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Ampicillin Susceptibility and Ampicillin-Induced Killing Rate of *Escherichia coli*

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The killing rate induced by ampicillin was determined in 20 strains of *Escherichia coli*. The apparent generation rate constant for each *E. coli* showed a characteristic concentration-dependent course. This course can be mathematically described and is determined by four parameters. Three of these parameters determine the speed of the process, and the fourth parameter determines a minimal concentration. The susceptibility of the strains, measured as the minimal inhibitory concentration by an agar dilution method, correlated with the minimal concentration and with a minimal inhibitory concentration calculated from the curve, but not with the rate-determining parameters.

The in vitro susceptibility of bacteria to antimicrobial agents is usually estimated by measuring the minimal inhibitory concentration (MIC), the minimal bactericidal concentration or both. Both methods give information about the effect of the antibiotic after a specified time interval. These parameters do not account for the kinetic processes that take place when bacteria are exposed to an antibiotic. This paper describes in mathematical terms the kinetics of the processes involved in the killing of bacteria by a β-lactam antibiotic.

From the data of the killing curves of *Escherichia coli* bacteria in contact with ampicillin, a mathematical description of the change in killing rate with the antibiotic concentration was developed. In addition, the parameters from the derived formula have been compared with the traditional susceptibility parameter (MIC) to see whether susceptibility and killing rate did correlate.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains were clinical isolates from patients with urinary tract infections.

Determination of the MIC. An agar dilution method was used for this purpose. First, an overnight culture of an *E. coli* strain, incubated without shaking in nutrient broth no. 2 (Oxoid Ltd.), was used to prepare a 10^3-times diluted bacterial suspension in normal saline. Next, a Steers replicator was used to transfer about 0.003 ml of suspension to a diagnostic sensitivity test agar (Oxoid Ltd.) plate which contained ampicillin. In this way, about 10^3 colony-forming units (CFU) were transferred per inoculation site. The plates contained ampicillin concentrations of 0.2, 0.4, 0.6, 0.8, etc., up to a maximum of 10 μg/ml. For the control, one diagnostic sensitivity test agar plate without antibiotic was included in each experiment. After 18 h of incubation at 37°C, the plates were read. A concentration of five colonies or less per inoculation site was used as the criterion for the MIC.

Determination of the killing rate. Proceeding from an overnight culture of an *E. coli* strain grown in nutrient broth, the optical density at 700 nm of the suspension was adjusted to 0.1, using nutrient broth. Next, the suspension was diluted 3×10^3 times with nutrient broth and preincubated at 37°C for 2 h. The culture was then divided into several subcultures, and ampicillin was added to all except the controls. Table 1 lists the ampicillin concentrations used with each *E. coli* strain. The subcultures thus obtained were incubated for 2 h, during which samples were taken every 20 min (starting at time zero, immediately after addition of ampicillin). In the samples, the number of CFU per milliliter was determined with the aid of the spiral plate maker system. Counting was done as described in the instructions for the use of petri dishes with a diameter of 14 cm. This means that a nutrient medium is inoculated in a spiral pattern with a continuously diminishing volume of the bacterial suspension. After incubation, the number of colonies is counted on a specific suitable part of the plate. By dividing this number by the volume of the fraction inoculated on the agar area counted, the number of CFU per milliliter is obtained. To eliminate the influence of the high ampicillin concentrations on counting, inoculation was preceded by penicillinase treatment when these high concentrations were added. Killing curves were obtained by plotting the logarithm of the number of CFU per milliliter at a given ampicillin concentration against time for each *E. coli* strain.

