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Adaptive Resistance of *Pseudomonas aeruginosa* Induced by Aminoglycosides and Killing Kinetics in a Rabbit Endocarditis Model

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Adaptive resistance following the first exposure to aminoglycosides is a recently described in vitro phenomenon in *Pseudomonas aeruginosa* and other aerobic gram-negative bacilli. We investigated the in vivo relevance of adaptive resistance in *P. aeruginosa* following a single dose of amikacin in the experimental rabbit endocarditis model. Rabbits with *P. aeruginosa* endocarditis received either no therapy (control) or a single intravenous (i.v.) dose of amikacin (80 mg/kg of body weight) at 24 h postinfection, after which they were sacrificed at 5, 8, 12, 16, or 24 h postdose. Excised aortic vegetations were subsequently exposed ex vivo to amikacin at 2.5, 5, 10 or 20 times the MIC for 90 min. In vivo adaptive resistance was identified when amikacin-induced pseudomonal killing within excised aortic vegetations was less in animals receiving single-dose amikacin in vivo than in vegetations from control animals not receiving amikacin in vivo. Maximal adaptive resistance occurred between 8 and 16 h after the in vivo amikacin dose, with complete refractoriness to ex vivo killing by amikacin seen at 12 h postdose. By 24 h postdose, bacteria within excised vegetations had partially recovered their initial amikacin susceptibility. In a parallel treatment study, we demonstrated that amikacin given once daily (but not twice daily) at a total dose of 80 mg/kg i.v. for 1-day treatment significantly reduced pseudomonal densities within aortic vegetations versus those in untreated controls. When therapy was continued for 3 days with the same total daily dose (80 mg/kg/day), amikacin given once or twice daily significantly reduced intravegetation pseudomonal densities versus those in controls. However, amikacin given once daily was still more effective than the twice-daily regimen. These data confirm the induction of aminoglycoside adaptive resistance in vivo and further support the advantages of once-daily aminoglycoside dosing regimens in the treatment of serious pseudomonal infections.

The aminoglycoside antibiotics represent an important part of the therapeutic arsenal against invasive infections caused by *Pseudomonas aeruginosa*. However, despite their many advantages, such as rapid and concentration-dependent killing, long postantibiotic durations of effects, and bactericidal synergy with antipseudomonal β-lactams (2, 4, 11, 19), aminoglycoside-based therapeutic regimens against *P. aeruginosa* have been limited by a number of factors (15, 18, 26). These factors include development of drug resistance during therapy (15), poor distribution in certain body sites (e.g., heart valve vegetations [3] and cerebrospinal fluid [25]), and reduced activities in the presence of low pH (13, 30), as is found in abscesses (13). The rate of the bactericidal action of aminoglycosides against *P. aeruginosa* and other aerobic gram-negative bacilli has been shown to be biphasic (9, 19). An initial phase of rapid bacterial killing is directly related to the initial drug concentration, while a second phase of slower bacterial killing is independent of the initial drug level. Bacteria surviving the first aminoglycoside exposure develop adaptive resistance to subsequent drug doses, an effect related to decreased aminoglycoside permeability (8, 16). The phenomenon of adaptive resistance in bacteria surviving the first exposure to aminoglycosides involves the development of an unstable, time-dependent refractoriness to aminoglycoside-mediated killing by subsequent drug doses (1, 8, 9, 14, 30). Therefore, adaptive resistance appears to be an additional factor which may adversely influence aminoglycoside-mediated killing of *P. aeruginosa.*

Our present studies were designed to examine the in vivo relevance of adaptive resistance using the experimental rabbit model of pseudomonal endocarditis.

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MATERIALS AND METHODS

Organism. The *P. aeruginosa* strain studied (PA 89-0933) has been described in detail elsewhere (30, 31). The MIC of amikacin for this strain is 2 μg/ml, as determined in cation-supplemented Mueller-Hinton broth at an inoculum of $\sim 5 \times 10^5$ CFU/ml. This inoculum encompasses the intravegetation pseudomonal densities observed in the experimental endocarditis model following a single intravenous (i.v.) amikacin dose of 80 mg/kg of body weight.

Antibiotic. Amikacin was supplied by Bristol Laboratories (Paris, France) and was reconstituted in sterile water.

