Susceptibility of Micro-organisms to Active Oxygen Species: Sensitivity to the Xanthine-oxidase-mediated Antimicrobial System

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Xanthine oxidase with acetaldehyde as substrate (the XOA system) generated superoxide anion and hydrogen peroxide, but this system had only weak bactericidal activity. Addition of Fe^{2+} and EDTA to the XOA system (XOA-Fe-EDTA system) increased bactericidal activity against *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhimurium*, although both *Mycobacterium tuberculosis* and *Candida albicans* remained highly resistant. Catalase (H_2O_2 scavenger) and mannitol (^1OH scavenger) almost completely inhibited the bactericidal activity of the XOA-Fe-EDTA system whereas SOD (O_2^- scavenger) was less inhibitory. Azide ($^1\text{O}_2$ scavenger) caused no such inhibition. The results suggest the possible role of ^1OH , H_2O_2 and O_2^- in the XOA-Fe-EDTA-mediated antimicrobial system, as effector molecules. There was no correlation between resistance of a given bacterium to active oxygen and the level of endogenous active oxygen-scavengers.

INTRODUCTION

Active oxygen species (Chance *et al.*, 1979; Krinsky, 1974; Rosen & Klebanoff, 1979*b*), the halogenation reaction (Klebanoff, 1967, 1968, 1982*a, b*; Klebanoff & Shepard, 1984), long-chain fatty acids (Kanai & Kondo, 1979; Saito *et al.*, 1984), and some cationic antibacterial peptides (Elsbach & Weiss, 1983; Pabst & Johnston, 1980) play important roles in the expression of microbicidal activity by macrophages and polymorphonuclear leucocytes. In particular, active oxygen species such as O_2^- , H_2O_2 , ^1OH and $^1\text{O}_2$ generated by these phagocytic cells in response to triggering by certain membrane perturbations and by some membrane activators (Dath & Karnovsky, 1975; Nathan, 1982; Oyanagui, 1981) exhibit a potent antimicrobial activity (Babior *et al.*, 1973). These active oxygen species can also be produced in a cell-free system consisting of xanthine oxidase (XO) and acetaldehyde (or xanthine) as a substrate (Rosen & Klebanoff, 1979*a*, 1981). The XOA system produces O_2^- , and H_2O_2 by spontaneous dismutation of the O_2^- . More toxic active oxygen radicals, ^1OH and $^1\text{O}_2$ (Hoidal *et al.*, 1979; Weiss *et al.*, 1977), are thought to emerge by the Haber-Weiss reaction: $\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow ^1\text{OH} + ^1\text{O}_2 + \text{OH}^-$. However, this reaction is very slow under physiological conditions (Halliwell, 1976), and the amount of ^1OH and $^1\text{O}_2$ generated spontaneously in the XOA system is negligible. Halliwell (1978*a, b*) reported that the addition of chelated iron such as Fe^{2+} -EDTA to the XOA system (XOA-Fe-EDTA system) resulted in a marked increase in the generation of ^1OH and $^1\text{O}_2$. The XOA-Fe-EDTA system is much more toxic to some bacteria, such as *Legionella pneumophila* and *Staphylococcus aureus*, than is the XOA system alone (Locksley *et al.*, 1982; Rosen & Klebanoff, 1981). Therefore, this active-oxygen-generating system is useful for studying the role of active oxygen, particularly ^1OH and $^1\text{O}_2$, in the intracellular killing of micro-organisms in phagocytic cells.

Abbreviations: SOD, superoxide dismutase; XO, xanthine oxidase; XOA, xanthine oxidase-acetaldehyde.

There are reports dealing with the antimicrobial activity of active oxygen in cell-free systems against *Staph. aureus* (Klebanoff, 1982*a*; Richmond *et al.*, 1981; Rister & Baehner, 1976; Rosen & Klebanoff, 1979*a*), *Escherichia coli* (Gregory *et al.*, 1973), *Candida* (Sasada & Johnston, 1980), *Legionella pneumophila* (Locksley *et al.*, 1982) and *Nocardia asteroides* (Filice *et al.*, 1980; Filice, 1983) as targets. However, no comparative study exists of the sensitivity of various organisms to active-oxygen-generating systems. In the present paper, we compare the sensitivity of *E. coli* and *Staph. aureus* (extracellular parasites), and of *Listeria monocytogenes*, *Salmonella typhimurium*, *Mycobacterium tuberculosis* and *Candida albicans* (intracellular parasites), to the XOA-Fe-EDTA system.

