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Early Stages of In Vitro Killing Curve of LY146032 and Vancomycin for *Staphylococcus aureus*

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The early stages of the time-killing curves of vancomycin and LY146032 have been studied, by use of short sampling intervals, for three strains of *Staphylococcus aureus*. Both vancomycin and LY146032 killed *S. aureus*, but the time-killing curves differed: the effect of vancomycin was slow, limited, and not related to the concentration of the drug, whereas that of LY146032 was rapid, extensive, and related to concentration. When strains ATCC 25923 and CIP 6525 were exposed to LY146032, the population decreased exponentially with time. The killing rate was constant and linked to the concentration by a Michaelis-Menten relationship. The maximum killing rate and the affinity constant of LY146032, estimated from the data transformed by the Lineweaver-Burk method, differed for the two strains. The concentration of the antibiotic at which killing theoretically begins (estimated by linear regression using the logarithm of the concentration) is of the same magnitude as the MIC of LY146032, which indicates the pure bactericidal mode of action of the drug. *S. aureus* ATCC 12600 was more resistant to the bactericidal effect of the two drugs, and its killing curve did not conform to the model described here.

LY146032 is a new antibiotic that belongs to a class of agents known as peptolides. It possesses almost the same activity as vancomycin against gram-positive isolates; like vancomycin, it inhibits the synthesis of peptidoglycan by these bacteria (5). LY146032 has been shown to be bactericidal against *Staphylococcus aureus* including methicillin-resistant strains (4, 7, 8, 10, 13).

In this study we compared the early-phase (between 0 and 8 h) of the time-killing curves of LY146032 and vancomycin on *S. aureus* strains. The antimicrobial effect of both drugs was studied with short sampling intervals so that the decrease of the bacterial population and the shape of the killing curves could be determined as accurately as possible. The phenomena observed are explained by use of mathematical modeling.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* ATCC 25923, ATCC 12600, and CIP 6525 (Institut Pasteur Type Collection, Paris) were used. Strains were deep frozen in liquid nitrogen until use and subcultured three times on Columbia blood agar (bio-Mérieux, Charbonnières les Bains, France) before use.

Bacterial counts. Bacterial counts were performed on Columbia agar after dilution, ranging from 10^{-1} to 10^{-6}, in sterile saline. In some steps undiluted samples were also used. A 0.1-ml sample of each dilution was plated onto agar, and counts were made in triplicate. Colonies were counted after 48 h of growth at 35°C. Means were calculated from plates with counts ranging from 20 to 300 CFU.

Antibiotics. Vancomycin and LY146032 were donated by Lilly Laboratories (Lilly France, Saint Cloud, France). A stock solution of the antibiotics was prepared each week and stored at 4°C until use.

Assessment of killing curve. Mueller-Hinton broth (bio-Mérieux), with the magnesium and calcium content adjusted, respectively, to 20 and 60 mg/liter, was used throughout the study. Each strain was grown overnight in identical broth. Organisms were harvested by centrifugation and suspended in saline. The inoculum was adjusted to 10^7 bacteria per ml by use of a nephelometer (Autobac; General Diagnostics, Div. Warner-Lambert Co., Morris Plains, N.J.); 0.1 ml of inoculum was added to 10-ml preheated broth tubes containing 10, 5, 2.5, or 1 mg of either LY146032 or vancomycin per liter. Tubes were then incubated at 35°C without shaking. Every 30 min between 0 and 8 h, the tubes were strongly agitated and a 0.1-ml sample was diluted and immediately plated; the tubes were immediately returned to incubation.

Assessment of growth. The procedure described above for assessment of the killing curve was followed, but antibiotics were omitted. Sampling was done every hour during a 4-h period.

Carryover of antibiotics. Carryover was estimated by plating a 0.1-ml sample of each antibiotic dilution onto Columbia agar. Plates were held at 35°C until resorption of the liquid was complete, and then approximately 100 CFU of strain CIP 6525 were introduced to each plate. Control plates without antibiotics were prepared in the same conditions. A CFU count was made after 48 h.

MIC determination. MICs were determined by a classical method on Mueller-Hinton agar with adjusted calcium and magnesium content (1, 5) and in Mueller-Hinton broth with 10^7 bacteria as for the assessment of the killing curves.

RESULTS

Growth curve. The three strains grew in an identical way: growth began with an accelerated phase of about 1 h preceding exponential growth. A generation time of 25 min was estimated graphically (11).

MIC. All strains were inhibited by 0.5 mg of LY146032 per liter and by 1 mg of vancomycin per liter. MICs were higher in broth (respectively, 1 and 2 mg/liter).

