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Adherence of organisms to silver-coated surfaces

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Pure silver-, silver oxide- and silver chloride-treated surfaces in comparison to polypropylene inhibited both growth and adherence from saline of *Serratia marcescens*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Candida albicans*. These same organisms demonstrated enhanced adherence to an Ion-Beam-Assisted-Deposited silver surface followed by loss of viability. This type of surface in contrast to the other silver surfaces did not produce zones of inhibition in agar diffusion tests.

Keywords: silver; bacterial adherence; antimicrobial silver

Introduction

Silver has been known for years for its broad spectrum antimicrobial properties. Colloidal silver was used in wound antisepsis and in combination with citrate salts for skin infections. One to two percent silver nitrate was employed almost 100 years ago for the treatment of ophthalmia neonatorum. Shortly thereafter silver acetate was formulated into eye lotions and creams, silver nitrate (0.01%) was used in bladder irrigations and silver lactate (1 : 100 to 1 : 2500) was employed as a general antiseptic [11]. Silver sulfadiazine, the non-ionized, water-insoluble powder (1%) was applied in creams for treatment of burn wounds and prevention of infections by Fox in 1968 [8] and is currently still in use. Colloidal preparations of silver salts are still used medically for local antiseptics on mucous membranes, silver acetate-containing antismoking lozenges, and coatings for breath mints. Other medical applications of silver involve silver-coated nylon fabrics that are used to treat and manage postoperative debridement wounds in cases of severe chronic osteomylitis and antimicrobial Foley catheters [3,9,11,14]. Aside from medical uses, silver is employed in water treatment partly because of concerns about the production of possible carcinogens during the disinfection of water with chlorine. Various silver-treated charcoal filters that remove odors and reduce organics in drinking waters are commercially available. Also, various ionization apparatus that produce low levels of silver ions ($<0.5\ \mu l\ ml^{-1}$) are manufactured for microbial control in recreational pool waters.

Silver occurs naturally in several oxidation states. The most common are elemental silver (Ag^0) and the monovalent silver ion (Ag^+). Compounds with higher oxidation states of silver (Ag^{+2} and Ag^{+3}) are also known. Antelman [1] proposed that silver could be prepared in a molecular crystal form (an oxide lattice where one pair of silver ions in the molecule is trivalent and another pair is monovalent). This preparation, tetrasilver tetroxide, is reportedly biocidal via a release of electrons. He reported 100% kills of bacteria (10^6 per ml) within 5 min at a concentration of Ag_4O_4 as low as 0.5 ppm.

The exact antimicrobial mode of action of silver is not known, but several possible mechanisms have been suggested. The oligodynamic effect of silver is attributed to the inactivation of enzymes via silver complexes with electron donors containing sulfur, oxygen or nitrogen (thiols, carboxylates, phosphates, hydroxyl, amines, imidazoles, indoles). Slawson *et al* [22] reported that silver ions inhibit the respiratory chain at two sites between cytochome *b* and cytochrome *d*; and between the site of substrate entry into the respiratory chain and flavoprotein in the NADH and succinate dehydrogenase regions. Silver ions at 86 μm inhibited oxidation of glucose, glycerol, fumarate, and succinate in *E. coli* [4]. Concentrations of 0.001 to 1.0 mM Ag^{2+} may alter enzyme conformations [18]. Ag ions also form complexes with bases contained in DNA [23] and are a potent inhibitor of fungal DNAases [19,20]. Coward *et al* [6], using electron microscopy, reported that silver sulfadiazine attacks the cell wall and forms blebs in the cell membrane. In spite of the historical recognition of the antimicrobial activity of silver, and its role in maintaining potability of water [7], there is little definitive information on the adherence of bacteria to silver or to other surfaces.

Insertion of prosthetic devices into the human body often leads to the formation of biofilms on the surface of the device [5,10,16,17]. The development of prosthetic devices with surfaces recalcitrant to biofilm formation would be of considerable value. Because silver is antimicrobial and relatively non-toxic for mammalian cells [2], its potential for further use in medical devices should be evaluated. This report examines the effect of some commercially available silver preparations on the primary adherence of *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *C. albicans* to solid surfaces.

