Enhanced Killing of *Acanthamoeba* Cysts with a Plant Peroxidase-Hydrogen Peroxide-Halide Antimicrobial System

Reanne Hughes, Peter W. Andrew, and Simon Kilvington*

*Department of Microbiology and Immunology, University of Leicester, Leicester LE1 9HN, United Kingdom*

Received 30 August 2002/Accepted 31 January 2003

The activity of H$_2$O$_2$ against the resistant cyst stage of the pathogenic free-living amoeba *Acanthamoeba* was enhanced by the addition of KI and either horseradish peroxidase or soybean peroxidase or, to a lesser degree, lactoperoxidase. This resulted in an increase in the cysticidal activity of 3% (wt/vol) H$_2$O$_2$, and there was >3-log killing in 2 h, compared with the 6 h required for comparable results with the peroxide solution alone ($P < 0.05$). With 2% H$_2$O$_2$, enhancement was observed at all time points ($P < 0.05$), and total killing of the cyst inoculum occurred at 4 h, compared with 6 h for the peroxide alone. The activity of sublethal 1% H$_2$O$_2$ was enhanced to give 3-log killing after 8 h of exposure ($P < 0.05$). No enhancement was obtained when KCl or catalase was used as a substitute in the reaction mixtures. The H$_2$O$_2$ was not neutralized in the enhanced system during the experiments. However, in the presence of a platinum disk used to neutralize H$_2$O$_2$ in contact lens care systems, the enhanced 2% H$_2$O$_2$ system gave 2.8-log killing after 6 h or total cyst killing by 8 h, and total neutralization of the H$_2$O$_2$ occurred by 4 h. In contrast, 2% H$_2$O$_2$ alone resulted in <0.8-log killing of cysts in the presence of the platinum disk due to rapid (<1 h) neutralization of the peroxide. Our observations could result in significant improvement in the efficacy of H$_2$O$_2$ contact lens disinfection systems against *Acanthamoeba* cysts and prevention of acanthamoeba keratitis.

Hydrogen peroxide (H$_2$O$_2$) is an effective microbial disinfectant, destroying pathogens by oxidation that results in protein denaturation (5). As such, it is widely used for disinfection processes in the food, water treatment, health care, and contact lens industries (5). In vivo, stimulated phagocytes produce H$_2$O$_2$ and then use it in the destruction of phagocytosed microorganisms (8). In neutrophils, at least, the process is further enhanced by the action of a myeloperoxidase that forms hypochlorous acid (HOCl) from chloride ions and H$_2$O$_2$ (17). The combination of H$_2$O$_2$ and HOCl is a potent antimicrobial combination that destroys cells by halogenation and oxidation of cell surface components (14, 17, 23, 28).

This innate myeloperoxidase-mediated antimicrobial system was first described by Klebanoff in 1967 (12). Subsequently, it was demonstrated that this mechanism can be reproduced in vitro to obtain enhanced hydrogen peroxide killing of a variety of pathogenic microbes, including bacteria (10, 13, 15, 24, 25), fungi (28), human immunodeficiency virus type 1 (16), and the parasitic helminth *Schistosoma mansoni* (11). Peroxidases other than myeloperoxidase have been used, including cosinophil peroxidase (27), lactoperoxidase, horseradish peroxidase (HRP), and catalase (2, 3, 9, 24). In addition, bromide and iodide have been shown to be capable of replacing chloride in the reaction (9, 11, 15). Until now, the effects of this potent antimicrobial system on protozoans have not been reported.

