Antibiotic Exposure and Its Relationship to Postantibiotic Effect and Bactericidal Activity: Constant versus Exponentially Decreasing Tobramycin Concentrations against Pseudomonas aeruginosa

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In vitro postantibiotic effects (PAEs) exhibited by a standard strain of Pseudomonas aeruginosa following exposure to tobramycin at constant concentrations were compared to those at exponentially decreasing concentrations. Exposure to a constant concentration showed more extensive bacterial killing and resulted in longer PAEs at comparable areas under the concentration-time curves above the MIC. This phenomenon suggests a significant contribution of pharmacokinetics to antimicrobial pharmacodynamics.

Postantibiotic effect (PAE) in vitro is the duration of continuous bacterial growth suppression following the abrupt removal of the test antibiotic. It has been shown that aminoglycosides display concentration-dependent bacterial killing and exhibit a long PAE against gram-negative organisms (1, 16). Conventionally, PAE is assessed by exposing the test organisms to constant concentrations of an antibiotic for a defined period of time, e.g., 1 or 2 h, prior to its removal. In previous studies, the area under the curve (AUC; concentration × exposure time) has been used to relate the different degrees of antibiotic exposure to the durations of PAE (3, 7, 16). However, exposure of microorganisms to constant antibiotic concentrations differs considerably from the in vivo situation, in that the organisms are subjected to continuously changing concentrations of the antibiotic in vivo. The present investigation aimed at studying the effects of two different modes of antibiotic exposure, i.e., constant versus exponentially decreasing concentrations, on PAE by using Pseudomonas aeruginosa as the test organism in an established in vitro kinetic model. Specifically, the relationships between PAE and bactericidal activity and antibiotic exposure under both conditions were assessed.

P. aeruginosa ATCC 27853 (Difco Laboratories Detroit, Mich.) was used as the test organism in the present study. Following initial isolation, the organism was maintained on agar slants at 4°C. Mueller-Hinton broth (BBL, Cockeysville, Md.) supplemented with 12.5 mg of Ca2+ per liter and 25 mg of Mg2+ per liter (MHB-S) and nutrient agar (BBL) were used as culture media throughout the experiments. Tobramycin sulfate was purchased from Sigma Chemical Co., St. Louis, Mo.

The MIC of tobramycin for P. aeruginosa was determined by the broth dilution technique (13) after incubation at 37°C for 18 h.

An overnight culture was prepared from the organism maintained on an agar slant. Prior to the experiments, this overnight culture was diluted with fresh MHB-S and was allowed to grow for 2 to 3 h at 37°C to attain logarithmic growth. The density of the actively growing culture was visually adjusted against a 0.5 McFarland standard. To start the experiments at time zero, an aliquot of the adjusted culture was introduced into MHB-S containing tobramycin at the designated concentrations. The initial inoculum of the test cultures was targeted to be approximately 10^8 CFU/ml for the experiments conducted with constant concentrations. A slightly higher initial inoculum (5 x 10^6 CFU/ml) was used for the experiments in which the in vitro kinetic model was used, to offset its intrinsic dilution effect.

After the organism was exposed to constant tobramycin concentrations (range, 0.25 to 8 times the MIC) for 1 h at 37°C, the antibiotic in the broth culture (10 ml) was removed by washing with saline (two times) and centrifugation (two times) at 1,200 × g for 10 min. Bacterial cells were resuspended in 10 ml of fresh MHB-S, and viable bacterial counts were determined at the time of inoculation, before and after antibiotic removal, and at hourly intervals afterward. The AUC above the MIC, an index for the degree of antibiotic exposure, was calculated as the product of the time of exposure (t) and the difference between the test concentration (C) and MIC, i.e., (C − MIC) × t.

The in vitro kinetic model described by Grasso et al. (9) was used to simulate the concentration-time profile observed for a one-compartment open pharmacokinetic model. The test organism was introduced into an airtight flask (flask A) containing 10 ml of MHB-S at an initial tobramycin concentration (C0) of 2 to 32 times the MIC. Flask A was placed in a shaking water bath calibrated at 37°C and was connected to another flask (flask B) containing antibiotic-free MHB-S. Flask A was also connected to a waste outlet for sample collection during antibiotic exposure. Antibiotic-free MHB-S in flask B was pumped into flask A by a peristaltic pump at a flow rate (F) adjusted according to the following equation: F = (V·0.693)/t1/2 (equation 1), where V is the volume of antibiotic-containing medium in flask A and t1/2 is the designated elimination half-life. Since flask A was airtight, the rates of inflow and outflow of the culture medium were equal and the culture volume was kept constant. Because the medium in flask A was continuously diluted, antibiotic concentrations in the culture decreased exponentially according to the t1/2 described in equation 1. Because of the wide range of tobramycin doses studied and to avoid complete eradication of the organism at high doses, a t1/2 of

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of 15 min was chosen. The performance of the model was monitored throughout by checking the rate of medium outflow during each sample collection and also by measuring the final volume of the culture medium prior to the antibiotic removal procedures.