Materials and methods

Organisms and culture conditions

Bacterial cells, *Staphylococcus epidermidis* SD23022B, *Pseudomonas aeruginosa* 3 and *Serratia marcescens* GSU 86-828 were obtained from corneal ulcer and ophthalmitis patients and maintained in a lyophilized state. Working cul-

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tures were kept on tryptic soy agar (TSA, BBL Microbiology Systems, Cockeysville, MD, USA), transferred every 2 months, and stored at 4° C. *Candida albicans* GSU 30 was grown in the same manner in Sabouraud Dextrose Broth (Difco Laboratories, Detroit, MI, USA).

Bacteria from working cultures were grown in shaken culture for 12–18 h at 37° C in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, USA). The cells were harvested by centrifugation and washed twice with sterile, phosphate buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.1% Na_2HPO_4, 0.02% g KH_2PO_4 L^{-1}). Bacterial and yeast cells were suspended in PBS to give 10^4 and 10^8 colony forming units (cfu) ml^{-1}, respectively.

Substrates

Silver oxide, silver chloride and pure silver were compared with an Ion-Beam-Assisted-Deposited (IBAD) silver coating. The silver oxide and silver chloride coatings were bonded to silicone and latex. The IBAD coating overlaid polyethylene, polypropylene and silicone. These materials were sectioned into discs usually 10 mm in diameter by 1.0 mm in thickness. Specially prepared polyethylene wells and wells coated with the IBAD silver coating were also studied. In certain experiments 5-mm cylinders or 10-mm squares of the above materials were examined. These various shapes will be subsequently referred to as sections. The processes for bonding the silver to the plastic surfaces and their exact composition were proprietary and unknown to us. Pure silver was obtained from a commercial source.

Biofilm challenge

Inocula (1 ml) were placed into polyethylene wells and IBAD-coated wells (volume 4 ml) and incubated at 25° C for 4, 24, and 48 h. The sections of other silver materials were also placed into polyethylene wells. After incubation for the above periods, the supernatant cell suspension was aspirated by pipette from the wells, serially diluted and plated onto enrichment agars. The wells were swabbed with separate sterile swabs and the swabs were streaked onto Tryptic Soy or Sabouraud Dextrose agar plates. The plates and tubes were incubated at 37° C and positive or negative growth results were recorded after 72 h. The sections of the other materials were removed from the polyethylene wells and treated similarly.

Radiolabel experiments

Cells from isolated colonies grown on TSA were inoculated into TSB and incubated at 37° C on a rotary shaker (150 rpm) for 12–18 h. Cells were harvested by centrifugation at 5000 × *g* for 5 min, washed twice in 0.9% saline (0.01 M) and suspended in minimal broth (0.1% g D-glucose, 0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.05% sodium citrate, 0.1% $(NH_4)_2SO_4$, and 0.01% $MgSO_4$ in 1 L distilled water). The D-glucose and $MgSO_4$ solutions were autoclaved separately. The minimal broth cultures were incubated with shaking at 25° C for 1 h. One to 3 μCi ml^{-1} of L-[3,4,5-^{3}H]leucine (NEN Research Products, Du Pont Company, Wilmington, DE, USA) with a specific activity of 153 Ci mM^{-1} were added and incubation was continued for 20

Table 1 Recovery of cells from inocula suspensions (10^8 cells) and from cells in biofilms in silver-coated wells and in non-coated (control) wells*

