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Surface Action of Gentamicin on *Pseudomonas aeruginosa*

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The mode of action of gentamicin has traditionally been considered to be at the 30S ribosomal level. However, the inhibition of bacterial protein synthesis alone appears to be insufficient to entirely explain the bactericidal effects. Bacteriolysis is also mediated through perturbation of the cell surface by gentamicin (J. L. Kadurugamuwa, J. S. Lam, and T. J. Beveridge, Antimicrob. Agents Chemother. 37:715–721, 1993). In order to separate the surface effect from protein synthesis in *Pseudomonas aeruginosa* PAO1, we chemically conjugated bovine serum albumin (BSA) to gentamicin, making the antibiotic too large to penetrate through the cell envelope to interact with the ribosomes of the cytoplasm. Furthermore, this BSA-gentamicin conjugate was also used to coat colloidal gold particles as a probe for electron microscopy to study the surface effect during antibiotic exposure. High-performance liquid chromatography confirmed the conjugation of the protein to the antibiotic. The conjugated gentamicin and BSA retained bactericidal activity and inhibited protein synthesis on isolated ribosomes in vitro but not on intact cells in vivo because of its exclusion from the cytoplasm. When reacted against the bacteria, numerous gentamicin-BSA-gold particles were clearly seen on the cell surfaces of whole mounts and thin sections of cells, while the cytoplasm was devoid of such particles. Disruption of the cell envelope was also observed since gentamicin-BSA and gentamicin-BSA-gold destabilized the outer membrane, evolved outer membrane blebs and vesicles, and formed holes in the cell surface. The morphological evidence suggests that the initial binding of the antibiotic disrupts the packing order of lipopolysaccharide of the outer membrane, which ultimately forms holes in the cell envelope and can lead to cell lysis. It is apparent that gentamicin has two potentially lethal effects on gram-negative cells, that resulting from inhibition of protein synthesis and that resulting from surface perturbation; the two effects in concert make aminoglycoside drugs particularly effective antibiotics.

Regardless of nephrotoxicity and ototoxicity, aminoglycosides are a class of antibiotics which are widely used in the treatment of serious infections by aerobic gram-negative bacteria (20). Although considerable research effort has been expended in determining the mechanism of action of these antibiotics, the precise biochemical events leading to bactericidal activity still remain a matter of controversy (1, 9, 23). The uptake of an aminoglycoside antibiotic such as gentamicin into *Pseudomonas aeruginosa* has been recognized as a multifactorial process involving an initial ionic interaction with the exterior of the cell followed by two energy-dependent phases requiring an energized cytoplasmic membrane (1, 4, 26). Once inside, it is believed that the drug works at the ribosomal level by inhibiting bacterial protein synthesis, and this effect appears to be a necessary part of the lethal event (2, 16). However, other antibiotics (e.g., chloramphenicol), also inhibit protein synthesis at least as effectively as do the aminoglycosides yet fail to cause death, causing only bacteriostasis (16). The inhibition of bacterial protein synthesis appears to be insufficient to entirely explain the rapid bactericidal effect of aminoglycosides. Similarly, the uptake of gentamicin through nonspecific channels created by the insertion of aberrant proteins into the membrane correlates imperfectly with lethality (16, 23).

It has been proposed that bactericidal aminoglycosides have other sites of action that help cause lethality (4, 8). Hancock (8) proposed a self-promoted uptake of aminoglycosides which involves rapid electrostatic binding of polycations such as gentamicin to outer membrane (OM) sites which are normally occupied by divalent cations used to cross bridge adjacent lipopolysaccharide (LPS) molecules. Aminoglycosides competitively displace the divalent cations, thereby perturbing the OM to promote the self-entry of the antibiotic. This event then could permeabilize the OM and could lead to cell death. Matsunaga and coworkers (19) have put forward another mechanism for the bactericidal action of aminoglycosides. They observed that the bactericidal aminoglycoside blocks the initiation of DNA replication and suggested this to be responsible for the lethal effect.

In a previous study, we demonstrated that ionic binding of gentamicin caused approximately a 50% loss in the viability in *P. aeruginosa* (14). These data together with previous results from our laboratory (18, 28) led us to believe that binding of gentamicin to the OM also contributes to cell death by weakening the entire cell envelope, which consists of the OM, peptidoglycan, and the plasma membrane. To further test this hypothesis, we chemically conjugated bovine serum albumin (BSA) to gentamicin so that the resulting complex was too large to pass through the cell envelope and the surface effect of gentamicin alone could be studied. In this way the surface binding sites of gentamicin and their distribution on bacteria could also be revealed, especially once colloidal gold was added to the complex to increase the electron scattering power for electron microscopy on intact bacterial cells before, during, and after death. This experimental technique allowed us to investigate how ionic binding contributed to the antibiotic's bactericidal action and to develop a better mechanistic view of the killing process of gentamicin in *P. aeruginosa*.

(Parts of this study were presented at the 93rd General Meeting of the American Society for Microbiology [13]).

