In Vitro Evaluation of the Determinants of Bactericidal Activity of Ampicillin Dosing Regimens against Escherichia coli

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An in vitro flow model was used to examine the influence of peak concentration (Cmax), the area under the antibiotic concentration-time curve (AUC), the magnitude of AUC above the MIC, and the aggregate time the antibiotic concentration exceeds the MIC (Tmax) on the bactericidal effect of ampicillin against Escherichia coli ATCC 12407. Bacteria in the log phase were exposed to therapeutically realistic drug regimens. Ampicillin concentration and bactericidal activity (CFU per milliliter) were measured over time. Four parameters reflecting bactericidal activity were quantitated: difference between initial and minimum and initial and final bacterial densities, area under the bacterial density-time curve, and a fourth parameter, Σ, which is a function of these three. Multiple regression analysis confirmed AUC as the major factor in predicting bactericidal activity. An AUC of >70 µg·h/ml correlated with the lack of emergence of resistance.

Since penicillin G was introduced in 1941, the dependence of therapeutic effect on antibiotic dose and administration schedule has been the focus of considerable investigative effort. The importance of this issue was demonstrated by Eagle et al. (5) and Miller et al. (20) who observed that the median effective total amount of penicillin G administered varied 100-fold depending on the size and frequency of individual doses. It is clear from such work that the time course of antibiotic concentration in serum is an important determinant of efficacy, but it has been difficult to identify a descriptor(s) of the β-lactam concentration-time curve with which antibacterial activity will be correlated. Among those considered have been the area under the antibiotic concentration-time curve (AUC), the magnitude of the AUC above the MIC (AUC > MIC), the total time that antibiotic concentration exceeds the MIC (Tmax), and the maximum antibiotic concentration attained during a dosing interval (Cmax) (3, 6, 9, 11, 12). These descriptors of dosing regimen are a function of dose and frequency of administration as well as clearance and volume of distribution.

Ampicillin is most commonly administered as a series of intermittent bolus doses. The regimen is designed to maintain antibiotic concentrations in serum above the MIC for some portion of the dosing interval, but it is not clear that time above the MIC should be considered a more important feature of the dosage regimen than other characteristics, e.g., Cmax or AUC (7, 8, 14, 19). Further, it is not clear whether there is an independent effect of half-life (which is a function of clearance and volume of distribution) or dosing interval or whether these factors are important only insofar as they affect AUC, AUC > MIC, Cmax, and Tmax.

We examined the dependence of the bactericidal activity of ampicillin on AUC, AUC > MIC, Tmax, and Cmax in in vitro studies with Escherichia coli ATCC 12407 in which ampicillin half-life and dosing interval were varied.

MATERIALS AND METHODS

Kinetic model. A modified Grasso in vitro kinetic dilution model was used to expose E. coli to various ampicillin concentration-time profiles (Fig. 1) (11). The flow of drug and bacteria-free medium into the bacterial flask in this model allows a range of drug half-lives to be simulated. However, flow also results in the dilution of bacteria, which, if not corrected for, could not be distinguished from the bactericidal activity of the antibiotic. The correction for flow is made with the following equation:

\[ N_f = \left( \frac{N_{\text{max}}}{N_{\text{max}} - N_f} \right) e^{-k_f t} + N_f \]

where \( N_f \) is the measured bacterial density, \( N_{\text{max}} \) is the maximum attainable bacterial density had there been no flow, \( k_f \) is the elimination rate constant, and \( N_f \) is the bacterial density which would have been observed had there been no flow (27). In each study, bacteria were exposed to the drug for 12 h. Doses of ampicillin were repeated as required by the dosing interval to maintain a 12-h total exposure. Dosing interval was varied to yield a range of AUC, Cmax, and Tmax.

