Role of the Superoxide Anion in the Myeloperoxidase-mediated Antimicrobial System*

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SUMMARY

An antimicrobial system has been previously described in the polymorphonuclear leukocyte which consists of myeloperoxidase, H₂O₂, and a halide. In the present study, H₂O₂ is replaced by xanthine and xanthine oxidase in the isolated myeloperoxidase-mediated antimicrobial system and the contribution of the superoxide anion (O₂⁻) and hydroxyl radicals (·OH) to the antimicrobial effect investigated.

Xanthine, xanthine oxidase, chloride, and myeloperoxidase have a marked microbicidal effect on Escherichia coli, Staphylococcus aureus, and Candida tropicalis. The requirement for H₂O₂ is indicated by the inhibitory effect of catalase. Superoxide dismutase, which catalyzes the conversion of O₂⁻ to O₂ and H₂O₂, inhibits microbicidal activity at high concentration (>88 μg per ml); however, this is probably due, in large part, to the nonspecific inhibitory effect of protein. Cytochrome c is reduced by O₂⁻ and thus inhibits the conversion of O₂⁻ to H₂O₂. The microbicidal activity of the xanthine-xanthine oxidase-chloride-myeloperoxidase system is inhibited by cytochrome c and superoxide dismutase, at low concentration (4.4 μg per ml), largely reverses this inhibition. Microbicidal activity in the presence of cytochrome c and superoxide dismutase is inhibited by catalase. The microbicidal activity of the reconstituted system is inhibited by catalase. The hydroxyl radical scavengers, benzoate and ethanol, also have an inhibitory effect on the microbicidal system at high concentration (>88 μg per ml), largely reverses this inhibition. These studies suggest that superoxide dismutase and scavengers of the O₂⁻ and ·OH radicals influence microbicidal activity through their effect on H₂O₂ formation.

EXPERIMENTAL PROCEDURE

Escherichia coli (ATCC no. 11775), Staphylococcus aureus (502A), and Candida tropicalis (hospital strain) were maintained on Trypticase soy agar (Baltimore Biological Laboratories, Baltimore, Md.) and transferred daily to Trypticase soy broth. Overnight cultures were washed twice with 0.1 M phosphate buffer pH 7.0 and suspended in the same solution to the required absorbance at

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540 nm. Canine myeloperoxidase was purified from pyometral pus to the end of Step 6 (17) and lactoperoxidase was prepared from bovine milk (18). Horseradish peroxidase (HRP) was obtained from Worthington Biochemical Corp., Freehold, N. J. Peroxidase activity was determined by the o-dianisidine method (19). One unit of activity is that causing an increase in absorbance of 0.001 per min at 490 nm in a Cary M 15 spectrophotometer. Xanthine oxidase (milk; specific activity approximately 0.4 unit per mg) was obtained as a suspension in 2.0 M ammonium sulfate, 0.1 M EDTA and 60 units of myeloperoxidase (MPO). Myeloperoxidase was re-
dialysed against 0.05 M acetate buffer, pH 3.8, containing 1 X 10^{-6} M and 1 X 10^{-4} M, respectively. Catalase (beef liver, crystalline, 54,500 units per mg) was obtained from Worthington Biochemical Corp. It was dialyzed against water prior to use. Superoxide dismutase (3000+ units per mg), obtained from Truett Laboratories, Dallas, Texas, was dialyzed against 0.1 M phosphate buffer, pH 7.0 for 60 min in a Cary M 15 spectrophotometer (Cary Instruments, Monrovia, Calif.).

Determination of Microbicidal Activity—The components of the reaction mixture (see legends) were incubated at 37° for 60 min in test tubes (10 X 76 mm) oscillating 80 times per min in an Eberbach water bath shaker. Aliquots (0.1 ml) were diluted with water and the viable cell count determined by the pour plate method using Trypticase soy agar.

RESULTS

Microbicidal Activity of Xanthine-Xanthine Oxidase-Chloride-Myeloperoxidase System—Incubation of E. coli or S. aureus with xanthine, xanthine oxidase, chloride, and myeloperoxidase at pH 7.0 for 60 min resulted in a marked fall in the viable cell count under the conditions employed in Table I. A lesser but statistically significant (p < 0.01) inhibitory effect of the complete system on C. tropicalis also was observed. Each of the components of the system (xanthine, xanthine oxidase, chloride, myeloperoxidase) was required for optimum microbicidal activity, and the effect was abolished by heat inactivation of either xanthine oxidase or myeloperoxidase or by the addition of azide or cyanide at 1 mM concentration. Myeloperoxidase could not be replaced by lactoperoxidase or horseradish peroxidase at equal (Table I) or 10-fold greater concentration (o-dianisidine units per ml). The activity of the xanthine-xanthine oxidase-chloride-myeloperoxidase system is greater at pH 7.0 than at pH 5.0 (Fig. 1). This is in contrast to the more acid pH optimum observed when the xanthine oxidase system is replaced by reagent H_{2}O_{2} (4) and presumably reflects the pH optimum of xanthine oxidase.

