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Time-Survival Studies for Quantifying Effects of Azlocillin and Tobramycin on *Pseudomonas aeruginosa*

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Time-survival studies were conducted to estimate the effects of azlocillin and tobramycin on *Pseudomonas aeruginosa* NCIMB 8295 (in the exponential phase of growth) at concentrations ranging from one-quarter to twice the MIC. The effects of the individual agents and their combinations were determined by measuring the viable counts (CFU per milliliter) over a 24-h period. The typical pattern observed from the plot of the logarithm of the CFU per milliliter against time was an initial rapid killing; this was followed by a period of stasis and regrowth. Initial rates of killing by tobramycin were concentration dependent, whereas this was not the case with azlocillin. From the time-survivor plots, the area under the curve for viable bacteria was also calculated. It offered a useful method of interpreting the results of time-kill studies, taking the overall pattern of killing and regrowth into consideration. The area under the curve for viable bacteria was concentration dependent for both antibiotics. A 2^2 factorial experimental design was used to analyze the joint effects of azlocillin and tobramycin. In such a factorial experiment, an interaction between two factors, in this case, azlocillin and tobramycin concentrations, is shown by a change in the slope of the plot when the concentration of the interactant is changed. Analysis of variance showed that the combination was synergistic at low concentrations, but this was not significant when the concentration of either interactant was increased.

Treatment regimens involving a combination of an aminoglycoside and a beta-lactam antibiotic are recommended for a number of infections. In particular, tobramycin and azlocillin are frequently given in combination to treat *Pseudomonas aeruginosa* infections (16). This combination of antimicrobial agents is often claimed to be synergistic, with such claims being based on in vitro laboratory tests such as the checkerboard method (4, 19). An additional measure of synergism has been developed from time-kill curve analysis, in which the criterion for synergism is a 2-$\log_{10}$ difference in viable counts at 24 h in the presence of the combination compared with the viable counts in the presence of the most effective individual component. However, Watanakunakorn (20), using this approach, reported that synergy was rarely observed with the azlocillin-tobramycin combination.

Kinetic studies of the interaction between aminoglycosides and microorganisms have demonstrated a biphasic reaction, with an initial concentration-dependent rapid bactericidal phase and a slower bactericidal phase that was not dependent on concentration (15). Those bacteria that survive the first exposure develop adaptive resistance which is mediated by impermeability to all aminoglycosides (6). These observations have had implications for aminoglycoside therapy (18). In contrast, the bactericidal activity of azlocillin against *P. aeruginosa* was shown to be limited (21), and the therapeutic effects of beta-lactam antibiotics were found to be not strongly dependent on the peak concentration in serum (1).

In the study described here, we used time-survival methods to study the effects of azlocillin and tobramycin alone and in combination on *P. aeruginosa*. Rates of killing and the area under the curve for viable bacteria (AUC-VB) were calculated from time-survival plots and were used to investigate the effects of drug concentration. A factorial design experiment was used to study the interactions of the two antibiotics.

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MATERIALS AND METHODS

Organism. *P. aeruginosa* NCIMB 8295 (ATCC 10145), a standard strain for antibiotic testing, was used. For all experiments, 1 ml of an overnight culture was inoculated into 100 ml of Iso-Sensitest broth (ISB; Oxoid), the preferred susceptibility testing medium in the United Kingdom (22), in a 250-ml flask, and the mixture was incubated in an orbital incubator (37°C, 150 rpm) for approximately 75 min. The final culture had an optical density at 540 nm of 0.1 (equivalent to a viable count of 10^8 CFU/ml) and was in the early exponential phase of growth.

Antibiotics. Azlocillin and tobramycin solutions were prepared in water from Securopen powder (Bayer UK Ltd.) and Nebcin solution (Eli Lilly & Co. Ltd.), respectively, and were sterilized by double filtration through a 0.22-μm-pore-size membrane filter. Solutions were prepared so that the addition of 1-ml volumes to 100 ml of ISB provided the required test concentration.