The organism was thus exposed to tobramycin concentrations analogous to those observed after injection of an intravenous bolus. For individual experiments, the duration of antibiotic exposure was defined as the time required for the concentration to decrease from \( C_0 \) to the MIC. Antibiotic removal procedures identical to those described above for the constant-concentration-exposure experiments were used. The duration of antibiotic exposure (t) between \( C_0 \) and the MIC for each experiment was computed as follows: 
\[
 t = \frac{\ln(C_0) - \ln(MIC)}{F} \cdot \frac{1}{0.693} \tag{2} 
\]
The AUC above the MIC (\( AUC_{\text{MIC}} \)) over t was also used as an index for antibiotic removal and was determined by the following equation:
\[
 AUC_{\text{MIC}} = \frac{[C_0 - MIC] \cdot t_{\text{max}}}{0.693} \tag{3} 
\]
Similar to the constant-concentration-exposure experiments, viable counts were determined at time zero, before and after antibiotic removal, and at every hour thereafter. The viable bacterial count obtained at each sampling time (T) before antibiotic removal was corrected for the first-order loss to dilution by dividing the viable count of the sample measured at T by the factor \( e^{-0.693 \cdot \frac{T}{T_{\text{half}}}} \). The control cultures (unexposed to antibiotic) were treated in the same manner.

Under both study conditions, PAE was defined as the difference in time required for the antibiotic-exposed culture and the control culture to increase by 1 log unit following antibiotic removal, as defined by Craig and Gudmundsson (5).

The bactericidal activity demonstrated during the period of antibiotic exposure (t) was assessed by a simple procedure for both experimental conditions; the extent of bactericidal effect over time was estimated by taking the slope between the viable counts (transformed by the natural logarithm) measured at time zero and that determined immediately before antibiotic removal. The following equation (equation 4), previously used for the more rigorous description of the bactericidal rate (10, 11, 14), was also used in the present study:
\[
 E = \frac{E_{\text{max}} \cdot (AUC_{\text{MIC}})^{\gamma}}{E(AUC_{\text{MIC}}_{\text{app}}) + (AUC_{\text{MIC}})^{\gamma}} \tag{5} 
\]
where E is the effect measured, \( AUC_{\text{MIC}} \) is the antibiotic exposure from time zero to the MIC, \( E_{\text{max}} \) is the effect required to illicit 50% of the maximal response (\( E_{\text{max}} \)). This model has been customarily used to describe the different pharmacodynamic responses of many agents including antimicrobial agents (12, 14, 17). Since both bactericidal responses under study were individually related to antibiotic exposure by equation 5, the respective model parameters for PAE and S were abbreviated \( E_{\text{max}} \) and \( S_{\text{app}} \), \( P(AUC_{\text{MIC}}_{\text{app}}) \) and \( S(AUC_{\text{MIC}}_{\text{app}}) \) and \( n \) and \( \gamma \) for ease of identification. Parameter estimation was performed by nonlinear regression analysis (PCNONLIN, version 4.0; SCI Software, Lexington, Ky.).

Viable bacterial counts for samples collected throughout the study were quantitated in duplicate by the colony counting assay. The mean log CFU per milliliter value for each sample was reported. Prior to inoculation, samples were subjected to a serial 10-fold dilution with sterile saline. A volume of 10 \( \mu \)l of each diluted sample was inoculated onto the agar surface. For samples for which a low bacterial count (i.e., <300 CFU/ml) was anticipated, a larger sample volume, (e.g., 0.1 to 1 ml) was used without dilution. Viable counts were recorded after 20 h of incubation at 37°C. The limit of accuracy of the assay was about 1.5 log CFU/ml; viable counts below this value should be interpreted with caution.

The tobramycin MIC was measured to be 1 \( \mu \)g/ml for the test organism.

