Comparison of the Antimicrobial Effects of Chlorine, Silver Ion, and Tobramycin on Biofilm

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The systematic understanding of how various antimicrobial agents are involved in controlling biofilms is essential in order to establish an effective strategy for biofilm control, since many antimicrobial agents are effective against planktonic cells but are ineffective when they are used against the same bacteria growing in a biofilm state. Three different antimicrobial agents (chlorine, silver, and tobramycin) and three different methods for the measurement of membrane integrity (plate counts, the measurement of respiratory activity with 5-cyano-2,3-ditolyl tetrazolium chloride [CTC] staining, and BacLight Live/Dead staining) were used along with confocal laser scanning microscopy (CLSM) and epifluorescence microscopy to examine the activities of the antimicrobials on biofilms in a comparative way. The three methods of determining the activities of the antimicrobials gave very different results for each antimicrobial agent. Among the three antimicrobials, tobramycin appeared to be the most effective in reducing the respiratory activity of biofilm cells, based upon CTC staining. In contrast, tobramycin-treated biofilm cells maintained their membrane integrity better than chlorine- or silver-treated ones, as evidenced by imaging by both CLSM and epifluorescence microscopy. Combined and sequential treatments with silver and tobramycin showed an enhanced antimicrobial efficiency of more than 200%, while the antimicrobial activity of either chlorine or tobramycin was antagonized when the agents were used in combination. This observation makes sense when the different oxidative reactivities of chlorine, silver, and tobramycin are considered.

Bacterial biofilms are responsible for industrial biofouling, microbial regrowth in distribution systems, persistent infections (6, 9, 11, 12), and many other expensive and life-threatening problems. Therefore, the control of biofilms is now understood to be crucial. However, there are still few effective control strategies, and they are poorly understood in many contexts. Many antimicrobial agents that are effective against planktonic cells turn out to be ineffective against the same bacteria growing in a biofilm state (10, 12, 38). Whereas strong oxidizing biocides are usually reliably effective against planktonic cells, sometimes weak oxidants or nonoxidants are superior for controlling biofilms (20, 41). Planktonic and biofilm cells also exhibit different susceptibilities to a certain antimicrobial concentration. Bacterial adaptive responses play a role in the design of control strategies (31, 43). In microenvironments with the intensive and constant exposure of bacteria to antibiotics, “there is selective pressure for antibiotic-resistant bacteria to maintain those determinants, survive, and even dominate the bacterial populations” (43). The combined application of multiple antimicrobial agents may be a strategy to improve their performance and circumvent bacterial adaptation. This might involve the use of antimicrobials with different chemistries and modes of action together. Although only antibiotics or antiseptics are permitted for use in the human body, other biocides or combinations can be used to clean medical devices. Silver-coated medical devices are widely used (3, 19, 33), and the antiseptics chlorhexidine and silver sulfadiazine and the antibiotics minocycline and rifampin have been used as catheter coatings. These were reported to reduce the risk of catheter-related bloodstream infections (26). Water treatment lines used for dialysis have been cleaned with hypochlorite solution (bleach) and with dilute acid solutions (nitric, citric, peracetic acid, etc.) (27, 29).

In order to choose appropriate antimicrobial agents and to optimize the dosing strategy on a case-by-case basis, it is necessary to improve our understanding of the interaction between various antimicrobial agents and biofilm cells. Many studies have examined the efficacy of either antibiotics alone (5, 44, 48) or biocides alone (17, 40). However, very little work has been done on the efficacy of combinations of different categories of agents, for example, antibiotics and oxidative biocides. A quantitative literature survey of bacterial susceptibility to several antimicrobial agents, including oxidants, antibiotics, and other biocides, was conducted by Stewart and Raquepas (39); and some biofilm resistance factors were suggested to be a measurement of biofilm susceptibility to antimicrobial agents. However, there was considerable variability in the resistance factor even for the same microbial species and the same antimicrobial agents, because they were collected from various studies conducted under different experimental conditions. Thus, a comparison of the susceptibilities of biofilms to various single agents of different categories has yet to be completed.

In this study, we selected three different antimicrobial agents (chlorine, silver, and tobramycin) to examine and compare their antimicrobial behaviors on biofilms. Chlorine, the most common disinfectant, is moderately oxidative and reacts with various components of bacterial cells (46). Silver has no ox-
dizing capacity but is involved in rendering various enzymes inactive by binding to thiol (—SH) groups in a cell (24). Re- cently, interest in the antimicrobial efficiency of silver compounds has increased, and many studies on the use of silver and other inorganic compounds as biocides in industrial sys- tems and medical devices have been conducted (19, 30). Tobramycin is frequently used in biofilm studies and report- edly inhibits protein synthesis and kills both growing and non- growing cells (36). Three methods of assessment were used to compare the antimicrobial effects of chlorine, silver, and tobramycin on biofilm in this study: plate counts, 5-cyano-2,3- ditolyl tetrazolium chloride (CTC) staining, and BacLight Live/Dead staining. In addition, experiments with combined and sequential treatments with the three agents were con- ducted and the antimicrobial efficiencies were evaluated.