Determination of MIC. A broth macrodilution method was used to determine the MICs of azlocillin and tobramycin in ISB with a final inoculum of 10^5 CFU/ml. After incubation at 37°C for 24 h, the MIC was taken as the highest dilution which failed to show visible growth. Tests were carried out in duplicate.

Time-survival determination. Four flasks containing 100 ml of ISB (prewarmed to 37°C) alone or in the presence of the antimicrobial agents under investigation were inoculated with 1 ml of culture and were incubated with shaking (200 strokes per min). At specified time intervals, samples (1 ml) were removed and were serially diluted in 0.1% peptone water, and 1-ml aliquots were plated onto Iso-Sensitest agar (Oxoid) for viable count determination by the pour plate method. Plates were incubated at 37°C for 18 h, and the number of CFU was determined. No further increase in CFU occurred following

incubation for up to 3 days. Four independent replicates were carried out for each experiment.

Determination of rate of killing. Point estimates for the rates of killing were obtained by a process of sequential optimization. Step one involved the selection of three points on the linear portion of the kill curve, by visual inspection, and regressing the log viable count versus time. Additional points were included sequentially. With each addition, the r^2 value (variance explained) was recomputed. If this value increased, then the next observation was added to the analysis. The slope associated with the highest r^2 value was taken as the best point estimate of the rate of killing. No weighting was used in the regression because there was no clear monotonic quantitative association between observed variance and the viable count, although the counts were clearly heteroscedastic. In some experiments, an initial lag phase was apparent from the time-survival plot. When this occurred, a second rate of killing was determined by removing early datum points before the sequential optimization exercise.

Determination of AUC-VB. For each plot of viable count against time, AUC-VB was calculated according to the trapezoidal rule, based on the use of one of the following equations. When the viable count at 24 h did not exceed the initial inoculum level, AUC-VB1 = $[(\log_{10} VC_{t_0})\ (t_{24})] - [\Sigma 0.5(\log_{10} VC_{t_i} \log_{10} VC_{t_{i+1}})\ (t_{i+1} - t_i)]$, where VC_{t_0} is the viable count at time zero and VC_{t_i} is the viable count at time t_i. In such a situation, AUC-VB was always negative.

When regrowth at 24 h (t_{24}) was greater than the initial inoculum level, AUC-VB was calculated in two parts; (i) the negative area, in which the viable count was less than the initial inoculum level, which was calculated according to the equation given above, and (ii) the area of positive growth, in which the viable count exceeded the initial inoculum level, which was calculated according to the following equation: AUC-VB2 = $0.5[(t_{24} - t_x)\ (\log_{10} VC_{t_{24}} - \log_{10} VC_{t_x})]$, where t_x is the time at which the regrowth reached the initial viable count, assuming linear exponential regrowth. The final AUC-VB was calculated by combining the two AUC-VB values obtained in the two equations given above. In such a case, the total AUC-VB may be either negative or positive, depending on the regrowth rate.

If there was no observed killing and the viable count always exceeded the initial inoculum level, AUC-VB was determined as follows: AUC-VB = $[\Sigma 0.5(\log_{10} VC_{t_1} + \log_{10} VC_{t_{i+1}})\ (t_{i+1} - t_i)] - [(\log_{10} VC_{t_0})\ (t_{24})]$. In this case, AUC-VB was always positive.

Statistical analysis. A one-factor analysis of variance was used to test the significance of concentration to the rate of killing and the AUC-VB for each antibiotic alone.

A 2^2 factorial design was used to study the interaction between azlocillin and tobramycin, and the significance of the observed changes was tested by analyses of variance of the rate of killing and the AUC-VB data. The concentrations of azlocillin were 0 (control), 3.125 μg/ml (low), and 12.5 μg/ml (high), and those of tobramycin were 0 (control), 0.5 μg/ml (low), and 2 μg/ml (high). In total, 32 (4 × 8) kinetic runs were performed over 9 days. We have previously described the statistical basis for factorial experiments and their use in microbiology (9).