Requirement for H_{2}O_{2}—H_{2}O_{2} is formed during the aerobic oxidation of xanthine by xanthine oxidase and the requirement for this product of the xanthine oxidase reaction is indicated by the inhibitory effect of catalase on microbicidal activity (Table II). Catalase heated at 100° for 15 min was without effect. Xanthine and xanthine oxidase can be replaced by reagent H_{2}O_{2} under the experimental conditions employed in Table II.

Requirement for O_{2}−—Superoxide dismutase at high concentration inhibited the xanthine-xanthine oxidase-chloride-myeloperoxidase microbicidal system (Table III). The inhibition was increased by heating the preparation at 100° for 15 min and the apoenzyme retained inhibitory activity. This suggests that the inhibitory effect of superoxide dismutase at high concentration

<table>
<thead>
<tr>
<th>Supplements</th>
<th>E. coli organisms/ml x 10^{-5} (n)</th>
<th>Staph. aureus organisms/ml x 10^{-5} (n)</th>
<th>C. tropicalis organisms/ml x 10^{-5} (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.58 ± 0.16 (30)*</td>
<td>3.23 ± 0.42 (3)</td>
<td>1.89 ± 0.28 (4)</td>
</tr>
<tr>
<td>Complete system</td>
<td>0.000009 ±</td>
<td>0.000004 ±</td>
<td>0.70 ± 0.04 (4)</td>
</tr>
<tr>
<td></td>
<td>0.000009 (22)</td>
<td>0.000004 (3)</td>
<td></td>
</tr>
<tr>
<td>Xanthine omitted</td>
<td>4.75 ± 0.13 (3)</td>
<td>3.95 ± 0.20 (3)</td>
<td>1.85 ± 0.46 (4)</td>
</tr>
<tr>
<td>Xanthine oxidase omitted</td>
<td>5.81 ± 0.46 (3)</td>
<td>4.66 ± 0.37 (3)</td>
<td>2.38 ± 0.71 (4)</td>
</tr>
<tr>
<td>Chloride omitted</td>
<td>6.12 ± 0.19 (3)</td>
<td>3.93 ± 0.07 (3)</td>
<td>2.29 ± 0.32 (4)</td>
</tr>
<tr>
<td>MPO omitted</td>
<td>5.70 ± 0.14 (5)</td>
<td>4.16 ± 0.72 (3)</td>
<td>2.76 ± 0.36 (4)</td>
</tr>
<tr>
<td>MPO heated (15° at 100°C)</td>
<td>6.16 ± 0.19 (3)</td>
<td>4.91 ± 0.31 (3)</td>
<td>2.78 ± 0.54 (4)</td>
</tr>
<tr>
<td>Xanthine oxidase heated (15° at 100°C)</td>
<td>5.33 ± 0.10 (3)</td>
<td>4.85 ± 0.21 (3)</td>
<td>2.61 ± 0.59 (4)</td>
</tr>
<tr>
<td>Azide (1mM) added</td>
<td>6.20 ± 0.40 (3)</td>
<td>5.02 ± 0.50 (3)</td>
<td>2.67 ± 0.39 (4)</td>
</tr>
<tr>
<td>Cyanide (1mM) added</td>
<td>5.16 ± 0.36 (3)</td>
<td>4.15 ± 0.21 (3)</td>
<td>2.48 ± 0.35 (4)</td>
</tr>
<tr>
<td>MPO omitted, LPO added</td>
<td>6.28 ± 1.61 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPO omitted, HRP added</td>
<td>5.93 ± 0.82 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.E. of (n) experiments.
is not due to its enzyme activity. It may be partly due to a nonspecific inhibitory effect of protein since crystalline bovine albumin at comparable protein concentrations also is inhibitory (Table III). Superoxide dismutase did not inhibit the microbialid system at concentrations of 88 μg per ml or below. This concentration is considerably higher than that required to inhibit other reactions which required the superoxide anion.

