation was blocked by superoxide dismutase, although the NADPH-oxidase did not produce detectable amounts of O\(_2\) without PMA. The results indicate that myeloperoxidase had Nbs-oxidase activity that was inhibited by superoxide dismutase but which was independent of O\(_2\) production by the NADPH-oxidase.

The amount of enzyme added in Table II was equal to the amount contained in the cells. That is, if neutrophils (2 \(\times\) 10\(^6\)/ml) secreted their entire content of myeloperoxidase, the concentration in the medium would be 0.1 μM (29). Therefore, the RSH-oxidase activity of the endogenous myeloperoxidase was sufficient to contribute to Nbs oxidation. However, the enzyme had only a weak GSH-oxidase activity and no ascorbate-oxidase activity.

Stimulated neutrophils oxidized Nbs equally well at pH 5.5 or 7.4, although the rate of O\(_2\) production by the NADPH-oxidase at pH 5.5 is only 10% that at pH 7.4 (29, 36). Nbs was oxidized much faster than GSH or ascorbate at low pH, and oxidation was inhibited by Cl\(^-\) or azide. These observations indicate that oxidation at low pH was due primarily to the Nbs-oxidase activity of myeloperoxidase. Experiments with the purified enzyme indicated that Nbs-oxidase activity was much greater at low pH.

The metal-ion-binding protein lactoferrin is another of the major lysosomal components secreted by neutrophils. Adding 10 μM lactoferrin to chelate any free Fe\(^{3+}\) had no effect, indicating that Fe\(^{3+}\) was not required (Table II). Similarly, Fe\(^{3+}\)-lactoferrin did not stimulate, indicating that Fe\(^{3+}\)-lactoferrin did not have RSH- or ascorbate-oxidase activity.

Role of Mn\(^{2+}\)—Omitting Mg\(^{2+}\) or substituting Ca\(^{2+}\) for Mg\(^{2+}\) had no effect (Table II). However, Nbs oxidation was partially inhibited by chelating agents for divalent cations. Chelator concentrations as low as 3 μM gave the maximum amount of inhibition, whereas concentrations up to 0.3 mM did not inhibit O\(_2\) production by the NADPH-oxidase. Chelators had no effect on GSH oxidation in Cl\(^-\)-free medium, but inhibited up to 32% when GSH oxidation was stimulated by adding Cl\(^-\). In contrast, chelators had no effect on ascorbate oxidation with or without Cl\(^-\). The results suggest that RSH oxidation was promoted by an endogenous divalent cation.

Adding Mn\(^{2+}\) promoted oxidation of RSH compounds but not ascorbate, suggesting that Mn\(^{2+}\) was the endogenous cation (Table II). Mn\(^{2+}\) did not promote RSH oxidation in the absence of neutrophils but did promote Nbs oxidation by unstimulated neutrophils. Therefore, the small amount of myeloperoxidase released by unstimulated cells was sufficient to catalyze Nbs oxidation provided that Mn\(^{2+}\) was added.

Mn\(^{2+}\) has superoxide dismutase activity (37), which results from oxidation of Mn\(^{2+}\) to Mn\(^{3+}\) by O\(_2\) followed by reduction of Mn\(^{3+}\) to Mn\(^{2+}\) by a second O\(_2\). Mn\(^{2+}\)-catalyzed dismutation of O\(_2\) resulted in inhibition of cyt c reduction by stimulated neutrophils or the O\(_2\)-generating system xanthine oxidase/acetalddehyde. Mn\(^{2+}\) at 2 μM inhibited reduction of 0.5 mM cyt c by 50%, indicating that O\(_2\) reacted over 100-times faster with Mn\(^{2+}\) than with cyt c. Despite the potent dismutase activity of Mn\(^{2+}\), Mn\(^{2+}\) promoted O\(_2\)-dependent RSH oxidation and had little effect on ascorbate oxidation.

Reaction of O\(_2\) with Reductants or Mn\(^{2+}\)—To determine whether O\(_2\) reacts with the reductants or with some other substance in the incubation mixture, concentrations of the reductants and superoxide dismutase were varied. If the reductant competes with superoxide dismutase for reaction with O\(_2\), then more enzyme will be required to inhibit oxidation when the reductant concentration is increased. Superoxide dismutase inhibited ascorbate oxidation by 50% (Fig. 3). When ascorbate was increased 4-fold from 0.5 to 2 mM, the amount of enzyme required for half the maximum effect (25% inhibition) increased 4-fold from 3.5 to 14 μg/ml. In contrast, when ascorbate was held constant at 0.5 mM and the number of cells was increased 4-fold, the amount of enzyme required for half the maximum effect remained nearly constant at 2.7–3.5 μg/ml. Therefore, oxidation resulted from the direct reaction of O\(_2\) with ascorbate rather than the reaction of O\(_2\) with an enzyme or factor contributed by the cells.