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PAO1 (14) and *Escherichia coli* DH5α (GIBCO BRL, Ontario, Canada) were grown in Mueller-Hinton broth (MHB) (Difco Laboratories, Detroit, Mich.) to the mid-exponential growth phase on an orbital shaker at 37°C.

Conjugation of gentamicin to BSA. Conjugation of gentamicin to BSA was performed by the method of Lewis et al. (15), with slight modifications. Five hundred one milligrams of gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) and 100 mg of BSA fraction V (Boehringer Mannheim, Montreal, Canada) were dissolved in 10 ml of 0.15 M NaCl–0.01 M sodium phosphate buffer (PBS) (pH 7.5) containing 0.1% (wt/vol) sodium azide (Fisher Scientific Co., Toronto, Canada). Coupling of the amine groups of gentamicin to the carboxyl groups of BSA was accomplished by the dropwise addition of 3.1 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Sigma) in 10 ml of distilled water at pH 7.5 over a 30-min period, with constant stirring. The mixture was incubated for 1 h at room temperature followed by 3 days at 4°C. The conjugate was dialyzed to remove unbound reactants from gentamicin-BSA at 4°C against 4 liters of PBS, with daily changes over a 4-day period, and then chromatographed on a column of Bio-Gel P6-DG (2.5 by 25 cm; Bio-Rad Laboratories, Ltd., Toronto, Canada), with water as the eluent at a flow rate of 2 ml/min. Fractions with an A_{280} were collected and lyophilized. Similarly, BSA was reacted with carbodiimide, but without gentamicin in the reaction mixture, to serve as a control in some experiments.

Preparation of gentamicin-BSA-gold. For electron microscopic localization of gentamicin binding sites, 15 nm of colloidal gold was prepared by the method of Frens (7) and subsequently reacted with the conjugated gentamicin-BSA solution by the method of Roth et al. (25).

Antibiotic susceptibility test. The MICs of gentamicin and the gentamicin-BSA complex were determined by a dilution method in MHB (22).

Bactericidal activities of gentamicin and gentamicin-BSA on *P. aeruginosa*. Exponential-growth-phase cultures were diluted in MHB to produce a bacterial suspension of 10^8 CFU/ml and were incubated at 37°C with subinhibitory (one-fifth the MIC) and inhibitory (the MIC or twice or four times the MIC) concentrations of the antimicrobial agents. Identical bacterial cultures with similar concentrations of BSA or BSA reacted with carbodiimide served as controls. After the cultures were exposed to antimicrobial agents for 0, 1, 2, 3, 4, and 18 h, the numbers of viable bacteria in the postexposure test and control cultures were determined as previously described (14). All assays were performed in triplicate, and each datum point in Fig. 1 to 3 is the average of at least three experiments.

Effect of gentamicin-BSA on an *E. coli* ribosomal S30 extract. The effect of gentamicin-BSA on protein synthesis was investigated by assaying β-galactosidase enzyme activity in the *E. coli* S30 coupled transcription-translation in vitro system (Promega Corp., Toronto, Canada) according to the manufacturer's instructions.

Assay for the penetration of gentamicin-BSA into the cytoplasm of *E. coli* DH5α (pGEMβgal) whole cells. The plasmid containing *lacZ* gene pGEMβgal (Promega) was transformed into *E. coli* DH5α according to the manufacturer's (GIBCO BRL) instructions. Successful transfer was monitored by antibiotic resistance and with Qiagen plasmid midipreparations (Qiagen, Inc., Chatsworth, Calif.). Exponentially growing *E. coli* organisms in MHB containing 100 μg of carbenicillin (Sigma) per ml were diluted to approximately 10^6 CFU/ml in fresh broth containing 5×10^{-4} M isopropyl-β-D-thiogalactoside (Boehringer Mannheim) and one-half the MIC of gentamicin or gentamicin-BSA. Identical cultures without the antibiotic served as controls. Test and control cultures were incubated at 37°C for 12 to 18 h. Intracellular β-galactosidase activity was measured with *o*-nitrophenyl-β-D-galactopyranoside (Sigma) as the substrate. Briefly, 1.0 ml of the cell suspension was pelleted by centrifugation at 12,000 × *g* for 10 min and the pellet was resuspended in 0.25 ml of 0.85% (wt/vol) sodium chloride containing 10 μl of toluene. After incubation for 10 min at 37°C, 0.25 ml of 0.4% (wt/vol) *o*-nitrophenyl-β-D-galactopyranoside in 2.5 mM PBS (pH 7.0) was added and the mixture was reincubated for a further 10 min. The cells that were not exposed to the antibiotic were considered to have 100% enzyme activity and were used as the reference. The enzyme activity was measured by monitoring the A_{420} and expressed as percent activity per weight of bacteria.