These studies suggest that the superoxide anion is not directly involved in the killing of E. coli by the complete peroxidase system. However, the superoxide anion is an intermediate in H₂O₂ formation by xanthine and xanthine oxidase and may therefore be involved indirectly. Ferricytochrome c reacts with the superoxide anion and inhibits its conversion to H₂O₂. Table IV demonstrates the inhibitory effect of ferricytochrome c on the xanthine-xanthine oxidase-chloride-peroxidase microbiocidal system and the partial reversal of this inhibition by superoxide dismutase at a concentration of 4.4 μg per ml. This effect of superoxide dismutase is decreased by heating the preparation at 100° for 15 min. The microbiocidal activity of the complete system in the presence of ferricytochrome c and superoxide dismutase is completely inhibited by catalase. The concentration of ferricytochrome c employed in Table IV (70 μg per ml) is that which gave the maximum superoxide dismutase-reversible inhibition of microbiocidal activity. Although an increase in the ferricytochrome c concentration produced a greater inhibition, this inhibition was progressively less sensitive to reversal by superoxide dismutase, suggesting a nonspecific inhibition of the microbiocidal system by high concentrations of ferricytochrome c similar to that of albumin or superoxide dismutase (Table III).
ferrocytochrome c is employed. The products of the xanthine oxidase-mediated antimicrobial system occurs in part via the intermediate formation of \( \text{O}_2^- \), since the microbicidal activity of the complete system depends on chloride and myeloperoxidase, and superoxide dismutase at concentrations up to 88 \( \mu \text{g} \) per ml did not inhibit the xanthine oxidase-chloride-myeloperoxidase antimicrobial system.

The mechanism of action of these agents is not clear. Their inhibitory effect is partially reversed by superoxide dismutase, and catalase inhibits the microbicidal activity of the complete system in the presence of benzoate (or ethanol) and superoxide dismutase. The mechanism of action of these agents is not clear. Their inhibitory effect is partially reversed by superoxide dismutase, and catalase inhibits the microbicidal activity of the complete system in the presence of benzoate (or ethanol) and superoxide dismutase. This supports the need for \( \text{H}_2\text{O}_2 \) for microbicidal activity and is compatible with its limitation by scavengers of hydroxyl radicals such as benzoate or ethanol. The formation of \( \text{H}_2\text{O}_2 \) by the xanthine oxidase system can occur by the univalent reduction of oxygen with the intermediate formation of \( \text{O}_2^- \) and by the direct divalent reduction of oxygen without apparent \( \text{O}_2^- \) production (22). A third mechanism for the formation of \( \text{H}_2\text{O}_2 \) may also occur. The superoxide anion reacts with \( \text{H}_2\text{O}_2 \) to form hydroxyl radicals and catalase inhibited the microbicidal activity of the complete system even though an inhibition of the formation of \( \text{O}_2^- \) would be expected. The nonenzymatic nature of this inhibition was emphasized by its potentiation by heat treatment.

Benzoate and, to a lesser degree, ethanol inhibit the xanthine-xanthine oxidase-chloride-myeloperoxidase antimicrobial system. The formation of \( \text{H}_2\text{O}_2 \) by the xanthine oxidase system can occur by the univalent reduction of oxygen with the intermediate formation of \( \text{O}_2^- \) and by the direct divalent reduction of oxygen without apparent \( \text{O}_2^- \) production (22). A third mechanism for the formation of \( \text{H}_2\text{O}_2 \) may also occur. The superoxide anion reacts with \( \text{H}_2\text{O}_2 \) to form hydroxyl radicals (Reaction 2) which interact with high efficiency to form \( \text{H}_2\text{O}_2 \) (Reaction 3)

\[
\text{OH} + \text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O}_2
\]  

\[(3)\]

In this sequence \( \text{H}_2\text{O}_2 \) is both utilized and formed. Scavengers of hydroxyl radicals would be expected to interfere with the formation of \( \text{H}_2\text{O}_2 \) by this mechanism without decreasing its degradation. Indeed the rate of degradation of \( \text{H}_2\text{O}_2 \) may be increased by the more efficient removal of a product of its interaction with \( \text{O}_2^- \). These data do not support the hydroxyl radical as the primary microbicidal agent since microbicidal activity is dependent on chloride and myeloperoxidase, and superoxide dismutase at concentrations up to 88 \( \mu \text{g} \) per ml did not inhibit the microbicidal activity of the complete system even though an inhibition of the formation of \( \text{O}_2^- \) by Reaction 2 would be expected. The formation of \( \text{OH} \) by another mechanism which