METHODS

Organisms. The following microbial strains were used (source in parentheses): *Escherichia coli* 81 and *Staphylococcus aureus* Smith (A. Matsumae, Kitasato Institute, Tokyo, Japan); *Listeria monocytogenes* EGD (K. Takeya, Kyushu University, Fukuoka, Japan); *Salmonella typhimurium* HKB-1 and *Candida albicans* NIH A207 (M. Kuwabara, Hiroshima Prefectural Hospital, Hiroshima, Japan); *C. guilliermondii* ATCC 9058, *C. krusei* Nagao 7493, *C. parapsilosis* ATCC 6295, *C. pseudotropicalis* IFO 0942, *C. stellatoidea* IFO 0692 and *C. tropicalis* IFO 0618 (S. Kodaira, Yakult Central Institute for Microbiological Research, Tokyo); *Mycobacterium tuberculosis* H₃₇Rv, *M. kansasii* ATCC 12478, *M. intracellulare* ATCC 13950 and *M. chelonae* subsp. *abscessus* ATCC 19977 (A. Y. Tsang, National Jewish Hospital and Research Center, Denver, Colorado, USA).

Chemicals. Xanthine oxidase (XO) (grade III), superoxide dismutase (SOD) (type I), catalase (from bovine liver, thymol-free), cytochrome *c* (type III) and horseradish peroxidase (type II) were purchased from Sigma. Acetaldehyde was purchased from Merck. All other chemicals were purchased from Wako Pure Chemical.

Assay for antimicrobial activity of the XOA-Fe-EDTA system. *E. coli*, *Staph. aureus*, *L. monocytogenes* and *S. typhimurium* were cultured in tryptose broth (Eiken Chemical Co.), with occasional shaking, at 37 °C for 18 h. *Candida* spp. were cultured in Sabouraud dextrose broth (Eiken) at 37 °C for 2 d and *Mycobacterium* spp. in Dubos Tween-albumin medium (Eiken) at 37 °C for 3–7 d. Cells were harvested by centrifugation (1000 *g*, 15 min), washed twice with 0.1 M-sodium sulphate, and suspended in 0.1 M-sodium phosphate buffer (pH 7.0) or 0.2 M-sodium acetate buffer (pH 5.5). The OD₅₄₀ of the microbial suspension was adjusted to the desired values using a Hitachi model 100-10 spectrophotometer. The reaction mixture (2 ml), consisting of 20 µg XO ml⁻¹, 10 mM-acetaldehyde, 0.1 mM-ferrous sulphate, 0.1 mM-EDTA and microbial suspension (5×10^5 ml⁻¹) in 38 mM-sodium phosphate (pH 7.0) or 40 mM-sodium acetate buffer (pH 5.5) was incubated at 37 °C with shaking for 60 min. The resultant microbial suspension was then serially diluted 10-fold with 0.15 M-saline, and the number of c.f.u. was determined on tryptose agar (Eiken) for *E. coli*, *Staph. aureus*, *L. monocytogenes* and *S. typhimurium*, Sabouraud dextrose agar (Eiken) for *Candida* species and Middlebrook 7H10 agar (Difco) for mycobacteria.

Antimicrobial effect of H₂O₂. Organisms suspended in 0.1 M-sodium phosphate buffer (pH 7.0) containing various concentrations of H₂O₂ (determined by absorbance at 230 nm) were incubated with shaking in a water bath at 37 °C for 1 h. The number of c.f.u. was determined as mentioned above.

Measurement of active oxygen concentration. OH production in the XOA-Fe-EDTA system was assayed by the method of Richmond *et al.* (1981). Briefly, the reaction mixture (4 ml), containing 2.5 mM-sodium salicylate, 0.1 mM-ferrous sulphate, 0.1 mM-EDTA, 10 mM-acetaldehyde and 20 µg XO ml⁻¹ in 38 mM-sodium phosphate buffer (pH 7.0), was incubated with shaking at 37 °C for 60 min. After incubation, 60 µl 11.6 M-HCl was added and cold diethyl ether extraction carried out. The extract was dissolved in 1 ml cold double-distilled water, to which 0.13 ml 10% (w/v) trichloroacetic acid in 0.5 M-HCl, 0.25 ml 10% (w/v) sodium tungstate and 0.25 ml 0.5% (w/v) sodium nitrite were added. The preparation was left to stand for 5 min, 2 ml 0.5 M-KOH was added and the A₅₁₀ measured. An appropriate standard curve was prepared using 2,3-dihydroxybenzoate.

