Aminoglycosides (e.g., gentamicin) are often used clinically in combination with other antimicrobial agents such as beta-lactams or glycopeptides for the treatment of serious infections with gram-negative and gram-positive organisms. Previous studies have repeatedly demonstrated a concentration-dependent killing effect of aminoglycosides against gram-negative bacteria (5, 6, 14, 25); optimal patient outcomes and suppression of resistance emergence are associated with peak concentration (maximum concentration of drug in serum $\left[C_{\text{max}}\right]$)/MIC ratio (3, 13, 15) or area under the concentration-time curve (AUC)/MIC ratio (16). There is also strong evidence suggesting that the first dose of an aminoglycoside is the most important in the course of therapy. Adaptive resistance is a phenomenon in which bacteria exhibit down-regulation of drug uptake upon frequent and repeated exposures to antimicrobial agents (26). Consequently, the first dose of aminoglycosides has the most bactericidal effect on the bacterial population. It has also been reported that attainment of a pharmacodynamic target ($C_{\text{max}}$/MIC $\geq 10$) within 48 h of therapy is associated with an early therapeutic response (12). Since the likelihood of aminoglycoside-induced nephrotoxicity is believed to be dependent on the cumulative drug exposure and/or concentration above a certain threshold (8, 17, 20, 24), achieving a pharmacodynamic target early may shorten the duration of therapy and thus reduce the likelihood of drug-induced adverse effects. Consequently, once-daily (or extended-interval) administration of aminoglycosides has been widely adopted in many hospitals in the United States (4).

While the approach is intuitive and consistent with pharmacodynamic principles, limited data are available to describe the pharmacodynamic activity of aminoglycosides against gram-positive bacteria (e.g., Staphylococcus aureus, viridans group streptococci, and Enterococcus spp.). Questions remain if the same dosing strategy should be used for severe infection with gram-positive bacteria (e.g., endocarditis), and evaluation of bacterial killing with various aminoglycoside exposures is essential to optimize dosing strategies in the clinical setting. We explored the impact of increasing gentamicin concentrations and various dosing regimens on the drug’s activity against different bacteria. The objective of the study was to compare the in vitro pharmacodynamics of gentamicin against S. aureus and Pseudomonas aeruginosa.

(This study was presented in part at the Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., December 16 to 19, 2005.)

**MATERIALS AND METHODS**

**Antimicrobial agent.** Gentamicin powder was purchased from Sigma (St. Louis, MO). Stock solutions of 1.024 mg/liter in sterile water were prepared, aliquoted, and stored at $-70^\circ$C. Prior to each susceptibility test, an aliquot of the drug was thawed and diluted to the desired concentrations with cation-adjusted Mueller-Hinton II broth (Ca-MHB) (BBL, Sparks, MD).

**Microorganisms.** Five strains of S. aureus were examined. Standard wild-type strain ATCC 29213 (American Type Culture Collection, Manassas, VA) and four clinical isolates (two oxacillin susceptible [strains 55 and 60] and two oxacillin resistant [strains 25 and 62]) were used. All clinical isolates of S. aureus used were wild type and were found to be clonally unrelated, as determined by
randomly amplified polymorphic DNA testing (19). *P. aeruginosa* ATCC 27853 was used for comparison. The bacteria were stored at −70°C in Protect (Key Scientific Products, Round Rock, TX) storage vials. Fresh isolates were subcultured twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) for 24 h at 35°C prior to each experiment.

**Susceptibility studies and mutation frequencies.** Gentamicin MICs/minimum bactericidal concentrations (MBCs) were determined for all bacterial strains in Ca-MHB using a modified broth macrodilution method, as described by CLSI (formerly NCCLS) (18). The final concentration of bacteria in each broth macrodilution tube was approximately 5 × 10⁷ CFU/ml of Ca-MHB. Serial twofold dilutions of drugs were used. The MIC was defined as the lowest concentration of drug that resulted in no visible growth after 24 h (instead of 16 to 18 h, as recommended by CLSI) of incubation at 35°C in ambient air. Samples (50 μl) from clear tubes and the cloudy tube with the highest drug concentration were plated on Mueller-Hinton agar (MHA) plates (Hardy Diagnostics, Santa Maria, CA). The MBC was defined as the lowest concentration of drug that resulted in ≥99.9% killing of the initial inoculum. The drug carryover effect was assessed by visual inspection of the distribution of colonies on medium plates. The studies were conducted in duplicate and repeated at least once on a separate day. Mutation frequency of resistance for each isolate was determined by plating approximately 1 × 10⁶ CFU/ml (200 μl) of bacteria on MHA plates with and without gentamicin supplementation at 3 times the MIC. Since susceptibility testing was performed in twofold dilutions and one tube (2× concentration) difference is commonly accepted as reasonable interday variation, quantitative cultures on drug-supplemented medium plates (at 3 times the MIC) would allow reliable detection of bacterial subpopulations with reduced susceptibility. The medium plates were incubated at 35°C for up to 24 h (total population) and 72 h (subpopulation with reduced susceptibility), and then bacterial density from each sample was estimated as described below.

