Relationship between the Susceptibility of Various Bacteria to Active Oxygen Species and to Intracellular Killing by Macrophages

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The susceptibilities of six micro-organisms to active oxygen species generated in the xanthine oxidase-mediated bactericidal system were as follows: Escherichia coli 81 > Listeria monocytogenes EGD > Salmonella typhimurium HKB 1 > Staphylococcus aureus Smith > Mycobacterium tuberculosis H37 Rv ≈ Candida albicans NIH A207 (the last two organisms were essentially resistant to this system). The H2O2–Fe-mediated halogenation system exhibited a higher microbicidal activity. When the micro-organisms were compared for their sensitivity to bactericidal activity of resident mouse peritoneal macrophages (Mφs), C. albicans, Staph. aureus and E. coli were killed rapidly, whereas M. tuberculosis, L. monocytogenes and S. typhimurium were more resistant. In tests for the ability to trigger an oxidative burst in mouse peritoneal Mφs (as measured by chemiluminescence), Staph. aureus showed the highest activity followed by the other organisms in the following order: C. albicans > E. coli > L. monocytogenes  ≈ M. tuberculosis. S. typhimurium exhibited no triggering activity. The high susceptibility of Staph. aureus and E. coli to Mφ bactericidal activity, and the partial resistance of L. monocytogenes and M. tuberculosis, correlated with their susceptibility to active oxygen and the H2O2–Fe-mediated halogenation reaction.

INTRODUCTION

In the foregoing paper (Yamada et al., 1987), we reported that O2− and ·OH play important roles in the expression of antimicrobial activity by the xanthine oxidase–acetaldehyde (XOA)–Fe–EDTA system, which enzymically generates active oxygen species. This antimicrobial system readily killed Staphylococcus aureus, Escherichia coli, Listeria monocytogenes and Salmonella typhimurium, but Candida albicans and Mycobacterium tuberculosis were highly resistant to it. Antimicrobial activity of active oxygen species can be demonstrated directly, but also by H2O2-mediated halogenation reactions such as the myeloperoxidase–H2O2–halide-mediated bactericidal system in polymorphonuclear leucocytes (PMNs) (Klebanoff, 1968, 1974; Klebanoff and Sheppard, 1984; Locksley et al., 1982). Although macrophages (Mφs) have no myeloperoxidase (Klebanoff, 1980), iron at physiological concentrations (Flitter et al., 1983; Gutteridge et al., 1981; Halliwell & Gutteridge, 1984) or endogenous catalase can catalyse the halogenation system (Gee et al., 1970; Klebanoff, 1969, 1982a; Klebanoff & Hamon, 1975; LeVitz & Diamond, 1984). At the acid pH achieved within the phagolysosome, the iron-mediated halogenation system acts more efficiently and has a more potent antimicrobial activity than at neutral and alkaline pH (Klebanoff, 1982a; LeVitz & Diamond, 1984). Therefore, H2O2–Fe-mediated halogenation is thought to be important in the microbicidal mechanism of Mφs. In this study, we examined the susceptibility of various organisms to the H2O2–Fe-mediated halogenation system and to the antimicrobial action of mouse peritoneal Mφs.

Abbreviations: i.p., intraperitoneal(ly); Mφ, macrophage; PEC, peritoneal exudate cells; PMA, phorbol myristate acetate; PMN, polymorphonuclear leucocyte; XOA, xanthine oxidase–acetaldehyde.
METHODS

Animals. Four-week-old female ddY mice were purchased from the Shizuoka Laboratory Animal Center, Shizuoka, Japan. At the time of experiment, they were 6 to 8 weeks old.

Organisms. The test organisms and their sources were as follows: *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), bacillus Calmette-Guérin (BCG) (Japan BCG Laboratory, Tokyo). Organisms were cultured as described previously (Yamada et al., 1987).

Chemicals. All reagents were purchased from Wako Pure Chemical.