Fitting of experimental data. The experimental data from the killing curves, i.e., the generation rate constant at uninhibited growth (k_0) and the apparent generation rate constants (k_a) at the different ampicillin concentrations were fitted to function with the aid of a computer program (Curfit). This program weighed the points (statistical weighing function, $1/Yi$) and

TABLE 1. Survey of the parameters determined in killing experiments, in a liquid medium, of *E. coli*

E. coli strain	k_0 (h^{-1})	$(k_0 - k_a)_{max}$ (h^{-1})	α (ml/μg)	C_0 (μg/ml)	cMIC (μg/ml)	MIC (μg/ml)
TO3	2.0	8.6	0.7	0.7	1.0	1.8
TO4	2.2	7.6	0.3	2.3	3.4	5.7
TO5	2.6	10.7	0.5	1.2	1.7	3.6
TO6	2.1	7.2	0.5	1.3	2.0	3.6
TO8	2.1	7.5	0.6	3.6	4.2	6.9
TO10	1.9	8.1	0.8	0.6	0.9	1.3
TO13	2.4	10.0	1.6	0.5	0.7	1.1
TO15	2.3	7.8	2.3	0.6	0.8	1.6
TO19	2.1	6.9	1.2	0.3	0.6	1.8
TO21	1.8	6.2	0.4	1.2	2.0	3.1
TO30	2.4	8.6	0.6	1.2	1.8	2.6
TO31	2.3	9.0	1.2	0.9	1.2	2.3
TO33	2.2	8.7	0.5	0.7	1.3	3.2
TO34	1.3	9.1	0.4	1.0	1.3	3.2
TO35	1.8	8.0	0.4	1.1	1.7	2.7
TO37	2.2	10.9	0.4	0.9	1.5	3.0
TO40	2.3	9.6	2.0	0.9	1.0	1.9
TO41	2.2	8.8	1.1	1.2	1.6	2.8
TO42	2.4	10.5	0.6	1.2	1.6	3.2
TO45	2.4	10.5	0.7	0.4	0.8	2.0

fitted them to function by searching for the least-squares deviation.

RESULTS

The killing curves of the strains studied all showed more or less the same characteristic course (Fig. 1A). A latent period is followed by an adaptation period in which growth responds to the antibiotic. The duration of this period varies per strain and per ampicillin concentration. The maximum duration observed was 1 h. The adaptation period is followed by a period in which the number of bacterial cells shows a logarithmic course in time. Depending on the ampicillin concentration, the bacteria grow or die during this period. Further incubation leads to a deflection of the curves, whereupon growth resumes.

By determining the apparent generation rate constant (k_a) of the killing curves during the period of logarithmic growth or death, the behavior of the bacteria at a given ampicillin concentration can be quantified.

A characteristic pattern is obtained when k_a is plotted against the ampicillin concentration per strain. For practical reasons (i.e., to avoid negative values) we plotted, instead of k_a, the value of the difference between the generation rate constant at uninhibited growth (k_0) and k_a. This gave rise to an accumulation of points in which a pattern was discernible that can be described mathematically with the use of equation 1: $k_0 - k_a = (k_0 - k_a)_{max} \{1 - \exp - \alpha (C - C_0)\}$ for $C \geq C_0$, where α is the constant which determines the steepness of the function course; k_{amax} is the maximum value of k_a attainable, and C_0 is the maximum concentration at which, in a liquid medium, ampicillin does not influence growth.