RESULTS

Effect of concentration of individual antibiotic. The MICs were 12.5 μg/ml for azlocillin and 2 μg/ml for tobramycin. Time-survival profiles of *P. aeruginosa* in the presence of azlocillin and tobramycin concentrations greater than and less than the MIC are presented in Fig. 1 and 2, respectively. The

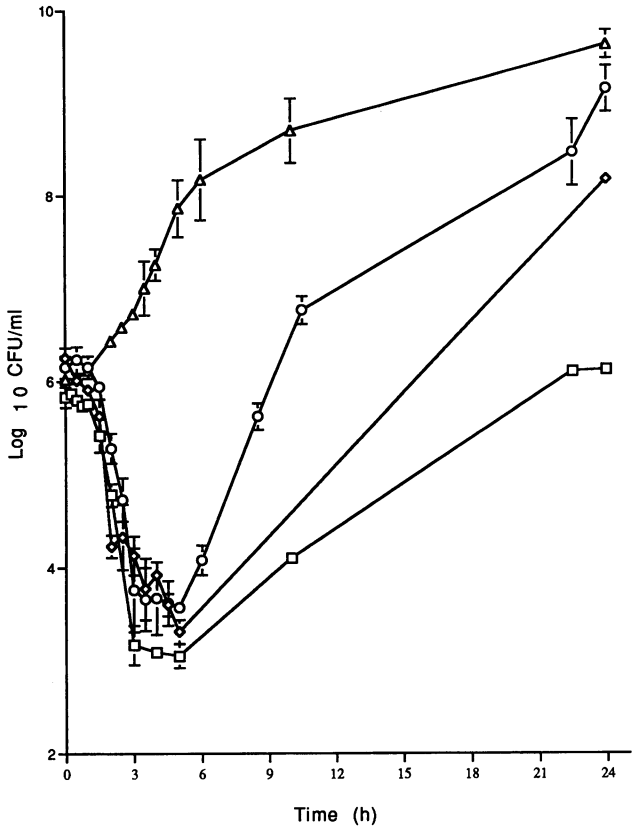

FIG. 1. Survival of *P. aeruginosa* in ISB at 37°C exposed to azlocillin at concentrations of 3.125 (△), 6.26 (○), 12.5 (◇), and 25 (□) μg/ml (errors bars are standard deviations [n = 4]).

growth of *P. aeruginosa* in ISB in the absence of antimicrobial agents consisted of a short lag phase of about 30 min; this was followed by a period of rapid exponential growth for up to 5 h, after which growth continued at a slower rate up to the 24-h sample time. During the exponential phase, the growth rate of the control was 0.696 ± 0.0312 $\log_{10}$ CFU/ml/h and the AUC-VB was 74.3 ± 2.27 $\log_{10}$ CFU · h/ml; these values were calculated from four replicate experiments. With azlocillin at 3.125 μg/ml, a reduction in the rate of growth but no bactericidal phase was observed. For all other concentrations of both azlocillin and tobramycin tested, the typical pattern consisted of an initial rapid bactericidal phase and then a period of stasis; eventually, regrowth became the predominant event. For tobramycin, the duration of the bactericidal phase and rate of killing ($P \leq 0.001$) were concentration dependent (Table 1), whereas for azlocillin, the initial rate of killing by azlocillin was not influenced by concentration ($P = 0.5$; Table 2). In addition, with azlocillin, there was an initial lag phase which decreased with increasing drug concentration. The rate of killing by azlocillin was recalculated by omitting the data from the lag phase. However, while this gave a better estimate of the rate of killing, as indicated by the improved r^2 values, the rates of killing were still independent of the concentration. The dramatic difference in the effects of the concentrations of tobramycin and azlocillin on the rate of killing is illustrated in Fig. 3.