Different results were obtained with RSH compounds. Superoxide dismutase inhibited GSH oxidation by up to 60–70%, and enzyme at 0.1 μg/ml was required for half maximal inhibition with 2 or 6 mM GSH (Fig. 4A). Varying the number of cells also had no effect (not shown). Similarly, superoxide dismutase inhibited Nbs oxidation by 80–90%, and half the maximum effect was obtained with 0.2–0.3 μg/ml enzyme regardless of Nbs concentration or the number of cells (Fig. 4B). Instead, more enzyme was required when Mn\(^{2+}\) was added. With 10 or 100 μM Mn\(^{2+}\), superoxide dismutase at 1.2 or 8 μg/ml was required for half the maximum effect. These results suggest that the first step in O\(_2\)-dependent RSH oxidation is the reaction of O\(_2\) with Mn\(^{2+}\). The results also
Superoxide-dependent Oxidation

O2 Uptake—Incubation of stimulated neutrophils with reductants resulted in increased rates of O2 uptake that were proportional to reductant concentration and the number of neutrophils. The increase in O2 uptake was blocked by superoxide dismutase (Table III), although this enzyme only partially inhibited oxidation.

In the absence of reductants, catalase caused nearly a 50% decrease in the observed rate of O2 uptake (Table III) due to the dismutation of two H2O2 to yield water and one O2. Adding azide to inhibit endogenous catalase caused a small increase in the rate of O2 uptake, and the rate with azide was exactly 2 times the rate with added catalase. Similarly, O2 uptake with reductants and catalase was about 50% slower than uptake with reductants alone or reductants and azide. Therefore, the increase in O2 uptake that accompanied RSH or ascorbate oxidation was the result of increased H2O2 production.

Significant rates of O2 uptake were obtained with unstimulated neutrophils in the presence of Mn2+ and RSH compounds, particularly Nbs. This O2 uptake was due to the Mn2+-dependent RSH-oxidase activity of myeloperoxidase. Mn2+ did not promote O2 uptake by neutrophils alone or RSH compounds alone.

Products of Oxidation—Nbs was oxidized to the disulfide (RSSR) compound Nbs2 as indicated by the relation between loss of absorbance at 409 nm and the increase at 323 nm. There was no loss of 323 nm absorbance when neutrophils were incubated with Nbs2 in Cl- -free medium and no increase in O2 uptake when neutrophils were incubated with Nbs2 or other RSSR compounds. Therefore, RSH or RSSR was not oxidized to a higher oxidation state such as the sulfonic or sulfonic acid (RSO3H or RSO2H).

One-electron oxidation of ascorbate yields monodehydroascorbate, which dismutates to yield ascorbate and dehydroascorbate, whereas two-electron oxidation yields dehydroascorbate directly (38). However, the product of ascorbate oxidation could not be converted to ascorbate by reduction with dithiothreitol, indicating that dehydroascorbate did not accumulate during ascorbate oxidation. Dehydroascorbate would not be expected to accumulate because it undergoes rapid hydrolysis at pH 7.4 to diketogulonate (35). In addition, H2O2 and HOCl oxidize dehydroascorbate (35) and diketogulonate.

Table IV shows that ascorbate oxidation by neutrophils resulted in accumulation of diketogulonate, although the yield of this product was less than the amount of ascorbate oxidized. The low yield was due to oxidation of dehydroascorbate and/or diketogulonate, as indicated by incubating neutrophils with dehydroascorbate and measuring the yield of diketogulonate.