*Acanthamoeba* is a genus of free-living amoebae that occur in virtually all soil and aquatic habitats (20). These organisms are characterized by a motile feeding and replicating trophozoite that can form a highly resistant cyst stage in response to adverse conditions (20). *Acanthamoeba* is an opportunistic pathogen of humans, causing fatal encephalitis in immunocompromised hosts and, more frequently, a potentially blinding infection of the cornea, termed acanthamoeba keratitis, in previously healthy persons (18). Contact lens wearers are most at risk from infection and account for 90% of reported cases (21). Hydrogen peroxide (3%) is a commonly used contact lens disinfectant and has been shown to be effective against *Acanthamoeba* cysts, giving a 3-log reduction in viability, provided that an exposure time of at least 4 to 6 h is used prior to neutralization (7). Hydrogen peroxide is toxic to the cornea and must be neutralized before lens wear to avoid pronounced stinging, lacrimation, hyperemia, and possible corneal damage (6, 26). One-step hydrogen peroxide systems which do not require a separate neutralization step are available. In these systems, neutralization is achieved in a storage case during disinfection by using a platinum-coated disk or soluble catalase tablet which catalyzes the decomposition of hydrogen peroxide to water and oxygen. However, the rapid neutralization in such systems results in little or no cysticidal efficacy (7).

In the present study we investigated the potential for enhancing the cysticidal activity of H$_2$O$_2$ through a plant peroxidase-hydrogen peroxide-halide antimicrobial system. The finding that use of this system results in significant enhancement of H$_2$O$_2$ killing of *Acanthamoeba* cysts, either alone or in the presence of a platinum disk used in one-step peroxide disinfection of contact lenses, prompted this report.

**MATERIALS AND METHODS**

*Acanthamoeba* strains and culture. *Acanthamoeba polyphaga* strain Ac-Ros isolated from a keratitis case in the United Kingdom in 1991 was used in all
developmental experiments. The pathogenic species Acanthamoeba castellanii and Acanthamoeba culbertsoni (strain ATCC 30715) and four Acanthamoeba strains from recent keratitis cases (Ak-1 to Ak-4) were also studied. Although the latter strains were not identified to the species level, they were most closely related to type II strains, which account for the majority of Acanthamoeba strains causing keratitis (20). Two nonpathogenic species, Acanthamoeba castellanii (strain Neff [- CCAP 1501/1a]) and Acanthamoeba palestiensis (strain CCAP 1547/1), were also tested. Trophozoites were maintained in a semidefined axenic broth medium as previously described (7). Cysts were prepared from the trophozoite cultures by using Neff’s chemically defined encystment medium or in 0.25% sodium hypochlorite for 30 min at room temperature. Ac-Ros cysts was then tested with the additional ceytialidal activity (results not shown). KI, HRP, or KI-HRP alone showed no cysticidal activity (results not shown). Hydrogen peroxide killing. The cysticidal activity of H2O2 alone in 0.25× Ringer’s solution (pH 4.31 to 5.62) against A. polyphaga Ac-Ros is shown in Fig. 1. Hydrogen peroxide at a concentration of <1% showed little cysticidal activity, giving <1-log killing in 6 h (results not shown). After 4 h of exposure, H2O2 at concentrations of 1, 2, and 3% gave values of 1.10 ± 0.31, 2.35 ± 0.49, and 2.75 ± 0.00 logs for killing of cysts, respectively. By 6 h the values were 1.67 ± 0.38, 3.46 ± 0.08, and 4.33 ± 0.08 logs, respectively (Fig. 1). Similar results were obtained with H2O2 tested in PBS (pH 6.55 to 7.13), and there was no statistical difference between this cysticidal activity and that in 0.25× Ringer’s solution (P > 0.05) (results not shown).

Enhanced hydrogen peroxide killing. Addition of KI to H2O2 enhanced the cysticidal activity of H2O2 (Fig. 1). The checkerboard experiments showed that maximum activity occurred with 50 μM KI and 50 U of HRP ml−1 for 1% H2O2, with 150 U of HRP ml−1 for 2% H2O2, and with 200 U of HRP ml−1 for 3% H2O2 (Fig. 1). Omission of either the KI or HRP from the reaction mixture resulted in no enhancement of cysticidal activity (results not shown). KI, HRP, or KI-HRP alone showed no cysticidal activity (results not shown).