**MATERIALS AND METHODS**

**Bacteria and media.** *Pseudomonas aeruginosa* PAO1 was grown in tryptic soy broth (TSB) at 37°C. For the experiments with planktonic cells, the cells were harvested by centrifugation at 1,000 × g for 10 min and washed twice with phosphate-buffered saline (PBS; pH 7.0). A PAO1 suspension was prepared by resuspending the cell pellet in 50 ml of PBS.

**Biofilm growth and biofilm reactors.** Biofilms were grown in Centers for Disease Control and Prevention (CDC) reactors (Biosurface Technologies Inc., Bozeman, MT) (7). The CDC reactor contains eight rods which each hold three glass coupons. An overnight culture was prepared by incubating PAO1 in 1/10- strength TSB for 20 h at 37°C. The sterile reactor was inoculated with 3.5 ml of an overnight culture that had been added to 350 ml of 1/100-strength TSB. The initial PAO1 population in this batch medium was about 10^9 CFU per milliliter. The reactor was operated in batch mode for 24 h at 100 rpm and room temper- ature. After 24 h in batch mode, the reactor was connected via a nutrient feed 0.5 M NaCl solution for1 hour1 h by a magnetic stirrer and the flasks were capped during stirring. The disinfection efficacy of each sample was evaluated only by plate counting.

**Fluorescent stains.** In order to evaluate cell membrane integrity, the BacLight Live/Dead bacterial viability kit (L-7012; Molecular Probes) was used. The kit contains Syto9 and propidium iodide to differentiate between cells with intact membranes (live) and membrane damaged cells (dead), respectively (15, 28). The stain was prepared by dilution of 3 μl of each component into 1 ml of distilled water. The respiratory activity of the cells was determined by staining with 1.6 μg/ml of CTC (Polysciences, Inc.) (37). Respiring cells were identified by the presence of intracellular, red CTC-formazan crystals. DAPI (10 μg/ml; Polysciences, Inc.) was used for the enumeration of the total cells (37).

For CLSM, the biofilm coupons were stained with 0.1 ml of each staining solution for 1 h in the dark. CTC-stained samples were incubated at 37°C. For epifluorescence microscopy, the cells were filtered onto black, polycarbonate membranes and then set on stain-soaked filter supports for 20 min.

**CLSM and epifluorescence microscopy.** Biofilm samples were imaged with a Leica AOBBS-SP2 confocal laser scanning microscope (Leica Microsystems, Bannockburn, IL). A water immersion objective lens (63 by 0.9 numerical aper- ture). When these preparations were analyzed, at least 2,000 cells were scored per sample. The image stacks collected by CLSM were analyzed with MetaMorph software (Molecular Devices Corporation, Downingtown, PA) and Imaris software (Bitplane, Zurich, Switzerland).

**Normalization and statistics.** All results were expressed as normalized cell ratios by setting the ratio for an untreated biofilm equal to 100%. The total cell density, plate count cell density, CTC-respiring cell density, and BacLight live cell density were defined as the number of DAPI-stained cells per unit area (number of cells/m²), the number of CFU per unit area (number of CFU/m²), the number of CTC-stained cells per unit area (number of cells/m²), and the number of Syto9-stained cells per unit area (number of cells/m²), respectively. In addition, we use the terms culturability ratio, respiratory ratio, and BacLight live cell ratio to refer to the plate count density/total cell density, the CTC-respiring cell density/total cell density, and the BacLight live cell density/total cell density, respectively. All ratios were averaged and expressed as the average ± standard error.

All antimicrobial experiments were repeated two or three times. Plate count analysis was conducted in triplicate, and more than five images per sample were taken for microscope analysis. The Student t test (two tailed) was performed for statistical analysis. A P value of less than 0.05 was used to indicate a significant difference, and a P value of more than 0.5 was used to indicate a similarity.

