Mechanism of Resistance to Silver Ions in *Klebsiella pneumoniae*

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The uptake of silver by an experimentally derived silver-resistant *Klebsiella pneumoniae* strain was three to four times lower than the uptake by a susceptible strain. Spheroplasts of the two strains showed no difference in uptake. AgNO₃ at a concentration of 40 µg/ml decreased the succinate dehydrogenase activity in susceptible and resistant strains by 100 and 18%, respectively. More than one resistance mechanism may be involved.

The use of silver compounds as antimicrobial agents is becoming increasingly common in topical burn and wound therapy (4–6). However, there have been reports of plasmid-mediated resistance to silver in clinical isolates (8, 11). It appears that resistance to silver is accompanied by cell surface-associated changes and is halide ion dependent (14–16). Since *Klebsiella* spp. are important in burn wound pathogenesis (12) and in nosocomial infections (7), we studied the mechanism of resistance to silver in a silver-resistant (Ag⁺) *Klebsiella pneumoniae* strain that was derived from a wild-type silver-susceptible (Ag⁻) strain.

*K. pneumoniae* strain B-5-1, which is susceptible to silver (10 µg/ml), was obtained from Medical College, Rohtak, India. Silver resistance was developed in a derivative of this strain by adaptation to increasing concentrations of AgNO₃; 10 or 11 transfers were required to achieve resistance to 70 µg of silver per ml. Silver resistance was retained after growth for more than 50 generations in the absence of AgNO₃. No concurrent development of resistance to other metal ions (Hg²⁺, Zn²⁺, Cd²⁺, Cu²⁺, Pb²⁺) or to antibiotics, except nalidixic acid (2 to 5 µg/ml), was observed.

The uptake of a radioisotope of silver (¹¹⁰AgNO₃; Bhaba Atomic Research Centre, Bombay, India) by Ag⁺ and Ag⁻ cells was studied in nutrient broth containing the desired concentration of ¹¹⁰AgNO₃. Samples (3.0 ml) from shaking growth cultures were centrifuged at 3-h intervals. The cells were washed twice with distilled water, and the radioactivity was measured. Cell growth was measured regularly. Binding of ¹¹⁰Ag to cell sonic extracts and isolated cell walls was also studied. A 1.0-ml portion of a cell sonic extract or cell wall preparation was incubated with 10 µg of ¹¹⁰Ag at 37°C for 30 min, and 2.5 ml of 10% trichloroacetic acid was added. The fractions were analyzed for radioactivity. When necessary, the cell extracts, cell walls, and resting cell suspensions were prepared in distilled water instead of buffers to avoid precipitation of silver salts.

Figure 1 shows the correlation between growth and uptake of silver by cells grown in the presence of 10 µg of silver nitrate per ml. The uptake by Ag⁺ cells was one-half the uptake by Ag⁻ cells at the onset of incubation. This difference was maintained for 9 h of incubation, while the optical density at 540 nm of the Ag⁻ strain was stable and the viable count fell by 2 logs. Despite a severalfold increase in cell mass, the uptake by the Ag⁻ strain after 6 h was still less (3.0 µg; optical density, 6.5) than the uptake by the Ag⁺ culture (4.0 µg; optical density, 0.07). A comparative study of dry weights revealed that the levels of uptake by Ag⁺ cells (growing in the presence of 5 µg of ¹¹⁰AgNO₃ per ml) after 3, 6, and 24 h of incubation were 6.1, 3.0, and 1.9 µg/mg (dry weight), respectively, while the levels of uptake by the Ag⁻ cells were 2.3, 1.0, and 0.52 µg/mg (dry weight), respectively. However, the cell extracts and cell walls from the two strains bound roughly equal amounts of ¹¹⁰Ag (1.5 to 1.6 and 0.85 to 0.87 µg/mg [dry weight], respectively).

To evaluate the contribution of cell walls in silver resistance, spheroplasts from the susceptible and resistant *K. pneumoniae* strains were prepared by penicillin treatment (10), and their levels of susceptibility to AgNO₃ and uptake of ¹¹⁰Ag were determined. The uptake of silver by spheroplasts suspended in sucrose broth containing 0.7 µg of ¹¹⁰AgNO₃ per ml was greater than the uptake by intact cells (Table 1). Ag⁺ spheroplasts bound 93 to 94% of the isotope accumulated by Ag⁻ spheroplasts, whereas the uptake by Ag⁻ cells was about one-half the uptake by Ag⁺ cells under similar conditions. The susceptibility of spheroplasts to AgNO₃ was tested, and we found that Ag⁺ spheroplasts were only three times less susceptible to the action of silver than Ag⁻ spheroplasts.