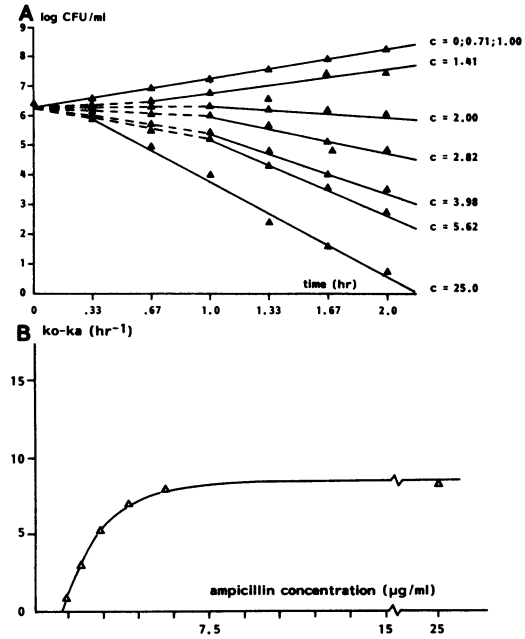

FIG. 1. Results of a killing experiment with *E. coli* TO30, indicating (A) the killing curves in response to various concentrations of ampicillin (c) and (B) the course of the $k_0 - k_a$ values with the ampicillin concentration. The drawn line in (B) is the course calculated from equation 1.

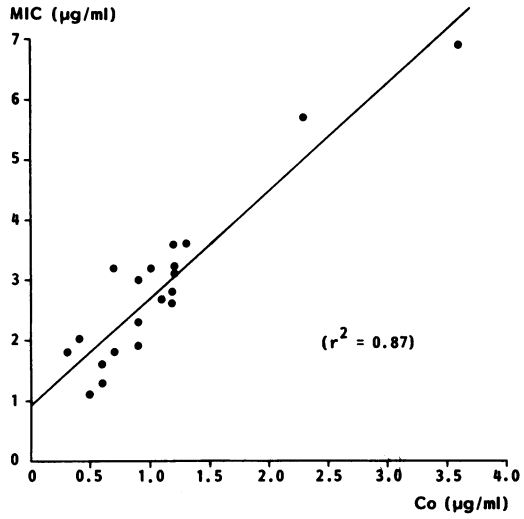

FIG. 2. Comparison between the MIC and the maximal concentration at which, in liquid medium, ampicillin does not influence growth (C_0). Linear regression: MIC = 0.93 + 1.78 × C_0.

The characteristic pattern of k_0-k_a versus ampicillin concentration was thus determined for each *E. coli* strain by the parameters k_0, $k_{a\max}$, α, and C_0.

Figure 1A and B plots the killing curves of strain *E. coli* TO30 and the course of k_0-k_a against the ampicillin concentration. The k_0-k_a values observed coincide very well with the calculated curve for the course (Fig. 1B). The course of k_0-k_a versus C shows three distinguishable phases: (i) the concentration segment $0 \rightarrow C_0$, in which the value of k_a does not differ from that of k_0; (ii) the segment in which k_a diminishes with the concentration; and (iii) the segment above a given concentration, in which the value of k_a no longer changes. The 20 tested *E. coli* strains showed without exception similar curves to that of *E. coli* TO30, the values of the parameters $(k_0-k_a)_{\max}$, α, and C_0 of the tested strains are presented in Table 1. For the sake of completeness, Table 1 also gives the value of k_0. Table 1, column 6, shows the calculated minimal inhibitory concentration (cMIC). This concentration was calculated from the curve for point $k_a = 0$, and can be regarded as the MIC resulting from this test system. After all, the MIC is the minimal concentration at which growth is inhibited, which is to say $k_a = 0$. Table 1, column 7, indicates the MIC of the strains as determined by an agar dilution method. It is quite evident that the killing pattern varies per strain. Not only do the values of C_0 vary, but the rate-determining factors α and $(k_0-k_a)_{\max}$ vary significantly per strain (α, 0.3 → 3.6 ml/μg; and $(k_0-k_a)_{\max}$, 6.2 → 10.9 h^{-1}). Comparison of the minimal concentration C_0 with both the cMIC and the MIC reveals good linear relations among these variables. Regression analysis discloses that the variables show the following interactions: MIC = 0.93 + 1.78 × C_0 (r^2 = 0.87); MIC = 0.52 + 1.51 × cMIC (r^2 = 0.92); and cMIC = 0.27 + 1.18 × C_0 (r^2 = 0.95). These relations are graphically represented in Fig. 2, 3, and 4. No relations were found between the parameters k_0, $k_{a\max}$, α, and C_0.