Experimental endocarditis. Female New Zealand White rabbits (weight, 2 to 2.5 kg) were housed in individual cages and given free access to food and water. Aortic endocarditis was induced as described elsewhere (23). Briefly, anesthetized animals underwent retrograde transaortic valvular catheterization with a sterile polyethylene tube to induce sterile aortic valve vegetations. Twenty-four hours post-catheter placement, pseudomonal endocarditis was induced by the i.v. inoculation of 10^8 CFU of *P. aeruginosa* via the marginal ear vein. Such procedures induced infective endocarditis in all animals with proper catheter placement. The catheter remained in place for the duration of the experiment.

Treatment regimens. Twenty-four hours after infection, animals were randomized to received either (i) no therapy (control), (ii) single-dose amikacin (80 mg/kg i.v.), (iii) amikacin (40 mg/kg/dose i.v. for a total of two doses), (iv)

TABLE 1. Induction of unstable adaptive resistance in vivo following single dose of amikacin in experimental rabbit endocarditis due to *P. aeruginosa*

Regimen (no. of rabbits)[a]	Time (h) of sacrifice[b]	Extent of bacterial killing (Δlog CFU/ml/90 min)			
		2.5 × MIC	5 × MIC	10 × MIC	20 × MIC
Control (6)	24	0.79 ± 0.23	1.61 ± 0.50	2.53 ± 0.53	3.28 ± 0.84
AMK (8)	5	0.78 ± 0.26	1.11 ± 0.45	1.56 ± 0.52	1.80 ± 0.68
AMK (6)	8	0.45 ± 0.39	0.78 ± 0.46	1.51 ± 0.80	1.92 ± 1.30
AMK (8)	12	0.00	0.54 ± 0.45	1.44 ± 0.70	1.81 ± 1.12
AMK (7)	16	0.43 ± 0.16	0.78 ± 0.27	1.54 ± 0.29	2.42 ± 0.39
AMK (6)	24	0.58 ± 0.43	1.14 ± 0.37	1.96 ± 0.35	2.81 ± 0.52

[a] AMK, amikacin at 80 mg/kg i.v. once.
[b] After the in vivo dose.

amikacin once daily (80 mg/kg/day i.v.) for 3 days, or (v) amikacin twice daily (40 mg/kg/dose i.v.) for 3 days.

Sacrifice. For assessment of therapeutic efficacy, control rabbits were sacrificed either 48 or 96 h after inoculation. Rabbits administered 1- or 3-day amikacin regimens were sacrificed at least 12 h after the last amikacin dose to prevent antibiotic carryover effects in vivo (10). Animals were euthanized by rapid i.v. sodium pentobarbital overdosage (100 mg/kg). At sacrifice, the hearts were removed, and all vegetations from individual animals were excised, pooled, weighed, and then homogenized in 0.5 ml of sterile normal saline solution. Vegetation homogenates were serially diluted with a spiral plater (Spiral System, Interscience, Saint Nom la Breteche, France) onto Trypticase soy agar plates, which were then incubated for 24 h at 37°C. Bacterial densities were expressed as $\log_{10}$ CFU per gram of vegetation (± standard deviation), with a lower limit of detection of 20 CFU/ml of homogenate. Mean vegetation densities in various treatment groups were compared to evaluate therapeutic efficacy. Culture-negative vegetations were assigned a value of <2.06 to 3.04 $\log_{10}$ CFU/g based on this detection limit and the actual vegetation weight.

Adaptive resistance design in vivo. In studies parallel to those of the treatment protocol described above, the experimental rabbit endocarditis model was used to evaluate the relevance of adaptive resistance in vivo. Control animals with pseudomonal endocarditis remained untreated and were sacrificed at 48 h postinfection. Rabbits were treated with a single dose of amikacin (80 mg/kg i.v.) and then sacrificed at 5, 8, 12, 16, or 24 h postdose. The vegetations of all animals were excised, pooled, weighed, and homogenized as described above. The homogenized vegetations were then exposed to 2.5, 5, 10, or 20 times the MIC of amikacin for 90 min at 37°C. Quantitative colony counts of vegetation homogenates were determined before and after amikacin exposure ex vivo on Trypticase soy agar plates containing polyanethole sulfonic acid (1%) and cysteine (0.01 M) designed to inactivate the aminoglycoside and prevent antibiotic carryover effects (10). The difference between the $\log_{10}$ CFU per milliliter of homogenates before and after the 90-min amikacin exposure ex vivo was defined as the $\Delta\log_{10}$ CFU per milliliter. The $\Delta\log_{10}$ CFU per milliliter for animals receiving amikacin in vivo were compared to those for controls receiving no amikacin in vivo. In vivo adaptive resistance was defined at a specific sacrifice time point when the $\Delta\log_{10}$ CFU per milliliter in animals receiving amikacin in vivo was less than that observed in control animals receiving no amikacin in vivo.