### Table V

**Effect of ethanol and benzoate**

<table>
<thead>
<tr>
<th>Supplements</th>
<th>E. coli viable cell count</th>
<th>organisms/mL × 10^4 (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.58 ± 0.16 (30)</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>0.00009 ± 0.00009 (22)</td>
<td></td>
</tr>
<tr>
<td>Benzoate (0.015 M) added</td>
<td>0.27 ± 0.06 (19)</td>
<td></td>
</tr>
<tr>
<td>Benzoate and superoxide dismutase (4.4 ( \mu \text{g/ml} )) added</td>
<td>0.16 ± 0.05 (18)</td>
<td></td>
</tr>
<tr>
<td>Benzoate, superoxide dismutase and catalase (6 ( \mu \text{g/ml} )) added</td>
<td>4.99 ± 0.41 (14)</td>
<td></td>
</tr>
<tr>
<td>Ethanol (1 M) added</td>
<td>0.88 ± 0.38 (9)</td>
<td></td>
</tr>
<tr>
<td>Ethanol and superoxide dismutase added</td>
<td>0.003 ± 0.002 (5)</td>
<td></td>
</tr>
<tr>
<td>Ethanol, superoxide dismutase, and catalase added</td>
<td>0.52 ± 0.20 (1)</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.E. of (n) experiments.

### DISCUSSION

The studies reported here are summarized diagrammatically in Fig. 3. Xanthine, xanthine oxidase, chloride, and myeloperoxidase form a potent microbicidal system, and the requirement for \( \text{H}_2\text{O}_2 \) is indicated by the inhibitory effect of catalase and by the replacement of xanthine and xanthine oxidase with reagent \( \text{H}_2\text{O}_2 \). The formation of the \( \text{H}_2\text{O}_2 \) required for the myeloperoxidase-mediated antimicrobial system occurs in part via the intermediate formation of \( \text{O}_2^- \) since the microbicidal activity of the complete system is inhibited by cytochrome c and this inhibition is partially reversed by superoxide dismutase. A superoxide dismutase-reversible inhibition is observed when either ferri- or ferrocyanochrome c is employed. The products of the xanthine oxidase system both reduce and oxidize cytochrome c; ferricytochrome c is reduced by \( \text{O}_2^- \) and ferrocyanochrome c is oxidized by \( \text{H}_2\text{O}_2 \) (and myeloperoxidase). Oxidation of ferrocyanochrome c by hydroxyl radicals also may occur (21).
utilizes chloride and myeloperoxidase cannot be excluded. Benzene and ethanol may inhibit the microbicidal system by a mechanism other than the scavenging of hydroxyl radicals. They do not inhibit microbicidal activity when xanthine and xanthine oxidase are replaced by H₂O₂, suggesting that their effect is not directly on the peroxidase system.

Although an antimicrobial effect of the xanthine oxidase system in the absence of chloride and myeloperoxidase was not apparent under the conditions employed here, the studies do not preclude an antimicrobial effect under different experimental conditions. Indeed, an inhibition of the growth of X. aureus by the xanthine oxidase system due to the formation of H₂O₂ has been reported (23). Further, the microorganisms employed here (E. coli, S. aureus, C. tropicalis) grow well under aerobic conditions and such organisms as a group, contain higher levels of superoxide dismutase than do obligate or aerotolerant anaerobes (24, 25). Organisms deficient in superoxide dismutase may be more sensitive to the toxic effect of the superoxide anion than are the organisms employed in this study. Thus, E. coli B grown in an iron-deficient aerobic medium are not (26).

What the findings reported here emphasize is that the antimicrobial activity of the products of the xanthine-xanthine oxidase system is increased many orders of magnitude by the addition of chloride and myeloperoxidase. Although the superoxide anion can react directly with myeloperoxidase to form an enzyme-substrate complex (27), the inhibitory effect of catalase on microbicidal activity and the absence of an inhibition by superoxide dismutase at concentrations up to 88 μg per ml suggest that H₂O₂ is the substrate for peroxidase in this system. The O₂⁻, however, is an intermediate in the reaction and the interplay of scavengers of O₂⁻ and superoxide dismutase offers an opportunity for control of the over-all microbicidal process through control of H₂O₂ formation.

The report of O₂⁻ formation by intact polymorphonuclear leukocytes (15), its increase by phagocytosis (15) and its absence from the leukocytes of patients with chronic granulomatous disease (28r raises the possibility that this radical is an intermediate in the formation of H₂O₂ in the intact cell. If so then the activity of the peroxidase system in situ may be influenced by scavengers of the oxygen radicals and by superoxide dismutase in the same manner as the model system studied here.

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Role of the Superoxide Anion in the Myeloperoxidase-mediated Antimicrobial System
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