KINETICS OF BACTERICIDAL ACTIVITY OF AMINOGLYCOSIDES DURING DYNAMIC DILUTION

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The time courses for viable microorganism count after addition of aminoglycosides were investigated in exponentially decreasing concentrations in *in vitro* using a continuous flow culture system. When aminoglycosides were added to the incubation medium containing *Escherichia coli*, the growth rate began to decrease after a lag phase and recovered gradually after the concentration fell below its effective level. To simulate this time course, the following equation including the retardation function was proposed;

$$\frac{dN}{dt} = (k_0 - k_d)N - k_0 P_c N \int_0^t C_0 e^{-k_d(t-\theta)} k_r e^{-k_r \theta} d\theta$$

where N is the number of viables, k_0 is the generation rate constant, k_d is the dilution rate constant, P_c is the bactericidal coefficient per unit concentration characteristic to the individual antibiotic, k_r is the reciprocal of the retardation time and C_0 is the initial concentration of the antibiotic.

P_c and k_r were calculated using the nonlinear least square method and the calculated time course agreed with the observed experimental data indicating the appropriateness of this equation. P_c has a negative relationship to the minimum inhibitory concentration for six aminoglycosides studied, kanamycin, amikacin, kanamycin B, tobramycin, dibekacin and habekacin. The values of k_r ranged between 3.12×10^{-2} to $6.40 \times 10^{-2} \text{ min}^{-1}$ and are thought to correlate with the mechanism of antibiotic actions.

Keywords — aminoglycoside; lag phase; continuous flow culture; kanamycin; amikacin; kanamycin B; tobramycin; dibekacin; habekacin

INTRODUCTION

In the clinical use of antibiotics, it is important to adjust the dosing schedule to keep the maximum and minimum concentrations in blood within the treatment range, *i.e.* between the minimum effective concentration (MEC) and the toxic concentration. The MEC is based on the minimum inhibitory concentration (MIC) determined by *in vitro* experiments, keeping the drug concentration at graded levels. Since the blood concentration of a drug in patients always changes, use of a static concentration to estimate the efficacy of antibiotics is questionable. In practice, aminoglycosides such as dibekacin and amikacin are given once or twice per day, even though the half life of aminoglycosides is known to be so short that it is

probable that blood concentration in patients may fall below the MIC.

Grasso *et al.*¹⁾ used the biexponential time course to simulate the serum level curves and discussed the activity of antibiotics, but did not analyze the time course of the effect of antibiotics on the growth of microorganisms. Garrett and Won²⁾ reported that the plot of the number of viable microorganisms *versus* time showed a lag phase after the addition of antibiotics before the onset of the cidal effect, and they calculated the cidal rate after the lag phase. These lag phases indicated that the cidal effect did not correlate directly with the drug concentration. In this study, therefore the retardation function was introduced to account for both the lag phases of the appearance of the cidal effect and

the recovery of bacterial growth during the dynamic condition of decreasing drug concentration. The information obtained will be useful to define correct and appropriate dose schedules of antibiotics and to choose effective concomitant use in clinical therapy.

MATERIALS AND METHODS

Materials — Six aminoglycosides (structures and names are shown in Chart 1 and Table I) were generous gifts from Meiji Seika Kaisha Ltd., Tokyo, Japan. The concentration of aminoglycosides is expressed as μg of its base per ml. All other reagents used were commercial products of reagent grade.

Culture of Microorganisms — The microorganism studied was *Escherichia Coli* strain B/r which was kindly provided by Central Research Laboratories, Meiji Seika Kaisha Ltd., Japan. A slant was used to inoculate replicate agar slants using nutrient agar (Difco, U.S.A.). These slant were then stored in a refrigerator. Fresh slants were inoculated at regular intervals each week.