Pharmacokinetics. Serum samples for pharmacokinetic analysis were obtained from rabbits with pseudomonal endocarditis which received either a 40- or an 80-mg/kg single i.v. bolus of amikacin. Blood samples were obtained at 5, 10, 15, 30, 60, 120, 180, 240, 360, 480, 600, and 720 min postdose. Serum amikacin concentrations were determined by fluorescence polarization immunoassay (TDK assay; Abbott Laboratories) (sensitivity limit, 0.8 μg/ml). Coefficients of variation ranged from 2.01 to 6.11% within runs; the day-to-day coefficients of variation ranged from 3.26 to 7.5%, depending on the amikacin level. Area under the curves (AUC) and serum half-lives ($t_{1/2}$) were determined for the two amikacin dose regimens by using a two-compartment computer model (27).

Statistical analyses. For comparing bacterial densities in vegetations in the different therapy groups, the Kruskal-Wallis test was used, with the Tukey posthoc modification for multiple comparisons. A P of <0.05 was considered significant.

RESULTS

In vivo adaptive resistance. The results of in vivo adaptive resistance of *P. aeruginosa* to amikacin in experimental rabbit endocarditis are shown in Table 1. For all groups, the net bactericidal effects of amikacin were shown to be dependent on the time elapsed between in vivo and ex vivo amikacin exposures. For example, in controls receiving no amikacin in vivo, the addition of 2.5 to 20 times the MIC of amikacin ex vivo to homogenized vegetations yielded a $\Delta\log_{10}$ CFU per milliliter of 0.79 to 3.28. In contrast, for animals receiving a single dose of amikacin in vivo, variable degrees of adaptive resistance were identified, with maximal refractoriness to ex vivo amikacin killing observed at 8 to 16 h post-in vivo dose and the least refractoriness to ex vivo amikacin killing observed at 5 and 24 h post-in vivo amikacin dose. In addition, for all vegetation homogenates (both from animals receiving in vivo amikacin and from controls), the magnitude of the $\Delta\log_{10}$ CFU per milliliter was clearly dependent on the concentration of amikacin to which pseudomonal cells within homogenates were exposed.

Treatment studies (Table 2). In rabbits treated with amikacin (80 mg/kg/day i.v.) once daily for 1 or 3 days, there were significant reductions in vegetation densities versus those in untreated controls ($P < 0.001$ and $P < 0.00001$, respectively). In contrast, for animals receiving amikacin at 40 mg/kg i.v. twice daily for 1 day, intravegetation pseudomonal densities were not significantly different from those in controls. For animals receiving amikacin (40 mg/kg i.v. twice daily) for 3 days, there was a significant reduction in vegetation densities versus those in untreated controls ($P < 0.05$). However, rabbits receiving once-daily amikacin had lower pseudomonal vegetation densities than those receiving amikacin twice daily at both 1 day and 3 days of therapy, although these differences did not reach statistical significance.

Pharmacokinetics. As expected, the AUC was higher and the $t_{1/2}$ was longer for the 80- than for the 40-mg/kg bolus dose of amikacin (289.7 ± 38.2 mg · h/liter and 56.6 ± 13.8 min versus 223.7 ± 16.6 mg · h/liter and 45.9 ± 7.8 min, respectively).

DISCUSSION

A recent emphasis in the literature advocates the use of once-daily aminoglycoside dosing (as opposed to multiple-dose regimens) in order to both minimize renal toxicity and ototoxicity (24, 29) and maintain clinical efficacy (12, 20, 28). The ability to utilize once-daily aminoglycoside dose regimens with an efficacy equivalent to that of multidose regimens has several theoretical foundations. First, since aerobic gram-negative bacilli are killed by aminoglycosides in a concentration-dependent (rather than a time-dependent) manner, the higher peak levels in serum obtained by once-daily dosing regimens (versus multidose regimens) tend to maximize killing. This concept has been supported by clinical data from Moore et al. (21), who showed a correlation between aminoglycoside efficacy in seri-

