

THE IRON-H₂O₂-IODIDE CYTOTOXIC SYSTEM*

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Myeloperoxidase (MPO), hydrogen peroxide (H₂O₂), and a halide form a potent antimicrobial system that appears to contribute to the antimicrobial activity of neutrophils and monocytes (1, 2). A new antimicrobial system is described here in which MPO is replaced by Fe²⁺ and the properties of the Fe²⁺ + H₂O₂ + halide system are compared with those of the MPO-catalyzed system.

Materials and Methods

Special Reagents. Myeloperoxidase was prepared and assayed as previously described (3). Catalase (bovine liver, 6.1 mg/ml, 60,000 U/mg), obtained from Worthington Biochemical Corp., Freehold NJ, was dialyzed against water before use and superoxide dismutase (SOD) (bovine erythrocytes, lyophilized powder, 12,300 U/mg), obtained from Miles Laboratories Inc., Miles Research Products, Elkhart, IN, was dissolved in water (5 mg/ml) and stored at -20°C. Catalase was heated at 100°C for 15 min and SOD was autoclaved at 120°C for 30 min where indicated. Water was deionized to a resistance of $>1.8 \times 10^7$ ohm/cm at 25°C, and all stock buffer and sodium sulfate solutions were passed twice over a Chelex-100 ion exchange column to remove trace metals (4).

Bactericidal activity. *Staphylococcus aureus* 502A was maintained on blood agar plates and transferred daily to trypticase soy broth (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, MD). Overnight 37°C cultures were washed twice and suspended in 0.1 M sodium sulfate to the required optical density at 520 nm. The bacteria were incubated at 37°C for the periods indicated with the components described in tables and figures, and the viable cell count was determined by the pour plate method (3) using trypticase soy agar. Colony-forming units were counted after 1-2 d incubation at 37°C and the results expressed as the geometric mean. Statistical analyses were performed as described (3) with not significant (NS) $P > 0.05$.

Results

MPO-H₂O₂-Halide System. The properties of the MPO system were as anticipated from prior studies. Bactericidal activity was dependent on each component of the system, inhibited by catalase but not by SOD, unaffected by the hydroxyl radical (OH·) scavengers mannitol and ethanol at 0.1M, inhibited by azide and cyanide at concentrations down to 10⁻⁵ M, and unaffected by EDTA at 10⁻⁴ M (Table I). H₂O₂ could be replaced by the H₂O₂-generating system glucose + glucose oxidase. The 0.2 M acetate buffer, pH 5.5, routinely used could be deleted or replaced by 0.02 M phosphate or lactate buffer, pH 5.5, with comparable results. When buffer was deleted, the pH of the reaction mixture was 6.4. Iodide could be replaced by 10⁻³-10⁻⁶ M bromide, 10⁻¹-10⁻⁴ M chloride, and 10⁻⁴-10⁻⁶ M thyroxine (Table II). Bactericidal activity was rapid (Fig. 1) and was optimal at pH 5.0, with activity falling sharply as the pH of the acetate buffer was increased to 6.5 (Fig. 2). Bactericidal activity was readily observed in phosphate buffer at neutral pH when the H₂O₂ and iodide concentrations were increased (data not shown).

* Supported by grant AI07763 from the U. S. Public Health Service and by a grant from the Rockefeller Foundation.

Fe²⁺-H₂O₂-Iodide System. When MPO was replaced by Fe²⁺, bactericidal activity was still apparent; however, the properties of the two systems differed in several respects. As with the MPO system, bactericidal activity was dependent on each component of the Fe²⁺-H₂O₂-iodide system and was inhibited by catalase but not SOD. Heat-treated catalase retained some inhibitory activity for both the MPO and Fe²⁺ systems, presumably due to the scavenging effect of protein. Fe²⁺ could not be replaced by an equimolar concentration of Fe³⁺; H₂O₂, however, could be replaced by glucose and glucose oxidase. In contrast to the MPO system, mannitol was inhibitory at 10⁻² M, ethanol at 10⁻¹ M and EDTA at 10⁻⁵ M. Azide also was inhibitory; however a higher concentration (10⁻⁴ M) was required for inhibition of the Fe²⁺ system than was needed for the MPO system (10⁻⁵ M). The inhibitory effect of cyanide was comparable in the two systems. When Fe²⁺ was used, 0.02 M acetate buffer pH 5.5 could not be replaced by phosphate or lactate buffer at the same concentration and pH (Table I); the latter buffers inhibited the bactericidal activity of the acetate-containing system (data not shown). The acetate buffer, however, could be deleted with little loss of activity; the decreased bactericidal activity observed