Sample preparation for thin-section electron microscopy. *P. aeruginosa* cells were harvested in the exponential growth phase, washed, and suspended in MHB to 10^8 CFU/ml. Cells were incubated with the gentamicin-BSA-gold suspension at concentrations ranging from the MIC to four times the MIC for 30 min at room temperature, enrobed in molten 2% (wt/vol) agar, and immediately fixed with a solution containing 2.5% (vol/vol) glutaraldehyde and 4% (wt/vol) formaldehyde in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma) buffer (pH 6.8) for 2 h. Cells were washed in HEPES buffer and were fixed for a further 1 h at room temperature in HEPES buffer containing 1% osmium tetroxide, washed in distilled water, incubated for 30 min in 2% aqueous uranyl acetate, dehydrated in an ethanol series (25, 50, 95, and 100% [vol/vol]), and embedded in Epon 812 (Can EM, Guelph, Canada). Thin sections were then cut with a Reichert OM U4 Ultracut ultramicrotome. Sections were collected on carbon- and Formvar-coated copper grids and poststained with uranyl acetate and lead citrate. Cells incubated with BSA-gold, colloidal gold, carbodiimide-reacted BSA-gold, or gentamicin-gold served as controls.

Unstained whole mounts of bacteria for electron microscopy. Cells were prepared as described above, and 20 μl was placed on carbon- and Formvar-coated nickel grids for 30 min and rinsed with distilled water. All samples were examined with a Philips EM300 transmission electron microscope operating at 60 kV under standard conditions with a liquid nitrogen cold trap in place.

Analytical chromatography. In order to determine the molar ratio of the antibiotic to the protein, high-performance liquid chromatography (HPLC) was performed with a Dionex series 4500 system (Dionex Canada, Ltd., Mississauga, Canada) with gentamicin, gentamicin-BSA, and BSA (1.0 to 3.0 mg) after acid hydrolysis in 4 M HCl at 100°C for 12 h in vacuo (5). The protein concentrations in gentamicin-BSA complexes were determined by amino acid analysis with a Beckman Gold amino acid analyzer (Beckman Instruments, Mississauga, Canada) with postcolumn ninhydrin detection. Samples of protein (approximately 0.15 mg each) were hydrolyzed in vacuo with 6 M HCl at 110°C for 16 h.

RESULTS

Conjugation of gentamicin to BSA. Analysis by HPLC was performed to confirm the conjugation of the antibiotic to

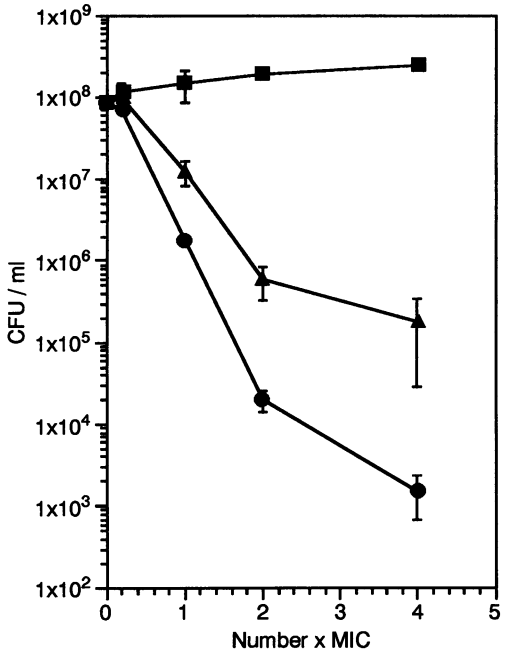

FIG. 1. Viability of *P. aeruginosa* PAO1 incubated with different concentrations of gentamicin and gentamicin-BSA. Symbols: ■, control; ●, gentamicin; ▲, gentamicin-BSA. The values are the means ± the standard deviations of three independent determinations.

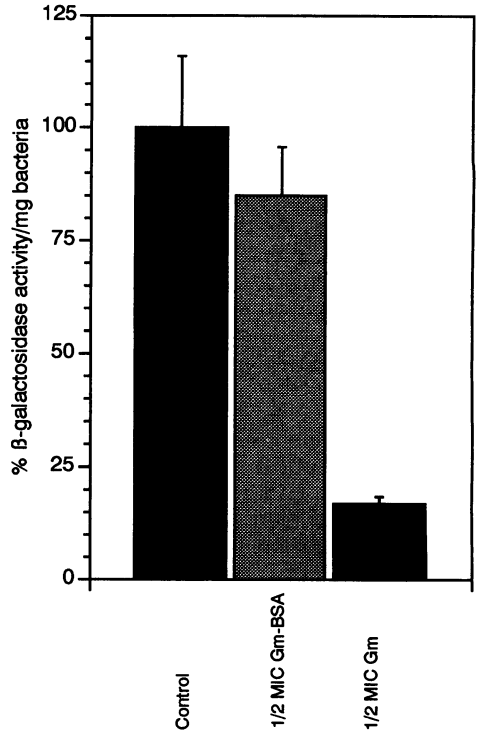

FIG. 2. Effects of gentamicin (Gm) and gentamicin-BSA (Gm-BSA) on protein synthesis, determined by assaying intracellular β-galactosidase activity in intact *E. coli*. Data are the means ± the standard deviations of three independent experiments.