Buffered Broth Medium — The composition

CHART 1. Structure of Aminoglycoside

of the broth medium was as follows; Mueller Hinton Broth (Difco), 21 g; Na_2HPO_4 , 1.71 g; KH_2PO_4 , 1.09 g and the total volume was adjusted to 1000 ml with purified water. The medium was autoclaved at 121 °C for 15 min, was adjusted to pH 7.00 with HCl or KOH and was sterilized by filtration through a membrane filter of a pore size 0.45 μm (type HA, Millipore Co., U.S.A.). The medium was buffered and its pH was adjusted to 7.00 carefully because aminoglycosides have a $\text{p}K_a$ around pH 7 attributed to their amino groups and because their bactericidal activities change considerably near their $\text{p}K_a$. It was determined that when the organism population increased to 2×10^7 cells/ml, the pH of the medium did not change.

Bacterial Culture — One drop of the culture medium from a fresh slant was inoculated into a 20 ml of fresh broth medium, and the culture was allowed to grow for 18–20 h at 37 °C. The growth of the culture was followed up to 2×10^9 cells/ml. The temperature of the cultures was maintained at 37 °C in a water bath equipped with a shaker.

The Determination of MIC — Broth medium containing an antibiotic at graded concentrations of two-fold series was inoculated to give about 10^5 cells/ml. The MIC was defined as the concentration of antibiotic to yield no visible multiplication of organism after incubation at 37 °C for 18–20 h.

The Determination of Bactericidal Activity Using a Continuous Flow Culture System — An

TABLE I. Names and Structure of Aminoglycosides

Aminoglycoside		R ₁	R ₂	R ₃	R ₄	M.W. ^{b)}
Kanamycin	(KM)	OH	OH	OH	NH ₂	484.5
Amikacin	(AMK)	OH	OH	OH	HABA ^{a)}	585.6
Kanamycin B	(AKM)	NH ₂	OH	OH	NH ₂	483.5
Tobramycin	(TOB)	NH ₂	H	OH	NH ₂	467.5
Dibekacin	(DKB)	NH ₂	H	H	NH ₂	451.5
Habekacin	(HBK)	NH ₂	H	H	HABA ^{a)}	552.6

a) HABA = $\text{NHCOCH(OH)CH}_2\text{CH}_2\text{NH}_2$.

b) Molecular weight of the base form.

aliquot (1 ml) of culture medium was diluted to achieve 10^8 cells/ml. An aliquot (1 ml) of this diluted culture was aseptically transferred into a 300 ml sponge rubber capped conical flask containing 50 ml of broth medium. The continuous flow culture system is shown in Fig. 1. Fresh medium was pumped into the flask from a reservoir at a constant rate of 0.86 ml/min by a peristaltic pump (MP 12, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and the medium in the flask was allowed to out at the same rate to keep the medium volume constant. The temperature of the cultures was maintained at 37 ± 0.01 °C in a water bath equipped with a shaker. After incubation for 70 min, removal of samples was started and the initial removal was noted as zero time. An antibiotic solution (0.4 ml) was injected into the flask at 80 min to achieve preplanned concentration. Periodical samplings of 0.1 ml medium were done at scheduled times.

Viable Organisms Count — The sample of 0.1 ml was transferred into a centrifuge tube which contained 5 ml of isotonic 1/75 M phosphate buffer (pH 6.8). The suspension of organism was centrifuged at 1800 *g* for 2 min and the precipitate was re-suspended to an appropriate volume in the same buffer to yield countable numbers of colonies. Samples (40–200 μ l) of the suspension were plated on Mueller Hinton Agar plates and the plates were incubated at 37 °C for 18–20 h.

FIG. 1. Continuous Flow Culture System

The number of colonies was then counted. The above centrifugation step was necessary to remove the antibiotic. In preliminary experiments, the effect of centrifugation on the generation rate and the recovery of organisms were determined.