**RESULTS**

**Antimicrobial efficacies of chlorine, silver, and tobramycin against planktonic and biofilm cells.** In order to compare the treatment efficacies of the various antimicrobial agents against planktonic cells versus those against biofilm cells, inactivation curves were compared, as shown in Fig. 1. The data were derived from the plate count results. In Fig. 1a, for planktonic cells, the data for 1 and 10 mg/liter were averaged. For the biofilm experiment whose results are shown in Fig. 1b, only the data for 10 mg/liter were used since the 1-mg/liter treatment failed to cause any significant biofilm inactivation (less than 0.2 log unit of inactivation in 5 h). The inactivation efficiency of each antimicrobial agent was significantly different depending upon which state the cells were in: planktonic or biofilm (P < 0.00001). Overall, the CT values for biofilm cells were a factor of 10 greater than those for planktonic cells. As expected, chlorine was the most effective among the three antimicrobial agents against planktonic cells, as shown in Fig. 1a. It required a CT value of 0.05 mg · min/liter to inactivate 1 log (90%);
$CT_{90}$ of the planktonic cells with chlorine. On the other hand, the $CT_{90}$ of the biofilm cells in chlorine was more than 300 mg·min/liter (Fig. 1b). A $CT_{90}$ of 20 mg·min/liter was needed for planktonic cells with both silver and tobramycin. These $CT$ values are much larger than the $CT$ value of chlorine, indicating that silver and tobramycin are less effective antimicrobial agents than chlorine for planktonic cells. However, the $CT_{90}$ values of silver and tobramycin for biofilm cells were similar to the $CT$ value of chlorine ($P < 0.84$). As shown in Fig. 1b, the three different antimicrobial agents had roughly the same inactivation efficiencies against PAO1 biofilm cells ($P > 0.77$), even though they had significantly different antimicrobial efficiencies against planktonic cells.

**Total, culturable, respiring, and BacLight Live/Dead live cell densities of untreated biofilm.** An untreated biofilm was examined for total, culturable, respiring, and BacLight live cell densities (Fig. 2). The average total cell areal density was $2.5 \times 10^{11}$ cells/m²; and the average plate count cell density was roughly half of that, which means that half of the cells in the biofilm lost their culturability. For CTC-respiring cells, $2.3 \times 10^{11}$ cells/m² was observed. The BacLight live cell density was about $1.8 \times 10^{11}$ cells/m². Overall, for the untreated biofilm, 50% of the total cells maintained their culturability and 90% and 70% of the total cells were respiring and maintained intact cell membranes, respectively. It has been reported that biofilm cells can retain significant respiratory activity, even though they fail to form colonies (37).

The total cell density of the biofilm was $2.5 \times 10^{11}$/m², and the total growth surface area, which includes both sides of a coupon, was 1.9 cm² (coupon diameter, 1.1 cm). Thus, $4.8 \times 10^7$ cells were treated in total for each biofilm experiment. Planktonic cell experiments were done with $5 \times 10^7$ cells per test volume ($10^6$ CFU/ml $\times 50$ ml). The populations of both biofilm and planktonic cells were so similar that comparison conditions were satisfied.

**Comparison of biofilm inactivation efficiencies by three methods: determination of plate counts, respiration activity, and membrane integrity.** The inactivation curves for the biofilms treated with chlorine, silver, and tobramycin were evaluated by three methods of measurement, shown in Fig. 3. For chlorine, the plate count curve declined sharply over the treatment time. After 80 min of treatment, a reduction of 1.4 log units was observed in the plate count curve. The CTC-respiring and BacLight live cell curves almost overlapped, as cell reductions of 0.9 log unit were observed for both curves after 80 min. The inactivation curve of the plate counts decreased most sharply with silver treatment, while the CTC-respiring and BacLight live cell curves decreased more smoothly. A total of 1.6 log units of cells in the plate count experiments were inactivated during 80 min of silver treatment. For both CTC-respiring and BacLight live cells, 1.0-log-unit and 0.5-log-unit cell reductions were shown, respectively. In the experiments with tobramycin, the qualitative trends were similar to those of silver inactivation, although the magnitudes were different. After 50 min of tobramycin treatment, the cells in the plate count experiments were reduced 1.5 log units, and 0.9-log-unit and 0.2-log-unit cell reductions were observed for the CTC-respiring and BacLight live cell ratios, respectively.

**Comparison of respiration activity and membrane integrity of biofilms treated with three antimicrobial agents.** In order to compare the different results obtained with each antimicrobial agent more easily, the data in Fig. 3 were reorganized according to the CTC-respiring cell ratios and the BacLight live cell ratios. The plate count cell ratio is shown on the y axis, and the CTC-respiring cell ratio and the BacLight live cell ratio are shown on the x axis in Fig. 4a and b, respectively. For example,
mycin-treated biofilm maintained their cell membrane integ-

rity. In the case of the silver-treated biofilm, 50% of the cells retained their respiring activity, while 10% of the cells had damaged cell membranes. These results were visualized by CLSM imaging, detailed in the following section.