The extent to which regrowth occurred was affected by the concentration of antibiotic. The AUC-VBs were calculated from the time-survival data and were significantly influenced

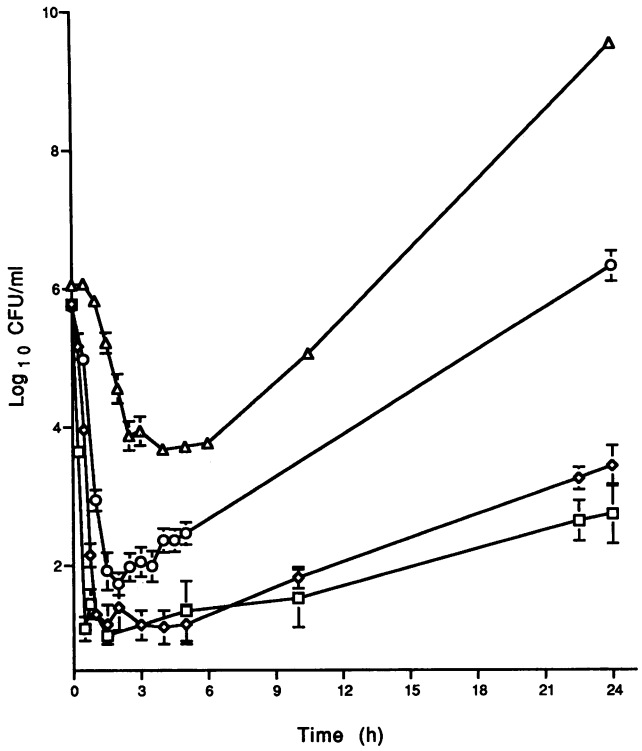

FIG. 2. Survival of *P. aeruginosa* in ISB at 37°C exposed to tobramycin at concentrations of 0.5 (△), 1.0 (○), 2.0 (◇), and 4.0 (□) μg/ml (error bars are standard deviations [n = 4]).

by the concentration of either agent ($P \leq 0.001$; Tables 1 and 2). The AUC-VB became increasingly negative as the concentration of either azlocillin or tobramycin increased, but this effect was more marked for tobramycin.

Effect of combinations of antibiotics. A 2^2 factorial design was used to investigate the effects of combinations of azlocillin and tobramycin on *P. aeruginosa*. The time-survival curves of *P. aeruginosa* treated with combinations of azlocillin and tobramycin were compared with the time-survival curves of *P. aeruginosa* treated with the individual components (Fig. 4). At the lower concentration of tobramycin (0.5 μg/ml), the combinations with both the high and the low concentrations of azlocillin were more effective than the individual components (Fig. 4a and b). However, the higher concentration of tobramycin (2.0 μg/ml) alone was as effective as tobramycin in combination with azlocillin (Fig. 4c and d). These observations are supported by the comparison of survival at 24 h. For tobramycin at 0.5 μg/ml combined with azlocillin at 3.125 or 12.5 μg/ml, the differences in the quantities of survivors (log CFU per milliliter) between those obtained with the combination and those obtained with the most effective single agent were 4.66 and 3.38, respectively. For the combinations of tobramycin at 2.0 μg/ml with azlocillin at 3.125 or 12.5 μg/ml, the respective differences were −0.25 and −0.15. These nega-

TABLE 1. Rate of killing and AUC-VB determined from time-survival plots of *P. aeruginosa* treated with tobramycin

Concn of tobramycin (μg/ml)	Duration of bactericidal phase (h)[a]	Rate of killing ($\log_{10}$ CFU/ml/h)[a,b]		AUC-VB ($\log_{10}$ CFU · h/ml [mean ± SD; n = 4])[b]
		Mean ± SD (n = 4)	r^2	
4.0	0.5	−9.42 ± 0.22	1.00	−92.4 ± 7.88
2.0	1.0	−4.80 ± 0.28	0.97	−85.9 ± 4.10
1.0	1.5	−2.76 ± 0.10	0.96	−41.7 ± 2.65
0.5	2.5	−1.14 ± 0.09	0.91	−0.72 ± 2.38

[a] Determined from the regression line that gave the highest r^2 value for consecutive datum points.
[b] Significant difference in rate of killing ($P \leq 0.001$) and AUC-VB ($P \leq 0.001$) with concentration determined by one-factor analysis of variance.