RESULTS

Hydrogen peroxide killing. The cysticidal activity of H2O2 alone in 0.25× Ringer’s solution was determined using Reed-Muench computations as previously described for Acanthamoeba cyst viability (4, 22). The reduction in the number of viable cysts was plotted as the change in the log viability for each time point compared to the zero-time viability. A statistical analysis was performed by using one-way analysis of variance with means and standard errors of the means of triplicate experiments.

FIG. 1. Enhanced killing of A. polyphaga cysts with the peroxidase-hydrogen peroxide-halide system. Symbols: ○, 1% H2O2; ■, 1% H2O2, 50 U of HRP ml−1; ▲, 2% H2O2; ▼, 2% H2O2, 150 U of HRP ml−1; △, 3% H2O2; ●, 3% H2O2, 200 U of HRP ml−1; and 50 μM KI. The error bars indicate standard errors of the means from triplicate experiments. In control experiments with only 0.25× Ringer’s solution, a >0.5-log reduction in cyst viability occurred (data not shown).
alone. Significant cysticidal activity was also observed with 3% H₂O₂ at all times (P < 0.05), and this activity resulted in total killing at 4 h, compared with 6 h for the peroxide solution alone.

**Modifications to the enhanced H₂O₂ system.** Replacing KI with KCl (50 μM to 200 μM) resulted in no enhancement of the activity of 1 to 3% H₂O₂ with concentrations of HRP up to 200 U ml⁻¹ (results not shown). Replacing HRP with SBP resulted in similar statistically significant enhanced cysticidal activity with 50 μM KI and 2 or 3% H₂O₂ (P < 0.05). No difference in efficacy was observed between the two plant-derived peroxidases. For example, 2% H₂O₂ and 50 μM KI used with 150 U of HRP ml⁻¹ gave levels of killing of 3.50 ± 0.25 logs after 4 h, compared to 3.89 ± 0.45 logs when 150 U of SBP ml⁻¹ was used. These values were compared with the value obtained with 2% H₂O₂ alone at this time, 2.33 ± 0.49 logs. With 2% H₂O₂, 50 μM KI, and 150 U of LPO ml⁻¹, levels of killing of 1.98 ± 0.27 logs were observed after 2 h and levels of killing of 3.56 ± 0.44 logs were observed after 4 h. These values were compared with the levels of killing obtained with 2% H₂O₂ alone at these times, 1.28 ± 0.13 and 2.42 ± 0.10 logs, respectively. Although there was some suggestion that the activity was enhanced, the findings for LPO were not statistically significant (P > 0.05).

In contrast, substituting catalase (50 to 200 U ml⁻¹) for HRP resulted in decreased cysticidal activity. For example, 2% H₂O₂ with 100 U of catalase ml⁻¹ and 50 μM KI gave levels of killing of 0.20 ± 0.36 log at 4 h, compared with 2.33 ± 0.49 logs with 2% H₂O₂ alone at that time. With 3% H₂O₂, 100 U of catalase ml⁻¹, and 50 μM KI, levels of killing of 0.51 ± 0.27 log occurred after 4 h, compared with 2.75 ± 0.00 logs with the peroxide alone. Replacing KI with KCl (50 to 200 μM) did not alter this effect with catalase (results not shown).

Substituting PBS (pH 6.55 to 7.13) for 0.25× Ringer’s solution (pH 4.31 to 5.62) in the optimized HRP-H₂O₂-KI reactions did not affect the efficacy of the enhanced cysticidal activity observed with 1 to 2% peroxide (results not shown) (P > 0.05). However, with 3% peroxide in PBS, the HRP-H₂O₂-KI optimized system gave levels of cyst killing of 2.84 ± 0.10 logs at 2 h, compared with 4.42 ± 0.08 logs in 0.25× Ringer’s solution (P < 0.05); the differences at the other times were not significant, and killing values similar to those obtained with 0.25× Ringer’s solution were obtained (results not shown) (P > 0.05).