We also studied uptake of ¹¹⁰Ag by toluene-treated cells (a cell suspension in water containing 0.3% [vol/vol] toluene and 0.7 µg of ¹¹⁰AgNO₃ per ml). Addition of toluene to Ag⁺ cells enhanced the level of uptake from 60 to 90% of the total isotope in the reaction mixture after 30 min at 37°C.

To determine whether development of resistance to silver altered the susceptibility of certain specific respiratory enzymes to silver, succinate dehydrogenase, an -SH-containing respiratory enzyme was studied, and the enzyme activity in cell extracts preincubated with 20 and 40 µg of AgNO₃ per ml for 15 min at 37°C were estimated colorimetrically (9). 20 and 40 µg of AgNO₃ per ml reduced the enzyme activity in Ag⁺ cells by 42 and 100% respectively, while the enzyme activity in Ag⁻ cells was inhibited by only 18% in both cases.

The decreased uptake by the Ag⁺ culture compared with the Ag⁻ culture was an indication of altered permeability or some other cell surface-associated changes, so that binding of ¹¹⁰Ag to Ag⁺ cells was decreased. However, the differences in the levels of uptake and susceptibility to AgNO₃ of Ag⁺ and Ag⁻ cells suspended in distilled water were not as significant as the differences observed with growing cells (unpublished data). It is possible that a resistance mechanism involving less uptake or binding needs a mediator, which is provided by the growth medium. Silver (14) proposed a halide ion-dependent silver resistance mechanism such that Ag⁺ cells do not extract Ag⁺ from an AgCl₂ precipitate, whereas Ag⁻ cells do. However, in our study, we observed no loss of silver resistance when nutrient broth without sodium chloride was used. Also, when we used
tryptone-yeast extract broth, which did not contain any NaCl, no significant decrease in the level of silver resistance was observed. Hence, it appears that chloride ions alone play little, if any, role in silver resistance, though NaCl does alleviate the toxicity of silver in susceptible cells. It is possible that other moieties, such as -SH group-containing proteins or peptides alone or in combination with NaCl, might be responsible for the phenomenon described above, since the addition of an -SH-binding reagent, such as N-ethylmaleimide resulted in a significant loss of silver resistance (unpublished data).

That the levels of binding of $^{110}$Ag to cell extracts of Ag$^+$ and Ag$^-$ strains were the same is significant since the levels of uptake by intact cells during growth were vastly different. Also, the levels of uptake by Ag$^+$ and Ag$^-$ spheroplasts were equivalent. Hence, an alteration in the cell envelope might be responsible for the lower level of uptake or binding of $^{110}$Ag by Ag$^-$ cells. The increased uptake of $^{110}$Ag after toluene was added suggests that lipids are involved in regulating the uptake and binding of $^{110}$Ag. Similar data are available for Cu$^{2+}$ and Hg$^{2+}$ resistance (2, 3, 13, 17). Preliminary studies on cell surface changes have revealed higher amounts of lipids in the cell walls of the resistant cells (unpublished data).

Silver ions affect respiration in bacteria (1), and prevention of respiration inhibition by -SH-containing compounds has also been reported (1). In this study AgNO$_3$ was found to inhibit succinate dehydrogenase, an -SH-containing respiratory enzyme. However, the level of inhibition was less in the Ag$^+$ strain, which suggests that there may be a structural alteration in the enzyme or some change in the enzyme environment which renders the enzyme less susceptible to AgNO$_3$. Since a K. pneumoniae Ag$^+$ cell extract offered no protection to the succinate dehydrogenase of Ag$^-$ cells, the alteration might well be in the enzyme structure, although further work will be needed to answer this question. In view of the observations described above, it appears that the decrease in permeability as a mechanism of resistance in the Ag$^+$ strain may be accompanied by other changes, which enable the strain to withstand high levels of silver in the medium.

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**LITERATURE CITED**