TABLE 2. Rate of killing and AUC-VB determined from time-survival plots of *P. aeruginosa* treated with azlocillin

Concn of azlocillin (μg/ml)	Duration of bactericidal phase (h)	Rate of killing ($\log_{10}$ CFU/ml/h)		AUC-VB ($\log_{10}$ CFU · h/ml [mean ± SD; n = 4])[a]
		Mean ± SD (n = 4)	r^2	
25	3.0[b]	−0.864 ± 0.003[b]	0.85	−27.73 ± 1.07
	1.0–2.0[c]	−0.133 ± 0.114[c]	0.98	
12.5	3.5[b]	−0.780 ± 0.120[b]	0.91	−18.0 ± 3.83
	1.5–3.5[c]	−0.762 ± 0.138[c]	0.72	
6.25	3.5[b]	−0.810 ± 0.144[b]	0.89	−8.95 ± 2.05
	1.5–3.5[c]	−1.218 ± 0.222[c]	0.96	
3.125	6.0[b]	+0.378 ± 0.084[b]	0.96	58.9 ± 6.97
	2.0–6.0[c]	+0.462 ± 0.114[c]	0.98	

[a] Significant difference in AUC-VB ($P \leq 0.001$) with concentration determined by one-factor analysis of variance.
[b] Determined from the regression line that gave the highest r^2 value for consecutive datum points starting at time zero.
[c] Determined from the regression line that gave the highest r^2 value for consecutive datum points excluding those from the initial lag phase.

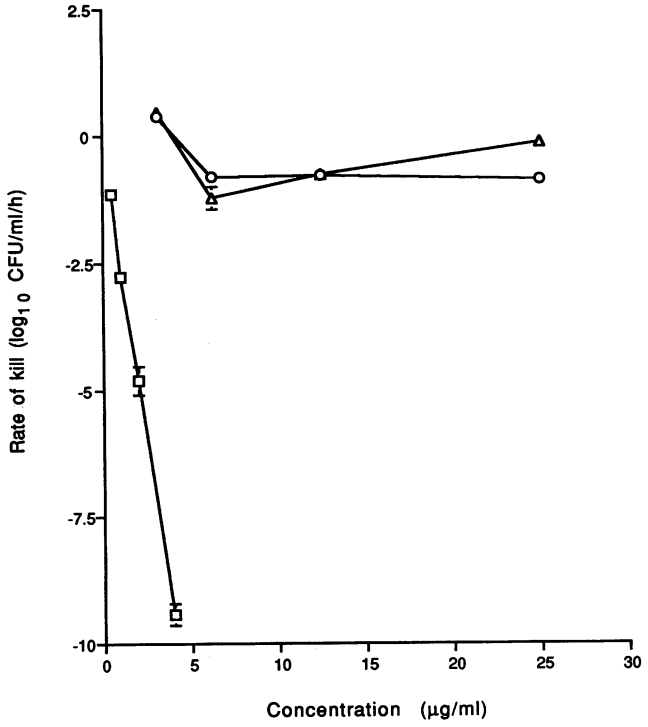

FIG. 3. Relationship of concentration of antibiotic and rate of killing calculated from the linear decline obtained in time-survival studies of *P. aeruginosa* in ISB at 37°C exposed to tobramycin (□) or azlocillin with the lag included (○) or the lag deleted (△) (error bars are standard deviations [n = 4]).