TABLE 2. Effect of dosing regimen of amikacin for 1 and 3 days of therapy in reducing the number of bacteria in vegetations caused by *P. aeruginosa* in experimental rabbit model[a]

Therapy group	Mean ± SD $\log_{10}$ CFU/g of vegetation (no. of rabbits)		No. of rabbits with sterile vegetations/total no. of animals (%)	
	1 day	3 days	1 day	3 days
Control	7.89 ± 0.37 (10)	7.84 ± 0.48 (4)	0/10 (0)	0/4 (0)
AMK at 80 mg/kg i.v. once daily	4.99 ± 1.53 (9)	4.19 ± 1.87 (10)	0/9 (0)	4/10 (40)
AMK at 40 mg/kg i.v. twice daily	7.06 ± 0.65 (7)	6.01 ± 1.51 (11)	0/7 (0)	1/11 (9)

[a] One-day treatment comparisons: amikacin once daily versus control, $P < 0.001$. Three-day treatment comparisons: amikacin once daily versus control, $P < 0.00001$; amikacin twice daily versus control, $P < 0.05$.

ous human infections and the magnitude of the peak serum drug level:MIC ratio. Second, even after serum aminoglycoside levels fall below the MIC of the infecting strain, the magnitude of the post-antibiotic growth inhibition duration (postantibiotic effect) is also related to the peak aminoglycoside:MIC ratios (4, 5). Third, during the terminal elimination phase following a once-daily aminoglycoside dose regimen (~16 to 24 h postdose in patients with normal renal function [22]), the target cells for aminoglycoside toxicity (e.g., the cochlear hair cells) are likely to be exposed to exceedingly low drug levels for a substantial time prior to subsequent doses. This drug-free duration is felt to be important in limiting end organ toxicities (29).

An additional theoretical advantage for once-daily aminoglycoside dosing was recently identified by Daikos et al. (9) and others (1, 8, 30, 31) with delineation of the phenomenon of in vitro adaptive resistance. These investigators observed that, following an initial exposure of *P. aeruginosa* to aminoglycosides, bacterial cells entered a prolonged period of relative refractoriness to further bactericidal effects during subsequent aminoglycoside exposures (1, 8, 9, 30). This phenotypic trait was unstable and disappeared following passage of such refractory cells in aminoglycoside-free media (1, 8, 9, 30). In addition, the longer the time following the initial aminoglycoside exposure (usually at >16 h), the lower the adaptive resistance response of bacterial cells.

Our current study was designed to examine the potential relevance of adaptive resistance in vivo. Several interesting findings emanated from this study. With intravegetation pseudomonal cells from animals receiving no in vivo amikacin (controls), the anticipated concentration-dependent killing induced by amikacin exposure ex vivo was observed (Table 1). Similarly, concentration-dependent killing of intravegetation pseudomonal cells was also seen ex vivo following a first exposure to amikacin in vivo in the experimental endocarditis model. However, the degree of concentration-dependent killing of such pseudomonal cells was less than that observed in controls. Paralleling previous data (1, 8, 30), this difference in the degree of concentration-dependent killing (controls versus aminoglycoside preexposed cells) could be clearly ascribed to the occurrence of adaptive resistance. Thus, intravegetation pseudomonal cells exposed to a single dose of amikacin in vivo exhibited variable refractoriness to a second amikacin exposure ex vivo, depending on the time interval between the first and second drug exposures. Maximal adaptive resistance occurred between 8 and 16 h following the first amikacin dose, with complete refractoriness observed at 12 h postdose. In contrast, the lowest degree of adaptive resistance was observed at time intervals of 5 h between amikacin exposures, especially at the lowest MIC multiple (2.5 times the MIC). This suggests an important concentration-dependent effect on the degree of bacterial killing even during the period of adaptive resistance. Moreover, as observed in vitro (1, 8, 30), adaptive resistance was unstable, with pseudomonal cells showing partial reversibility to a more amikacin-susceptible phenotype by 24 h following the initial amikacin exposure. We point out that the duration of adaptive resistance delineated in the current in vivo studies (~16 h) is substantially longer than we and others have observed in vitro (1, 8, 30). This disparity may well relate to the persistence of the aminoglycoside within cardiac vegetations in vivo or the low metabolic state of intravegetation bacteria. Also, it is quite likely that intravegetation bacteria are not exposed equally to aminoglycosides. Despite uniform distribution of radiolabeled aminoglycosides throughout experimentally induced cardiac vegetations (6, 7), Bayer et al. (3) showed by computer modeling that aminoglycoside concentrations are approximately four- to eightfold higher at the peripheries of experimental aortic vegetations than at the center of such lesions.