CLSM visualization of untreated and treated biofilms with 60% culturability inactivated by chlorine, silver, and tobramycin. Figures 6 and 7 show the CLSM images, analyzed by using Imaris software, of untreated and treated biofilms with 60% culturability. BacLight Live/Dead-stained biofilms were imaged and are shown in Fig. 6. Live cells, which have intact cell membranes, are stained with Syto9 and emit a green fluorescence when they are stained with the BacLight Live/Dead stain. Otherwise, cells with damaged membranes stained with propidium iodide and showed a red fluorescence. As shown in Fig. 6, the untreated biofilm was stained mostly green, with a few red cells being present. In the chlorine-treated biofilm, most cells were red, which means that chlorine intensively damaged the cell membrane integrity. On the other hand, there were half green cells and half red cells in the silver-treated biofilm and mostly green cells with a few red cells in the tobramycin-treated biofilm. The interpretation is that the cell membrane integrity was less damaged by silver and tobramycin than by chlorine.

Biofilms stained with CTC-DAPI were visualized through CLSM (Fig. 7). Respiring cells can reduce the redox stain CTC and fluoresce red, while nonrespiring cells are stained only by DAPI and fluoresce blue. The untreated biofilm showed mostly red-stained cells and a few blue cells (Fig. 7). Similarly, most cells in the chlorine-treated biofilm were red, which means that cell respiring activity was not as affected by chlorine. In contrast, the silver-treated biofilm showed some blue fluorescent cell clumps surrounded by red cells. Very few red cells were visible in the tobramycin-treated biofilm, which indicates that tobramycin reduced the cell respiring activity more than silver and chlorine did.

Enhanced efficiencies in combined or sequential antimicrobial treatment of biofilm. In order to compare the antimicrobial efficiencies of single treatments, combined treatments, and sequential treatments, the log inactivation results based on the plate cell count after 30 min of each treatment are summarized in Table 1. In the combined and sequential treatments, most treatments showed enhanced activity compared to the activity of the single treatments; the exception was the combination of
chlorine and tobramycin. Overall, silver and tobramycin, applied either in combination or sequentially, was the most effective at inactivating biofilm cells. This combination treatment showed almost 300% enhanced efficiency. Enhancements of 290% and 222% were observed when silver was used as the primary and as the secondary agent, respectively ($P = 0.00006$). Chlorine and silver combined showed a mild enhancement of 118%. The sequential application of chlorine and silver was more effective than the use of chlorine and silver combined, and the enhancements were 257% (when chlorine was used as the primary agent) and 197% (when chlorine was used as the secondary agent) ($P = 0.00008$). On the other hand, the antimicrobial activities of chlorine and tobramycin were decreased when they were used in combination.

**FIG. 6.** CLSM images of untreated and chlorine-, silver-, and tobramycin-treated PAO1 biofilms stained with BacLight Live/Dead stain. The culturability of each treated biofilm was 60%.

**FIG. 7.** CLSM images of untreated and chlorine-, silver-, and tobramycin-treated PAO1 biofilms stained with CTC-DAPI. The culturability of each treated biofilm was 60%.
efficiency when they were used in combination (53%) was much less than the sum of the individual efficiencies (100%). Similar antienhancing effects were observed when chlorine and ciprofloxacin or chlorine and carbencillin were combined (data not shown). A slightly enhanced efficiency of 109% was observed only when tobramycin was applied before chlorine was applied.

**DISCUSSION**

**Biofilm resistance to various antimicrobial agents.** We observed significantly increased levels of resistance of biofilm cells to chlorine (an oxidant), silver ion (a biocide), and tobramycin (an antibiotic) compared with those of planktonic cells. We also observed different mechanisms of action based upon the antimicrobial agent used. It is well known that the susceptibility of cells to antimicrobial agents is diminished in biofilms (1, 12, 13, 38, 40); and a literature survey of the susceptibilities of biofilms to several antimicrobial agents, including oxidants, antibiotics, and other biocides, was conducted (39), even though a direct comparison of biofilm susceptibilities to various agents can be made only when tobramycin was applied before chlorine and 29 for silver (16).