H₂O₂ production was measured by oxidation of phenol red according to Pick & Keisari (1980). The reaction mixture (4 ml), containing 0.25 mM-phenol red, 50 µg horseradish peroxidase ml⁻¹, 10 mM-acetaldehyde and 20 µg XO ml⁻¹ in 38 mM-sodium phosphate buffer (pH 7.0), was incubated with shaking at 37 °C for 60 min. After incubation, 0.1 ml 1 M-NaOH was added, and after boiling for 3 min, oxidation of phenol red was quantified spectrophotometrically at 610 nm.

The assay of O₂⁻ was based on its ability to reduce cytochrome *c*, according to the method of Babior *et al.* (1973). The reaction mixture (2 ml), containing 160 µM-cytochrome *c*, 60 µg catalase ml⁻¹, 10 mM-acetaldehyde and 20 µg XO ml⁻¹ in 38 mM-sodium phosphate buffer (pH 7.0), was incubated with shaking at 37 °C for 60 min. and then reduction of cytochrome *c* was quantified spectrophotometrically at 550 nm.

Measurement of endogenous SOD and catalase. Organisms were harvested by centrifugation (1000 *g*, 15 min), washed and suspended in 0.15 M-saline. The microbial cells were disrupted with an ultrasonic disintegrator (Insonator, model 200M, Kubota Shoji Co.) at 2 A for 2 min and centrifuged at 20000 *g* for 15 min. The supernate

was assayed for SOD and catalase, and its protein concentration was determined by the Lowry method. The assay for SOD, as described by Rister & Baehner (1976), was based on the capacity of SOD to inhibit cytochrome *c* reduction mediated by O_2^- generated in the XOA system. The reaction mixture (2 ml), consisting of 5 mM-acetaldehyde, 40 μg XO ml^{-1} , 60 μg catalase ml^{-1} and microbial cell extract in 50 mM-sodium phosphate buffer (pH 7.8), was incubated at 25 °C, and the change in the A_{550} was recorded. Bovine SOD was used as a standard. Catalase was assayed spectrophotometrically by the method of Beers & Sizer (1952). The reaction mixture (2 ml), consisting of 15 mM- H_2O_2 and microbial cell extract in 50 mM-sodium phosphate buffer (pH 7.0), was incubated at 25 °C, and the change in A_{240} was recorded. Bovine catalase was used as a standard.

RESULTS

Antimicrobial activity of the XOA-Fe-EDTA system

Table 1 shows representative results (similar experiments were done at least four times) on the microbicidal activity of the XOA system supplemented with a fixed concentration (100 μM) of EDTA and varying concentrations of Fe^{2+} at pH 7.0 and 5.5.

Although the XOA system alone exhibited little microbicidal activity against the test organisms, the activity was markedly augmented by simultaneous addition of EDTA and Fe^{2+} . At pH 7.0, *E. coli*, *Staph. aureus*, *L. monocytogenes* and *S. typhimurium* were killed in the presence of 10–1000 μM - Fe^{2+} ; 100 μM - Fe^{2+} (equimolar to the EDTA) was optimum for the expression of microbicidal activity. Similar results were obtained in the XOA-Fe-EDTA system at pH 5.5, except for the following. First, *Staph. aureus*, *L. monocytogenes* and *S. typhimurium* were more readily killed at the optimum concentration of Fe^{2+} (100 μM) at pH 5.5 than at pH 7.0. Second, excess amounts of Fe^{2+} over 100 μM caused a more marked reduction in the activity of the antimicrobial system at pH 5.5 than at pH 7.0.

The XOA-Fe-EDTA system failed to kill any of the *Mycobacterium* or *Candida* species tested (see Methods), even in the presence of an optimum dose of Fe^{2+} , at pH 7.0 or pH 5.5.