Microbicidal activity of \( \text{H}_2\text{O}_2-\text{Fe}-\text{iodide} \) system. Microbicidal activity of the \( \text{H}_2\text{O}_2-\text{Fe}-\text{iodide} \) system was measured according to the method of Klebanoff (1982a). The reaction mixture (2 ml), consisting of 10 or 100 \( \mu \text{M} \)-sodium iodide and \( \text{H}_2\text{O}_2 \), various concentrations of ferrous sulphate, 20 \( \mu \text{M} \)-acetate buffer (pH 5.5), and test organisms (final concentration \( 5 \times 10^5 \text{ c.f.u. ml}^{-1} \)), was incubated in a shaking water bath at 37 °C for 60 min. The number of c.f.u. of the resultant microbial suspension was then determined on appropriate agar media after serial 10-fold dilutions with 0.15 M-saline (Yamada et al., 1987). Fe-mediated halogenation system was measured according to the method of Klebanoff (1982a). The reaction mixture (2 ml), consisting of 10 or 100 \( \mu \text{M} \)-sodium iodide and \( \text{H}_2\text{O}_2 \), various concentrations of ferrous sulphate, 20 \( \mu \text{M} \)-acetate buffer (pH 5.5), and test organisms (final concentration \( 5 \times 10^5 \text{ c.f.u. ml}^{-1} \)), was incubated in a shaking water bath at 37 °C for 60 min. The number of c.f.u. of the resultant microbial suspension was then determined on appropriate agar media after serial 10-fold dilutions with 0.15 M-saline (Yamada et al., 1987).

Microbicidal activity of host peritoneal MΦs. Mice were given intraperitoneally (i.p.) viable (0-01 mg per mouse) and heat-killed (0-1 mg per mouse) BCG 21 d and 3 d before microbial challenge, respectively. Normal and BCG-treated mice were infected i.p. with \( 1 \times 10^6 \) test organisms suspended in saline. Two hours later, micro-organisms present in the peritoneal cavity were harvested in Hanks' balanced salt solution (HBSS) and sonicated for 20 s with a Handy Sonic model UR-20P (Tomy Seiko Co., power 10) to free and disperse the phagocytosed micro-organisms, and the number of c.f.u. was determined. To determine the virulence of test organisms, mice were injected i.p. with \( 1 \times 10^6 \) organisms per mouse and survival of the infected animals was observed for up to 4 weeks.

Measurement of MΦ oxidative burst. This was done by a chemiluminescence method. Mouse peritoneal exudate cells (PEC) were harvested with HBSS 4 d after i.p. injection of 1 mg yzmosan A per mouse. The PEC contained more than 85% of MΦs, with 9% contamination by PMNs. The incubation mixture (1 ml), consisting of PEC (10⁶ cells in 0-9 ml) and 0-1 ml 0-1 mM-luminol, was measured for background chemiluminescence at 37 °C in a Toyo Kagaku lumicounter ATP-958. After addition of various concentrations of microbial suspension (0-1 ml), light emissions were counted for 10 s at 2 min intervals for up to 10 min. After the incubation, PEC were smeared on a glass slide, stained with Giemsa stain and the number of MΦ-associated organisms was counted microscopically.

RESULTS

Susceptibility of various micro-organisms to the \( \text{H}_2\text{O}_2-\text{Fe}-\text{mediated} \) halogenation system

Table 1 shows the susceptibility of the test organisms to microbicidal activity of \( \text{H}_2\text{O}_2-\text{Fe}-\text{iodide} \)-mediated halogenation system. In the presence of 10 \( \mu \text{M} \) each of \( \text{H}_2\text{O}_2 \) and iodide but in the absence of \( \text{Fe}^{2+} \), considerable killing of *L. monocytogenes* was noted, whereas the other organisms were not killed. Addition of 10 \( \mu \text{M} \)-Fe\(^{2+}\) markedly augmented the microbicidal activity of this system, and killing of the test organisms was observed in the following order: *Staph. aureus* \( \simeq *E. coli* \( \simeq *S. typhimurium* > *L. monocytogenes* > *M. tuberculosis*. Although *C. albicans* was most resistant to this system, reductions of two orders of magnitude in the numbers of c.f.u. of the organisms was observed in the presence of equimolar amounts of \( \text{Fe}^{2+} \) and iodide. In the presence of 100 \( \mu \text{M} \) each of \( \text{H}_2\text{O}_2 \) and iodide, rapid killing of *Staph. aureus, E. coli, S. typhimurium* and *L. monocytogenes* was noted, even in the absence of \( \text{Fe}^{2+} \). In contrast, *M. tuberculosis* and *C. albicans* were killed in the halogenation system with 100 \( \mu \text{M} \) each of \( \text{H}_2\text{O}_2 \) and iodide only when \( \text{Fe}^{2+} \) was added at concentrations over 10 \( \mu \text{M} \). In summary, the sensitivity of test micro-organisms to the \( \text{H}_2\text{O}_2-\text{Fe-mediated} \) halogenation system was in the order *S. typhimurium* \( \simeq *E. coli* \( \simeq *Staph. aureus* > *L. monocytogenes* > *M. tuberculosis* > *C. albicans*.