Carryover. Differences observed between bacterial counts on the control plates and those inoculated with 0.1 ml of the

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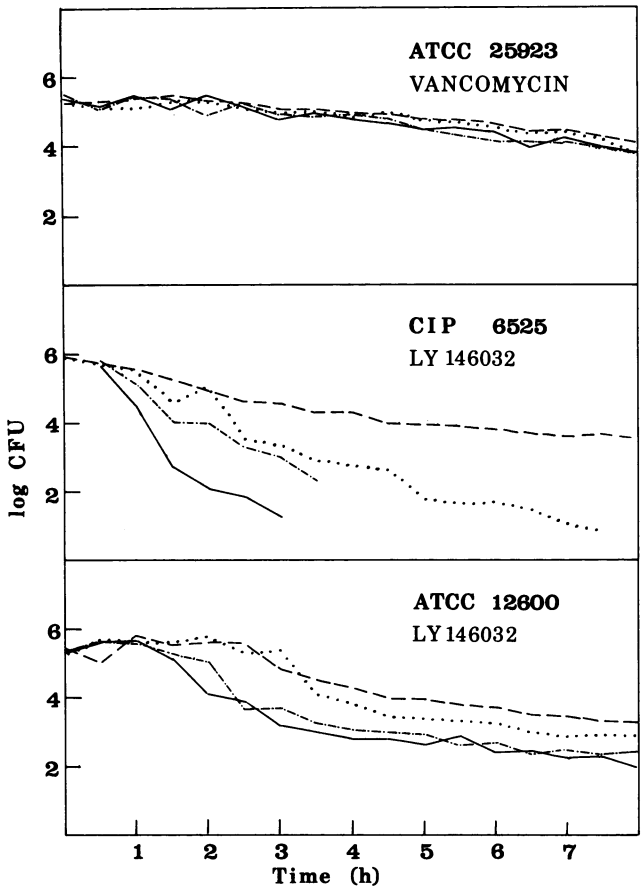

FIG. 1. Time-killing curve of *S. aureus* ATCC 25923, CIP 6525, and ATCC 12600 exposed to vancomycin or LY146032 at concentrations of 10 mg/liter (—), 5 mg/liter (– · –), 2.5 mg/liter (.....), and 1 mg/liter (----). The effect of vancomycin is slow, limited, and not related to the concentration, whereas that of LY146032 is rapid, extensive, and related to the concentration. The killing curves of *S. aureus* ATCC 12600 do not conform to the model of the two other strains.

1-, 2.5-, and 5-mg/liter concentrations of each antibiotic were within the limits of biological variation. Impregnation of plates with 0.1 ml of a solution of 10 mg of antibiotic per liter induced inhibition of about 80% of the bacterial population. Results of undiluted samples containing 10 mg of antibiotic per liter have therefore been rejected to avoid overestimation of the activity of both drugs.

Killing curve. Enumeration of bacteria was difficult after overnight growth, especially for strains exposed to vancomycin; consequently all bacterial counts were made after 48 h of incubation. A decrease of the bacterial population from

TABLE 1. Killing rate (*m*) obtained by orthogonal linear regression fo the time-killing curve of *S. aureus* exposed to LY146032

LY146032 concn (mg/liter)	Absolute value of *m* (1/h)	
	ATCC 25923	CIP 6523
10	3.51	4.04
5	2.78	2.54
2.5	2.30	1.65
1	1.45	0.69

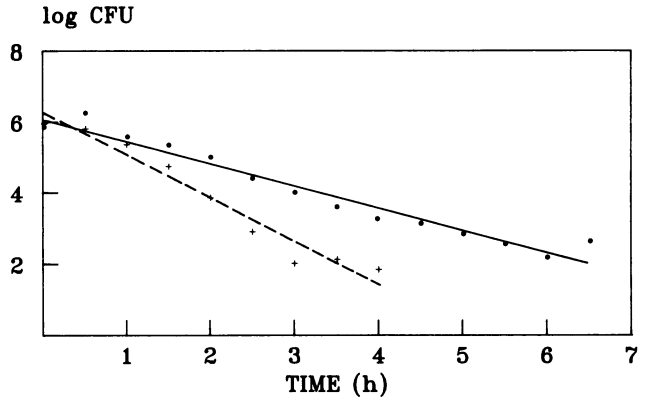

FIG. 2. Time-killing curve of *S. aureus* ATCC 25923 exposed to LY146032 at 1 mg/liter (——) and 5 mg/liter (----); the regression lines were fitted to experimental data.