Age of biofilm and species	Planktonic		Biofilm	
	IBAD silver	Control	IBAD silver	Control
4 hours				
S. marcescens	0[a]	9.0×10^5	0	10^7–10^8
P. aeruginosa	1.4×10^6	1.9×10^6	10^8	10^7–10^8
S. epidermidis	9.1×10^6	2.0×10^6	10^8	10^7–10^8
C. albicans	9.8×10^6	2.3×10^7	TNTC[b]	225
C. albicans[c]	0	<100	<100	<100
24 hours				
S. marcescens	0	1.4×10^6	0	10^7–10^8
P. aeruginosa	0	5.8×10^4	0	10^7–10^8
S. epidermidis	4.2×10^7	$21. \times 10^6$	10^8	10^7–10^8
C. albicans	3.1×10^7	2.6×10^7	TNTC	TNTC
C. albicans[c]	0	<100	<100	<10[d]
48 hours				
S. marcescens	0	1.7×10^6	0	10^7–10^8
P. aeruginosa	0	2.9×10^6	0	10^7–10^8
S. epidermidis	0	2.5×10^6	<100	10^7–10^8
C. albicans	1.4×10^7	2.0×10^7	TNTC	TNTC
C. albicans[c]	<10	<200	<100	<300

*Average results of three experiments
[a]0 = no cells recovered, less than 1 cell ml^{-1}
[b]Too numerous for accurate counts
[c]Inoculum suspension is 10^4
[d]<10 = 1–9 colonies recovered

min. These cells were washed four times and suspended in saline to a concentration of about 10^8 cells ml^{-1}. Sections of test material were incubated with 3 ml of the radiolabeled cell suspension for 2 h at 25° C. The sections were removed from the cell suspension with forceps and immersed five times in each of three successive changes of saline (250 ml). The sections were shaken free of excess saline and transferred to 20-ml glass scintillation vials. Ten milliliters of Opti-Fluor scintillation cocktail (Packard Instrument Co, Downers Grove, IL, USA) were added to each vial; the vials were vortexed, and counted in a liquid scintillation counter (LS-7500, Beckman Instruments, Inc, Fullerton, CA, USA). Scintillation counts were converted to actual cell numbers with a calibration curve relating disintegration per min (DPM) to viable cell counts mm^2 of surface area.

In variations of the procedure, sections were incubated for 18 h in 3 ml of minimal broth that was inoculated with 10^4 cells ml^{-1}. The non-radiolabeled bacteria were allowed to adhere first to the sections which were then exposed to radiolabeled leucine. The cells undergo growth in the minimal broth during the 18 h to densities estimated to be from 10^7 to 10^9 ml^{-1}. Because of this amplification, in these experiments a relative degree of adherence was provided (as indicated by differences in DPM) rather than cells/surface area. After incubation, the samples were immersed five times in each of three successive changes of saline (250 ml). Three changes of saline removed all nonspecific radioactivity associated with the sections.

Scanning electron microscopy

Sections were incubated with 10^8 cells ml^{-1} of Gram-negative bacteria in saline at 37° C for varying time periods up to 24 h. The sections were placed in glass vials and rinsed for 5 min with 3 ml of Sorensen's phosphate buffer pH 7.4. The sections were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 1 h at room temperature, and rinsed three times for 10 min in 0.1 M phosphate buffer. Postfixation was in 1% OsO_4 in 0.1 M phosphate buffer for 1 h at room temperature. The sections were then rinsed three times in 0.1 M phosphate buffer for 10 min, dehydrated in a graded ethanol series, and dried in the presence of CO_2 in a critical point dryer (Balzer's CPD 020, Balzer's Ltd, Hudson, NH, USA). The sections were sputter coated with 7–9 nm Au/Pd and examined in a JEOL-35C scanning electron microscope (Leica Cambridge Ltd, Cambridge, UK) operating at 15 kV.

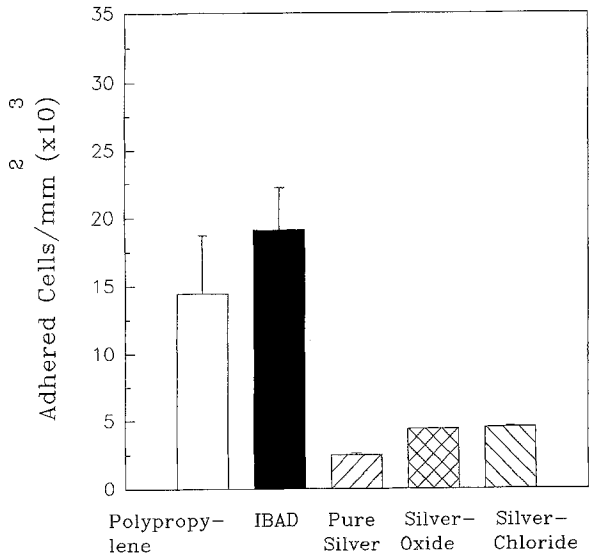

Figure 1 Adherence after 2 h of *S. marcescens* from saline to various surfaces. Vertical bars represent the standard error of the mean (n = 6)