TABLE I
Comparison of the Bactericidal Activities of the MPO-H₂O₂-Iodide and Fe²⁺-H₂O₂-Iodide Systems*

Supplements	Viable cell count			
	MPO + H ₂ O ₂ + I ⁻		Fe ²⁺ + H ₂ O ₂ + I ⁻	
	<i>organisms/ml</i> × 10 ⁻⁶			
None	2.66‡		2.93	
Complete system	0.00005	<0.001§	0.00003	<0.001
-Iodide	2.52	NS	3.65	NS
-H ₂ O ₂	2.42	NS	3.20	NS
-MPO	3.28	NS	—	—
-Fe ²⁺	—	—	3.28	NS
-Fe ²⁺ , + Fe ³⁺	—	—	2.92	NS
+SOD	0.00009	<0.001	0.0002	<0.001
+Catalase	1.94	NS	3.45	NS
+Heated catalase	0.004	<0.05	0.004	<0.001
+Mannitol (10 ⁻¹ M)	0.00001	<0.001	2.66	NS
+Mannitol (10 ⁻² M)	—	—	3.98	NS
+Mannitol (10 ⁻³ M)	—	—	0.002	<0.01
+Ethanol (10 ⁻¹ M)	0.00002	<0.001	2.46	NS
+Ethanol (10 ⁻² M)	—	—	0.006	<0.001
+Azide (10 ⁻⁴ M)	2.30	NS	2.88	NS
+Azide (10 ⁻⁵ M)	2.00	NS	0.00004	<0.001
+Azide (10 ⁻⁶ M)	0.00003	<0.001	—	—
+Cyanide (10 ⁻⁵ M)	2.02	NS	4.05	NS
+Cyanide (10 ⁻⁶ M)	0.00003	<0.001	0.0001	<0.001
+EDTA (10 ⁻⁴ M)	0.0005	<0.001	2.77	NS
+EDTA (10 ⁻⁵ M)	0.0006	<0.001	1.35	NS
+EDTA (10 ⁻⁶ M)	0.00006	<0.01	0.0004	<0.001
-H ₂ O ₂ , + glucose and GO	0.00003	<0.001	0.00002	<0.001
-Acetate buffer	0.016	<0.02	0.003	<0.001
-Acetate, + phosphate buffer	0.00017	<0.001	2.38	NS
-Acetate, + lactate buffer	0.00034	<0.001	1.97	NS

* The reaction mixture contained 0.02 M sodium acetate buffer pH 5.5, 3.5 × 10⁸ *E. coli*, water to a final volume of 0.5 ml and the supplements indicated below where indicated: MPO, 8 mU; ferrous sulfate (Fe²⁺), 10⁻⁵ M; ferric sulfate (Fe³⁺), 10⁻⁵ M; H₂O₂, 10⁻⁵ M; sodium iodide, 10⁻⁶ M; SOD, 62 U/ml (5 µg/ml); catalase, 360 U/ml (6 µg/ml); and mannitol, ethanol, azide, cyanide, and EDTA at the concentrations indicated. Where indicated H₂O₂ was replaced by 10⁻⁴ M glucose and 2.8 U/ml glucose oxidase and acetate buffer was deleted or replaced with 0.02 M sodium phosphate or 0.02 M sodium lactate buffer pH 5.5. Incubation time, 60 min.

‡ Geometric mean of three to seven experiments.

§ The probability value for the difference from the control ("None").

|| Glucose oxidase.