![Fig. 3. Inhibition of ascorbate oxidation by superoxide dismutase.](image)

![Fig. 4. Inhibition of RSH oxidation by superoxide dismutase.](image)

| TABLE III  
| O2-dependent O2 uptake |  
| Complete (+ Cl-) | Nbs | GSH | Ascorbate | O2 uptake, μM/min |
|---------------------|-----|-----|----------|---------------|
| No reductant | 11.6 | 21.9 | 14.8 | 19.6 |
| + Superoxide dismutase | 11.0 | 12.5 | 11.3 | 11.5 |
| + Catalase | 6.3 | 11.9 | 7.3 | 19.5 |
| + Superoxide dismutase, + catalase | 6.2 | 6.4 | 7.7 | 6.8 |
| + Azide | 12.6 | 24.6 | 16.3 | 24.2 |
| - PMA | 0.2 | 0.5 | 0.6 | 0.6 |
| - PMA, + Mn2+ | 0.2 | 4.1 | 1.3 | 0.6 |
| - Cells | 0 | 0.2 | 0.2 | 0.3 |
| - Cells, + Mn2+ | 0 | 0.2 | 0.2 | 0.5 |

The complete system consisted of neutrophils (2 × 106/ml) and PMA with or without 1 mM reductant. When added, superoxide dismutase was 15 μg/ml, catalase was 5 μg/ml, azide was 1 mM, and Mn2+ was 10 μM.
Oxidation was partially inhibited by catalase, indicating that part of the H₂O₂ or HOCl formed during ascorbate oxidation would be consumed in reactions with ascorbate oxidation products. Oxidation was also partially inhibited by superoxide dismutase, suggesting that dehydroascorbate was oxidized by O₂⁻ although at a much slower rate than ascorbate. Oxidation of dehydroascorbate was stimulated by Mn²⁺, but the effect of Mn²⁺ was suppressed when ascorbate was present. The inability of Mn²⁺ to stimulate or inhibit oxidation may be due to the chelating activity of ascorbate (39).

**Model Systems**—Similar oxidation of ascorbate and dehydroascorbate was obtained with stimulated neutrophils or with O₂⁻ added as a solution of KO₂ in dimethyl sulfoxide (Table IV). Oxidation of either compound by KO₂ was partially inhibited by superoxide dismutase or catalase, and Mn²⁺ stimulated the oxidation of dehydroascorbate but not ascorbate. Greater oxidation of dehydroascorbate was obtained with KO₂ than with neutrophils, probably because dehydroascorbate reacts slowly with H₂O₂ and thus competes poorly with H₂O₂-consuming activities in neutrophils. The results indicate that O₂⁻ reacts with ascorbate and dehydroascorbate at significant rates and that no enzyme or other factor is required for O₂⁻-dependent oxidation.

Nbs was oxidized when incubated with the hemoproteins myeloperoxidase, eosinophil peroxidase, and catalase (Table V). Therefore, these metalloproteins had RSH-oxidase activity that was independent of an external source of O₂⁻. Nevertheless, oxidation was inhibited by superoxide dismutase, indicating that O₂⁻ was produced when the enzymes were incubated with Nbs and that O₂⁻ was an intermediate in Nbs oxidation. Oxidase activity of the peroxidases or catalase was abolished by heating, as was the ability of catalase or superoxide dismutase to inhibit oxidation.

The metalloprotein xanthine oxidase did not have RSH-oxidase activity, but Table V shows that Nbs was oxidized when incubated with the O₂⁻-generating system xanthine oxidase/acetaldehyde. These results were obtained at a level of xanthine oxidase that produced O₂⁻ at 30-40 nmol·ml⁻¹·h⁻¹. About 350 μM Nbs was oxidized, so that about 10 Nbs were oxidized per O₂⁻, and this oxidation was inhibited by superoxide dismutase.

Halide ions inhibited the RSH-oxidase activity of the peroxidases but had little effect on the other systems (Table V).
dependence on enzyme concentration was observed with other halides or SCN\(^{-}\), and with eosinophil peroxidase. Because the concentration of enzyme secreted by neutrophils at 2 × 10\(^6\)/ml would be less than 0.1 \(\mu\)M, halides would not necessarily block the oxidase activity of myeloperoxidase in experiments with neutrophils. Similarly, the peroxidase-inhibitor azide would not block completely. Azide at 20 \(\mu\)M inhibited by 50\%, but complete inhibition was not obtained even at 1 mM azide (Table V).

Azide caused a 2- to 3-fold increase in the RSH-oxidase activity of catalase (Table V), although azide inhibits catalase activity. The results suggest that the two activities of catalase are distinct and that azide promoted RSH oxidation by preventing the destruction of \(\text{H}_2\text{O}_2\).

Chelators for divalent cations caused partial or complete inhibition of the oxidase activity of peroxidases or catalase (Table V), although they do not inhibit peroxidase or catalase activity. Partial inhibition was also obtained with xanthine oxidase/acetaldehyde, although chelators do not inhibit \(\text{O}_2\) production. Maximum inhibition was obtained with 1-3 \(\mu\)M chelator. Oxidation was promoted by Mn\(^{2+}\), suggesting that the chelators inhibited by sequestering a small amount of contaminating Mn\(^{2+}\).