Sodium perborate (1 to 3% [wt/vol] in 0.25× Ringer’s solution, yielding 0.17 to 0.5% H₂O₂) was not cysticidal even after 8 h of contact time (<1-log killing), and addition of HRP (5 to 20 and 150 U ml⁻¹) and KI or KCl (50 μM) did not result in increased cysticidal activity (results not shown).

The peroxide levels (1 to 2% [wt/vol]) in the HRP-H₂O₂-KI and SBP-H₂O₂-KI systems remained constant throughout the experiments. However, rapid H₂O₂ neutralization occurred when catalase was used in place of peroxidase, and the levels fell to 0.2% after the first hour and then remained at this level thereafter (results not shown).

**Enhanced activity against other Acanthamoeba species and strains.** When tested in 0.25× Ringer’s solution, all additional *Acanthamoeba* species and strains showed enhanced killing with 2% H₂O₂, 150 U of HRP ml⁻¹, and 50 μM KI (Fig. 2). The differences were statistically significant at 4 h compared to the values for H₂O₂ alone (P < 0.05) for strains Ak-3, Ak-Ros, and Ak-4, as well as *A. castellanii* Neff and *A. palestinensis* CCAP 1547/1. By 6 h the enhanced system had achieved total killing (>3 logs) for all species and strains studied, compared with values of <3 logs for 2% H₂O₂ alone (results not shown).

**Peroxidase-hydrogen peroxide-halide system with platinum neutralization.** The efficacy of 2% H₂O₂ with 150 U of HRP ml⁻¹ and 50 μM KI in the presence of a platinum neutralizing disk is shown in Fig. 3. Little cysticidal activity was found with 2% H₂O₂ alone in the presence of the platinum disk, and
<0.8-log killing of cysts occurred after 6 h of exposure. In contrast, the HRP-H$_2$O$_2$-KI system with the platinum-coated disk gave 1.4-log killing after 1 h, 2.4-log killing after 4 h, and 2.8-log killing after 6 h. Total cyst killing (>3 logs) was achieved by 8 h (results not shown). Less than 1-log killing occurred with the platinum disk alone in 0.25 Ringer’s solution, KI, HRP, or KI-HRP (results not shown).

The hydrogen peroxide levels in the mixtures fell to <0.5 ppm within 1 h with H$_2$O$_2$ and the platinum-coated disk. However, with HRP-H$_2$O$_2$-KI and the platinum-coated disk, the H$_2$O$_2$ levels were 0.5% after 1 and 2 h and <0.5 ppm at 4 h (Fig. 3).

DISCUSSION

Hydrogen peroxide (3%) is an effective disinfectant against *Acanthamoeba* cysts, giving at least a 3-log reduction in viability provided that an exposure time of at least 4 to 6 h is used (7). In this study, we demonstrated that the activity of H$_2$O$_2$ against *Acanthamoeba* cysts can be enhanced by addition of the halide I$^-$ and a plant-derived peroxidase. This resulted in an increased rate of cysticidal activity and total killing of the cyst inocula by 2 h with 3% H$_2$O$_2$ and by 4 h with 2% H$_2$O$_2$. The system also enabled a sublethal H$_2$O$_2$ concentration (1%) to produce 3-log killing within 6 h. Furthermore, this finding indicates that the previously observed in vitro activity of the peroxidase-peroxide-halide system against pathogenic viruses, bacteria, fungi, and the helminth *S. mansoni* can be extended to include the pathogenic protozoan *Acanthamoeba* (10, 11, 13, 15, 16, 24, 28).