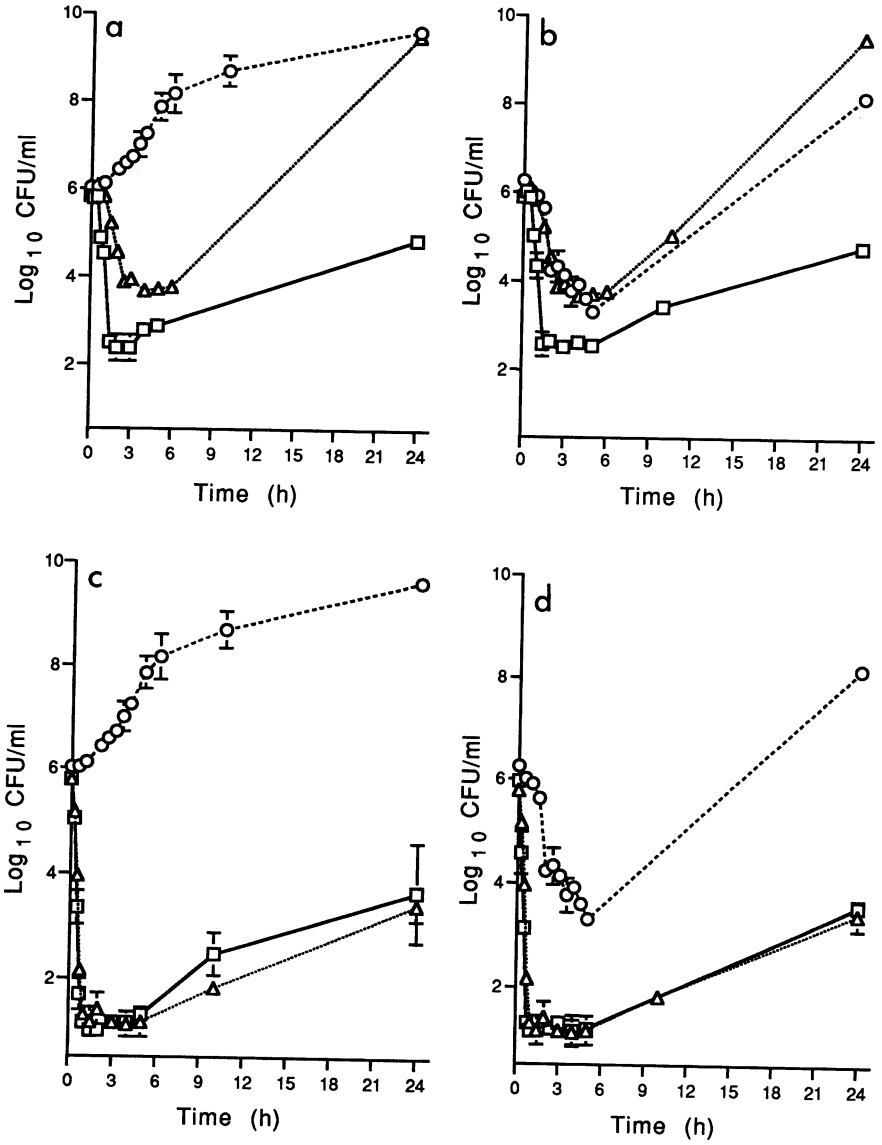

FIG. 4. Survival of *P. aeruginosa* in ISB at 37°C exposed to azlocillin (○) and tobramycin (△) alone and in combination (□). (a) Azlocillin at 3.125 μg/ml and tobramycin at 0.5 μg/ml. (b) Azlocillin at 12.5 μg/ml and tobramycin at 0.5 μg/ml. (c) Azlocillin at 3.125 μg/ml and tobramycin at 2.0 μg/ml. (d) Azlocillin at 12.5 μg/ml and tobramycin at 2.0 μg/ml. Error bars are standard deviations (n = 4).

tive values reflect the increased activity of the higher tobramycin concentration. Rates of killing were calculated for the initial linear portions of these time-survivor plots (Table 3). An increase in the tobramycin concentration (with the azlocillin concentration kept constant) resulted in an increase in the rate of killing, whereas an increase in the azlocillin concentration (with the tobramycin concentration kept constant) produced little or no increase in the rate of killing. A short lag period of 30 min was apparent in combinations that included the lower concentration of tobramycin. The change in the tobramycin concentration had a greater effect on the AUC-VB than the change in the azlocillin concentration did (Table 3).

In a factorial experiment, an interaction between two factors, in this case, azlocillin and tobramycin concentrations, may be shown by a change in the slope of the AUC-VB or the rate of killing and concentration plot when the concentration of the interactant is changed. The absence of an interaction is conversely shown by parallel lines (5). Both the rate of killing and AUC-VB against concentration plots (Fig. 5b) showed a marked change in slope for the combination of low concentrations, whereas only a slight change was observed for high concentrations (Fig. 5a). Analysis of variance on AUC-VB showed that the effect of the combination was synergistic at low concentrations (AUC-VB, $P < 0.001$; rate of killing, $P < 0.001$) but was not significant when the concentration was increased.