Lactoferrin did not inhibit, indicating that Fe\(^{3+}\) was not involved, and Fe\(^{3+}\)-lactoferrin did not stimulate (Table V). Free iron as ferric chloride or ferrous ammonium sulfate at concentrations up to 0.1 mM did not catalyze oxidation of 1 mM Nbs, GSH, or ascorbate. Mn\(^{2+}\) did not cause Fe\(^{3+}\), lactoferrin, or Fe\(^{3+}\)-lactoferrin to take on oxidase activity, and no oxidation was obtained with Mn\(^{2+}\) alone or Mn\(^{2+}\) plus xanthine oxidase or acetaldehyde.

Myeloperoxidase had greater RSH-oxidase activity with Nbs, cysteine, or dithiothreitol than with GSH or 2-mercaptoethanol and had no ascorbate-oxidase activity (Table V). A similar pattern was observed with eosinophil peroxidase, catalase, or xanthine oxidase/acetaldehyde. Fig. 5 shows that Mn\(^{2+}\) did not alter this specificity and that Mn\(^{2+}\) caused similar stimulation of RSH oxidation by myeloperoxidase or xanthine oxidase/acetaldehyde. This figure also provides an estimate of Mn\(^{2+}\) contamination. By extrapolation from the effect of added Mn\(^{2+}\), the endogenous Mn\(^{2+}\) was about 0.1 \(\mu\)M.

Myeloperoxidase and xanthine oxidase have dissimilar active sites and enzymatic mechanisms. Therefore, it is unlikely that the specificity of RSH oxidation was due to selective binding of RSH compounds by the enzymes. Instead, the results suggest that xanthine oxidase/acetaldehyde and myeloperoxidase/RSH acted as \(\text{O}_2\)-generating systems and that differences in rates of oxidation were due to differing inter- actions of RSH compounds with \(\text{O}_2\) and Mn\(^{2+}\).

Fig. 5 shows that there was little GSH oxidation by xanthine oxidase/acetaldehyde at this low level of enzyme. However, 180 \(\mu\)M GSH was oxidized when xanthine oxidase was increased to a level (8 \(\mu\)g/ml) that produced \(\text{O}_2\) at 200 nmol/ml\(^{-1}\)h\(^{-1}\), which is equivalent to neutrophils at 1 × 10\(^6\)/ml. This oxidation was inhibited by catalase but not by superoxide dismutase. Mn\(^{2+}\) caused a 3-fold increase in GSH oxidation to 540 \(\mu\)M, and this oxidation was inhibited 50\% by superoxide dismutase. The maximum rate of GSH oxidation was obtained at 10 \(\mu\)M Mn\(^{2+}\). Therefore, oxidation of three GSH per \(\text{O}_2\) was observed when the rate of \(\text{O}_2\) production was high and sufficient Mn\(^{2+}\) was added.

**DISCUSSION**

\(\text{O}_2\) as an Oxidant—The results indicate that neutrophils can use \(\text{O}_2\) as an oxidant to eliminate GSH or ascorbate from the extracellular phase. The amount of reductant eliminated in this way is up to 3 times greater than would be obtained if \(\text{O}_2\) was reduced directly to \(\text{H}_2\text{O}_2\), which explains the utility of releasing \(\text{O}_2\) rather than \(\text{H}_2\text{O}_2\).

Oxidation of extracellular GSH by stimulated neutrophils is consistent with oxidation of GSH by \(\text{O}_2\), reduction of \(\text{O}_2\) to \(\text{H}_2\text{O}_2\), and oxidation of GSH by \(\text{H}_2\text{O}_2\).

\[
\begin{align*}
2\text{H}^+ + 2\text{O}_2 \rightarrow 2\text{H}_2\text{O}_2 + \text{GSSG} \\
2\text{H}_2\text{O}_2 + 4\text{GSH} \rightarrow 4\text{H}_2\text{O} + 2\text{GSSG}
\end{align*}
\]

net:

\[
2\text{H}^+ + 2\text{O}_2 + 6\text{GSH} \rightarrow 4\text{H}_2\text{O} + 3\text{GSSG}
\]

Intermediates such as Mn\(^{2+}\), the thyl radical (GS), and sulfenic acid (GSOH) may be involved, and the second reaction may be mediated by myeloperoxidase-catalyzed oxidation of Cl\(^{-}\) to HOCl and the oxidation of GSH by HOCl. Therefore, these equations summarize multistep reactions, although the result is oxidation of up to three GSH per \(\text{O}_2\). In the presence of catalase, one GSH would be oxidized per \(\text{O}_2\), so that catalase inhibits by 67\%. Similarly, superoxide dismutase inhibits by 67\%.