BSA as well as to calculate the molar ratio of the protein to the antibiotic. After acid hydrolysis of gentamicin and gentamicin-BSA, a peak with a retention time of 4.73 ± 0.04 min was a common component of both gentamicin and the complex but was not a component of the BSA acid hydrolysate. This confirmed the chemical coupling of the antibiotic to the protein. The protein concentration in gentamicin-BSA was estimated by analysis of the amino acid content; this allowed the estimation of the molar ratio of the protein to the antibiotic, which ranged from 1:25 to 1:53. In a previous study, Lewis et al. (15) observed this binding ratio to be 1:25 to 1:50.

Bactericidal activities of gentamicin and gentamicin-BSA against *P. aeruginosa* PAO1. As a result of charge interaction of the amino group(s) of gentamicin during conjugation to BSA (together with potential steric interference), the antibacterial activity of the conjugate was less than that of gentamicin alone. However, gentamicin-BSA retained some bactericidal activity and was inhibitory to growth of PAO1 at 1,200 μg/ml, compared with 2 μg/ml for gentamicin. The killing effects on PAO1 exposed to different concentrations of gentamicin and gentamicin-BSA for 4 h are shown in Fig. 1. Concentration-dependent decreases in the numbers of viable bacteria are seen with both compounds. At concentrations of one-fifth the MIC, both agents prevented bacterial growth. Cultures exposed to the MICs of gentamicin-BSA and gentamicin displayed bacterial killing of approximately 1 and 1.7 $\log_{10}$ CFU/ml, respectively, while at twice the MICs the bactericidal activities of the two compounds were 2 and 4 $\log_{10}$ CFU/ml, respectively. Both compounds demonstrated maximal decreases in growth at four times the MIC, with gentamicin killing a greater percentage of the initial inoculum. Rapid decreases in viable bacteria due to the drug concentration were also seen in the early phases of the killing curves (i.e., after 1, 2, and 3 h). The rates of decline in viable cells at these points with the two compounds were similar to those shown for 4 h. After 18 h of incubation, the numbers of viable bacteria slightly increased in cultures exposed to one-fifth the MICs of gentamicin and gentamicin-BSA.

Effect of gentamicin-BSA on protein synthesis in intact *E. coli*. Since it was not possible to transfer the *lacZ* gene to *P. aeruginosa*, we were obliged to compare the in vivo and in vitro activities of gentamicin-BSA in *E. coli* DH5α (pGEMβgal), which is a more easily transformed system. In order to determine that gentamicin-BSA does not enter cells to inhibit protein synthesis and that the bactericidal activity of gentamicin-BSA on whole cells is due to surface binding, we examined cytoplasmic β-galactosidase activity in *E. coli* that had been transformed with pGEMβgal. Measurement of β-galactosidase activity is a reliable and convenient method for analyzing protein synthesis in intact cells. The MICs of gentamicin and gentamicin-BSA for *E. coli* DH5α (pGEMβgal) were 1 and 625 μg/ml, respectively. Exponentially growing cultures, after their densities were adjusted to 10^6 CFU/ml, were incubated for 4 h with one-half the MIC of either gentamicin or gentamicin-BSA. These concentrations of the antibiotics exerted a bacteriostatic effect on *E. coli* cells during the incubation period. As shown in Fig. 2, the synthesis of β-galactosidase was reduced approximately by 15% in bacteria exposed to the gentamicin-BSA complex in comparison to the control cells. In contrast, the synthesis of β-galactosidase was inhibited almost 75% in cells exposed to gentamicin. This suggests that, though gentamicin-BSA could inhibit protein synthesis in vitro on isolated ribosomes, the complex was not readily able to pass through

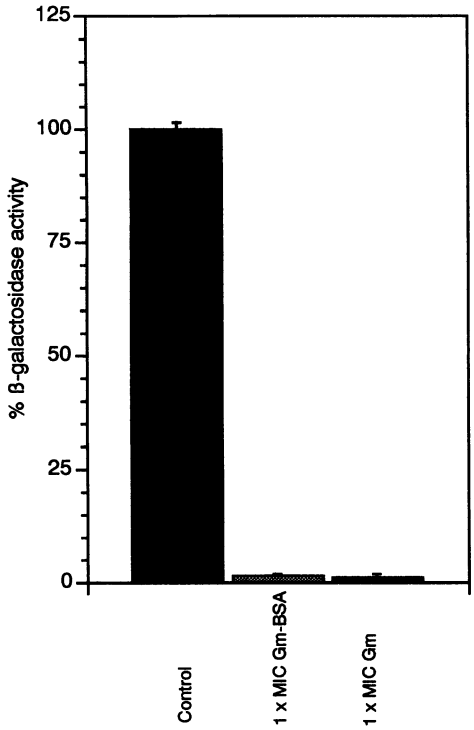

FIG. 3. Effects of gentamicin (Gm) and gentamicin-BSA (Gm-BSA) on protein synthesis, determined by assaying β-galactosidase activity in the *E. coli* S30 ribosome system. Data are the means ± the standard deviations of three independent experiments.

the cell envelope to inhibit ribosomal protein synthesis in vivo.

