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Postantibiotic Effect of β-Lactam Antibiotics on *Escherichia coli* Evaluated by Bioluminescence Assay of Bacterial ATP

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The in vitro postantibiotic effects (PAE) of aztreonam, ceftazidime, cefuroxime, imipenem, and piperacillin on *Escherichia coli* ATCC 25922 were studied by a bioluminescence assay of bacterial ATP. In parallel with the PAE investigation, viability and morphology studies were performed. The strain was exposed for 2 h to different concentrations of β-lactam antibiotics. The antibiotic activity was eliminated by 10^{-4} dilutions, and regrowth of bacteria was monitored hourly by the bioluminescence assay of bacterial ATP. The length of PAE was dose dependent for ceftazidime (0.5 to 2.6 h), cefuroxime (0.4 to 2.6 h), and imipenem (0.3 to 4.5 h). The long PAE for these antibiotics at higher concentrations was associated with a potent initial killing and the presence of spheroplasts. Aztreonam and piperacillin produced a short, non-dose-dependent PAE (0.4 to 0.95 h). Short PAEs (below 1 h) were seen concomitantly with production of filaments, except in the case of imipenem, which only produced spheroplasts. The bioluminescence method was not jeopardized by filament formation, in contrast to the viable count assay which is normally used for PAE investigations. This makes it possible to study PAE for β-lactam antibiotics on gram-negative bacteria with bioluminescence.

The persistent suppression of bacterial growth after short antimicrobial exposure is called the postantibiotic effect (PAE). By definition, there should be no subinhibitory concentrations of antimicrobial agent left when the PAE starts.

A long PAE provides the potential for administering the antimicrobial agent with longer intervals between doses. This fact has stimulated intensified research concerning the PAE phenomenon during the last decade.

β-Lactam antibiotics have consistently shown PAEs against various gram-positive cocci (1, 3–5). In contrast to gram-positive cocci, there are marked differences in PAE caused by β-lactam antibiotics on gram-negative bacteria (2, 3, 5, 13, 16). Gudmunsson and Craig did not see any PAE and frequently noted a negative PAE when *Escherichia coli* and *Pseudomonas aeruginosa* were exposed to β-lactam antibiotics (3). Similar results with differences in the PAEs for β-lactam antibiotics on gram-positive cocci and gram-negative bacteria have also been observed by other investigators (1, 4, 16). The only reported exception to the absence of PAE when β-lactam antibiotics and gram-negative bacteria are combined is imipenem (2, 3, 10, 13).

Regrowth after drug removal has generally been monitored by viable count. This is a laborious and indirect method. In this study we have used a new direct method to evaluate the PAE which is based on a bioluminescence assay of bacterial ATP.

MATERIALS AND METHODS

Bacterial strain. The strain used was *E. coli* ATCC 25922.

Antibiotics. Aqueous stock solutions of active drugs were prepared from aztreonam (964 μg/mg; Squibb Institute for Medical Research, Princeton, N.J.), ceftazidime (976 μg/ml; Glaxo, London, England), cefuroxime (925 μg/mg; Glaxo), piperacillin (1,010 μg/ml; Lederle Inc., Carolina, P.R.), and imipenem (964 μg/ml; MSD International, Rahway, N.J.).

Growth medium. Mueller-Hinton broth (GIBCO Limited, Renfrewshire, Scotland) supplemented with 25 mg of Mg^{2+} and 25 mg of Ca^{2+} per liter was used as the growth medium, giving a total of 0.86 mM Mg^{2+} and 1.02 mM Ca^{2+}. Osmolality was 323 mosM per liter.

MIC determinations. Serial twofold dilutions of antibiotics were prepared in growth medium. Samples of these dilutions (0.5 ml) were added to series of test tubes. Bacterial strains in logarithmic phase were diluted to approximately 2×10^5 CFU/ml, and 0.5-ml aliquots of these cultures were added to the tubes, which were then incubated at 37°C. Visible growth was recorded after 24 h.

Determination of bacterial viability. Bacterial numbers were determined before and after 2 h of exposure to the antibiotic as CFU per milliliter by plating after serial dilution (12) (see Fig. 1).

Bioluminescence assay of intracellular bacterial ATP. (i) Analytical equipment. Light emission from the bioluminescence assay was measured in a 1250 Luminometer (LKB-Wallac, Turku, Finland) and recorded on a 1250 Display (LKB-Wallac). The extraction of bacterial ATP was performed in a LKB-Biocal 2073 incubator (LKB Products, Bromma, Sweden).

