Postantibiotic Effect Assessments for Antibiotics Exhibiting a Wide Range of Bactericidal Activities by Using a Modified Total-Cell-Counting Method

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We recently described a total-cell-counting method for postantibiotic effect (PAE) assessments that performs well with weakly bactericidal antibiotics. This note presents a modified method for the study of PAE with extended capability to cover a broad range of bactericidal activities.

A number of indirect methods have been proposed for postantibiotic effect (PAE) assessments. These methods include CO_2 production (3), ATP (4), and optical density (7) measurements. Recently, we have described a total-cell-counting (TCC) method using the Coulter counter as a more practical alternative to the conventional pour plate technique (5). The TCC method has been established for antibiotics showing low degrees of bactericidal activity.

A recent attempt to study by the TCC method the PAE induced by ciprofloxacin (a highly bactericidal antibiotic) on *Pseudomonas aeruginosa* revealed a significant reduction in the precision of viable-cell-count estimates due to the generation of a large number of dead cells. Dead cells, in the context of the conventional pour plate technique, are the cells that lose their ability to multiply into countable colonies. Since the viable-cell-count estimates obtained by the TCC method at various times subsequent to antibiotic removal are computed as the differences between the total cell counts measured by the

TABLE 1. Comparison of the PAE data obtained by the two methods for the six antibiotic-bacterium combinations at various concentrations studied

Organism/antibiotic	MIC (μg/ml)	Concn tested (μg/ml)	Bactericidal activity[a]	PAE (h) by:	
				Pour plate technique	Modified TCC method[b]
P. aeruginosa/ciprofloxacin	1	0.5	0.81	0.12	0.28
		1	1.53	1.70	1.64
		2	2.92	2.15	1.84
		4	3.83	4.24	4.81
		8	3.71	5.14	5.54
		16	3.63	5.11	5.60
P. aeruginosa/tobramycin	0.5	0.25	0.81	0.97	0.81
		0.5	2.19	2.96	3.22
		1	2.91	2.70	2.27
		2	4.05	4.03	3.97
		4	4.77	4.68	4.35
		8	5.89	5.38	5.03
S. faecalis/trimethoprim	0.5	0.25	0.28	0.60	0.60
		0.5	0.35	0.78	0.92
		1	0.40	0.88	0.95
		2	0.49	0.93	0.80
		4	0.90	0.99	0.81
P. aeruginosa/piperacillin	8	8	1.06	−0.23	−0.54
		32	0.80	0.29	0.30
E. coli/tetracycline	2	8	1.49	0.55	0.68
		16	1.57	1.72	1.76
S. aureus/gentamicin	1	4	5.42	7.60	7.45

[a] Expressed as difference in log viable cell density (CFU per milliliter) relative to the control value; determined prior to antibiotic removal at 1 h.
[b] By extrapolation.

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Coulter counter and the number of dead cells generated during the period of antibiotic exposure, it is necessary to define this dead cell count. Such count can be determined by subtracting the viable cell count (pour plate technique) from the total cell count (Coulter counter) measured simultaneously at the time of antibiotic removal. For highly bactericidal antibiotics, a large number of dead cells approaching that of the total cell count is generated. Because of this, any small variations in the total-cell-count measurements will cause a significant degree of error in the viable-cell-count estimates. As a result, a modified approach using the TCC method by extrapolation has been developed. This method is now applicable to the study of PAE for antibiotics showing a wide range of bactericidal activities.