Theoretical Methods — From the fact that the cidal effect showed a lag phase after the addition of an antibiotic, the following equation which considers the lag phase was proposed;

$$\frac{dN}{dt} = (k_o - k_d)N - k_o P_c R(t)N \quad (1)$$

where

N = number of viable microorganisms

k_o = generation rate constant (min^{-1})

k_d = dilution rate constant; 0.0172 min^{-1}

P_c = bactericidal coefficient per unit concentration characteristic to the individual antibiotic ($\text{ml}/\mu\text{g}$)

$R(t)$ = retardation convolution;³⁾

$$\int_0^t C(t-\theta) k_r e^{-k_r \theta} d\theta \quad (\mu\text{g}/\text{ml}) \quad (2)$$

k_r = reciprocal of retardation time (min^{-1})

$C(t)$ = concentration of antibiotic in medium;

$$C_o e^{-k_d t} \quad (\mu\text{g}/\text{ml})$$

Then Eq. 1 becomes

$$\frac{dN}{dt} = (k_o - k_d)N - k_o P_c k_r C_o N (e^{-k_d t} - e^{-k_r t}) / (k_r - k_d) \quad (3)$$

In integrated form;

$$\ln N = \ln N_o + (k_o - k_d)t -$$

$$k_o P_c C_o (1 - \frac{k_r e^{-k_d t} - k_d e^{-k_r t}}{k_r - k_d}) / k_d \quad (4)$$

where N_o was the number of viable organisms when the antibiotic was added.

In Eq. 4, N , N_o , k_o , k_d , t and C_o could be observed and determined in experiments, while P_c and k_r had to be calculated by the nonlinear least square method.

RESULTS AND DISCUSSION

The results of the effect of centrifugation on generation rate and recovery of microorganisms are shown in Table II. The generation rate constant was not affected by centrifugation. The recovery percent of microorganisms after centrifugation was 94.9%. Thus, the centrifugation

TABLE II. *Effect of Centrifugation on Generation Rate Constant and Recovery of Microorganisms^{a)}*

	Generation rate constant (10^{-2} min^{-1})	Recovery (%)
Control	3.47 ± 0.15	100
With centrifugation	3.48 ± 0.17	94.9 ± 3.2

a) Results are given as the means $\pm$ S.D. of six experiments.

step was employed on all the samples in this study.

The observed viable microorganism counts *versus* time are plotted in Fig. 2 with the calculated lines according to Eq. 4 for three C_0 levels of six aminoglycosides. It was noted that the viable count decreased gradually after the addition of antibiotic and increased gradually with the decrease of the antibiotic concentration. The close agreement between the experimental

results and the calculated results supports our development and use of Eq. 4.

MIC and the estimated parameters, P_c and k_r , in graded initial concentration levels (C_0) are shown in Table III for six aminoglycosides. Values of P_c increased with C_0 at lower C_0 values and were almost constant at higher C_0 , while k_r increased with C_0 . These results show that P_c may be a function of C_0 for a wide range of C_0 and that Eq. 4 was valid in the limited

FIG. 2. *Effect of Antibiotics on Time Course of Viable Microorganism Count*

a) KM, b) AMK, c) AKM, d) TOB, e) DKB, f) HBK. Arrows show the time when aminoglycoside solution was added. The lines were calculated according to Eq. 4. (O), (Δ) and (□) were in the order of the initial concentration of antibiotics (C_0) marked with b) in Table III.

range of concentration. P_c and k_r from three C_0 levels marked with *b*) were averaged and are shown in the same Table. There was a negative relationship between MIC and the averaged P_c , while k_r did not correlate with MIC for all antibiotics tested.

The coefficient, P_c is the bactericidal activity per unit concentration characteristic for a given antibiotic and represents the probability of kill-

ing the organisms during the generation phase. P_c was determined by using decreasing concentrations of aminoglycosides. It is reasonable to assume that P_c correlates with MIC which is the static bactericidal activity and is affected by the substitution of the aminoglycoside with some side groups. The rate constant, k_r , that was introduced to explain the lag phase of the onset of the cidal effect, is the reciprocal of the retardation