Effect of gentamicin-BSA on protein synthesis in *E. coli* S30 extract. The effect of gentamicin-BSA on protein synthesis in *E. coli* S30 extract was examined by comparison with gentamicin in an *E. coli* ribosomal system, and the results are presented in Fig. 3. The expression of functional β-galactosidase enzyme was assayed colorimetrically with the MICs of the two compounds. Approximately 98% inhibition was observed with both compounds, suggesting that if gentamicin-BSA can gain access to functional ribosomes, it interferes with protein synthesis in a manner similar to that of gentamicin in vitro. The inhibitory effects of β-galactosidase activity at one-half the MICs of the two compounds were also examined, and β-galactosidase activity was equally as effective at the MICs.

Electron microscopy. Whole mounts of *P. aeruginosa* either not treated (Fig. 4a) or treated with the gentamicin-BSA-gold probe (Fig. 4b and c) are shown. Numerous gentamicin-BSA-gold particles were clearly seen on whole mounts that were exposed to the probe (85% of the cells were labelled, with a mean average of 23 particles per cell; cell count = 100), and background labelling was negligible (Fig. 4b and c). Cells that were exposed to the gentamicin-BSA-gold probe formed many blebs of envelope material that were far above the numbers seen in untreated control cells. Some blebs were labelled with gold particles (Fig. 4d). The same increase in envelope blebbing was also seen with cells treated with gentamicin alone (12, 18).

In thin sections, the gentamicin-BSA-gold probe was found to be exclusively associated with the OM surface and the probe was never seen in the cytoplasm. This was in good agreement with our finding (Fig. 2) that the smaller gentamicin-BSA complex also did not penetrate to the cytoplasm and did not inhibit protein synthesis. Untreated control cells showed intact cell envelopes with few OM blebs (or vesicles) emanating from them (Fig. 5a). Examination of a number of thin sections of gentamicin-BSA-gold-treated cells suggested that the gentamicin-BSA-gold did, over time, perturb the cell surface in the following sequence. First, the OM became loosely attached to the bacterium at specific sites which were labelled with the probe (Fig. 5b). These sites exfoliated to form small OM vesicles (Fig. 5c) which were eventually cast from the cell (Fig. 5d) to reside in the external milieu (Fig. 5e). These vesicles left small, transient holes in the membrane (Fig. 5c to f), and, eventually, these perturbations expanded more deeply into the envelope until the plasma membrane was breached (Fig. 5f). This, we believe, is a lethal event and contributes to the overall effectiveness of gentamicin's activity. It also exactly mimics the process seen with gentamicin alone (18). Incubation of cells with BSA-gold, colloidal gold, carbodiimide-activated BSA-gold, or gentamicin-colloidal gold alone showed virtually no binding activity in *P. aeruginosa* cells.

DISCUSSION

Gentamicin and indeed all other aminoglycoside antibiotics are potent inhibitors of protein synthesis in a wide range of bacteria. Yet the overall cationic nature of these antibiotics makes them also likely candidates for having a perturbing effect on natural lipid bilayers. Ca^{2+} and Mg^{2+} are commonly found as counterions for the phosphoryl groups of LPS and phospholipids and are necessary for salt-bridging these macromolecules within the bilayer matrix (6, 24, 27). It seems likely that gentamicin could compete with Ca^{2+} and Mg^{2+} for these bilayer sites, displace the natural divalent cations, and bind to the OM. If successful at binding, the large antibiotic cation would perturb the native lipid packing order and destabilize the OM. Thermodynamically, these regions of the OM would have to reconfigure their constituent macromolecules, resulting in an increase in blebbing of the OM into the external milieu. This explanation, although logical, is intuitive and has been difficult to unequivocally prove. The ribosomal effect of gentamicin cannot easily be separated from the membrane effect. Clearly, the concepts of envelope perturbation (14, 18, 24, 28) and self-promoted uptake (8) are not new, but our use of gentamicin-BSA has, for the first time, allowed the unequivocal separation of the two gentamicin effects. Furthermore, the use of gentamicin-BSA-gold has allowed us to visualize the chain of cell envelope events at the electron microscopic level. Both gentamicin complexes are too large to be taken up by the cytoplasm, and they therefore have only the membrane effect on intact cells.

The rationale for the design of our gentamicin-BSA probes was simple; use of a nontoxic, readily available protein (BSA) as a large ligand chemically linked to gentamicin to form a bulky gentamicin-studded macromolecular complex. BSA contains many aspartic acid and glutamic acid residues (95 in total), so carboxyl groups would be easily activated by carbodiimide for interaction with the amine groups of gentamicin. Strict control of reagent stoichiometry so that BSA is limiting would ensure that, overall, not all amine sites on the antibiotic would be neutralized by ligand attachment. Our goal was to retain as much gentamicin activity as possible (i.e., retention of the amine groups) on a macromolecular complex that could not be taken up by the cell.