10^5 to 10^4 CFU was obtained with vancomycin. When strains ATCC 25923 and CIP 6525 were exposed to vancomycin, the curve was almost linear. The killing rate was thus approximately constant and did not appear to be related to the vancomycin concentration. The decrease in viability of the cells was more complex for strain ATCC 12600. The effects of LY146032 appeared to be related to concentration (Fig. 1). The time-killing curves of strains ATCC 25923 and CIP 6525 exposed to the various LY146032 concentrations were straight in a semilogarithmic representation. The mortality of the bacteria exposed to LY146032 was thus exponential (9) (Fig. 2), and the constant killing rate was the slope of the regression line (Table 1). In most cases the correlation coefficient was close to 0.95. The dose-effect relationships for strain ATCC 25923 and CIP 6525 exposed to LY146032 are shown in Fig. 3. There was a linear relation between the killing rate and the logarithm of the concentration of the drug (Table 2). This relation was independent of time, and the threshold concentration C_0 could be calculated from it, giving a null mortality rate (C_0 is the theoretical concentration at which no bactericidal effect is observed, and in this case the bacteria can either multiply or be only inhibited [MIC]). C_0 is estimated to be of the same magnitude as the MIC. The curves shown in Fig. 3 resemble the saturation curves well known in enzymology where enzymatic degradation of synthesis rate is plotted against substrate concen-

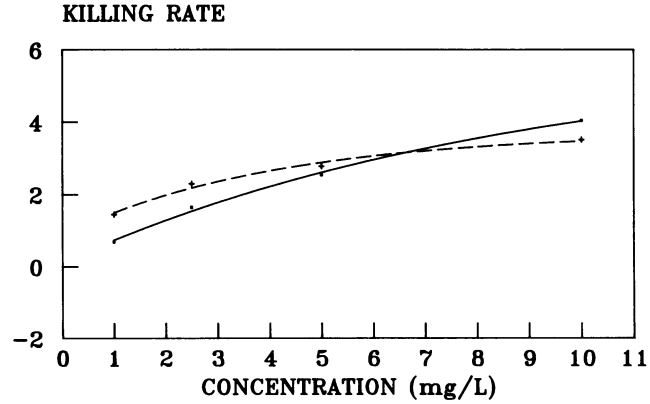

FIG. 3. Relationship between killing rate (1/hours) and concentration of LY146032 for *S. aureus* ATCC 25923 (----) and CIP 6525 (——).

TABLE 2. Parameters[a] estimated by orthogonal linear regression for the relation between killing rate of LY146032 on two strains of *S. aureus*

Transformation	Strain	*a*	*b*	r^2	C_0	K_c	m_{max}[b]
Logarithmic[c]	ATCC 25923	0.87	1.45	0.995	0.19		
	CIP 6525	1.42	0.51	0.969	0.69		
Lineweaver-Burke[d]	ATCC 25923	0.25	0.43	0.994		1.7	2.29
	CIP 6525	1.33	0.10	0.997		12.4	9.32

[a] r^2, Correlation coefficient; C_0, theoretical concentration without bactericidal effect; K_c, affinity constant; m_{max}, maximum killing rate.
[b] Absolute values.
[c] $m = b + a$ (log C).
[d] $1/m = b + a$ (1/C).

tration. The concentration was studied on the same theoretical grounds, by use of the classical Lineweaver-Burk plot after reciprocal transformation of the data (Fig. 4). For enzymes following the Michaelis-Menten equation, this led to a straight line, which is also observed here. The relationship between m and C can be expressed as follows: $m = m_{max}[C/(K_c + C)]$, where m_{max} is the maximum killing rate and K_c is the affinity constant of the bacteria to the antibiotic. The parameters m_{max} and K_c have the same meaning as in enzymology; their values are shown in Table 2.

DISCUSSION

Both vancomycin and LY146032 are known to exhibit bactericidal effects against *S. aureus* (3, 5, 6), but this has previously been studied over a longer time, usually 24 h, and with greater sampling intervals (1 to 4 h). The lack of data on the early stages of killing results from the fact that studies over 24 or 48 h appeared to be more representative of the effect of the drug during therapy; also, technical problems arise with short sampling intervals. The major difficulty is to minimize the processing time to maintain the sampling schedule and to study, for one strain, all of the concentrations in the same experimental conditions and on the same day. This complexity and the cost of conducting the experiment explain why the number of strains studied has been limited. It also explains the choice of reference strains, which are collection strains, used either for antibiotic susceptibility testing quality control (ATCC 25923) or taxonomy (ATCC 12600) or known as a prototype of methicillin-resistant strains (CIP 6525) (2). In the case of vancomycin and LY146032, the use of such reference strains is not critical because the clinical isolates are all susceptible.