TABLE III. MIC, P_c and k_r in Various Levels of C_0 for Six Aminoglycosides

	MIC ($\mu\text{g/ml}$)	C_0 ($\mu\text{g/ml}$)	P_c ($\text{ml}/\mu\text{g}$)	k_r (10^{-2} min^{-1})	Mean $\pm$ S.D. ^{a)}	
					P_c ($\text{ml}/\mu\text{g}$)	k_r (10^{-2} min^{-1})
KM	12.5	7.89	0.257	1.62	0.414 ± 0.024	4.17 ± 1.90
		9.29	0.352	2.15		
		10.9	0.424 ^{b)}	2.78 ^{b)}		
		12.8	0.432 ^{b)}	3.41 ^{b)}		
		14.2	0.387 ^{b)}	6.33 ^{b)}		
AMK	6.25	6.08	0.398	3.06	0.568 ± 0.013	6.40 ± 2.34
		6.92	0.469	4.04		
		7.09	0.569 ^{b)}	4.06 ^{b)}		
		9.13	0.580 ^{b)}	6.41 ^{b)}		
		10.2	0.555 ^{b)}	8.74 ^{b)}		
AKM	3.13	3.01	0.80	1.22	1.19 ± 0.01	3.12 ± 0.32
		3.20	1.10	2.72		
		3.31	1.19 ^{b)}	2.78 ^{b)}		
		3.62	1.18 ^{b)}	3.15 ^{b)}		
		4.11	1.19 ^{b)}	3.42 ^{b)}		
TOB	1.56	1.33	0.83	1.58	2.14 ± 0.04	3.89 ± 0.75
		1.71	2.04	2.08		
		1.78	2.19 ^{b)}	3.26 ^{b)}		
		2.13	2.14 ^{b)}	3.69 ^{b)}		
		2.72	2.11 ^{b)}	4.72 ^{b)}		
DKB	3.13	3.07	0.75	1.69	1.22 ± 0.05	3.57 ± 0.43
		3.27	0.91	1.65		
		3.50	1.21 ^{b)}	3.15 ^{b)}		
		3.94	1.28 ^{b)}	4.01 ^{b)}		
		4.69	1.18 ^{b)}	3.55 ^{b)}		
HBK	3.13	1.94	1.04	1.54	1.31 ± 0.01	3.25 ± 0.83
		2.50	1.14	2.92		
		2.63	1.30 ^{b)}	2.29 ^{b)}		
		2.79	1.31 ^{b)}	3.73 ^{b)}		
		3.47	1.30 ^{b)}	3.73 ^{b)}		

^{a)} Mean $\pm$ S.D. of last three data marked with *b*) for each aminoglycoside.

time that is used in the fields of electronics, kinetics and rheology. The retardation convolution of Eq. 2 yields the consequent output of the process of linear combination of $C(t)$ and $k_r e^{-k_r t}$.

The constant, k_r may relate to the mechanism of the antibiotic activity and is thought to be the transport rate constant through the bacterial wall or the nucleous membrane and/or some unknown biological process of the bactericide after the misreading of deoxyribonucleic acid (DNA) codes. The values of k_r ranged from 3.12×10^{-2} to $6.40 \times 10^{-2} \text{ min}^{-1}$ for the tested aminoglycosides. Each antibiotic with a different mechanism may have its appropriate k_r .

The term, $P_c R(t)$ is multiplied by k_o on the assumption that the cidal effect works in proportion to the size of k_o on organisms during their generation phase. Garrett and Won²⁾ proposed the apparent first order kinetic for the cidal effect of antibiotics on the assumption that the rate of loss of viable cells after lag and induction periods may be expressed as the difference between the rate of generation and that of killing, where the latter is a function of drug concentration, $C(t)$. The kill rate is proportional to the first power of drug concentration;

$$\frac{dN}{dt} = [k_o - k_c \{C(t) - C^*\}] N \quad (5)$$

where k_c is the kill rate constant and C^* is the minimum concentration above which antibacterial activity is manifested. C^* is MIC and should appear in Eq. 1 but was assumed to be zero for aminoglycosides in this paper because the

simulated lines for this assumption fit the observed data better than that for non-zero C^* .

We also tried their proposal as using the next equation assuming that k_c is independent of k_o .

$$\frac{dN}{dt} = (k_o - k_d) N - k_c R(t) N \quad (6)$$

The calculate k_c and k_r according to Eq. 6 varied considerably with the change of C_o indicating the inappropriateness of Eq. 6. The lag phases reported but not discussed by Garrett and Won²⁾ may be explained by the theory reported in this paper. In conclusion, Eq. 4 expresses the entire time course of the number of viable organisms after the addition of an antibiotic, including the lag phase.

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