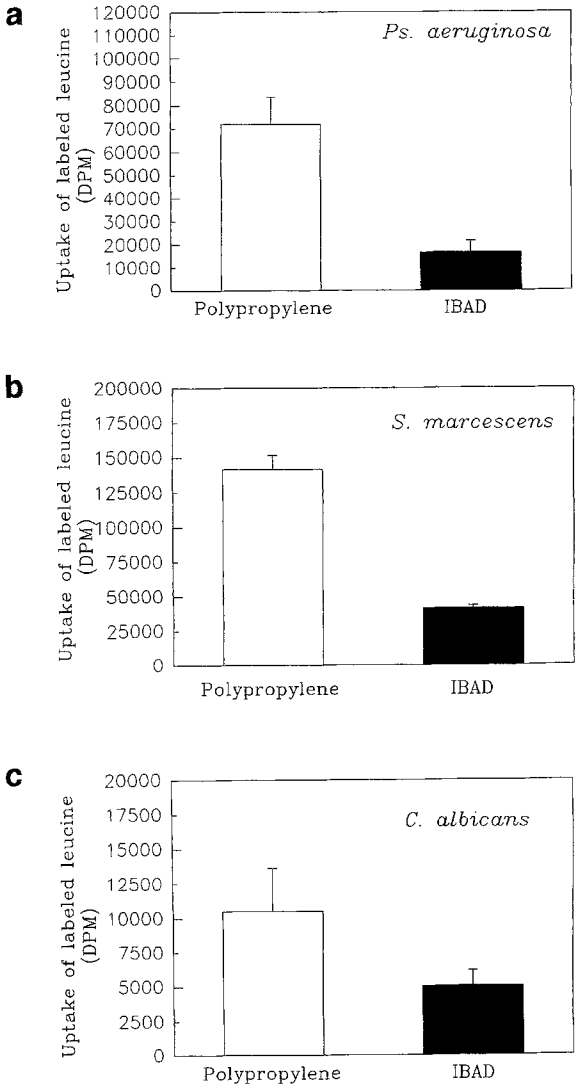

Figure 2 Uptake of radiolabeled leucine by microorganisms adhered to polypropylene (□) and IBAD silver (■). Cells were adhered from saline over 18 h at 22° C. Vertical bars represent the standard error of the mean (n = 6)

Silver leaching studies

IBAD silver-coated polypropylene wells and controls were filled with purified water and borate buffered saline. The wells were stored for 7–10 days and the contents analysed for total silver by Inductively Coupled Plasma Emission Spectroscopy [21]. Sections of the silver test materials were placed in petri dishes and overlayed with a thin film of half-strength TSA agar seeded with *S. marcescens* (10^6 cells