TABLE II
Effect of Halides*

Halide	Viable cell count			
	MPO + H ₂ O ₂ + I ⁻		Fe ²⁺ + H ₂ O ₂ + I ⁻	
None	2.52‡		3.65	
Iodide	(10 ⁻⁶ M)	0.00005	<0.001§	0.00003
	(5 × 10 ⁻⁷ M)	0.00006	<0.001	0.0001
	(2 × 10 ⁻⁷ M)	1.91	NS	2.89
	(10 ⁻⁷ M)	2.63	NS	3.30
Bromide	(10 ⁻³ M)	0.00001	<0.001	3.88
	(10 ⁻⁴ M)	0.00001	<0.001	3.96
	(10 ⁻⁵ M)	0.00001	<0.001	3.34
	(10 ⁻⁶ M)	0.00006	<0.001	3.76
Chloride	(10 ⁻⁷ M)	2.49	NS	—
	(10 ⁻¹ M)	0.00001	<0.001	3.12
	(10 ⁻² M)	0.00001	<0.001	3.22
	(10 ⁻³ M)	0.00001	<0.001	—
Thyroxine	(10 ⁻⁴ M)	0.0009	<0.001	—
	(10 ⁻⁵ M)	2.06	NS	—
	(10 ⁻⁴ M)	0.00004	<0.001	3.25
	(10 ⁻⁵ M)	0.00004	<0.001	2.58
(10 ⁻⁶ M)	0.004	<0.001	2.78	

* The complete system was as described in Table I except that the halide (or thyroxine) was varied as indicated.

‡ Mean of three experiments.

§ The probability value for the difference from no halide ("None").

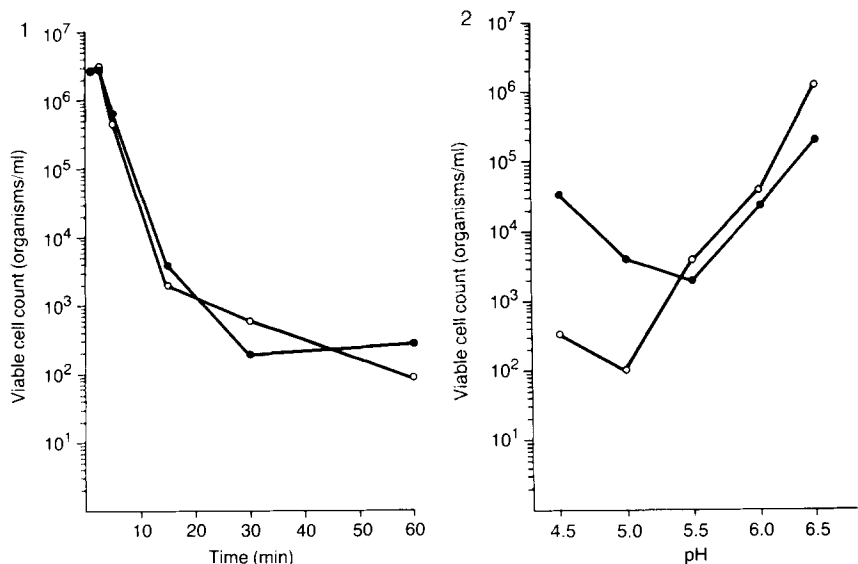


FIG. 1. Effect of incubation period. The reaction mixture was as described for the complete system in Table I (MPO + H₂O₂ + iodide (○); Fe²⁺ + H₂O₂ + iodide (●)); except that the incubation period was varied as indicated.

FIG. 2. Effect of pH. The reaction mixture was as described for the complete system in Table I (MPO + H₂O₂ + iodide (○); Fe²⁺ + H₂O₂ + iodide (●)); except that the incubation period was 15 min and the pH of the acetate buffer was varied as indicated.

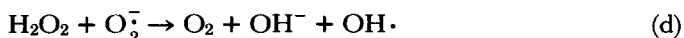
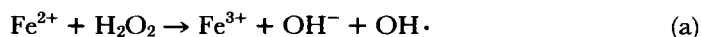
could be accounted for by the higher pH of the buffer-free mixture. In sharp contrast to the MPO system, iodide could not be replaced by bromide, chloride, or thyroxine (Table II). With both systems, iodide was effective at concentrations of 5×10^{-7} M or greater. The rate of fall in bacterial cell count by the two systems was comparable

(Fig. 1); the pH optimum of the Fe^{2+} - H_2O_2 -iodide system however was shifted slightly to 5.5 (Fig. 2).