Table 1. *Microbicidal effects of active oxygen species in the XOA system at pH 7.0 and pH 5.5*

The organisms ($5 \times 10^5 \text{ ml}^{-1}$) were incubated in the XOA system (2 ml) at pH 7.0 (a) or 5.5 (b), with or without 100 μM -EDTA and with various concentrations of Fe^{2+} . Numbers of surviving organisms after 60 min incubation were counted. The results are means of two determinations $\pm$ the range.

O_2^- - generating system	Addition		Survival [log (c.f.u. per incubation mixture)]					
	EDTA	Fe^{2+} (μM)	<i>Staph.</i> <i>aureus</i>	<i>L. monocyto-</i> <i>genes</i>	<i>E. coli</i>	<i>S. typhi-</i> <i>murium</i>	<i>M. tuber-</i> <i>culosis</i>	<i>C. albicans</i>
(a) Incubation at pH 7.0								
—*	—	—	5.91 $\pm$ 0.01	5.44 $\pm$ 0.02	5.78 $\pm$ 0.01	5.94 $\pm$ 0.01	5.64 $\pm$ 0.01	5.45 $\pm$ 0.01
XOA	—	—	5.72 $\pm$ 0.20	5.25 $\pm$ 0.07	5.69 $\pm$ 0.10	5.78 $\pm$ 0.15	5.53 $\pm$ 0.01	5.42 $\pm$ 0.01
XOA	+	—	5.52 $\pm$ 0.07	5.32 $\pm$ 0.02	5.49 $\pm$ 0.02	5.56 $\pm$ 0.01	5.52 $\pm$ 0.10	5.47 $\pm$ 0.01
XOA	+	1	5.27 $\pm$ 0.01	5.13 $\pm$ 0.05	4.60 $\pm$ 0.01	5.63 $\pm$ 0.04	5.35 $\pm$ 0.14	5.46 $\pm$ 0.01
XOA	+	10	5.16 $\pm$ 0.04	4.88 $\pm$ 0.04	1.69 $\pm$ 0.07	5.22 $\pm$ 0.04	5.52 $\pm$ 0.05	5.45 $\pm$ 0.01
XOA	+	10^2	3.70 $\pm$ 0	2.40 $\pm$ 0.11	0	2.56 $\pm$ 0.32	5.46 $\pm$ 0.01	5.40 $\pm$ 0.01
XOA	+	10^3	3.81 $\pm$ 0.12	2.25 $\pm$ 0.01	0	2.57 $\pm$ 0.24	5.40 $\pm$ 0.03	5.45 $\pm$ 0
(b) Incubation at pH 5.5								
—*	—	—	5.60 $\pm$ 0	5.83 $\pm$ 0.02	5.68 $\pm$ 0.01	5.88 $\pm$ 0	5.41 $\pm$ 0.07	5.89 $\pm$ 0.02
XOA	—	—	5.59 $\pm$ 0.06	5.46 $\pm$ 0.31	5.60 $\pm$ 0.04	5.84 $\pm$ 0.03	5.32 $\pm$ 0.06	5.77 $\pm$ 0.05
XOA	+	—	5.59 $\pm$ 0.07	5.73 $\pm$ 0.13	5.65 $\pm$ 0.04	5.82 $\pm$ 0.02	5.40 $\pm$ 0	5.73 $\pm$ 0.02
XOA	+	1	4.21 $\pm$ 0.10	5.40 $\pm$ 0.02	3.56 $\pm$ 0.05	4.37 $\pm$ 0.09	5.33 $\pm$ 0.07	5.69 $\pm$ 0.06
XOA	+	10	4.52 $\pm$ 0.20	5.08 $\pm$ 0.20	3.50 $\pm$ 0.02	4.32 $\pm$ 0.04	5.28 $\pm$ 0	5.74 $\pm$ 0.02
XOA	+	10^2	2.65 $\pm$ 0.16	0	0	1.73 $\pm$ 0.03	5.08 $\pm$ 0.03	5.74 $\pm$ 0.02
XOA	+	10^3	4.92 $\pm$ 0	2.75 $\pm$ 0.04	5.30 $\pm$ 0.04	5.79 $\pm$ 0.05	5.38 $\pm$ 0.13	5.83 $\pm$ 0.01

* Buffer alone.

Table 2. *Effects of active oxygen scavengers on the bactericidal activity of the XOA-Fe-EDTA system*

The organisms ($5 \times 10^5 \text{ ml}^{-1}$) were incubated for 60 min in the XOA-Fe-EDTA system ($100 \mu\text{M}$ each of EDTA and Fe^{2+}) in the presence or absence of the indicated scavengers. The activities of SOD and catalase were 2800 and 11000 units (mg protein^{-1}), respectively. The results are means of two determinations $\pm$ the range.