DISCUSSION

The β-lactam antibiotics, which include ampicillin, are bactericidal agents. This means that, by definition, these antibiotics kill the bacteria at a concentration which equals or exceeds the MIC. As this study confirms, this does not mean that they can produce only a bactericidal effect. At concentrations below the MIC, bacteriostatic effects are often observed (6). The influence of an antibiotic on the bacteria is expressed in a change in the structure and morphology of the cells (4), in inhibition of bacterial growth (1), and in bacterial death. For adequate insight into the action of an antibiotic, it is important to know not only to which extent the above effects occur but also at which speed they become manifest.

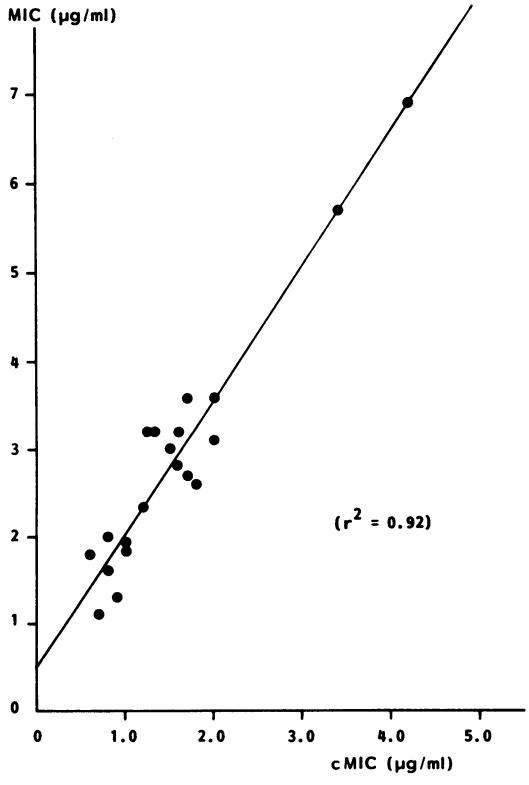

FIG. 3. Comparison between the MIC and the cMIC. Linear regression: MIC = 0.52 + 1.51 cMIC.

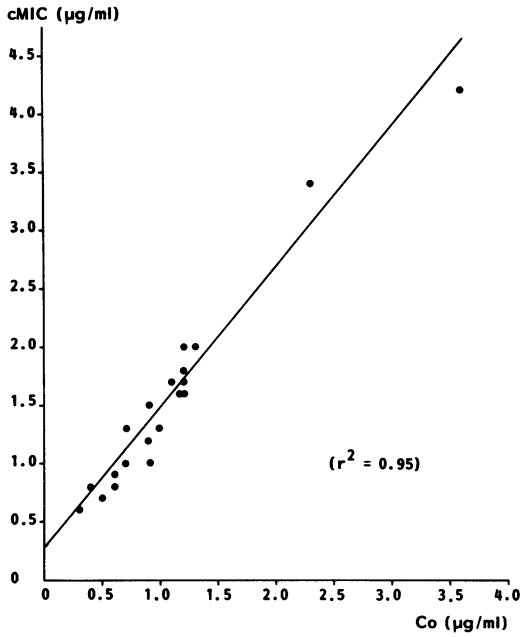

FIG. 4. Comparison between the cMIC and the maximal concentration at which, in liquid medium, ampicillin does not influence growth (C_0). Linear regression: cMIC = 0.27 + 1.18 × C_0.

Rolinson et al. have demonstrated that the rate of bacteriolysis at concentrations above the MIC is determined largely by the antibiotic concentration to which the bacteria are exposed (7).

For practical reasons, bacterial growth or death in response to antibiotics is studied in liquid media. This procedure is complicated by the fact that, at relatively low concentrations, the inhibitory or killing process is relatively soon overshadowed by the growth of adapted or selected resistant bacteria. This phenomenon is not observed on solid media on which a resistant mutant expresses itself as a single colony.