It should be noted that Fenton's reagent (5), i.e., $\text{Fe}^{2+} + \text{H}_2\text{O}_2$ (the complete system with iodide omitted in Table I) was ineffective under the conditions employed; indeed no staphylocidal activity was observed with a 10-fold increase in the Fe^{2+} and H_2O_2 concentrations to 10^{-4} M (data not shown).

Discussion

The demonstration of H_2O_2 formation by phagocytes (6) centered attention on this agent as an important contributor to the cytotoxic activity of these cells. The toxicity of H_2O_2 is increased many orders of magnitude by MPO and a halide, and this amplification mechanism appears to be operative in the neutrophil and monocyte (1, 2). The reactivity of H_2O_2 also can be increased by iron salts. In 1894, Fenton (5) described the strong oxidizing activity of a mixture of ferrous ion and H_2O_2 and it was subsequently proposed by Haber and Weiss (7) that the oxidant formed by Fenton's reagent was $\text{OH}\cdot$ as follows: $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}\cdot$ (a). Although alternative reactions have been proposed, as for example the formation of the ferryl radical (8) $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{FeO}^{2+} + \text{H}_2\text{O}$ (b), or a reactive intermediate such as the $\text{H}_2\text{O}_2 - \text{Fe}^{2+}$ complex (9) which have chemical properties similar to $\text{OH}\cdot$, it is the generally held view that $\text{OH}\cdot$ is generated by Fenton's reagent as shown in reaction (a). When the iron concentration is limiting, the reduction of the ferric iron formed is needed for the complete degradation of H_2O_2 . This can be accomplished by the superoxide anion (O_2^-) (Haber-Weiss reaction) as follows:



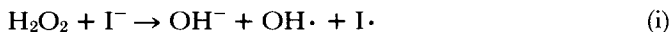
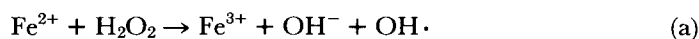
The formation of $\text{OH}\cdot$ by phagocytes by the mechanisms indicated above and their contribution to the cytotoxic activity of these cells has been reviewed (10).

Recently, we have observed that $\text{OH}\cdot$ can react with iodide to iodinate tyrosine residues of protein, and we have used this iodination reaction to define the optimum conditions for the generation of available $\text{OH}\cdot$ (11). Iodination by Fenton's reagent ($\text{Fe}^{2+} + \text{H}_2\text{O}_2$) occurred best in low concentrations (≤ 0.02 M) of acetate buffer pH 5.5. We used these conditions in an attempt to demonstrate the bactericidal activity of $\text{OH}\cdot$ generated by Fenton's reagent; no staphylocidal activity was observed at FeSO_4 and H_2O_2 concentrations of 10^{-4} M. However, when iodide was added, a marked fall in viable cell count was observed even when the Fe^{2+} and H_2O_2 concentrations were lowered to 10^{-5} M. This paper describes the properties of this antimicrobial system and compares them to those of the MPO- H_2O_2 -halide system. The composition of the two systems was identical except that MPO (8 mU) was replaced by ferrous sulfate (10^{-5} M).

It is clear that the MPO- and Fe^{2+} -dependent systems differ in major ways. In the MPO system, the peroxidase catalyzes the oxidation of a variety of halides by H_2O_2 and the cytotoxicity is mediated by the products formed. There is no evidence for $\text{OH}\cdot$ involvement and, although MPO contains heme iron, this iron appears firmly bound and unavailable to chelation by EDTA. In contrast, the Fe^{2+} - H_2O_2 -iodide system was inhibited by EDTA, iodide was the only effective halide, and our findings suggest that the bactericidal activity is dependent on the formation of $\text{OH}\cdot$. Thus, bactericidal

activity required both Fe^{2+} and H_2O_2 , which interact to form $\text{OH}\cdot$ (7), and the $\text{OH}\cdot$ scavengers mannitol and ethanol were inhibitory. The more stringent buffer requirements of the Fe^{2+} - H_2O_2 -iodide system may reflect its sensitivity to iron chelators and the scavenging effect of organic buffers. Azide and cyanide react with the iron of heme proteins, and their inhibition of the MPO-dependent system presumably results from this reaction. Both also inhibit the Fe^{2+} - H_2O_2 -iodide system, although a higher concentration of azide was required than was needed for inhibition of the MPO system. Azide and cyanide are chelating agents and also can react with $\text{OH}\cdot$ (12) and thus may inhibit the Fe^{2+} -dependent system by either or both of these mechanisms. Although $\text{OH}\cdot$ appears to be a necessary component of the Fe^{2+} - H_2O_2 -iodide system and this radical has been implicated as a toxic product of the xanthine oxidase, as well as other, antimicrobial systems (10), $\text{OH}\cdot$ generated solely by Fenton's reagent did not appear to be directly staphylocidal under our conditions; the addition of iodide was required.