Susceptibility of various organisms to the microbicidal activity of MΦs

Table 2 shows the susceptibility of test organisms to mouse peritoneal MΦ-mediated killing *in vivo*. As indicated in experiment 1, *C. albicans, Staph. aureus* and *E. coli* were readily eliminated from the peritoneal cavity of the normal mice during the first 2 h after i.p. injection, whereas *S. typhimurium, L. monocytogenes* and *M. tuberculosis* were less readily killed. Since resident MΦs were dominant in the PEC populations of the normal mice [nearly 60% of the PEC were MΦs with a very low capacity to produce \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) in response to phorbol myristate acetate (PMA)], and PMN contamination was low, the elimination of *C. albicans, Staph. aureus* and *E.
Table 1. Microbicidal effect of the \( \text{H}_2\text{O}_2 \)-iodide-Fe-mediated halogenation system (pH 5.5)

The results are means of two determinations ± the range. A value of 0 indicates that no survivors were detected. The initial values of log c.f.u. were as follows: \textit{Staph. aureus}, 5·80 ± 0·02; \textit{E. coli}, 5·74 ± 0·01; \textit{L. monocytogenes}, 5·88 ± 0·01; \textit{S. typhimurium}, 5·88 ± 0·01; \textit{M. tuberculosis}, 5·39 ± 0·01; \textit{C. albicans}, 5·85 ± 0·02.

<table>
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<tr>
<th>Addition (µM)</th>
<th>\textit{Staph. aureus}</th>
<th>\textit{E. coli}</th>
<th>\textit{L. monocytogenes}</th>
<th>\textit{S. typhimurium}</th>
<th>\textit{M. tuberculosis}</th>
<th>\textit{C. albicans}</th>
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<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>Iodide</td>
<td>Fe(^{2+} )</td>
<td>Survival (log c.f.u.)</td>
<td>Survival (log c.f.u.)</td>
<td>Survival (log c.f.u.)</td>
<td>Survival (log c.f.u.)</td>
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<td>0</td>
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<td>0</td>
<td>5·66 ± 0·01</td>
<td>5·68 ± 0·02</td>
<td>5·89 ± 0·01</td>
<td>5·90 ± 0·01</td>
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<td>10</td>
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<td>0</td>
<td>5·74 ± 0·02</td>
<td>5·72 ± 0·02</td>
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<td>5·69 ± 0·04</td>
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<td>0</td>
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<td>5·29 ± 0·03</td>
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<td>5·74 ± 0·18</td>
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<td>5·51 ± 0·31</td>
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Table 2. Susceptibility of the test organisms to microbicidal activity of Mφ

Mice were given i.p. 1 × 10⁶ each of the indicated organisms, and the number of viable organisms in the peritoneal cavity was measured 2 h later. The number of organisms at 0 h is fixed at 100%. The results are means ± standard error (n = 3).

<table>
<thead>
<tr>
<th>Survival (%)</th>
<th>\textit{Staph. aureus}</th>
<th>\textit{E. coli}</th>
<th>\textit{L. monocytogenes}</th>
<th>\textit{S. typhimurium}</th>
<th>\textit{M. tuberculosis}</th>
<th>\textit{C. albicans}</th>
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<tr>
<td>Expt 1: normal mice (resident Mφ)</td>
<td>9·3 ± 0·2</td>
<td>10·5 ± 0·9</td>
<td>81·3 ± 23·3</td>
<td>55·0 ± 5·2</td>
<td>93·7 ± 6·0</td>
<td>4·2 ± 0·1</td>
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<tr>
<td>Expt 2: BCG-treated mice (activated Mφ)*</td>
<td>8·6 ± 1·0</td>
<td>1·5 ± 0·3</td>
<td>2·8 ± 1·4</td>
<td>72·1 ± 34·9</td>
<td>28·0 ± 3·5</td>
<td>0·3 ± 0·1</td>
</tr>
</tbody>
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* Mice were injected i.p. with 0·01 and 0·1 mg viable and heat-killed BCG per mouse 21 and 3 d before bacterial challenge, respectively.