Bacteria. E. coli ATCC 12407 was used in these experiments. The general experimental procedure was as follows. An overnight culture of E. coli in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was diluted to 10² CFU/ml and allowed to grow to 10⁶ CFU/ml in the same medium. A 1-ml sample of the 10²-CFU/ml culture was added to the test flask, resulting in a density of 10⁵ CFU/ml. When the bacteria reached a density of 10⁶ CFU/ml (in the logarithmic growth phase), the dose of ampicillin required to obtain the desired initial drug concentration was added to the test flask and the peristaltic pump was started. Samples for determination of bacterial density were obtained at 0.5-h intervals. Bacterial density prior to the addition of drug was determined by measurement of A600. After the addition of ampicillin, bacterial density was determined by serially diluting samples in sterile phosphate-buffered saline contain-

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ing 0.1% gelatin. Triplicate 10-μl aliquots of each dilution and 100 μl of undiluted sample were streaked onto nutrient agar plates (Difco). The plates were incubated at 37°C for a minimum of 12 h. Preliminary studies determined that the small amount of ampicillin in the sample had no effect on the estimation of bacterial density (data not shown), most probably because of dilution of the drug during preparation of the sample prior to streaking on the plates. The MIC and MBC of ampicillin were determined by broth dilution by standard methods (25, 26). The MIC is defined as that ampicillin concentration which inhibited visibly detectable growth in broth containing 5 × 10⁸ CFU. The MBC was the ampicillin concentration which resulted in killing of ≥99.9% of the same inoculum. The MBC was the same as the MIC when tested as described above and at an inoculum of 10⁴ CFU. Rejection values of 3 and 11 were used for the inocula of 5 × 10⁸ and 1 × 10⁹ CFU, respectively (22).

**Determination of bactericidal parameters.** These studies required quantitative measures of bactericidal activity. The four parameters used were \( \Delta N_{\text{max}} \), \( \Delta N_{\text{tot}} \), BAUC, and \( \Sigma \), which (except for \( \Sigma \)) are illustrated in Fig. 2. The values reported for each parameter were determined over the entire 12-h period of exposure of bacteria to antibiotic. \( \Delta N_{\text{max}} \) is the difference between the initial and minimum bacterial densities and represents the initial decrease in viable cell density associated with antibiotic addition. \( \Delta N_{\text{tot}} \) is the difference between the bacterial density at the beginning and end of the 12-h study and represents the regrowth phase (in which the emergence of resistance becomes apparent) over two or more dosing intervals. BAUC is the area under the bacterial concentration-time curve divided by the initial inoculum size. BAUC is a time-integrated parameter and is affected by cell death, stationary, and regrowth phases. The parameter \( \Sigma \) is introduced in an attempt to obtain a single parameter which is a function of the three fundamental parameters. \( \Sigma \) is calculated from a rank of 10 to the lowest value of each respective fundamental parameter \( (\Delta N_{\text{max}}, \Delta N_{\text{tot}}, \text{ and BAUC}) \) observed in all studies conducted with a given strain of bacteria. A score of 1 is assigned to the maximum value of each parameter. Scores are assigned to other values of a given parameter by linear interpolation between these extremes, \( \Sigma \) is the sum of the ranks thus assigned to the values of the respective parameters. A value of \( \Sigma \) is calculated for each exposure condition.

**Ampicillin assay.** Ampicillin concentration was determined by a standard disk diffusion microbiological method (24). *Sarcina lutea* ATCC 9341 or *Bacillus subtilis* (Difco) was used as the assay organism (2, 15). Ampicillin standards (Sigma Chemical Co., St. Louis, Mo.) were prepared in...
Mueller-Hinton broth. The lower limit of detection was 0.5 μg/ml, and the coefficient of variation of the slope of the standard curve was 6.6% over a 2-year period.

**Determination of pharmacokinetic parameters.** The in vitro system is designed such that antibiotic concentration will decline monoexponentially. The actual elimination rate constant, \( k_e \), and maximum concentration, \( C_{\text{max}} \), were determined by linear regression. AUC and AUC > MIC were calculated by the trapezoidal rule over the 12-h period of each study (10). The aggregate time during which concentrations were above the MIC for the initial inoculum was calculated by \( T_{\text{MIC}} = k_e^{-1} \ln(C_{\text{max}}/\text{MIC}) \).

**Statistical analysis.** Multiple regression analysis was performed to determine the importance of each of the descriptors of the antibiotic concentration-time profile as a determinant of \( \Delta N_{\text{max}}, \Delta N_{\text{tot}} \), BAUC, and \( \Sigma \). Multiple regression was performed by the stepwise linear regression procedure with a significance level of 0.05 (17). Stepwise regression is an improved version of forward regression which permits reexamination at every step of the variables incorporated in the model in previous steps. At each step, a partial F test for each variable presently in the model is made, treating it as though it were the most recent variable entered. This procedure allows removal of variables that were entered at an early stage which have become superfluous because of their relationship with other variables subsequently evaluated.