It is known that in vivo myeloperoxidase plays a crucial role in killing phagocytosed bacteria in neutrophils by reacting with H$_2$O$_2$ to form an enzyme substrate complex that oxidizes halide to produce even more toxic components (13, 14). The primary agent involved is hypohalous acid, which destroys cells by halogenation and oxidation of cell surface components (14, 17, 23, 28). It has been suggested that the peroxidase-peroxide-halide system acts preferentially against pathogenic rather than nonpathogenic organisms through binding to the cell surface: the target-bound peroxidase then catalyzes halide oxidation and facilitates the disproportionation of peroxide to singlet molecular oxygen at the surface of the target microbe (1, 2, 3). As the lifetime of singlet molecular oxygen is short and its diffusion potential is proportionally limited, the target organism is killed with minimal damage to any nonpathogenic microbes or host cells (1).

The precise mode of action of the peroxidase-hydrogen peroxide-halide system described here against *Acanthamoeba* cysts is unclear, but it appears to be equally effective against both pathogenic and nonpathogenic species (*A. castellanii* Neff and *A. palestinensis* CCAP 1547/1). However, it seems probable that under the reaction conditions described here, this results in the formation of HOI, which enhances the cysticidal activity of the H$_2$O$_2$. Whether this reaction is localized through primary binding of the peroxidase on the cyst wall surface or occurs within the cyst has yet to be elucidated.

Replacing KI with KCl in the system did not result in enhanced cysticidal activity. This is in accordance with other studies, which have shown that I$^-$ is the most effective halide, followed by Br$^-$ and then Cl$^-$ (11, 13, 15). We also found that the enzyme SBP could replace HRP in the system with comparable cysticidal enhancing activity. Enhanced activity was also observed with LPO, although this enzyme was not as efficacious as the plant peroxidases. Replacing these peroxidases with bovine liver catalase resulted in no cysticidal activity, as the H$_2$O$_2$ was rapidly neutralized within 1 h. Using lower levels of catalase and replacing KI with KCl did not result in enhanced H$_2$O$_2$ activity. This is in contrast to the findings for SBP or HRP when the H$_2$O$_2$ levels remained constant throughout the experiments. Replacing H$_2$O$_2$ with sodium perborate (3% [wt/vol], yielding 0.5% H$_2$O$_2$) in the system re-
sulted in no cysticidal activity, presumably due to the low peroxide-generating capacity of the latter chemical (7).

Hydrogen peroxide is commonly used for disinfection of contact lenses, although it must be neutralized before the lenses are worn to avoid corneal damage (6, 26). One-step hydrogen peroxide systems which do not require a separate neutralization step are available; neutralization is achieved during disinfection in the storage case by using a platinum-coated disk or a soluble catalase tablet which catalyzes the decomposition of hydrogen peroxide. However, in one-step systems this neutralization process occurs too rapidly for cysticidal activity to occur (7). In the peroxidase-hydrogen peroxide-halide system developed here, which exhibits enhanced cysticidal activity, neutralization of the H2O2 does not occur during the reaction. However, when the reaction was performed in the presence of a platinum disk, not only was the enhanced killing maintained, but the peroxide was neutralized so that the concentration was <0.5 ppm by 4 h. Levels of H2O2 of 2 to 5 ppm are not likely to cause irritation to the eye; 30 ppm has been reported to induce cytotoxicity in the eye, and 100 ppm produces noticeable discomfort (26).

Acanthamoeba keratitis is a potentially sight-threatening infection that is most commonly seen among contact lens wearers (21). Although one-step hydrogen peroxide disinfection systems are convenient for contact lens wearers, they are not effective against the highly resistant cyst form of Acanthamoeba (7). The findings of this study demonstrate that the efficacy of H2O2 against Acanthamoeba cysts can be enhanced by addition of KI and a plant-derived peroxidase. When the reaction is performed in the presence of a platinum disk, the cysticidal activity is retained and complete neutralization of the H2O2 is obtained. This may result in a significant improvement in the efficacy of one-step H2O2 systems against Acanthamoeba cysts and, possibly, other contact lens-related ocular pathogens.

ACKNOWLEDGMENT

This study was supported by research grant GA324 from the British Society for Antimicrobial Chemotherapy.

REFERENCES