**Time-kill studies.** Time-kill studies were conducted in duplicate on separate days with different and escalating gentamicin concentrations. Six clinically achievable concentrations of gentamicin were used, normalized to 0 (control), 0.5, 1, 2, 4, 8 and 16 times the MIC. An overnight culture of the isolate was prepared as described above. The experiment was conducted for 5 days in a humidified incubator set at 35°C. The bacteria were subjected to various gentamicin exposures, simulating unbound steady-state pharmacokinetic profiles resulting from two different gentamicin dosing regimens with identical daily doses (once-daily dosing to achieve a peak concentration of 24 μg/ml and 3-times-daily dosing to achieve a peak concentration of 8 μg/ml). A third system was set up as placebo control. Gentamicin elimination half-lives of 2.5 h were simulated in all systems.

**Pharmacokinetic validation.** Serial samples were obtained from the infection models on days 0 and 2. Gentamicin concentrations in these samples were assayed by a validated bioassay method as described below. The concentration-time profiles were modeled by fitting a one-compartment linear model to the observations using the ADAPT II program (7).

**Bioassay.** Gentamicin concentrations were determined by a microbioassay utilizing Klebsiella pneumoniae ATCC 13883 as the reference organism. The bacteria were incorporated into 30 ml of molten cation-adjusted MHA (at 50°C) to achieve a final concentration of approximately 1 × 10⁷ CFU/ml. The agar was allowed to solidify in 150-mm medium plates. Size 3 cork borer was used to create nine wells in the agar per plate. Standards and samples were tested in duplicate with 40 μl of the appropriate solution in each well. The gentamicin standard solutions ranged from 1 to 32 μg/ml in Ca-MHB. The medium plates were incubated at 35°C for 24 h, and the zones of inhibition were measured. The assay was linear (correlation coefficient ≥ 0.99) using zone diameter versus the log of the standard drug concentration. The intraday and interday coefficients of variation for all standards were <4% and <6%, respectively.

**Microbiologic response.** Serial samples were also obtained at baseline; at 4, 8, and 24 h (predose) and daily thereafter in duplicate from each hollow-fiber system, for quantitative culture to define the effect of various drug exposures on the bacterial population. The samples (0.5 ml) to quantify the bacterial population were processed as described above.

The modeling estimation process involved two steps. For each bacterium, the intrinsic bacterial growth rate and maximal bacterial population size (to account for contact inhibition) were first determined from placebo (control) experiments, using the ADAPT II program (7). Using these parameter estimates, the parameter values in the kill function were subsequently determined using data from all active treatment experiments simultaneously. An unweighted (least-squares) error structure for the log-transformed data was used.

**Hollow-fiber infection model.** The schematic of the system has been described previously (1). The drug was directly injected into the central reservoir to achieve the peak concentration desired. Fresh (drug-free) growth medium (Ca-MHB) was infused continuously from the diluent reservoir into the central reservoir (180 ml) to dilute the drug, in order to simulate drug elimination in humans. An equal volume of drug-containing medium was removed from the central reservoir concurrently to maintain an isovolumetric system. Bacteria were inoculated into the extracapillary compartment of the hollow-fiber cartridge (Fibercell Systems, Inc., Frederick, MD); they were confined in the extracapillary compartment but were exposed to the fluctuating drug concentration in the central reservoir by means of an internal circulatory pump in the bioreactor loop. The optional absorption compartment of the system was not used.

**Experimental setup.** For validation purposes, only the two standard ATCC strains of *S. aureus* and *P. aeruginosa* were used. Bacterial inocula (20 ml) were prepared as described above. The experiment was conducted for 5 days in a humidified incubator set at 35°C. The bacteria were subjected to various gentamicin exposures, simulating unbound steady-state pharmacokinetic profiles resulting from two different gentamicin dosing regimens with identical daily doses (once-daily dosing to achieve a peak concentration of 24 μg/ml and 3-times-daily dosing to achieve a peak concentration of 8 μg/ml). A third system was set up as placebo control. Gentamicin elimination half-lives of 2.5 h were simulated in all systems.

**RESULTS**

**Susceptibilities and mutation frequencies.** The susceptibility of the bacterial isolates to gentamicin were as shown in Table 1. Baseline resistant subpopulations were detected in all isolates. The mutation frequency of gentamicin resistance (more than 3 times the MIC) ranged from 1 in 3 × 10⁴ to 1 in 4 × 10⁵.

