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Effect of Oxygen-Dependent Antimicrobial Systems on *Legionella pneumophila*

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Legionella pneumophila was susceptible to the antimicrobial action of oxygen metabolites generated by both the myeloperoxidase-H_2O_2-halide and the xanthine oxidase systems.

Phagocytic cells have an impressive arsenal of antimicrobial agents at their disposal. Chemical agents implicated in the antimicrobial action of phagocytic cells include organic acids (5), cationic proteins (21), toxic oxygen metabolites (11), and myeloperoxidase (MPO) (13, 16). *Legionella pneumophila*, the human pathogen responsible for Legionnaires disease, survives intracellularly within phagocytes both in vivo (3, 19, 20) and in vitro (9, 10). The precise mechanism by which *L. pneumophila* circumvents phagocyte antimicrobial action has not been established.

In a previous communication, we have identified a low-molecular-weight toxin produced by *L. pneumophila* (7). Incubation of human polymorphonuclear leukocytes (PMN) with *L. pneumophila* toxin dramatically inhibits phagocytosing oxygen consumption and hexose monophosphate shunt activity in a dose-dependent manner at concentrations which do not impair phagocytosis (8). Bacterial iodination and killing are also significantly impaired in toxin-treated PMN (8). If *L. pneumophila* is susceptible to oxygen-dependent microbicidal mechanisms, the ability of *L. pneumophila* toxin to inhibit the phagocytosis-associated generation of toxic oxygen metabolites may be a critical determinant of its virulence. In the present study, we have examined the susceptibility of *L. pneumophila* to killing by the MPO-H_2O_2-halide system and by reactive oxygen derivatives produced by the xanthine oxidase system.

L. pneumophila Knoxville I was obtained from the Centers for Disease Control, Atlanta, Ga. Cultures were grown at 37°C on charcoal yeast extract agar slants in a 5% CO_2 atmosphere and were transferred weekly.

Avirulent *L. pneumophila* had been passed on agar slants for over two years. Virulent organisms were obtained by egg passage of the avirulent bacteria four times via the yolk sac route in 7-day-old embryonated hen eggs. After the final egg passage, yolk sac membranes were harvested and stored at −70°C. The egg 50% lethal dose as determined by the method of McDade and Shepard (17) was 1.4×10^6 bacteria per ml for avirulent *L. pneumophila* and 4×10^3 bacteria per ml for the virulent organism.

Bacteria grown 4 to 5 days on charcoal yeast extract agar slants were swabbed from the slants and suspended to a final concentration of 5×10^7 colony-forming units (CFU) per ml in either phosphate-buffered saline, pH 7.4, or 0.1 M potassium phosphate, pH 5. In the MPO microbicidal assay, 10^7 *L. pneumophila* cells were incubated for 30 min at 37°C in polypropylene tubes containing 2mM NaCl, 0.01 μM H_2O_2, and 150 *o*-dianisidine units of MPO in a final volume of 2 ml of 0.1 M potassium phosphate, pH 5. MPO, which was purified by the method of Agner (1), was generously supplied by Seymour J. Klebanoff (University of Washington, Seattle, Wash.). In the xanthine oxidase system, 5×10^6 bacteria were incubated for 60 min at 37°C in a final volume of 1 ml containing (1.5×10^{-4})M xanthine and 100 μg of xanthine oxidase (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) per ml with or without the scavengers histidine, mannitol, superoxide dismutase (SOD) (Sigma Chemical Co., St. Louis, Mo.), and catalase (Worthington Diagnostics, Freehold, N.J.). After incubation, samples were removed, serially diluted, and spread on charcoal yeast extract plates. Plates were incubated 5 to 7 days at 37°C, and colonies were counted.