This mechanism results in increased \(\text{O}_2\) uptake and \(\text{H}_2\text{O}_2\) production relative to what is observed in the absence of GSH. However, \(\text{O}_2\) is reduced only in the reaction catalyzed by NADPH-oxidase. Therefore, this is not an auto-oxidation reaction.

In the absence of GSH, the rate of \(\text{O}_2\) uptake is about half the rate of \(\text{O}_2\) production because spontaneous dismutation of two \(\text{O}_2\) yields one \(\text{O}_2\). When GSH is added, \(\text{O}_2\) uptake equals \(\text{O}_2\) production, so that \(\text{O}_2\) uptake increases 2-fold, although the rate of \(\text{O}_2\) production does not change. With GSH and catalase, one \(\text{O}_2\) it taken up per two \(\text{O}_2\), so that catalase inhibits by 50\%. Similarly, superoxide dismutase inhibits \(\text{O}_2\) uptake by 50\%, although either enzyme inhibits GSH oxidation by up to 67\%.

Ascorbate oxidation by neutrophils is more complex. \(\text{O}_2\) acts as an oxidant for ascorbate (AH\(_2\)), but part of the \(\text{H}_2\text{O}_2\) produced in this reaction is consumed in the oxidation of AH\(_2\) by \(\text{H}_2\text{O}_2\) or HOCl, where AH\(_2\) represents dehydroascorbate or

![Diagram](image-url)
Peroxidases and catalase have Mn²⁺-dependent RSH-oxidase of 0; on the RSH compounds. The result is that leukocyte that the peroxidase/RSH and catalase/RSH combinations act 2-keto-4-gulonate. 4H⁺ + 4O₂ + 2AH₂ → 4H₂O₂ + 2AH₂ 4H₂O₂ + 4H⁺ + 4Cl⁻ → 4H₂O + 4HClO 3HClO + 3AH₂ → 3H₂O + 3H⁺ + 3Cl⁻ + 3AH₂ HClO + AH₂ → H₂O + H⁺ + Cl⁻ + A net: 4H⁺ + 4O₂ + 5AH₂ → 5H₂O + 4AH₂ + A Three reducing equivalents are lost per O₂, which is the same as in O₂-dependent GSH oxidation. In these equations which do not include oxidation of AH₂ by O₂, four O₂ oxidize five AH₂, the yield of AH₂ is 80% of that predicted assuming oxidation of AH₂ to AH₃₂O, and superoxide dismutase or catalase inhibits by up to 60%. The experimental observations are that about one AH₂ is oxidized per O₂, the yield of AH₂ is 78%, superoxide dismutase inhibits by 50%, and catalase inhibits by 30%. Both AH₂ and AH₃₂O are scavengers for cytotoxic oxidants. Therefore, O₂-dependent oxidation eliminates two reductants that could block the cytotoxicity of H₂O₂ and oxidized halides. O₂-initiated Auto-oxidation—Results consistent with O₂-initiated auto-oxidation are obtained when GSH-oxidizing systems are incubated with certain RSH compounds, particularly Nbs and cysteine. The first step in RSH oxidation is the reaction of O₂ with Mn³⁺. O₂ may oxidize Mn²⁺ to Mn³⁺, and auto-oxidation would be initiated if Mn³⁺ oxidizes RSH to a product that reduces O₂ to O₂⁻. 2H⁺ + O₂ + Mn³⁺ → H₂O₂ + Mn²⁺ Mn³⁺ + RSH → Mn²⁺ + RS + H⁺ RS + O₂ + RSH → RSSR + H⁺ + O₂⁻ net: O₂ + 2 RSH → H₂O₂ + RSSR Both O₂⁻ and Mn³⁺ are catalysts, so that this sequence repeats n times with O₂ as the electron-acceptor. The H₂O₂ produced in each cycle also acts as an oxidant. nO₂⁻ + 2nRSH → nH₂O₂ + nRSSR nH₂O₂ + 2nRSH → 2nH₂O + nRSSR net: nO₂⁻ + 4nRSH ↔ 2nH₂O₂ + 2nRSSR The result is O₂⁻-initiated Mn³⁺-catalyzed RSH auto-oxidation. In this mechanism, catalase inhibits by 50%, and superoxide dismutase inhibits by a large percentage that depends on the value of n. O₂⁻ uptake in the presence of RSH is inhibited 50% by catalase, and superoxide dismutase eliminates all of the additional O₂⁻ uptake that is associated with RSH oxidation. Mn³⁺ catalyzes the oxidation of GSH by O₂⁻, but auto-oxidation is not observed. One possible explanation is that the reaction of two GS radicals with each other to produce GSSG is faster than the reaction of GS with O₂⁻ to produce O₂⁻. Mn³⁺ catalyzes O₂⁻-dependent auto-oxidation of other RSH compounds by stimulated neutrophils, xanthine oxidase/acetalddehyde, peroxidases, and catalase. These results suggest that the peroxidase/RSH and catalase/RSH combinations act as O₂⁻-generating systems and that Mn³⁺ catalyzes the attack of O₂⁻ on the RSH compounds. The result is that leukocyte peroxidases and catalase have Mn³⁺-dependent RSH-oxidase activities. Mn³⁺-dependent oxidase activities of hemoproteins have been studied extensively (40-45) particularly with horseradish peroxidase as the catalyst and NADH as substrate. These activities have caused confusion in studies on the neutrophil NADPH-oxidase. There are many reports of Mn²⁺-stimulated NAD(P)H oxidation and O₂⁻ uptake by subcellular fractions from neutrophils. In contrast, the "respiratory-burst oxidase" is not stimulated by Mn³⁺ or inhibited by azide, shows specificity for NADPH, and is present in cells lacking myeloperoxidase (1-5, 46).