DISCUSSION

Kinetic studies of the survival of *P. aeruginosa* following treatment with azlocillin and tobramycin provided quantitative information on the influence of the concentration of each individual agent on this interaction. The 2^2 factorial experimental design allowed the investigation of the influence of high

TABLE 3. Rate of killing and AUC-VB determined from time-survival plots of *P. aeruginosa* treated with a combination of azlocillin and tobramycin

Concn (μg/ml)		Duration of bactericidal phase (h)	Rate of killing (log_{10} CFU/ml/h)		AUC-VB (log_{10} CFU · h/ml [mean ± SD; n = 4])
Tobramycin	Azlocillin		Mean ± SD (n = 4)	r^2	
2.0	12.5	0.75[a]	−6.30 ± 0.714[a]	0.99	−89.3 ± 4.00
2.0	3.125	1.0[a]	−5.34 ± 0.534[a]	0.97	−77.6 ± 9.52
0.5	12.5	1.5[a]	−2.34 ± 0.132[a]	0.89	−52.7 ± 3.13
		0.5–1.5[b]	−3.30 ± 0.360[b]	1.0	
0.5	3.125	1.5[a]	−2.22 ± 0.066[a]	0.87	−50.0 ± 3.37
		0.5–1.5[b]	−3.24 ± 0.162[b]	0.98	

[a] Determined from the regression line that gave the highest r^2 value for consecutive datum points starting at time zero.
[b] Determined from the regression line that gave the highest r^2 value for consecutive datum points excluding those from the initial lag phase.

and low concentrations of both azlocillin and tobramycin on the observed interactions between these agents. Time-survival curve investigations of antimicrobial action are traditionally interpreted by making comparisons of rates of killing and survival at a defined time point. Guggenbichler et al. (11) proposed that the area under the concentration curve is an important parameter in the determination of antimicrobial action, although quantitative determinations were not undertaken. Goi et al. (10) calculated the AUC-VB of growth curves determined turbidimetrically in the presence of antimicrobial agents by the use of a digital planimeter. In the present study, AUC-VBs of plots of viable counts versus time were calculated according to the trapezoidal rule. This method allowed quantitative assessment of the results of time-kill studies of antimicrobial action, taking the overall pattern of killing and regrowth into consideration rather than basing interpretation on the effect observed at a single defined end point. In a time-survival experiment, the two chief processes are likely to be the destruction of a susceptible bacterial population and the emergence and regrowth of a resistant bacterial subpopulation. While the determination of AUC-VB gives a combined measure of these two events, it does not allow the contribution of each to be assessed independently.

Tobramycin produced a concentration-dependent log-linear rate of killing. The goodness of fit to a linear relationship was demonstrated by the r^2 values from the regression equation. The duration of the bactericidal phase was also concentration dependent. Other studies have demonstrated this concentration-dependent effect, based on the appearance of the kill curve over 6 h (13), although a biphasic bactericidal relationship has also been reported (15). The overall outcome of exposure to tobramycin determined by AUC-VB was also significantly dependent on the tobramycin concentration. It has been demonstrated that bacteria that survive the initial exposure to aminoglycosides develop adaptive resistance because of the emergence of slowly growing resistant subpopulations (7, 8). The bacterial transmembranous electrical potential is the driving force for aminoglycoside entry, and this is reduced in these resistant subpopulations, which have a lower proton motive force (2, 3).

Doubling of the concentration of azlocillin from 3.125 to 6.26 μg/ml resulted in a change from growth-inhibiting behavior to bactericidal behavior. Thereafter, azlocillin exhibited a bactericidal phase which was characterized by a lag phase of growth of the bacteria and which was not concentration dependent. The bacteria that survived this period were also able to regrow. The extent of regrowth was significantly influenced by the azlocillin concentration, as indicated by the AUC-VB. This pattern of killing and regrowth was reported by White et al. (21) and is in keeping with the view that the bactericidal activities of beta-lactam antibiotics are not concentration dependent.