MPO in the presence of H_2O_2 catalyzes the oxidation of halides by H_2O_2 to form reactive species, which are capable of halogenating and oxidizing the chemical constituents of bacterial membranes (4, 14). In addition, the products of the MPO-H_2O_2-halide system oxidatively decarboxylate amino acids and cleave peptide bonds (18). The bactericidal acitivity of the MPO-

TABLE 1. Effect of the MPO-halide-H_2O_2 system on *L. pneumophila*

Reaction mixture	Viable cell count (CFU/ml) ($\times 10^6$)[a]	
	Avirulent	Virulent
Complete	0.00001[b]	0.00001[b]
MPO omitted	4.8	4.6
H_2O_2 omitted	5.2	4.5
MPO and H_2O_2 omitted	5.0	5.0

[a] Results are expressed as the mean of three experiments. The number of viable organisms was considered statistically significant compared with the control (5×10^6 CFU/ml) when $P < 0.05$.
[b] $P < 0.001$.

mediated system is extremely potent, and this system is considered to be the predominant antimicrobial mechanism operative in PMN (15). Analysis of the data presented in Table 1 clearly indicates that *L. pneumophila* is sensitive to MPO-mediated killing processes. Avirulent and virulent *L. pneumophila* are equally susceptible to MPO-mediated antimicrobial action. The bactericidal activity of the MPO-H_2O_2-halide system for *L. pneumophila* is absolutely dependent on exogenously added H_2O_2. In contrast to catalase-negative microorganisms, catalase-positive microorganisms such as *L. pneumophila* fail to secrete H_2O_2. Although H_2O_2 is required for microbicidal activity, the low concentration of H_2O_2 used in these studies is not toxic in the absence of MPO.

To evaluate the susceptibility of *L. pneumophila* to other toxic oxygen metabolites generated independently of MPO, experiments with the xanthine oxidase system were performed. Xanthine oxidase catalyzes the oxidation of xanthine to hypoxanthine with the resultant production of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (6). These compounds may subsequently interact to form hydroxyl radicals (·OH) (2) and singlet oxygen ($^1O_2$) (12). The susceptibility of *L. pneumophila* to each of the aforementioned reactive oxygen species was assessed by incorporating specific inhibitors into the xanthine oxidase reaction mixture. Mannitol acts as a scavenger of (·OH), and singlet oxygen ($^1O_2$) is quenched by histidine. A reduction in H_2O_2 concentration is achieved by incorporating catalase in the xanthine oxidase reaction mixture. Likewise, the concentration of ($O_2^{\cdot-}$) may be decreased by the inclusion of SOD.

TABLE 2. Susceptibility of *L. pneumophila* to oxygen intermediates generated by the xanthine oxidase system[a]

Additive	Viable cell count (CFU/ml) ($\times 10^{-6}$)[b]	P[c]
None	1.35	<0.001
Catalase (100 μg/ml)	4.97	
SOD (200 μg/ml)	4.11	
Mannitol (10 mM)	4.04	
Histidine (10 mM)	1.55	<0.01
MPO (150 U)	0.00001	<0.001

[a] The data shown were obtained by using avirulent *L. pneumophila*; comparable results were obtained with virulent *L. pneumophila* (data not shown).
[b] Results are expressed as the mean of three experiments.
[c] Probability values are shown when the number of viable organisms was statistically significant ($P < 0.05$) compared with the control (5×10^6 CFU/ml).

L. pneumophila is susceptible to the oxygen metabolites generated by the xanthine oxidase system (Table 2). The killing is significantly impaired by the inclusion of SOD, catalase, or mannitol. These observations suggest that the production of (·OH) is predominantly responsible for the bactericidal activity of the xanthine oxidase system for *L. pneumophila*. Catalase and SOD influence the (·OH) concentration indirectly by decreasing the concentrations of the substrates used in (·OH) formation. The antimicrobial activity of the oxygen metabolites generated by the xanthine oxidase system is modest in comparison with the microbicidal activity of the MPO-H_2O_2-halide system. When MPO was added to the xanthine oxidase system, the antimicrobial activity of the system was dramatically augmented, and no viable colonies are detected (Table 2).

The results indicate that *L. pneumophila* is indeed susceptible to the microbicidal properties of oxygen metabolites generated in vitro. The in vivo production of toxic oxygen metabolites by PMN is initiated by interaction at the cell surface. *L. pneumophila* toxin selectively inhibits the activation of PMN oxidative metabolism (8). Accompanying the defect in the oxidative triggering of toxin-treated PMN is a corresponding impairment of the killing of catalase-positive microorganisms by the phagocytes (8). The ability of *L. pneumophila* toxin to selectively inhibit the activation of PMN oxidative metabolic processes represents a mechanism by which *L. pneumophila* which is susceptible to these systems in vitro may evade their phagocyte antimicrobial action in vivo.

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