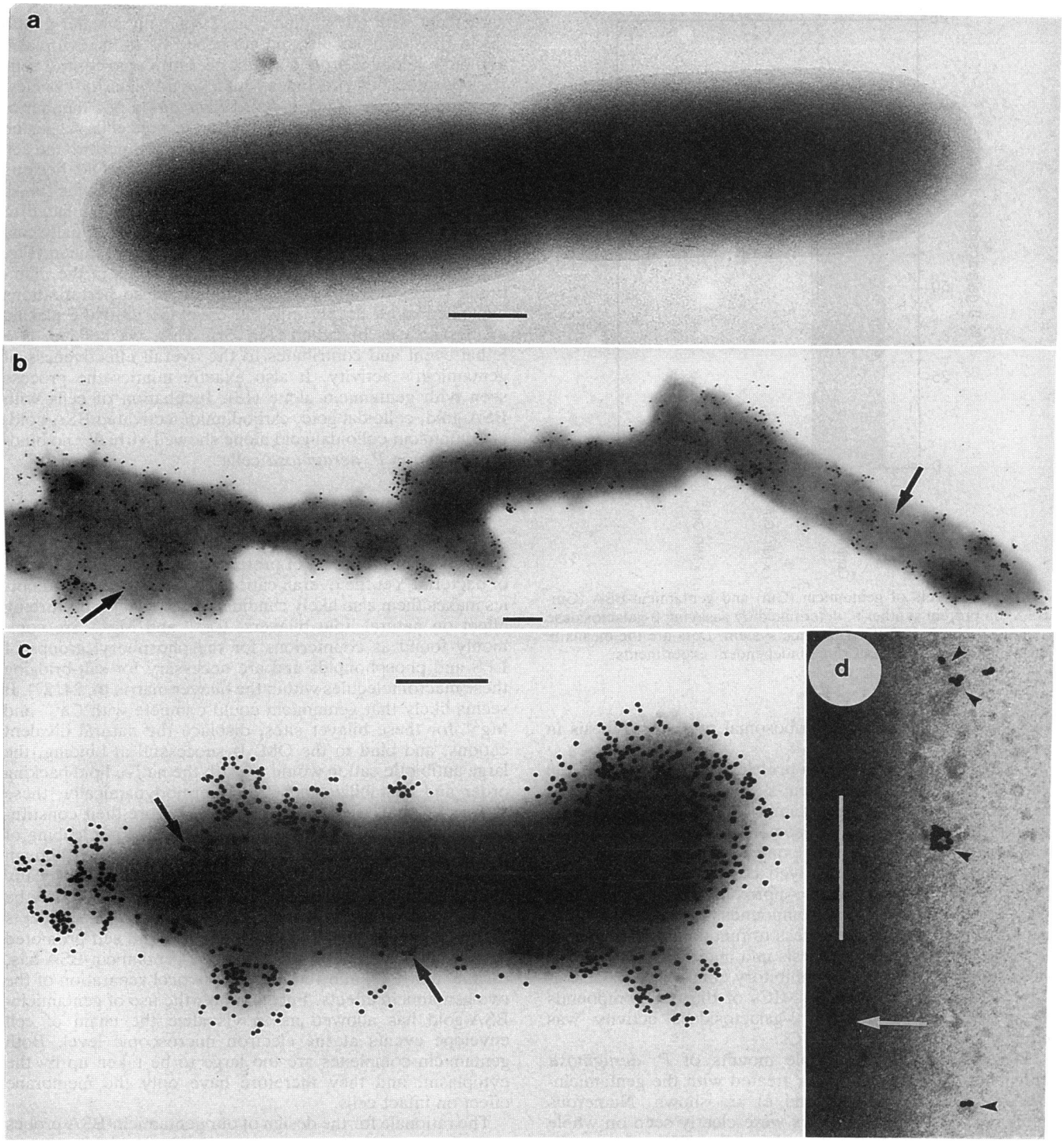

FIG. 4. Intact whole mounts of *P. aeruginosa* PAO1. (a) Control cell. (b and c) *P. aeruginosa* PAO1 treated with twice the MIC of gentamicin-BSA-gold. Note that numerous gold particles are observed on the cell (arrows) and several are associated with OM blebs (arrowheads in panel d). Few gold particles, which were always associated with OM vesicles or cytoplasmic material, can be seen in the background. Panels b and c are photographically developed to underexpose the image to better reveal the gold particles lying on top of the cell. Panel d is overexposed to show the peripheral OM blebs and their associated gold particles. Bar = 250 nm.