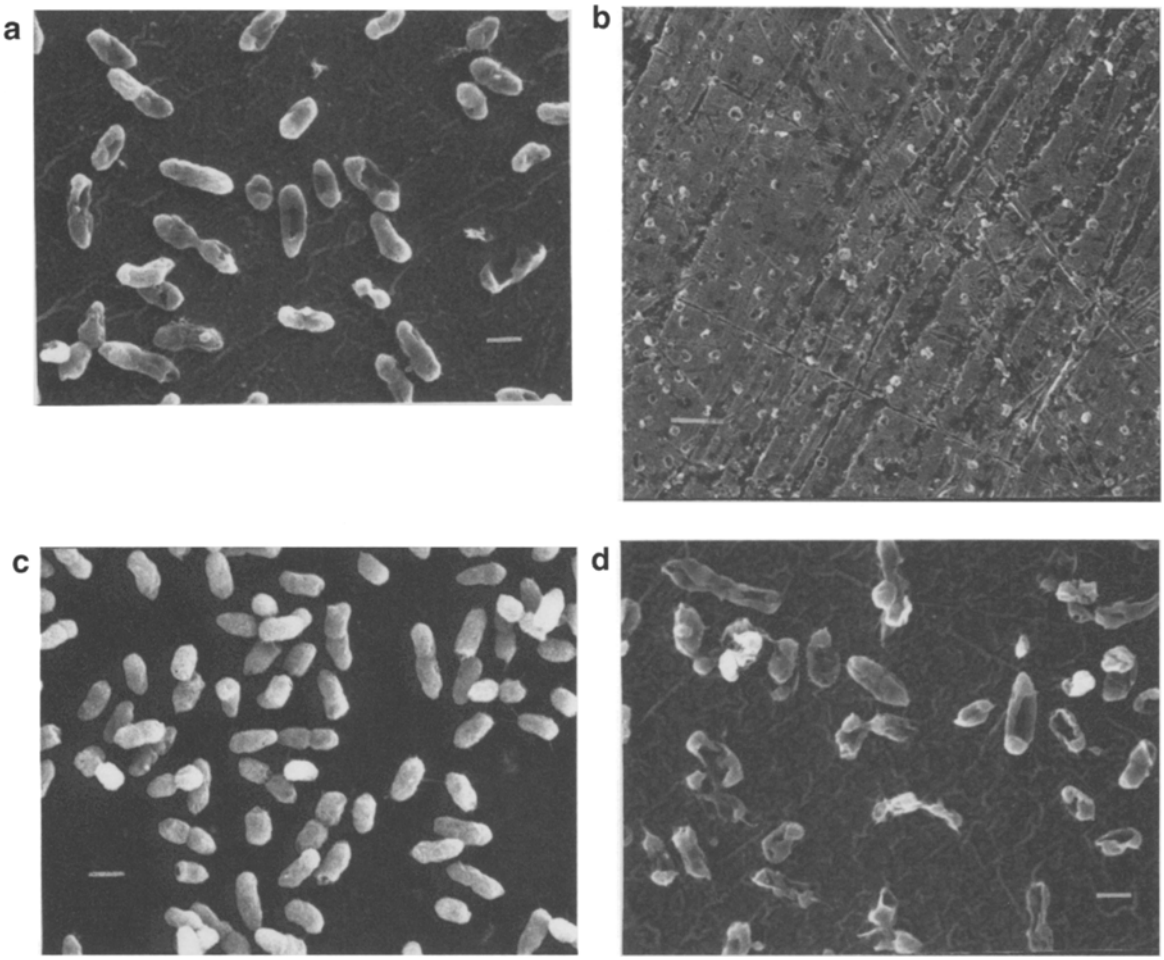

Figure 3 Scanning electron microscopy of adherence of *S. marcescens* to: (a) IBAD silver after 4 h (bar = 1.0 μm), (b) fine silver after 18 h (bar = 3.0 μm), (c) polypropylene control after 4 h (bar = 1.0 μm), (d) IBAD silver after 18 h (bar = 1.0 μm)

ml^{-1}). The plates were incubated at 22° C and examined at 24 and 48 h for zones of inhibition.

Statistical analyses

In all tests, at least five discs were examined and tests were performed at least in triplicate on separate testing days. Adherence to the various materials compared statistically with an unpaired '*t*-test'. Results were considered significant at $P<0.05$.

Results

Biofilm challenge

The numbers of bacteria recovered from saline solutions stored in the IBAD silver-coated wells decreased markedly with time. *S. marcescens* was the most sensitive bacterium and *S. epidermidis* was the most resistant. Data for a representative series of experiments are presented in Table 1. The time for the cell densities to decrease to zero recovery was inoculum-sensitive and varied in repeat experiments between 24 and 72 h. The dependence of the inhibition on inoculum level was most evident with *C. albicans*. At 10^7 cells ml^{-1}, reduction of recoverable yeast cells from the buffer and silver surfaces was negligible within 48 h, whereas at 10^4 cells ml^{-1} there was no recovery at 4 h from the buffer and only a few cells persisted on surfaces at 24 h (Table 1). In general, bacteria and yeast cells appeared to persist longer on the surfaces than in the saline.