The mechanism of the toxicity is not known. Iodide is converted to organic form when albumin is added to the Fe^{2+} - H_2O_2 -iodide system (11) and the substitution of a bulky iodide for hydrogen at crucial locations on the surface of the cell may contribute to the toxicity. However, the oxidation of essential components at or near the cell surface may be equally or more important. Oxidations initiated by Fenton's reagent are complex and incompletely understood (8, 12). The oxidation of halides by $\text{OH}\cdot$ generated by Fenton's reagent would be expected to proceed in two steps (12) which for iodide are as follows: $\text{OH}\cdot + \text{I}^- \rightarrow \text{OH}^- + \text{I}\cdot$ (e); $\text{I}\cdot + \text{I}^- \rightarrow \text{I}_2^-$ (f), with the possibility of competitive reactions of the intermediate species ($\text{I}\cdot$). Among the latter may be the following reaction (12): $\text{I}\cdot + \text{H}_2\text{O}_2 \rightarrow \text{IOH} + \text{OH}\cdot$ (g), with further generation of $\text{OH}\cdot$ as well as the potentially toxic hypoiodous acid. Reutilization of $\text{OH}\cdot$ for the oxidation of iodide (reaction e) would constitute a chain reaction resulting in the complete degradation of H_2O_2 with the formation of toxic iodide oxidation products. An alternative sequence of reactions is suggested by the studies of Weiss (13), who proposed that Fe^{3+} , which remains in solution at acid pH, can react with iodide with the generation of Fe^{2+} . This would suggest the following sequence:



However, our inability to replace Fe^{2+} with Fe^{3+} in the Fe^{2+} - H_2O_2 -iodide system would argue against this mechanism. It has been proposed that the product of iodide oxidation by peroxidase and H_2O_2 is reduced in the course of the oxidation of microbial components, with the regeneration of iodide for use by the peroxidase system (14). A similar reaction as a consequence of the action of the Fe^{2+} - H_2O_2 -iodide system on the target cell would allow iodide to be reused in a catalytic manner. The combined result of these, and presumably other, reactions is a system with potent cytotoxic activity.

The high reactivity and simple requirements of the Fe^{2+} - H_2O_2 -iodide system suggest that it may be useful for the generation of reactive oxidants effective against microorganisms as well as other cells and non-cellular systems, in solutions which lack high levels of radical scavengers or chelators. In addition to *S. aureus*, we have found the system to be toxic to *Escherichia coli* and to LSTRA mouse lymphoma cells (data

not shown). The breadth and magnitude of the oxidative activity of the Fe^{2+} - H_2O_2 -iodide system and its biological role, if any, however, remain to be delineated.

Summary

A potent antimicrobial system is described which consists of ferrous sulfate (Fe^{2+}), hydrogen peroxide (H_2O_2), and iodide in 0.02 M sodium acetate buffer pH 5.5. H_2O_2 could be replaced by the H_2O_2 -generating system glucose + glucose oxidase. This system, unlike the myeloperoxidase- H_2O_2 -halide system, was ineffective when iodide was replaced by bromide, chloride, or thyroxine, and was inhibited by EDTA, the hydroxyl radical scavengers mannitol and ethanol, and phosphate and lactate buffers at the same concentration and pH as the acetate buffer used. The acetate buffer, however, could be replaced by water. It is proposed that Fe^{2+} and H_2O_2 (Fenton's reagent) generate $\text{OH}\cdot$ (or a closely related substance), which interacts with iodide to form one or more toxic species.

The author gratefully acknowledges the skilled technical assistance of Ann Waltersdorph and the expert secretarial help of Caroline Wilson.

Received for publication 12 July 1982.

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