Addition	Survival rate (%)			
	<i>Staph. aureus</i>	<i>L. monocytogenes</i>	<i>E. coli</i>	<i>S. typhimurium</i>
SOD ($\mu\text{g ml}^{-1}$)				
0	<0.1	<0.1	<0.1	<0.1
10	<0.1	<0.1	<0.1	<0.1
100	8.0 ± 1.4	19.0 ± 3.7	56.0 ± 9.0	44.2 ± 0.9
Catalase ($\mu\text{g ml}^{-1}$)				
0	1.3 ± 0.3	0.6 ± 0.1	<0.1	<0.1
60	142.9 ± 11.0	57.7 ± 3.9	93.1 ± 5.2	112.5 ± 4.7
120	170.3 ± 16.5	84.6 ± 5.1	101.7 ± 0	114.1 ± 18.8
Mannitol (mM)				
0	0.4 ± 0.1	0.4 ± 0.1	<0.1	0.1 ± 0
10	73.4 ± 0.9	66.7 ± 1.7	64.6 ± 9.2	86.4 ± 2.0
100	120.9 ± 4.2	74.2 ± 0.9	81.6 ± 3.1	94.2 ± 0.7
Azide (mM)				
0	0.5 ± 0.3	0.7 ± 0	<0.1	<0.1
1	2.5 ± 0.1	6.3 ± 0.8	2.6 ± 0.2	6.5 ± 0.1
10	4.8 ± 0.5	10.1 ± 0.3	5.1 ± 0.3	7.0 ± 0.1

Roles of active oxygen species in the manifestation of antimicrobial activity of the XOA-Fe-EDTA system

Table 2 shows effects of various active oxygen-scavengers on killing of test bacteria in the XOA-Fe-EDTA system at pH 7.0. Representative results of four separate experiments are given. Bactericidal activity of the XOA-Fe-EDTA system against these bacteria was markedly inhibited by catalase (H_2O_2 scavenger) at 60–120 $\mu\text{g ml}^{-1}$ and by mannitol ($\cdot\text{OH}$ scavenger) at 10–100 mM. SOD (O_2^- scavenger) showed a lower but significant inhibitory activity against the XOA-Fe-EDTA system: at 50 $\mu\text{g ml}^{-1}$ it led to no appreciable reduction in the activity of the XOA-Fe-EDTA system, but a significant inhibition of the antimicrobial system was seen with 100 $\mu\text{g SOD ml}^{-1}$. In contrast, azide ($^1\text{O}_2$ scavenger) had no significant effect on the activity of the XOA-Fe-EDTA system, even at 10 mM. These results indicate that H_2O_2 , $\cdot\text{OH}$ and also O_2^- may play an important role in the expression of antimicrobial activity of the XOA-Fe-EDTA system, although mannitol and azide are not strictly specific scavengers of $\cdot\text{OH}$ and $^1\text{O}_2$, respectively. Similar results were also obtained at pH 5.5 (data not shown).

Fig. 1 shows representative results for the time course of active oxygen production in the XOA-Fe-EDTA system. O_2^- was generated rapidly ($1.1 \text{ nmol min}^{-1} \text{ ml}^{-1}$) and reached a plateau at 30 min. Generation of H_2O_2 , as a result of spontaneous dismutation of O_2^- , was somewhat slower ($0.75 \text{ nmol min}^{-1} \text{ ml}^{-1}$). The generation rate of $\cdot\text{OH}$ ($0.1 \text{ nmol min}^{-1} \text{ ml}^{-1}$) was much less than that of the former species. It should be noted that the apparent $\cdot\text{OH}$ generation is less than the actual one, since the $\cdot\text{OH}$ radical is very unstable (Babior, 1978). Fig. 2 shows effects of SOD on the generation of H_2O_2 and $\cdot\text{OH}$ in the XOA and XOA-Fe-EDTA systems (60 min incubation), respectively. The generation of both H_2O_2 and $\cdot\text{OH}$ significantly increased in the presence of SOD at 10–100 $\mu\text{g ml}^{-1}$. The production of $\cdot\text{OH}$ in the XOA-Fe-EDTA system was highest when EDTA and Fe^{2+} were present in approximately equimolar amounts (data not shown). This is consistent with the observation that the antimicrobial activity of the XOA-Fe-EDTA system was greatest when equimolar amounts of EDTA and Fe^{2+} were added (Table 1). This may suggest that $\cdot\text{OH}$ is important in the bactericidal mechanism of the XOA-Fe-EDTA system.