The significance of Mn²⁺-catalyzed reactions in leukocyte functions is unclear because the physiologic concentration of Mn²⁺ in forms that are active as redox catalysts is unknown. Ascorbate may bind and deactivate Mn²⁺. Furthermore, the Mn²⁺-dependent oxidase activities of leukocyte peroxidases are at least partially inhibited by physiologic levels of halides and SCN⁻. On the other hand, only small amounts of Mn²⁺ are required, the reactions may be favored at the acid pH of the phagolysosome, and microorganisms may release Mn²⁺ into phagolysosomes. Certain bacteria accumulate high levels of Mn²⁺ in forms that react with O₂⁻ (47). Therefore Mn²⁺ may catalyze O₂⁻-dependent oxidation of reductants in phagolysosomes. The Mn²⁺-dependent oxidase activities of leukocyte peroxidases are of particular interest because these activities could provide an alternative source of O₂⁻ and cytotoxic oxidants in cells that are deficient in NADPH-oxidase activity.

Role of O₂⁻ in Leukocyte Antimicrobial and Cytotoxic Activities—The results suggest that an important role for O₂⁻ in leukocyte function is to participate in oxidation of extracellular reductants. When these reductants are eliminated, H₂O₂ and oxidized halides are free to exert toxicity. O₂⁻-dependent oxidation also results in a large increase in H₂O₂ production, and this H₂O₂ could be used to attack target cells or to produce oxidized halides. However, H₂O₂ production increases only as long as the reductant is present, and most of the H₂O₂ or oxidized halides would be scavenged by the reductant. Predictions can be made that would provide further tests of the hypothesis. First, superoxide dismutase may block toxic activities of leukocytes when the system under study contains extracellular reductants. Such conditions would be encountered in vivo and in experimental systems containing plasma, serum, interstitial fluid, broken cells, or media conditioned by growing cells. Second, compounds that undergo rapid O₂⁻ oxidation may be ineffective in blocking toxicity or may exacerbate toxicity by promoting H₂O₂ production. Third, the combination of superoxide dismutase and a reductant may be much more protective than either agent alone. Many studies have reported protective effects of superoxide dismutase alone or with catalase. However, the role of extracellular reductants in these effects has not been considered. Results of this study also indicate that protective effects could easily be misinterpreted. Inhibition of leukocyte cytotoxicity by superoxide dismutase does not necessarily indicate that O₂⁻ reacts with the target cells. Inhibition by superoxide dismutase plus catalase does not necessarily indicate that iron and hydroxyl radicals are involved. Instead, superoxide dismutase may inhibit oxidation of extracellular reductants. When these reductants are protected against O₂⁻-dependent oxidation, they can detoxify H₂O₂ and products of peroxidase/H₂O₂/halide systems.

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