P. aeruginosa ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Streptococcus faecalis* ATCC 29212, and *Escherichia coli* ATCC 25922 were used as test organisms in this study. Sterilized Mueller-Hinton broth supplemented with 12.5 µg of Mg^{2+} per ml and 25 µg of Ca^{2+} per ml and nutrient agar were used as the culture media throughout the study. Both the lyophilized organisms and culture media were purchased from Difco Laboratories, Detroit, Mich. Tobramycin, gentamicin, trimethoprim, piperacillin, and tetracycline were acquired from Sigma Chemical Co., St. Louis, Mo. Ciprofloxacin was a gift from Bayer, Leverkusen, Germany. These antibiotics were chosen because of the wide range of bactericidal activities provided. The MICs of individual antibiotics for the respective microorganisms were determined by the macrodilution method (6). The experimental conditions employed in the present study were essentially the same as those mentioned previously (5). Briefly, individual organisms tested were exposed to the respective antibiotic at the designated concentrations (Table 1) for 1 h (2). Antibiotic was removed by centrifugation and repeated saline washing (two times). Viable counts were obtained by the conventional pour plate technique (reference method) at time zero, immediately before and after antibiotic removal, and 0.5- to 1-h intervals thereafter. In a direct contrast to these viable-cell-count data, total cell counts were monitored every 1 to 2 h by employing a similar schedule with a Coulter counter. When the bacterial culture entered the logarithmic growth phase following the period of static growth, total cell counts were taken more frequently, at 15-min intervals, to obtain an accurate estimate of the slope of bacterial growth. The total-cell-count data reported for the individual microorganisms tested were adjusted for the difference in counting efficiency via the standard curve, e.g., viable cell count measured with the Coulter counter versus that by the pour plate technique (5).

The basic principle for the operation of the modified TCC method is depicted in Fig. 1. Essentially, a line corresponding to the logarithm of the viable count measured by the pour plate technique immediately after drug removal (log N_0) was extrapolated horizontally. Nonweighted linear regression was applied to the total-cell-count data obtained in the logarithmic growth phase following the static period such that a best-fit line was obtained (Fig. 1). This regression line was back-extrapolated to intercept the horizontal line ($y = \log N_0$) as previously described. Two major time intervals over the PAE experiment were then defined; T is the time difference between the intersection point and the time of drug removal, and T' is the time for the bacteria to increase by 1 log unit from log N_0 to $1 + \log N_0$. With the measurement of N_0, the estimate of T' was determined directly from the regression equation. The same procedures were applied to the control culture to obtain the estimates of T and T' for the controls (C and C', respectively) (Fig. 1). Therefore, PAE was computed as follows: PAE = $(T + T') - (C + C')$. Similarly, the PAE derived from the viable-count
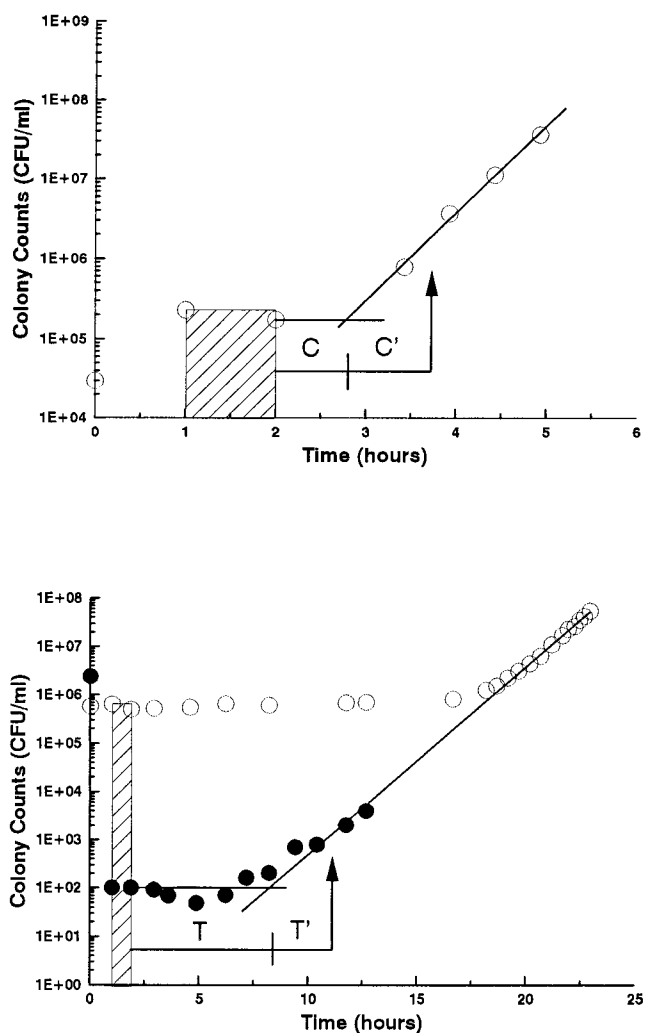