### RESULTS

The goal of this investigation required the simulation of a large number of dosing regimens. The general strategy was to evaluate ranges of dosing interval and half-life which are encountered clinically after intravenous ampicillin administration. \( C_{\text{max}} \) was varied from approximately 1 to 100 times the MIC. Bacterial density and ampicillin concentration-time curves from a representative study are shown in Fig. 3; parameter values calculated from these raw data are summarized in row 3 of Table 1. The dosing interval was 6 h and the half-life of ampicillin was approximately 1 h; this allowed drug concentration to exceed the MIC for 6 h of the 12-h study. Ampicillin was effective in killing bacteria (\( C_{\text{max}} \) was nine times the MIC and \( \Delta N_{\text{max}} \) was −3.3 log CFU/ml, i.e., \( 10^{-3.3} \text{CFU/ml} \)). However, there was a net growth of bacteria over the period of exposure; \( \Delta N_{\text{tot}} \) was 1.9 log CFU/ml. Net growth began 2 h after the first dose and 1 h after the second dose. Table 1 shows the reproducibility of these results in a series of four replicate studies. The replicates shown represent data collected over 1 year.

The results obtained from the various exposure conditions are summarized in Table 2. Data from individual studies were analyzed by multiple linear regression to determine the descriptor(s) of the antibiotic concentration-time profile most strongly associated with bactericidal activity.Log transformations of \( C_{\text{max}} \) and AUC, referred to as \( \log C_{\text{max}} \) and \( \log AUC \), were used in the analysis because the log transformations improved the correlation between these parameters and bactericidal efficacy. Log transformation did not improve the correlation between measures of efficacy and any other descriptors of dosage regimen (e.g., \( T_{\text{MIC}} \)).

The results of the regression analysis are shown in Table 3. A value of 0 for a coefficient means that inclusion of the parameter with which it is associated does not improve the overall correlation. The absolute value of the regression coefficient is a reflection of the relative importance of a parameter as a determinant of the bactericidal activity of a regimen. The relationship between the various bactericidal parameters and log AUC for *E. coli* is shown in Fig. 4.

The apparent emergence of resistance was encountered in

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<th>( t_{1/2} ) (h)</th>
<th>( C_{\text{max}} ) (μg/ml)</th>
<th>( T_{\text{MIC}} ) (h)</th>
<th>AUC (μg · h/ml)</th>
<th>AUC &gt; MIC (μg · h/ml)</th>
<th>( \Delta N_{\text{max}} ) (log CFU/ml)</th>
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* 1/2, Half-life.

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* Data represent the mean of two to four individual incubations when dosing interval and half-life (\( t_{1/2} \)) were constant and peak concentration varied less than 10%. \( n = 1 \) if no superscript.
more than half of the studies conducted. The frequency of mutation for resistance to 10 μg of ampicillin per ml was 6.18 \times 10^{-7} for *E. coli* ATCC 12407, as determined by the modified Luria-Delbruck fluctuation test (19). The frequency of mutation for resistance to ampicillin at 20 μg/ml was 1.1 \times 10^{-9}. Thus, resistance to ampicillin at 10 μg/ml apparently developed as a consequence of the emergence of a resistant clone present in the original inoculum of *E. coli* ATCC 12407 (23). The MIC was regularly determined over the study period. The initial population of *E. coli* ATCC 12407 (10^7 CFU/ml) was tested to determine what fraction of the population was resistant to ampicillin concentrations equal to the MIC, twice the MIC, four times the MIC, and eight times the MIC. Of the initial bacterial population, 0% was resistant at these ampicillin concentrations.