Fig. 1

Fig. 2

Fig. 1. Generation of active oxygen species in the XOA-Fe-EDTA system. Generation of O_2^- ($\blacktriangle$) and of H_2O_2 ($\blacksquare$) in the XOA system alone, and that of $\cdot OH$ ($\bullet$) in the XOA system supplemented with $100\ \mu M$ each of Fe^{2+} and EDTA, were measured as described in Methods. Each point indicates the mean $\pm$ the range ($n = 2$) (a horizontal bar indicates that the results were identical).

Fig. 2. Effect of SOD on the generation of active oxygen species in the XOA and XOA-Fe-EDTA systems. H_2O_2 ($\bullet$) and $\cdot OH$ ($\blacktriangle$) were generated in the XOA and XOA-Fe-EDTA systems ($100\ \mu M$ each of Fe^{2+} and EDTA) containing SOD at the indicated concentrations, respectively. Each point indicates the mean $\pm$ the range ($n = 2$).

Fig. 3. Antimicrobial activity of H_2O_2 against *L. monocytogenes* ($\circ$), *Staph. aureus* ($\triangle$), *E. coli* ($\square$), *S. typhimurium* ($\bullet$), *C. albicans* ($\blacksquare$) and *M. tuberculosis* ($\blacktriangle$). For details see Methods. Each point indicates the mean $\pm$ the range ($n = 2$) (a horizontal bar indicates that the results were identical).

Fig. 3 shows the antimicrobial activity of H_2O_2 . *Staph. aureus*, *E. coli*, *S. typhimurium*, *L. monocytogenes* and *C. albicans* were sensitive to H_2O_2 and almost completely destroyed by H_2O_2 at concentrations above $5\ mM$. In contrast, *M. tuberculosis* was resistant to H_2O_2 , even at $10\ mM$. However, it should be noted that all the test organisms, except *Staph. aureus*, were resistant to $0.5\ mM$ - H_2O_2 and that this amount is nearly ten times the concentration of H_2O_2 accumulated in the XOA-Fe-EDTA system during the 60 min incubation (Figs 1 and 2).

Table 3. *Content of endogenous scavengers in the test organisms*

Organism	Peroxidase-catabolizing enzyme* [units (mg protein) ⁻¹]	
	SOD	Catalase
<i>E. coli</i>	12.8 ± 0.3	6.7 ± 1.2
<i>Staph. aureus</i>	12.1 ± 1.4	60.0 ± 4.3
<i>L. monocytogenes</i>	39.7 ± 5.1	113.3 ± 12.3
<i>S. typhimurium</i>	12.1 ± 1.5	6.0 ± 0.5
<i>M. tuberculosis</i>	5.5 ± 0.3	0.8 ± 0.1
<i>C. albicans</i>	61.0 ± 9.0	1.9 ± 0.4

* The results are means ± standard error ($n = 3$).

Amount of endogenous active oxygen-scavengers in the test organisms

Table 3 shows the content of endogenous active oxygen-scavengers in the organisms used in this study. *C. albicans*, which was resistant to the XOA-Fe-EDTA system, had the highest content of SOD, followed by *L. monocytogenes*. Although *M. tuberculosis* had the least SOD, it was resistant to the XOA-Fe-EDTA system (Table 1). The catalase content differed markedly from species to species (Table 3). There was no statistical correlation (Student's *t* test, $P \geq 0.1$) between the content of SOD or catalase of a given microbe and its susceptibility to the XOA-Fe-EDTA system. Indeed, *C. albicans* and *M. tuberculosis*, resistant to the XOA-Fe-EDTA system, had a much lower content of catalase than the other organisms. Also there was no significant difference in the content of endogenous scavengers between the intra- and extracellular parasites.