Microbial adherence

Adherence of *S. marcescens* after 2 h exposure to IBAD silver-coated polypropylene, polyethylene or silicone was significantly enhanced compared to non-coated controls or to pure silver. Representative data in comparison to polypropylene are shown in Figure 1. Relatively few cells ($<10^4$ mm^2) adhered to silver oxide- and silver chloride-coated surfaces within 2 h. Adherence to polyethylene alone was essentially the same as that to polypropylene (data not shown). In general after 18 h exposure the number of cells on the surface of the IBAD silver capable of uptake of leucine was reduced significantly (Figure 2). The reduction in uptake of leucine by *S. marcescens* was noted as early as 4 h (data not shown).

SEM

Mostly collapsed and apparently lysed cells of *S. marcescens* were present on the IBAD silver after 4 h; only sparse cell debris or clumps of shrunken cells were present on pure silver (Figure 3). The damaged cells in concentrations similar to those observed at 4 h were also evident on the

IBAD silver at 18 h exposures (Figure 3). The cells of *P. aeruginosa* on IBAD appeared similar to those of *S. marcescens* but were most evident at 18–24 h exposures (data not shown).

Silver leaching

The release of ions from IBAD silver coatings into purified water and borate-buffered saline was negligible. The silver level in purified water was 0.06 ppm and in borate buffer 0.08 ppm; the detection limit for this assay was 0.05 ppm. Also, this material produced negligible zones of inhibition in studies which employed agar diffusion whereas zones from 3 to 5 mm were detected with pure silver, silver oxide- and silver chloride-containing materials.

Discussion

The antimicrobial activities of silver, AgCl and AgO are well documented. However, there are only vague reports on the role of silver in preventing bacterial adherence, although this may be implied from empirical observations [7,15]. Mclean *et al* [15], noted that multiple metal surface-film combinations such as Ag/Cu offer great promise in lowering the incidence of device-associated nosocomial infections. These authors showed that the viability of *S. epidermidis* and *S. aureus* was greatly reduced within 10 h of exposure to Ag/Cu sputter-coated catheter material, and eliminated within 24 h. However, they also noted that a Ag/Cu alloy complex did not produce a zone of inhibition in agar diffusion tests and that Ag ions were not released from the Ag/Cu coating.

Our studies indicate that silver surfaces that release Ag ions are not only biocidal but inhibit bacterial adherence to the silver-treated surface. In contrast, the IBAD silver-coated material, which could not be shown readily to release ions, was a surface that permitted bacterial adherence, followed by killing and detachment (possibly from ablation). The cause of this enhanced initial attachment to the IBAD surface is not clear. In separate studies (unpublished data), the efficacy of the IBAD surface against *S. marcescens* was maintained after multiple challenges.

Other investigations have noted antimicrobial activities at metal surfaces without apparent release of toxic metal ions. Heining [12] suggested that a silver-alumina surface could promote a catalytic interaction with oxygen, which resulted in bactericidal activity. Complexing of silver with various oxides, such as aluminum, palladium or titanium, is possible. Titanium oxide may serve as a photocatalyst for the production of toxic hydroxyl radicals [13]. This latter type of activity was proposed for a silver-alumina medium. Our inhibitory silver-coated materials however were incubated in the dark immediately after inoculation. Possibly, metabolic activities of attached cells resulted in uptake of Ag ions. Alternatively the IBAD silver surface with its enhanced binding of bacterial cells affected the conformation of cell membrane proteins resulting in cell lysis. This physical effect may have interacted with trace ion toxicity. IBAD-silver technologies represent a new era in disinfection research. Additional studies are necessary to better understand the dynamics of these metallic based systems with microorganisms.

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