Morioka et al. (21) used a BSA-gold approach to determine neomycin binding sites in glutaraldehyde-fixed Epon-embedded *E. coli* but could not differentiate between the surface and ribosomal effects since both the OM and cytoplasm were labelled on thin sections. They concluded that the observed binding sites were related to the drug's overall biological activity. Our results with our probe clearly illustrate the surface effectiveness of gentamicin-BSA for living intact bacteria and provide insight into the mechanism of how ionic binding of gentamicin could contribute to the

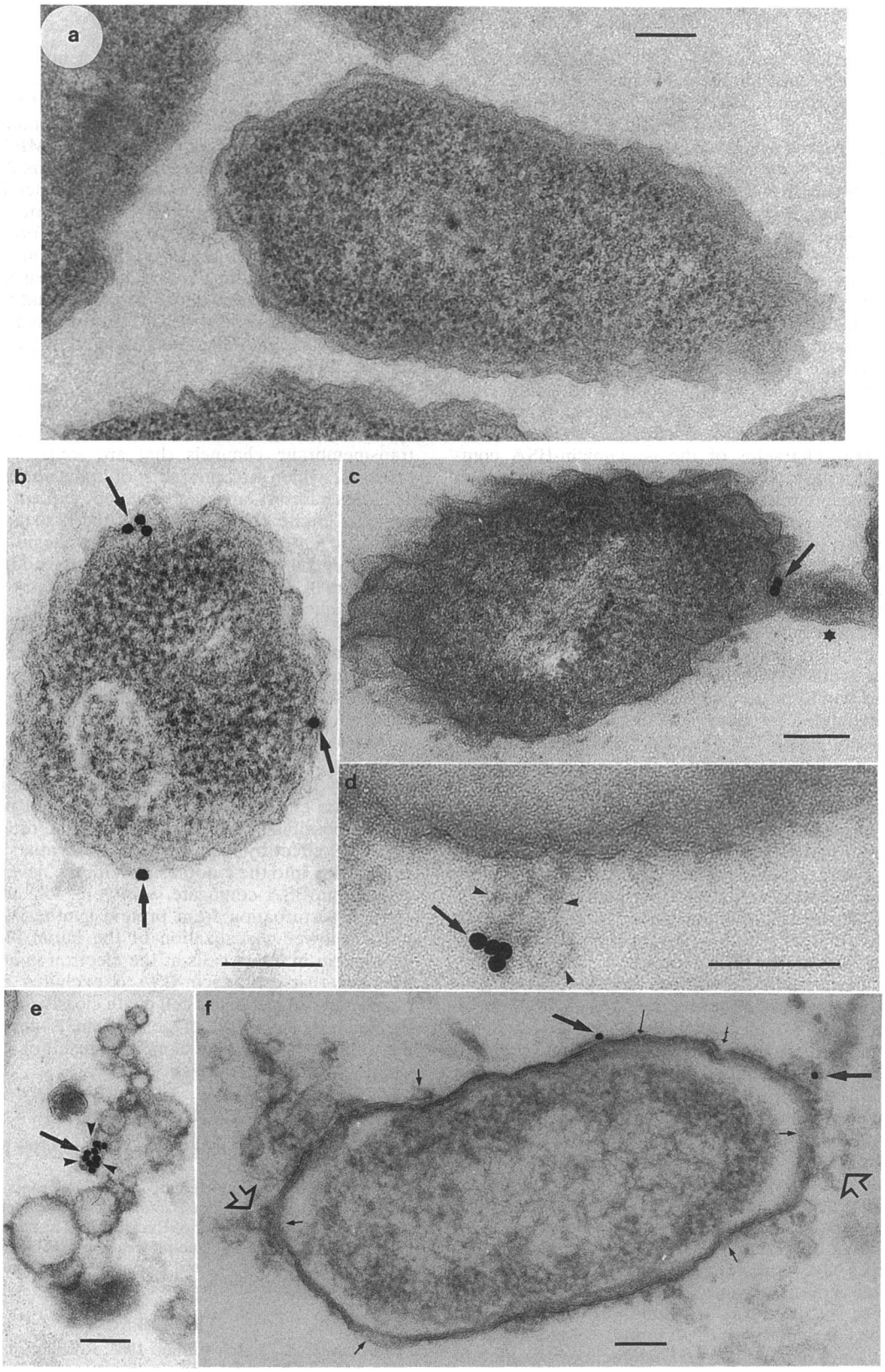

FIG. 5. Thin sections of *P. aeruginosa* PAO1. (a) Control cell. (b to f) *P. aeruginosa* PAO1 exposed to twice the MIC of gentamicin-BSA-gold. (b) Gold probe attached at multiple sites to the OM. Note the gold particles (arrows) on the OM, while the cytoplasm is devoid of such particles. (c) The cytoplasmic content is liberated in close proximity to the gold probe, and expansion of the OM (*) is visible. (d) Formation of numerous OM blebs (arrowheads) on the cell surface. (e) Cell from which OM blebs have eventually been cast. These vesicles appear to be associated with several gold particles. (f) Holes in the OM and perturbed plasma membrane (small arrows). Note that the cell is undergoing lysis and the cytoplasmic content is streaming out through the cracks of the cell envelope (open arrow). Bar = 100 nm.

antibiotic's bactericidal action. We have further observed a synergistic effect between gentamicin and gentamicin-BSA compared with gentamicin alone (12). These findings may lead to a better, rational design of less toxic, new drugs with enhanced effectiveness against *P. aeruginosa*.