coli from the peritoneal cavity is thought to be attributable to resident Mφs. Experiment 1 (Table 2) shows that the susceptibility of the test organisms to the microbicidal action of resident Mφs is in the following order: \textit{C. albicans} ≅ \textit{Staph. aureus} ≅ \textit{E. coli} > \textit{S. typhimurium} ≅ \textit{L. monocytogenes} ≅ \textit{M. tuberculosis}. In experiment 2 (Table 2), the resident Mφs in the peritoneal cavity were immunologically activated by double injections of viable and heat-killed BCG at 21 d and 3 d before microbial challenge, respectively [in this case, the PEC were mainly composed of Mφs (75%) and other cells such as lymphocytes; the resultant Mφs showed a high PMA response, releasing 430 nm-H₂O₂ in response to 10 ng PMA ml⁻¹ per 10⁶ cells; unpublished data]. In this case, \textit{C. albicans}, \textit{E. coli} and \textit{L. monocytogenes} were readily eliminated from the peritoneal cavity. Although \textit{M. tuberculosis} was also more rapidly killed by activated Mφs than by resident Mφs, the organisms was still relatively resistant. On the other hand, \textit{S. typhimurium} was highly resistant to both the resident and the activated Mφs. The high resistance of \textit{S. typhimurium} HKB-1 to the microbicidal activity of host Mφs was consistent with its high virulence in mice: 1 × 10⁶ organisms given i.p. to mice killed 90% of the host animals within 1 week, while the same number of the other micro-organisms killed only 20% or less of mice within 4 weeks.
Bacterial activation of the Mφ oxidative burst

Fig. 1 shows chemiluminescence of zymosan A-induced Mφs in contact with test organisms at m.o.i. values of 20 and 40. The test organisms were examined for their ability to trigger an oxidative burst by zymosan A-induced mouse PEC (composed of 88% Mφs, with low PMN contamination). Over the first 10 min, *Staph. aureus* triggered the highest chemiluminescence, followed by the other micro-organisms in the following order; *Staph. aureus* > *C. albicans* > *E. coli* > *L. monocytogenes* > *M. tuberculosis*. *S. typhimurium* failed to trigger chemiluminescence (Fig. 1d). Three groups of test organisms could be recognized, according to the light emission patterns of Mφs induced by contact with organisms: (1) chemiluminescence continued to increase during the course of measurement for up to 12 min (*Staph. aureus* and *E. coli*); (2) chemiluminescence reached a maximum in the early phase and declined thereafter (*C. albicans*); (3) chemiluminescence reached a plateau in the early phase (*L. monocytogenes*, *M. tuberculosis* and *S. typhimurium*). Patterns (2) and (3) suggest the release from the test organisms of inhibitory factors against Mφ chemiluminescence.

*S. typhimurium*, which lacked Mφ-chemiluminescence-triggering activity, was associated with Mφ cells to the same extent as the other organisms (data not shown), indicating that deficiency in the chemiluminescence-triggering ability of the organisms is not due to a lack of ability to contact the Mφ cell surface.
DISCUSSION

Although it is reported that Mφs have no myeloperoxidase (Klebanoff, 1980), there is evidence that the halogenation reaction is important for the bactericidal activity of these cells (Lehrer, 1975). There are at least three candidates for the mediator of the $\text{H}_2\text{O}_2$-dependent halogenation reaction in Mφs: first, myeloperoxidase molecules produced by PMN and taken up by Mφs (Hanker & Giammara, 1983); second, catalase, which exhibits peroxidase activity rather than $\text{H}_2\text{O}_2$-decomposing activity when $\text{H}_2\text{O}_2$ is maintained at a very low steady-state concentration, particularly at acid pH (Gee et al., 1970; Klebanoff, 1969; Klebanoff & Hamon, 1975); third, iron ions in phagolysosomes of phagocytic cells which are at a sufficient concentration under physiological conditions to support the halogenation reaction, as described here (about 5 µM; Flitter et al., 1983; Gutteridge et al., 1981; Halliwell & Gutteridge, 1984). Also, the $\text{H}_2\text{O}_2$–Fe-mediated halogenation reaction is thought to be important for mechanisms related to bacterial killing by phagocytes. When the microbicidal efficacy of the active oxygen-generating system (XOA–Fe–EDTA system), representing direct toxic effects of active oxygen species, is compared with the $\text{H}_2\text{O}_2$–Fe-mediated halogenation system, the latter seems to be more important for intracellular killing, particularly of *Mycobacterium* and *Candida*.