**Time-kill studies.** The killing profiles of gentamicin against *S. aureus* ATCC 29213 are as shown in Fig. 1A. A consistent trend was apparent for all five *S. aureus* isolates (see the supplemental data). Overall, the bactericidal activity appeared to be concentration dependent, as gentamicin concentration was increased from 0.5 to 4 times the MIC. However, the rate of killing seemed to plateau at concentrations beyond 4 to 8 times the MIC. These observations were in direct contrast to those observed with *P. aeruginosa* (Fig. 1B). A rapid reduction in...
bacterial burden was seen within 2 h of gentamicin exposure (all concentrations), which was followed by regrowth. With increasing gentamicin concentrations, a concentration-dependent trend was observed with respect to the suppression of regrowth, consistent with previous observations (5, 6).

**Pharmacodynamic modeling.** The model fits to the data were satisfactory. The $r^2$ for *S. aureus* ATCC 29213, 55, 60, 25, and 62 and *P. aeruginosa* ATCC 27853 were 0.915, 0.946, 0.942, 0.942, 0.900, and 0.956, respectively (Fig. 2 [ATCC strains only; for others see the supplemental data]). The final model parameter estimates are as shown in Table 1, and the relationships between gentamicin concentration and bactericidal activity are as shown in Fig. 3 (ATCC strains only; for others see the supplemental data). Against *S. aureus*, killing of the predomi-
nant bacterial population was the prominent feature observed (with minimal adaptation resulting in regrowth); the bactericidal activity observed was concentration dependent at low concentrations (less than 4 times the MIC), and further increase in killing activity became less substantial when the concentration was beyond 4 to 8 times the MIC (Fig. 3A). On the other hand, our modeling analysis revealed that maximal killing of the predominant (susceptible) \( P. \) aeruginosa population was readily achieved with all the gentamicin concentrations used, as reflected in a rapid decline in bacterial burden within 2 h of exposure in the time-kill studies (Fig. 1B). However, the prominent feature observed was the concentration-dependent relationship with respect to the most resistant subpopulation present at baseline (full adaptation) (Fig. 3B), leading to the differential propensity of increasing gentamicin concentrations in suppressing regrowth over time. The values of the sigmoidicity constants \( (H) \) between \( S. \) aureus and \( P. \) aeruginosa were also noted to be dissimilar, partially explaining the difference in their concentration-killing profiles.

**Pharmacokinetic validation in hollow-fiber infection models.** All simulated gentamicin exposures were satisfactory; the \( r^2 \) values for once- and three-times-daily dosing were 0.962 and 0.989, respectively (data not shown).

**Microbiologic responses in hollow-fiber infection models.** The effect of different concentration-time profiles of gentamicin on \( S. \) aureus ATCC 29213 and \( P. \) aeruginosa ATCC 27853 were as shown in Fig. 4. Against \( S. \) aureus, gentamicin dosing given 3 times daily appeared to be more bactericidal compared to once-daily administration, using identical daily doses (Fig. 4A). Both dosing regimens achieved substantial killing (approximately 5-log kill) at 24 h, but regrowth was apparent with repeated dosing over the next 4 days for the once-daily dosing regimen. On the other hand, sustained bacterial suppression over 5 days was observed with the 3-times-daily dosing regimen. This was in contrast to data for \( P. \) aeruginosa, in which the dosing schedule did not appear to have a significant impact on the killing activity of gentamicin. As long as the daily dose remained identical, the time courses of bacterial burden over 5 days were similar,
regardless whether the entire daily dose was given at once or over three doses (Fig. 4B).

**DISCUSSION**

Aminoglycoside pharmacodynamics has revolved primarily around the gram-negative bacteria. Studies have demonstrated positive outcomes utilizing an extended dosing interval of aminoglycosides in infections with gram-negative organisms (13, 20). However, limited clinical experience with infections with gram-positive organisms is available (21), and aminoglycoside use has been mostly based on theory. In this study we strived to improve our understanding of their pharmacodynamic properties against gram-positive bacteria, specifically, *S. aureus*. Therefore, similar to the earlier studies of efficacy against gram-negative bacteria (5, 6), investigations with monotherapy of gentamicin were undertaken. Once the pharmacodynamic properties of the aminoglycosides are well understood, more clinically relevant studies with various antimicrobial agent combinations could be performed subsequently to improve patient care.