**Different reactivities of antimicrobial agents cause different kinds of damage in biofilm cells.** After antimicrobial treatment, many cells retained significant respiratory activity or membrane integrity, even though they lost the ability to form colonies on agar medium, as shown in Fig. 2 to 4. A previous study reported that bacterial numbers based on plate counts were less than the numbers based on direct viable counts, bacterial luminescence, and CTC or Syto9 staining (8). Among the three antimicrobial agents used in this study, chlorine appeared to damage the cell membrane most effectively, while tobramycin affected cell respiratory activity. Silver had characteristics between those of chlorine and tobramycin. This might be because of their different reactivities with the cells. Chlorine destroys microorganisms by chlorinating the lipid protein substance in the bacterial cell wall to form toxic chloro compounds (14, 42) and induces the leakage of macromolecules from the cells. One of the major bactericidal functions of silver is its interaction with the ribosome and the ensuing inhibition in expression of the enzymes and proteins essential to ATP production (47). Silver is also reported to react with the respiratory chain and inhibit the oxidation of glucose, glycerol, fumarate, etc. (4) and to make an Ag-DNA complex, which was reported to exhibit antibacterial activity (2). Tobramycin is one of the aminoglycosides, which are hydrophilic sugars with multiple amino groups that are protonated at physical pH to function as polycations and target accessible regions of polyaminic 16S rRNA on the 30S ribosome (43).

According to previous studies, the effects of the limited penetration of antimicrobial agents (38), changes in the bacterial phenotype of biofilm cells (25, 32, 40), and biofilm cells in persist (21, 22, 36, 45), as well as the different reactivities of antimicrobial agents, were widely considered to be factors which affect biofilm resistance to antimicrobial agents. It was shown that chlorine reacted with the cellular
biomass fast enough that the diffusion of the disinfectant into the biofilm was limited and that tobramycin could penetrate the P. aeruginosa biofilm but failed to inactivate the bacteria (44). A silver penetration study has not yet been reported; and only the adsorption of heavy metal ions, such as Cu$^{2+}$ and Zn$^{2+}$, by biofilms has been reported (5). Therefore, limited penetration cannot explain the different antimicrobial actions of the three agents at present. In addition, the differences in the reactivities of different phenotypes, i.e., dormant or persist-er cells, with oxidative antimicrobial agents have not yet been reported, so our results cannot be explained by these factors until further studies are conducted.

**Effective combination of antimicrobial agents for biofilm control.** Combined or sequential treatment with silver and tobramycin, which have relatively similar antimicrobial behaviors among three agents, was the most effective for biofilm control (Table 1). A significant enhancement of activity might be achieved by targeting of the antimicrobial actions of agents in combination, although in this case, the actions of both agents involve respiratory activity. On the other hand, chlorine was found to be less effective in combination with antibiotics. It is possible that chlorine reacted with the antibiotics (tobra-mycin, carbenicillin, ciprofloxacin), decreasing the available concentrations of both chlorine and the antibiotics. When 10 mg/liter of chlorine and 10 mg/liter of tobramycin were mixed, the chlorine concentration decreased to 5 mg/liter after 5 min and remained at 5 mg/liter for 30 min. Chlorine at 10 mg/liter is 191 μM and tobramycin at 10 mg/liter is 21 μM, so it is possible that 4.5 molecules of chlorine reacted with a molecule of tobramycin. This is plausible because tobramycin has five secondary amine groups per molecule which can react with chlorine. Chlorine demand was observed with carbenicillin and ciprofloxacin. In sequential treatment, the total antimicrobial efficiency was found to be enhanced when the biofilm was treated with a more reactive and less selective agent (i.e., chlorine) prior to treatment with a less reactive and more selective agent (silver ion). This was also applicable to silver and tobramycin but not to chlorine and tobramycin because they react with each other. It is supposed that a more reactive and less selective agent could alter the cell structure physi- cally or chemically in such a way that the antimicrobial action of a secondary agent might be facilitated.

In conclusion, the antimicrobial activities of an oxidant, a metal ion, and an antibiotic (chlorine, silver ion, and tobramycin, respectively) on biofilm cells were investigated by three methods, each of which used a different analytical principle for the determination of antimicrobial activity. The resistance of the biofilm cells to an oxidant was increased almost 250 and 300 times compared with the resistance to the metal ion and the antibiotic, as assessed by viable plate counts. The compo-nents of biofilm cells principally affected were related to cell membrane integrity in the case of oxidant treatment and res-piratory activity in the case of antibiotic treatment. Metal ion treatment affected both components similarly. The use of combinations of agents which have similar antimicrobial behaviors but which are not too oxidative, i.e., silver and tobramycin, might be an effective strategy for preventing microbial adapt-ation and facilitating the antimicrobial actions of the agents. This study might contribute to a better understanding of the antimicrobial interactions between biofilm cells and antimicro-bial agents and help to establish better strategies for the use of antimicrobials against biofilms through the appropriate choice and the use of the appropriate combinations of agents.

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