In concert with the above sequential in vivo-ex vivo exposure studies of aminoglycoside adaptive resistance, our in vivo treatment studies of animals with established pseudomonal endocarditis further confirmed the existence of phenotypic adaptive resistance in vivo. Intravegetation pseudomonal densities were significantly reduced (by ~3 $\log_{10}$ CFU/g versus those in untreated controls) by single-dose amikacin (80 mg/kg i.v.) but not by the same total daily dose administered in a two-dose regimen (40 mg/kg i.v. twice daily). The improved in vivo efficacy of the single 80-mg/kg amikacin dose compared to the twice-daily 40-mg/kg dose after 1 day of therapy undoubtedly reflects two distinct events: (i) a more favorable pharmacokinetic profile (higher AUC and longer $t_{1/2}$) and (ii) a dosing interval (once per 24 h) that circumvents the time interval of maximum adaptive resistance (8 to 16 h postdose). Similarly, in animals with pseudomonal endocarditis that receive longer-term therapy (3 days), both once- and twice-daily-dose amikacin treatment significantly reduced intravegetation pseudomonal densities versus those in controls ($P < 0.00001$ and $P < 0.05$, respectively). In addition, we observed a trend toward enhanced rates of vegetation sterilization in the once-daily versus twice-daily amikacin regimen after 3 days of therapy, although this trend did not reach statistical significance. It is also important that with longer durations of the twice-daily amikacin regimen (i.e., 3 days versus 1 day), a trend toward progressive reductions in vegetation densities was seen. This finding suggests that in vivo adaptive resistance may be overcome by extending the duration of intermittent dose strategies. We are currently evaluating the efficacy of twice-daily amikacin regimens administered for 6 to 12 days in this same pseudomonal endocarditis model.

The mechanisms of unstable adaptive resistance have not been clearly defined to date. However, we previously showed that alterations in pH in vitro can substantially influence the duration of adaptive resistance, a result related to pH-dependent effects on the magnitude of bacterial killing by the first aminoglycoside exposure (30). Recently, Karlowsky et al. (16) demonstrated that during the period of adaptive resistance in vitro, pseudomonal cells exhibit significant impairment of aminoglycoside uptake as well as a reduction in the net transmembrane proton-motive force. This finding is consistent with data from the present study in which the magnitude of bacterial killing during the adaptive response period was enhanced by progressively increasing the amikacin concentrations from 2.5 to 20 times the MIC, likely increasing the net amikacin uptake. Karlowsky et al. observed no alterations of either cell envelope lipopolysaccharide patterns or outer membrane protein profiles in comparing pseudomonal cells prior to and during the adaptive resistance period. In contrast, a number of distinct alterations in the cytoplasmic membrane protein repertoire were found (16). These investigators speculated that such cytoplasmic membrane perturbations may represent alterations in one or more membrane proteins associated with electron transport chain components. Importantly, this same investigative group recently showed that during adaptive resistance in vitro, pseudomonal cells appear to up-regulate a two-component genetic system involving enhanced expression of the nitrite reductase gene (*denA*) via derepression of the nitrite reductase gene activator (*anr*) (17). These genetic events facilitate terminal electron transport acceptance in the anaerobic respiratory pathway. Interestingly, identical genetic up-regulation occurred when pseudomonal cells were grown anaerobically in vitro. It is thus reasonable to hypothesize that since

anaerobiosis results in reduced uptake of and bacterial killing by aminoglycosides (17), a common mechanism underlying adaptive resistance may exist. This mechanism may well be associated with enhanced expression of pseudomonal genes in its anaerobic respiratory pathway, activated in response to initial aminoglycoside exposures.

The results of previous in vitro studies concerning adaptive resistance (1) as well as our current in vivo studies in the experimental endocarditis model further support the concept of single-daily dosing of aminoglycoside antibiotics. We emphasize that most in vitro and in vivo data on adaptive resistance have been specifically derived from *P. aeruginosa*. Whether or not the concepts described above are broadly applicable to other aerobic gram-negative bacilli awaits further experimental and clinical corroborations.

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