DISCUSSION

The XOA-Fe-EDTA system has been used as a model of oxygen-dependent bactericidal mechanisms of phagocytes (Rosen & Klebanoff, 1979*a*). The XOA system generates O_2^- and H_2O_2 and, in the presence of iron-EDTA, also $\cdot OH$ and 1O_2 (Rosen & Klebanoff, 1981). This system had a potent microbicidal effect on all the organisms tested in the present study, except for *C. albicans* and *M. tuberculosis*. H_2O_2 at the concentration achieved in the XOA system (about 50 μM) failed to kill any of the organisms except *Staph. aureus* (Fig. 3), suggesting that H_2O_2 is unlikely to play a central role in the XOA-Fe-EDTA system as an effector molecule. However, the role of H_2O_2 in this microbicidal system cannot be excluded, because the microbicidal activity of H_2O_2 is known to increase when H_2O_2 is continuously generated (Klebanoff, 1969). As shown in Table 1, both bactericidal activity and production of $\cdot OH$ in the XOA-Fe-EDTA system were highest in the presence of equimolar amounts of Fe^{2+} and EDTA. This suggests that the $\cdot OH$ radical is one of the important species in the bactericidal mechanism of the XOA-Fe-EDTA system. However, the finding that $\cdot OH$ generation in the XOA-Fe-EDTA system increased two- to fourfold in the presence of SOD at 10–100 $\mu g\ ml^{-1}$ (Fig. 2) is inconsistent with the observation that 100 μg SOD ml^{-1} caused a significant reduction in the microbicidal activity of the system (Table 2). Thus, it seems that not only $\cdot OH$ but also O_2^- can play an important role in the microbicidal system. The reduction in the bactericidal activity of the XOA-Fe-EDTA system by catalase may be attributed to the almost complete removal of H_2O_2 by the enzyme, hence a cessation of $\cdot OH$ generation.

The present observations indicate an important role for $\cdot OH$ in the microbicidal mechanisms of our XOA-Fe-EDTA system. $\cdot OH$ is also thought to be important in the intracellular killing of bacteria by macrophages and polymorphonuclear leucocytes, in which XOA and EDTA may be replaced by NADPH oxidase-NADPH and probably tryptophan or ADP as an iron-chelating agent, respectively (Oyanagui, 1981). There are reports that the $\cdot OH$ radical participates in the oxygen-dependent microbial killing of these phagocytic cells (Babior, 1978; Hoidal *et al.*, 1979; Rosen & Klebanoff, 1979*b*), although other investigators have proposed an important role for

O_2^- (Yost & Fridovich, 1974), H_2O_2 (Boveris *et al.*, 1980; Dockrell & Playfair, 1984; Murray *et al.*, 1980; Murray, 1981), and 1O_2 (Krinsky, 1974) as direct effector molecules against micro-organisms rather than $^{\cdot}OH$, since $^{\cdot}OH$ is too unstable to pass through the cell wall into the cytoplasm.

We found no tendency for the intracellular parasites to be more resistant to the direct toxic effect of active oxygen radicals. However, *Mycobacteria* and *Candida* species were completely resistant. Active oxygen species presumably oxidize certain essential proteins or enzymes (Chance *et al.*, 1979). *Mycobacteria* contain abundant lipid, and lipid content correlates well with their resistance to active oxygen radicals (Goren *et al.*, 1974*a, b*). Therefore, these lipids in the mycobacterial cell wall may be able to trap toxic active oxygen radicals before any oxidation of membrane proteins occurs. If so, this may account for the high resistance of *Candida* species to the XOA-Fe-EDTA system; *C. albicans* also has a relatively large amount of lipid in its cell surface (Sato *et al.*, 1968).

Although there are reports that the endogenous scavenger (SOD and catalase) content of bacteria correlates with their resistance to active oxygen species (Amin & Olson, 1968; Gregory *et al.*, 1973; Howard, 1981; Mandell, 1975; Murray *et al.*, 1980; Murray, 1981), our results are closer to the observations of Filice *et al.* (1980), Filice (1983) and Gangadharam & Pratt (1984), who found that the SOD and catalase contents of *Nocardia asteroides*, *M. intracellulare* and *M. tuberculosis* did not correlate with their resistance to killing by H_2O_2 or by the lactoperoxidase- H_2O_2 -iodide system. In addition, we found no obvious difference between intra- and extracellular parasites, in terms of their oxygen scavenger content. The level of endogenous scavengers for active oxygen species is probably not the only factor determining the sensitivity of a micro-organism to active oxygen species.

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