Table 4 shows that the emergence of resistance (defined by a minimum of a threefold increase in MIC) was associated with an ampicillin AUC of <70 μg·h/ml. In variants of the

Grasso model, regrowth of bacteria has been shown under certain conditions by Haag et al. (13) to be a consequence of adherence of bacteria to the wall of the incubation flask, forming a thin film. The film sheds bacteria into the medium, resulting in apparent regrowth. We evaluated the potential role of this artifact in our system by conducting (in duplicate) incubations without drugs and with a medium flow rate such that the drug half-life would have been 15 min. Results (mean values of the duplicate studies) are shown in Fig. 5. Bacterial density declined log linearly for the first 4 h of the incubation and then became constant at approximately 3.5 log CFU/ml. The results suggest that some bacteria adhere to the model (in that bacterial density should have been reduced to 1 log CFU/ml at this flow) but that adherence per se cannot account for the regrowth observed in our studies with ampicillin. In addition, growth rate constants at ampicillin concentrations below the MIC were dependent on medium flow rate. The growth rate constant at a medium flow rate of 1.2 ml/min was 0.58 ± 0.13 h^{-1} (range, 0.3 to 0.74 h^{-1}), and at a medium flow rate of 0.25 ml/min, it was 1.0 ± 0.16 h^{-1}.

![Graph showing correlation between AUC and log CFU](image)

**FIG. 4.** Scatter plots illustrating the correlation between the various measures of bactericidal efficacy and log AUC.
In vitro studies with several strains of bacteria and antibiotics, Blaser et al. (4) observed that dosage regimens which produced a peak antibiotic concentration eightfold greater than the MIC effected substantial cell killing and inhibited the emergence of resistance. In our studies, \( C_{\max} \) was identified as an important determinant (although secondary to AUC) of \( \Delta N_{\text{tot}} \), the bactericidal parameter which is most directly affected by the emergence of resistance. \( C_{\max} \), of course, is simply the ampicillin concentration at a single point in time and provides virtually no information concerning concentrations after that time. A \( C_{\max} \) of \( >10 \) times the MIC can result in a net gain in bacterial concentration (positive \( \Delta N_{\text{tot}} \)) if the half-life is short, whereas the same \( C_{\max} \) coupled with a longer elimination half-life can result in a net loss of bacteria (negative \( \Delta N_{\text{tot}} \)) (Table 2). The different signs of the coefficients of AUC and \( C_{\max} \) (Table 3) are apparently due to the frequent association of a high \( C_{\max} \) with a short half-life. A contribution of \( C_{\max} \) to the other bactericidal parameters may have been masked by the correlation between \( C_{\max} \) and AUC, \( r^2 = 0.56 \).

AUC was also found to be an important determinant of the emergence of resistance, defined by a threefold increase in MIC or by a positive value of \( \Delta N_{\text{tot}} \) (Table 2). There was no such result with \( C_{\max} \). This observation is consistent with that reported by Grasso et al. (11).

As mentioned above, Haag et al. (13) showed that the emergence of resistance could be an artifact of in vitro systems owing to the adherence of bacteria to the glass walls of incubation flasks. A similar experiment conducted with \( E. coli \) ATCC 12407 indicated that the regrowth observed in these experiments was not an artifact of the in vitro system (Fig. 5). In addition, resistance was documented by changes in MIC, and the growth rate constants at an ampicillin concentration of \(<1 \mu g/ml\) (the MIC for the original inoculum) in our studies were a function of medium flow rate (i.e., ampicillin half-life), whereas Haag et al. (13) pointed out that a constant growth rate as a function of medium flow rate is a hallmark of apparent resistance owing to adherence of bacteria to glass.

Although the emergence of resistance is a problem encountered more commonly in vitro than in vivo, suboptimum antibiotic therapy has been shown to allow the emergence of resistant strains in animal studies and in certain patients (1, 18, 21). The emergence of resistance is of particular concern when the host is immunocompromised (9). In immunocompromised patients, the results of kinetic in vitro studies may be directly applicable. The observation that resistance is least likely to emerge when AUC is high means that resistance will be least likely when relatively large doses are given frequently. A large dose, frequent administration, or a long half-life alone may be insufficient.

Maintenance of antibiotic concentration above the MIC for the initial inoculum also appears to be insufficient to prevent the emergence of resistance of \( E. coli \) to ampicillin. Indeed, a constant ampicillin concentration just above the MIC is likely to allow the emergence of resistant \( E. coli \), whether present initially or arising from a mutation during exposure to the antibiotic. The results of this and other studies (4) suggest that the emergence of resistance will be discouraged if a dosage regimen is adopted which is designed to eradicate the least susceptible organism present at the beginning of therapy.

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LITERATURE CITED