FIG. 1. Principle of operation of the modified TCC method by extrapolation. In this example, data are shown for either the untreated control *S. aureus* culture (top) or the culture exposed to gentamicin at 4× MIC (bottom). The delay in resumption of bacterial growth is estimated as $C + C'$ for the control and $T + T'$ for the antibiotic-treated culture. Total cell counts and viable cell counts are represented by open and closed circles, respectively. PAE is computed as $(T + T') - (C + C')$. The centrifugation and washing procedures are depicted by the shaded bars.

data, by convention, is the time required, relative to the control, for the bacteria to increase by 1 log unit following drug removal.

In order to optimize performance of the TCC method by extrapolation, the following conditions have to be considered. (i) Contribution of the number of dead cells to the estimation of T' via the regression analysis has to be minimal. Data obtained in the present study suggest that a >0.5-log-unit increase in total cell count above that measured immediately after antibiotic removal would have satisfied this condition. (ii) An upper limit of $10^{7.5}$ CFU/ml was imposed on the total cell counts in the logarithmic growth phase to avoid the impact of stagnant bacterial growth as a result of nutrient depletion on the regression analysis.

To demonstrate the applicability of the modified method, data from the PAE experiments conducted at the two different levels (low and high) of bactericidal activity by exposing *P. aeruginosa* to tobramycin at 1× and 16× MIC are shown in Fig.
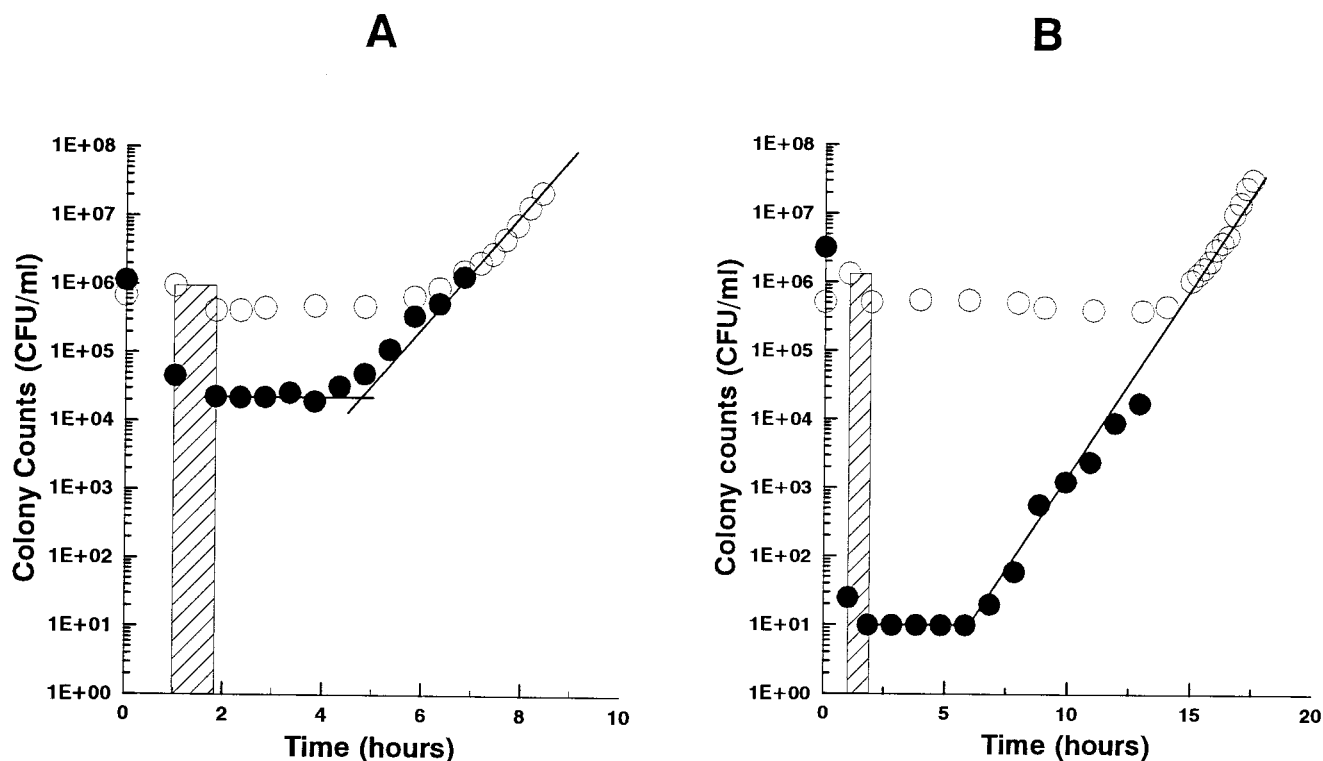
FIG. 2. Illustration of the ability of the modified TCC method to assess the PAE of tobramycin against *P. aeruginosa* at two levels of bactericidal activities: 1× MIC (A) and 16× MIC (B). Open and closed circles depict the total cell counts and viable cell counts, respectively. The shaded bars represent antibiotic removal by centrifugation and cell washing.