Interpretation of the effects of using a combination of two antibiotics is more problematic. Hallander et al. (12) considered that the inhibition of regrowth at 24 h in a culture containing a combination of antimicrobial agents compared with that in a culture containing the most effective constituent was the most reliable indicator of synergism, as determined by the killing curve method. The regrowth pattern in the presence of antimicrobial agents singly or in combination was also taken into consideration in studies by Konig et al. (14), who used time-kill curve analysis. In time-kill studies of the interaction between azlocillin and tobramycin (17, 20), a 2-log_{10} difference in the viable count at 24 h in the presence of the combination compared with that in the presence of the most effective individual component was adopted as the definition of synergistic behavior. By applying this criterion to the results obtained in the present study, synergism was demonstrated for combinations in which the tobramycin concentration was low. However, a claim for synergy cannot be made if one applies the additional restriction that one of the drugs must be present in a concentration which does not affect the growth curve of the test organism. While the lowest concentration of azlocillin was

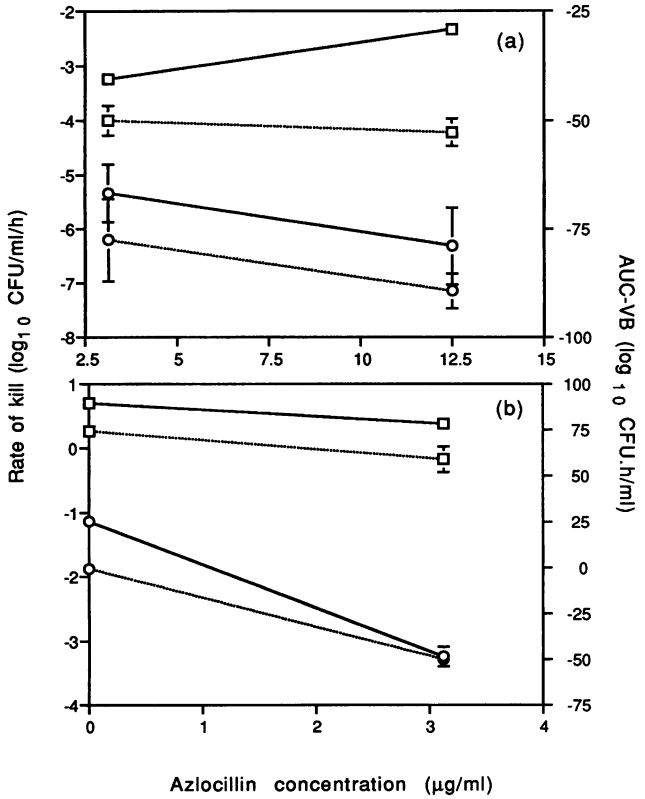

FIG. 5. Results of a factorial design experiment to evaluate the effects of tobramycin at 0.5 (□) and 2.0 (○) μg/ml and azlocillin at 3.125 and 12.5 μg/ml (a) and the effects of tobramycin at 0 (□) and 0.5 (○) μg/ml and azlocillin at 0 and 3.125 μg/ml in combination (b) on *P. aeruginosa* by using rate of killing (solid line) and AUC-VB (dotted line). Error bars are standard deviations (n = 4).

not bactericidal, it nevertheless reduced the growth rate compared with the growth rate of the control, and AUC-VB was also reduced.

The 2^2 factorial experimental design allowed investigation of an alternative approach to the assessment of the interaction between these antimicrobial agents in terms of the calculated AUC-VB and the rate of killing, whereby the influences of high and low concentrations of both azlocillin and tobramycin were determined. Analysis of variance showed that the interaction was significant only for the combination of azlocillin at 3.125 μg/ml and tobramycin at 0.5 μg/ml, i.e., the low concentrations tested. Combination therapy therefore appears to be of benefit in the presence of levels of azlocillin and tobramycin less than the individual MICs of the drugs. However, the overall effect of the higher concentration of tobramycin was greater than the overall effect of lower doses of tobramycin used in combination with azlocillin.

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