Because Mφs are dominant in the peritoneal cell populations in normal and in BCG-treated mice, the present results reflect essentially the susceptibility of test organisms to resident and activated Mφs. *S. typhimurium* and *M. tuberculosis*, and also *L. monocytogenes*, all of which are intracellular parasites, were highly or markedly resistant to Mφ-mediated killing. In contrast, *C. albicans*, which is highly resistant to the XOA–Fe–EDTA system was rapidly killed by host Mφs. Also *C. albicans* was more resistant to the $\text{H}_2\text{O}_2$–Fe-mediated halogenation system than were the other microbes. Thus, the mechanisms of intracellular killing of *C. albicans* may involve other factors, such as cationic proteins, unsaturated fatty acids and lysosomal hydrolases (Martinez & Carroll, 1980; Miller, 1969) rather than oxygen-dependent mechanisms, as proposed by Lehrer (1972, 1975). Our group (Saito et al., 1984) and Kanai & Kondo (1979) have suggested the importance of unsaturated free fatty acids (arachidonic, oleic, palmitoleic, etc.) in the intraphagosomal killing of mycobacteria, which are resistant to active oxygen species (Yamada et al., 1987) and show considerable resistance to $\text{H}_2\text{O}_2$–Fe-mediated halogenation (Table 1). A similar proposal was made earlier by Hemsworth & Kochan (1978).

In the presence of luminol, chemiluminescence originates from $\text{OH}$ and $\text{O}_2$ rather than from $\text{O}_2$ and $\text{H}_2\text{O}_2$ (Babior, 1978; Brummer et al., 1985; Johnston, 1978), and thus we can recognize more complex intracellular events in Mφs after the onset of the respiratory burst by the chemiluminescence assay than by measurement of $\text{O}_2$ and $\text{H}_2\text{O}_2$ generation. In fact, we previously found that in infections due to *Mycobacterium intracellulare* or *L. monocytogenes*, the responsiveness of host Mφs to PMA in the form of the oxidative burst (which correlates well with the extent of Mφ activation: Nathan & Root, 1977; Tomioka & Saito, 1980) was better represented by chemiluminescence than by $\text{H}_2\text{O}_2$ release (Saito et al., 1986). In addition, $\text{OH}$, which is detectable by the chemiluminescence assay, is very important for the expression of direct microbicidal activity of active oxygen species (Yamada et al., 1987) and also for halogenation reaction system (Klebanoff, 1982b). Therefore, the chemiluminescence assay is a more reliable indicator of the $\text{O}_2$-dependent microbicidal function of a given Mφ cell than the measurement of $\text{O}_2$ and $\text{H}_2\text{O}_2$ generation.

All the intracellular parasites tested here, except *C. albicans*, were less active than the extracellular parasites in triggering a Mφ oxidative burst. This finding is consistent with the observations by other investigators that virulent species (or strains) of *Candida* (Sasada & Johnston, 1980), *Salmonella* typhi (Kossack et al., 1981; Miller et al., 1972) and *M. intracellulare* (Gangadharam & Edwards, 1984) trigger a reduced respiratory burst in Mφs and PMNs. In this study, *S. typhimurium* HKB-1 showed a high resistance to Mφ-mediated bacterial killing, although it was highly sensitive to the XOA–Fe–EDTA system (Yamada et al., 1987) and to the $\text{H}_2\text{O}_2$–Fe-mediated halogenation system (Table 1). The inability of this microbe to trigger a Mφ oxidative burst (Fig. 1) may partly explain this enigmatic status. Alternatively, it is possible that this microbe inhibits phagolysosomal fusion of Mφs after phagocytosis as in the case of *M. tuberculosis* (Lowrie, 1983).
REFERENCES


Bacterial susceptibility to active oxygen