2. Direct comparison of the PAE data obtained by both methods demonstrates the practicality of this method (Table 1). When the PAE data collected at the different concentrations for all six antibiotic-bacterium combinations were plotted against those by the reference pour plate technique, a regression line ($n = 22$, $r = 0.992$) with a slope of 1.015 (95% confidence interval: 0.956 to 1.074) and an intercept of -0.045 (95% confidence interval: -0.234 to 0.144) was obtained. The slope and intercept values of this regression line were not statistically significantly different ($P < 0.01$) from the line of unity. Table 1 shows the ability of this method to cover a wide range of PAE data (-0.5 to 7.5 h) and various degrees of bactericidal activity (0.28- to 5.89-log-unit decrease in viable counts relative to the control value). In addition, the ability to detect the negative PAE exhibited by the piperacillin-*P. aeruginosa* combination further reinforces the application of the present method. Also apparent from these data was the concentration dependency of the bactericidal effect exhibited by the antibiotic-bacterium combinations tested. Moreover, the increase in the duration of PAE with respect to antibiotic concentration appeared to be less than proportional at higher concentrations. This observation is in agreement with that observed previously for penicillin G, erythromycin, and rifampin against *S. aureus* (1). For individual antibiotics, a general trend was also observed for an increase in PAE with increasing level of bactericidal activity in the concentration range studied (Table 1).

Results from the present study demonstrate the applicability of the modified TCC method by extrapolation for PAE assessments of antibiotics showing a wide range of bactericidal activities. This is a significant improvement over the TCC method reported earlier (5). As total-cell-count measurements during the logarithmic growth phase and a single viable count immediately after antibiotic removal are the only requirements for this method, efforts spent on performing PAE experiments can be reduced. Indeed, planning of the experiment and scheduling of sampling times can be more flexible because continuous sampling over the entire experiment is no longer necessary. With a high degree of accuracy, this modified method can be a more complete and practical alternative to other techniques for PAE studies; most importantly, this method is applicable to antibiotics exhibiting a wide range of bactericidal activities.

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