Our data revealed that the killing profiles of gentamicin against *S. aureus* and *P. aeruginosa* were different. First, in time-kill studies, the killing profiles of gentamicin against different strains of *S. aureus* were comparable but different from that observed with *P. aeruginosa*. The often-cited concentration-dependent killing was observed only in *P. aeruginosa*, not in *S. aureus*, over the (clinically relevant) concentration range examined. In addition to comparing the killing profiles against different bacteria qualitatively (visually), we modeled the experimental data mathematically in order to provide an objective and quantitative evaluation. The merits of our mathematical modeling approach over conventional pharmacodynamic modeling have been discussed previously (22). While the adherence of bacteria to the surface of the conical flasks was not considered in the evaluation of these in vitro experiments, the interpretation of the modeling results was consistent with the observations. We recognized that all *S. aureus* strains examined in the study had similar susceptibilities to gentamicin (0.5 to 1 µg/ml); using isolates with a broader range of susceptibility to gentamicin might have further enhanced the generalizability of our findings.

Since the drug concentrations in time-kill studies are static (constant over time), we felt that the clinical relevance of the results might not be very evident. Therefore, a hollow-fiber infection model (in which drug concentration fluctuates over time, resembling human elimination and repeated dosing) was used to provide further clinical insights of our findings. In the hollow-fiber infection models, the impact of dosing schedules of gentamicin on the bacteria was somewhat dramatic. Based on the modeling analysis, the killing activity of gentamicin against *S. aureus* began to plateau at 4 to 8 times the MIC (Fig. 3A). Consequently, high peak concentrations (beyond 8 times the MIC) resulting from once-daily administration would be unlikely to result in a substantial increase in bacterial killing. Coupled with a prolonged period in which drug concentration was negligible, once-daily dosing might not suppress/eradicate the bacteria as readily as a regimen with more frequent dosing. On the other hand, we found a concentration-dependent effect of gentamicin in suppressing regrowth of *P. aeruginosa* (up to at least 32 times the MIC, as shown in Fig. 3B). An enhanced bacterial killing rate against the resistant subpopulation was anticipated from high peak concentrations (approximately 12 times the MIC) associated with once-daily administration, which was offset by a prolonged period in which drug concentration was negligible (minimal killing). Therefore the overall extents of killing were comparable regardless of the dosing schedules (once versus thrice daily) as long as the total daily doses (drug exposure) remained identical. These observations from the hollow-fiber infection models were again consistent with our expectations and previous studies (2, 11). We verified the simulated gentamicin exposures were reasonable in the hollow-fiber infection models, but the mechanism of regrowth was not explicitly investigated. From the modeling perspective, regrowth after initial decline of the bacterial population was empirically attributed to adaptation in this study. Under antimicrobial selective pressure, a preexisting resistant subpopulation gradually replaced the entire population as the dominant clone over time; regrowth and the emergence of resistance were observed as a result. Furthermore, as long as the relationship between drug concentration and bactericidal activity is not saturable (concentration-dependent killing), the AUC/MIC ratio (primarily determined by the daily dose, regardless of dosing schedule) would be closely related to the cumulative killing over time. The postantibiotic effect of gentamicin was not specifically investigated in this study, in view of the doubtful clinical relevance reported in previous studies (9, 10).

In contrast to our data, in a recent in vitro study, the activ-
ities of gentamicin given once and 3 times daily against S. aureus in simulated endocardial vegetations were investigated in combination with daptomycin and vancomycin (23). The authors concluded that gentamicin given as a single large dose was superior to three smaller doses in combination with daptomycin or vancomycin. The experimental design of this study and our study differed in several ways, which prohibited direct comparison of the findings. Firstly, the bactericidal activity of gentamicin was examined in combination with other agents (daptomycin and vancomycin); therefore the pharmacodynamics of gentamicin might have been confounded by interaction (e.g., synergy or antagonism) with these agents used concurrently. Secondly, a simulated-infected-endocardial-vegetation model was used in the previous study (23); the observed killing in these simulated fibrin clots was (at least partially) dependent on the penetration (concentration achieved) of the antimicrobial agents inside the vegetations. Finally, the previous study was not strictly a dose fractionation study, as we have conducted. The total daily doses of gentamicin were not identical in the once-daily (simulating a human equivalent dose of 5 mg/kg of body weight every 24 h) and 3-times-daily (simulating a human equivalent dose of 1 mg/kg every 8 h) regimens. Therefore, the overall levels of killing by gentamicin might be expected to be different. In view of these differences, the pharmacodynamics of gentamicin against S. aureus might not have been interpreted concordantly by the two groups.

In conclusion, we found that gentamicin exhibited distinct killing profiles against S. aureus and P. aeruginosa. The well-accepted concentration-dependent bactericidal activity of the aminoglycosides may not be applicable against all bacteria. These results may guide optimal dosing strategies of gentamicin in staphylococcal infections and warrant further investigations.

ACKNOWLEDGMENTS

This study was supported partially by a GEAR (grant to enhance and advance research) award from the University of Houston and a research grant from the Johns Hopkins Center for Alternatives to Animal Testing.

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