For direct comparison, the changes in viable counts over time in the test cultures with respect to those for the control are presented in Fig. 1A and B for exposures under constant and exponentially decreasing concentrations, respectively. It is readily noticeable from Fig. 1A and B that the extent of bacterial killing and the length of PAE were dependent on the degree of antibiotic exposure. However, for the data obtained by the in vitro kinetic model, bactericidal activity exhibited by the antibiotic was reduced rapidly with decreasing doses (Fig. 1B). This may be due to the lower degree of antibiotic exposure and a shorter exposure time for the test organism at lower doses. With respect to measured PAE, its duration was also considerably shorter at the highest level of exposure to exponentially decreasing antibiotic concentrations compared to that at constant concentrations, i.e., 2.8 versus 4.7 h. Furthermore, a clear nonlinear dependence of PAE on antibiotic exposure was observed in both cases, with an apparent plateau or \( PAE_{\text{max}} \) (Fig. 2A). The estimates for \( PAE_{\text{max}} \), \( P(AUC_{\text{MIC}})_{\text{app}} \) and \( n \) by equation 5 were 5.5 and 3.3 h, 0.2 and 22.6 mg · min/1, and 0.86 and 0.49 for the exposures at constant and exponentially decreasing concentrations, respectively. Indeed, this clear continuous trend of nonlinear PAE data indicated in Fig. 2A further supports the validity of the data that were obtained.

The degree of bactericidal activity was also comparatively lower with respect to exposure at continuously decreasing concentrations (Fig. 2B). The bactericidal activity-versus-antibiotic exposure profile was similar to that observed for PAE; both sensitivity and the maximal bactericidal effect attainable were lower when antibiotic concentrations decreased exponentially (Fig. 2B). The estimates for \( S_{\text{app}} \), \( S(AUC_{\text{MIC}}_{\text{app}}) \), and \( \gamma \) derived from equation 5 were 15.7 and 8.4 h⁻¹, 22.6 and 39.4 mg · min/liter, and 1.0 and 2.1 for the exposures at constant and continuously decreasing concentrations, respectively.

Our study demonstrates that at a similar level of antibiotic exposure, the organisms exposed to constant tobramycin concentrations appear to be relatively more susceptible (higher sensitivity) to the bactericidal effect as well as the growth suppression effect (longer PAE) of the antibiotic. The reason for this phenomenon is unclear. Because the direction of changes in both bacterial responses, regardless of the mode of antibiotic exposure, appear to parallel each other (Fig. 2A and B), an association between the two responses, if any, was further tested by regression analysis of all data collected under both conditions. A significant correlation (\( r = 13; r = 0.95; P < 0.01 \)) was observed, suggesting a strong dependence of PAE on the degree of bactericidal activity. Such agreement of data on the regression line pertaining to both experimental conditions confirms the functionality of the in vitro kinetic model for PAE studies.
Although the limitations imposed on the selection of doses and $t_{1/2}$s for rapidly bactericidal antibiotics are intrinsic problems associated with the use of in vitro models, the experimental conditions used in the present study conformed to the tobramycin pharmacokinetics observed in mice and other smaller rodents (15). Therefore, data obtained with in vitro models can help supplement and support some of the in vivo data observed in preclinical studies. Interestingly, the 2- to 3-h PAE observed at the two higher doses with the in vitro model compares favorably to the PAE observed in vivo in a mouse thigh infection model after administration of a tobramycin dose of 4 mg/kg of body weight when the exposure time above the MIC is about 1 h in both cases (5). Current data also suggest that the AUC above the MIC would be an important parameter for assessments of both bactericidal activity and PAE. This interpretation agrees with the results of a previous animal study on the therapeutic efficacies of two aminoglycosides (6, 15).

Several pharmacokinetic parameters including the peak antibiotic concentration, AUC, and time above the MIC have been proposed for the evaluation of in vitro and in vivo efficacy data for antimicrobial agents (2, 4, 6, 8, 15). However, from a pharmacokinetic perspective, these parameters often relate mathematically to one another; e.g., alterations in dose can cause parallel changes in peak concentration and AUC in linear pharmacokinetic systems (see equations 2 and 3). Among these parameters, AUC integrates both time and intensity of the drug concentrations observed in vivo and is physiologically more relevant as an index of drug exposure. Another advantage associated with the use of AUC is that it renders the need for standardized antibiotic exposure time not necessary.

To the best of our knowledge, this is the first study to specifically evaluate the effects of exponentially decreasing antibiotic concentrations on PAE with an in vitro kinetic model. The most significant finding from this study is the possible overestimation of bactericidal activity and PAE by conventional testing at constant concentrations compared to the activity in the more physiological environment of continuously changing concentrations. Hence, without assessing the possible impacts on the dynamic system, transposing pharmacokinetic parameters between experimental conditions may lead to erroneous interpretations. Only limited data are presented in this study; application to other antibiotic-bacterium combinations will be the subject of further studies.

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