In this study, a decrease of the bacterial population from 10^5 to 10^4 CFU was obtained within 8 h with vancomycin. This is approximately the same as observed in previous studies with *S. aureus*. The minor differences may be due to the use of an inoculum prepared from overnight culture (11), even when growth began quickly in our conditions; the use of a 4-h inoculum was not possible here because of the total duration of one experiment (9 h). LY146032 was more bactericidal and was rapidly active. Its bactericidal activity was also demonstrated on the methicillin-resistant strain CIP 6525. These facts are in accordance with previous results concerning these two antibiotics (3, 4, 8, 10, 13). The killing of bacteria by LY146032 is demonstrated quickly, at the beginning of the growth, and it is very rapid at a high concentration (10 mg/liter) (10, 13).

The method used in this study enabled further observations not possible with longer intervals of sampling, especially a precise description of the shape of the killing curves. The mortality of the strains ATCC 25923 and CIP 6525 exposed to LY146032 is thus exponential, and the constant killing rate is the slope of the regression line.

There is a linear relation between the killing rate and the logarithm of the concentration on LY146032, and the threshold concentration C_0 (giving $m = 0$) is estimated to be of the same magnitude as the MIC for the two strains, which indicates the almost pure bactericidal effect of LY146032. The biological meaning of the relation between m and logarithm of C is, however, difficult to understand because the killing rate increases infinitely when concentration increases and we consider it only as a useful descriptive tool.

The relationship between the killing rate and concentration is better described by a Michaelis-Menten-type relation. This expression is satisfactory, since the fitting of the regression curve to the data is good and the killing rate does not increase over m_{max}. The shape of the saturation curve should also be considered in therapeutics, because increasing the concentration does not greatly affect the killing rate except at lower concentrations (saturation effect).

No previously published works have described such a model of bacterial killing. The model of Tosh et al. (W. Tosh, H. Buol, and O. Zak, Proc. 13th Int. Congr. Chemother., p. 50/10–50/30, 1983) is different, since it considers that the killing rate is not a constant; it also lacks a simple biological explanation and remains only descriptive, well adaptated to the killing effect of penicillins but not to the action of LY146032 on the strains used in this study.

With respect to strain ATCC 12600, the effect of the antibiotics was different; the phase of active killing was

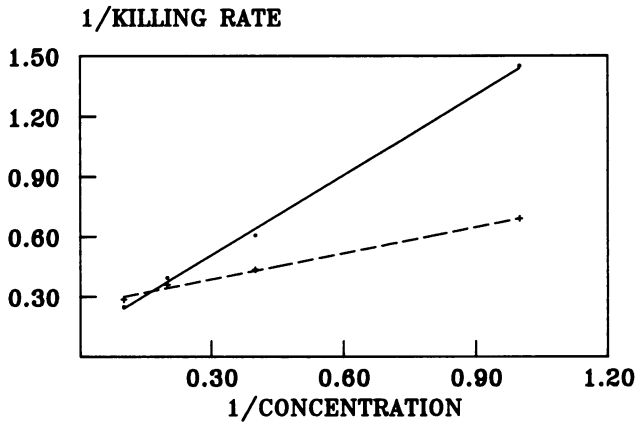

FIG. 4. Relationship between killing rate and concentration of LY146032 after Lineweaver-Burk transformation of the data for *S. aureus* ATCC 25923 (----) and CIP 6525 (——).

reduced to half an hour for vancomycin and to 1 h for LY146032. The decrease in population was also limited, and the general shape of the curve is sigmoidal. This has been verified also in two other experiments and can be considered as typical of the strain as is also its original time-killing curve with vancomycin. No satisfactory mathematical model of the phenomenon has been found, and no explanation of the differences observed between strain ATCC 12600 and the two other strains is possible.

This study of time-killing curves of *S. aureus* exposed to vancomycin and LY146032 gives information on phenomena arising in the early phase of antibiotic contact. For strains CIP 6525 and ATCC 25923, the killing phase with vancomycin occurred after 2 to 4 h and had a constant rate not related to concentration. For the same strains exposed to LY146032, the killing rate was constant and linked to the concentration by a Michaelis-Menten relationship. This observation enables comparison of the bacterial mortality with enzymatic processes. The killing rate was limited, as was the speed of an enzymatic reaction; increasing the concentration did not result in an increased killing rate. One strain (ATCC 12600) was less susceptible to the killing effect of the two antibiotics, and the mathematical model describing mortality of this strain is more complex. Two antibiotics with many properties in common thus differ in their ways of killing the bacteria, and this is clearly demonstrated by use of simple mathematical modeling.

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