The killing curves determined in this study clearly demonstrate that strains of a single bacterial species can give rise to curves which differ in configuration. The drug-induced adaptation period shows marked differences between various strains and, within the strain, between different concentrations. This period probably reflects, on the one hand, the process of ampicillin distribution over the bacteria and, on the other hand, the time which the antibiotic requires to exert its influence on the bacterium. The adaptation period is followed by a period characterized by a semilogarithmic relation between the number of CFU per milliliter and time. When the antibiotic concentration is not too high and the observations are continued for a sufficient period of time, the linear course is deflected, and a phase of resumed exponential growth follows. Garrett and Won (3) have demonstrated that two factors play a role in this respect: this growth results from overgrowth by more resistant bacteria from the population, and the antibiotic concentration diminishes as a result of absorption or degradation.

Mattie described a model which can be used to describe the killing curves for β-lactam antibiotics from the moment of their addition to the medium until the moment at which overgrowth becomes manifest (5). For this purpose, Mattie makes use of the following equation: $\ln n_t + \ln n_0 + k_0 t - \frac{1}{2}at^2$, where n_t is the number of CFU per milliliter at time t; n_0 is the number of CFU per milliliter at time zero, k_0 is the growth constant; and a is the inhibition constant. With this function, the course of the curves is approximated by a parabole. The killing curves obtained in our study, however, could not be approximated with this function. The observations deviated too much from the proposed curve.

However, by disregarding part of the information obtained, and focusing solely on the phase in which logarithmic growth or death was observed, it was possible to construct the growth or killing curve for each concentration with the aid of one parameter: the apparent generation rate constant k_a. Between this parameter and the ampicillin concentration, a relation proved to exist that could be described by equation 1.

Garret and Won (3), who studied the influence of penicillin G on *E. coli*, and Elkhouly and Führer (2), who studied the influence of ampicillin on *E. coli*, used relatively low concentrations of these drugs. Consequently, they failed to observe the saturation effect. Both groups, however, did find a specific minimal concentration, below which no effect occurred and a linear relation between $k_0 - ka$ and concentration above the value $C = C_0$ was evident. At first, there seems to be a discrepancy between their findings and those obtained in our study. However, when the proposed exponential function in equation 1 is replaced by the first term of the Taylor serial system (i.e., $\exp. -\alpha(C-C_0) \rightarrow 1 - \alpha(C-C_0)$, the following equation emerges: $k_a = k_0 - (k_0-k_a)_{max}\{1 - 1 + \alpha(C-C_0)\} = k_0 - (k_0-k_a)_{max}\,\alpha(C-C_0) = k_0 - \text{constant} \times (C-C_0)$. This equation is identical with the relation proposed by Garrett and Won.

With the proposed function the growth-death pattern could be determined by its parameters k_0, $k_{a\,max}$, α, and C_0. It was found by further analysis that no relations existed between the rate determining parameters k_0, $k_{a\,max}$, and α of the tested *E. coli* strains. A high value of the maximal apparent generation rate constant ($k_{a\,max}$) could be found with strains that either

showed high or low values of α. The question of whether there existed a relation between these parameters and the susceptibility parameter MIC was also studied. Again no relation could be demonstrated between the MIC and the rate determining parameters k_0, $k_{a\ max}$, and α. These findings warrant the conclusion that the rate of interaction between the bacteria and the antibiotic is not expressed in the MIC. Relations did, however, prove to exist between the minimal concentrations MIC, C_0, and cMIC. The differences between MIC and cMIC can perhaps be explained by the use of different media in the two test systems. Sherris et al. (8) demonstrated that results obtained by the agar dilution method can differ from those produced by the broth dilution method.

Because in this study we were concerned only with the interaction between *E. coli* and ampicillin, further studies are necessary to show whether this formula can also be applied to other combinations of bacteria and antibiotics.

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