It is important to attempt to understand how the surface effect of gentamicin-BSA compares with that of gentamicin alone. The net cationic charge on gentamicin based on the number of amine groups in the molecular structure is 5+. But the number of the charges at pH 7.4 measured by titrimetric means is 3.46+ (11). Clearly, we relied on a certain number of these charges as we chemically linked gentamicin molecules to each BSA molecule through the amines of the antibiotic, and the overall charge for each gentamicin molecule is therefore reduced. We estimate that the total number of gentamicin molecules bound to each BSA molecule varies from 25 to 53 (average number = 39 gentamicin molecules per gentamicin-BSA molecule); the mass and charge character of the gentamicin-BSA complexes must also vary, and this variability is dependent on the number of gentamicin molecules per BSA molecule. Presumably, since gentamicin was saturating during the coupling reaction and since the BSA molecular size is constant, the number of gentamicin molecules per BSA molecule ultimately depends on the number of BSA carboxyl groups neutralized by the amine functions of each gentamicin molecule. Those BSA molecules surrounded by the least number of gentamicin molecules (i.e., 25) would have fewer amine groups available on their outer shell of gentamicin molecules and would be less positively charged than BSA molecules surrounded by many gentamicin molecules. Because we do not know the absolute folding of the BSA molecule and its number of exposed carboxyl groups under the labelling conditions, and since the gentamicin charge per molecule could range from 3.46+ to 5+, it is not possible to estimate the range of charge on the gentamicin-BSA conjugate at this time. To compound the complexity of our system even further, commercial preparations of gentamicin contain three separate chemical varieties of the antibiotic, C_1, C_{1a}, and C_2, with M_rs of 478, 450, and 464. These species may possess subtly different BSA binding capabilities which may affect the final gentamicin-BSA product.

Neglecting these subtle differences among separate gentamicin-BSA complexes, assuming the M_r of BSA to be 67,000, the average M_r of gentamicin to be 464, and the number of gentamicin varieties in our commercial preparation to be equal, our gentamicin-BSA complex would have a M_r range of 78,600 (i.e., 25 gentamicin molecules) to 91,592 (i.e., 53 gentamicin molecules). The larger-M_r complexes would be more highly charged than the smaller ones. Suffice it to say that even though our gentamicin-BSA preparation possesses a range of chemical complexes of variable mass and charge, they are all highly cationic and they are all (at least) as OM reactive as gentamicin alone. Peterson et al. (24) have shown that aminoglycosides with greater net positive charges have a greater affinity for LPS and therefore greater OM disruption capacity. Indeed, this information correlated very well with our previous ultrastructural and biochemical observations with *P. aeruginosa* exposed to two different antibiotics (gentamicin and amikacin) with different charge characters (18, 28). Similarly, since gentamicin-BSA has a higher charge per molecule than gentamicin, gentamicin-BSA should have high OM disruption capacity. Loh et al. (17) have shown that as the aminoglycoside charge increases, so also does the MIC. Since the MIC for the uncomplexed antibiotic is a net result of its surface and cytoplasmic (ribosomal) activities, gentamicin-BSA would not be as effective, even with its greater OM disruption capacity. Comparing the results of our MICs for gentamicin (alone) and gentamicin-BSA (using 39 gentamicin molecules per BSA molecule) on a molar basis, the MIC of gentamicin is 4 μM and the MIC of gentamicin-BSA is 22 μM.

The pleotropic effects of aminoglycosides include inhibition of ribosomal function (1–3), membrane damage (3, 18, 28), and suppression of DNA initiation (19). It is believed that the inhibition of protein synthesis and an ability to cause codon misreading alone are not specific reasons for lethality (1, 8). Bryan and Kawan (1) proposed that the lethality of aminoglycosides is the result of a gradual disruption of cytoplasmic membrane integrity and subsequently of its function. This alteration occurs apparently when the aminoglycosides traverse the cytoplasmic membrane, at which time they enter the cell. Davis (4) suggested that the mechanism of bactericidal action of aminoglycosides is due to the transmembrane channels that are formed from aberrant proteins, which are products of translational misreading. This mechanism, however, does not explain how abnormal proteins create channels or how the first molecules of the antibiotic enter the cell before they begin to induce the autocatalytic process of increasing misreading and channel formation (4, 23). The present results seem to answer some of these questions.

In our previous study (14), we demonstrated how ionic binding could contribute to cell death of *P. aeruginosa* upon exposure to gentamicin. Highly charged LPS (i.e., B band) was most strongly affected, so much so that it significantly contributed to cell death. The postexposure removal of the antibiotic from the medium showed that a continuing supply of the drug other than that bound by initial exposure was not necessary for the bactericidal action, confirming the finding of Jackson et al. (10). However, it was not possible to rule out the possibility that the initially bound drug could induce the lethal effect by inhibiting protein synthesis following its penetration into the cytoplasm. With the development of the gentamicin-BSA conjugate, we were able to separate the surface perturbation from protein synthesis, and the gold probe allowed visualization of the initial binding sites of gentamicin in intact cells at the electron microscopic level. The fact that gentamicin-BSA is excluded from the cytoplasm and can still cause cell death clearly demonstrates that the bactericidal effect of gentamicin is partly due to the disruption of the cell envelope as a result of ionic binding of the drug to the cell surface.

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