(ii) Analytical reagents. ATP-monitoring reagent (LKB-Wallac) was used in the assay of ATP. Apyrase (purified grade I) (Sigma Chemical Co., St. Louis, Mo.) was used to eliminate extracellular ATP before the extraction of intracellular ATP. Other reagents were of analytical grade.

(iii) Elimination of extracellular ATP. A 50-μl sample from the culture was incubated for 10 min at 37°C with 50 μl of solution consisting of 0.04% apyrase in supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.).

(iv) Extraction of intracellular ATP. After elimination of extracellular ATP, 50 μl of the apyrase-treated sample was pipetted into 500 μl of boiling 0.1 M Tris buffer, pH 7.75, containing 2 mM EDTA. After being heated for 90 s, the extracts were cooled before the assay of ATP. This procedure inactivated the apyrase and disrupted the bacterial cells, causing them to release their ATP.

(v) Luciferase assay of ATP. Luciferase reagent (100 μl)

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was added to 550 μl of each extract, and the light intensity was recorded.

(vi) Calculation of assay results. Sample ATP levels were calculated by using assays of standard amounts of ATP as a reference. Correction was made for background luminescence. Known amounts of ATP added to the extracts were used as internal standards in order to correct for inhibition of the luciferase reaction by the extracts.

Determination of PAE with bioluminescence. Logarithmic-phase bacteria were obtained by incubating the bacterial strains in growth medium for 8 to 10 h without shaking. The bacterial numbers were monitored by the bioluminescence assay of bacterial ATP (10^{-6}M ATP corresponded to approximately 10^9 CFU/ml), and the cultures were diluted to approximately 2×10^7 CFU/ml. Samples of these cultures (0.5 ml) were added to tubes containing different concentrations of β-lactams in 0.5 ml of growth medium, and the tubes were incubated at 37°C for 2 h without shaking.

After samples for ATP determination were removed, the cultures were diluted 10^{-4} with prewarmed growth medium. Each experiment included at least one unexposed control culture prepared and treated as described above. Regrowth of bacteria was monitored each hour in the diluted cultures by the bioluminescence assay of bacterial ATP. In a growing culture, there is a good correlation between intracellular ATP and viability (11). Each experiment was repeated three or four times.

The PAE was calculated from the regrowth curves by the equation PAE = T − C, where *T* is the time required for the bacterial population in the test culture to increase by 1,000-fold after dilution of the drug, and *C* is the time required for bacterial population in the control culture to increase by 1,000-fold. The ATP levels in β-lactam-exposed cultures immediately after 10^{-4} dilution were below the detection limit. Therefore, the ATP was measured before dilution, and the values obtained (divided by 10^4) were used as the starting values in these calculations.

TABLE 1. Influence of residual concentrations of aztreonam on regrowth of *E. coli* ATCC 25922 after 10^{-3} and 10^{-4} dilutions monitored by bioluminescence

Concn of aztreonam (μg/ml [×MIC])[a]	Residual aztreonam concn after 10^{-3} dilutions (μg/ml [×MIC])	Time to suppression of growth after 10^{-3} dilutions (h)	Residual aztreonam concn after 10^{-4} dilutions (μg/ml [×MIC])	Time to suppression of growth after 10^{-4} dilutions (h)
32 (512)	0.032 (1/2)	7.13	0.0032 (1/20)	0.95
16 (256)	0.016 (1/4)	2.43	0.0016 (1/40)	0.85
8 (128)	0.008 (1/8)	1.45	0.0008 (1/80)	0.85
4 (64)	0.004 (1/16)	1.2	0.0004 (1/160)	0.85
2 (32)	0.002 (1/32)	1.25	0.0002 (1/320)	0.64

[a] Before dilution, the cultures were exposed to these aztreonam concentrations for 2 h.

Normally, the PAE is calculated on the basis of the time taken for the bacterial numbers to increase by 10-fold. However, the ATP values after 10-fold growth were below the detection limit, as we used 10^4 times dilution. We therefore chose to use the time taken for bacterial numbers, as measured by ATP, to increase by 1,000-fold, which we considered adequate since the growth curves for drug-treated and untreated bacteria were parallel once the growth had started.

Morphology. Parallel with the PAE and viability studies, we also performed morphological studies. After 2 h of antimicrobial agent exposure, the bacterial morphology was studied by microscopy at a magnification of ×1,250. The cultures were stained with acridine orange (9).

RESULTS

MICs. The MICs for *E. coli* ATCC 25922 were as follows: aztreonam, 0.0625 μg/ml; ceftazidime, 0.25 μg/ml; cefuroxime, 4 μg/ml; imipenem, 0.25 μg/ml; piperacillin, 2 μg/ml.

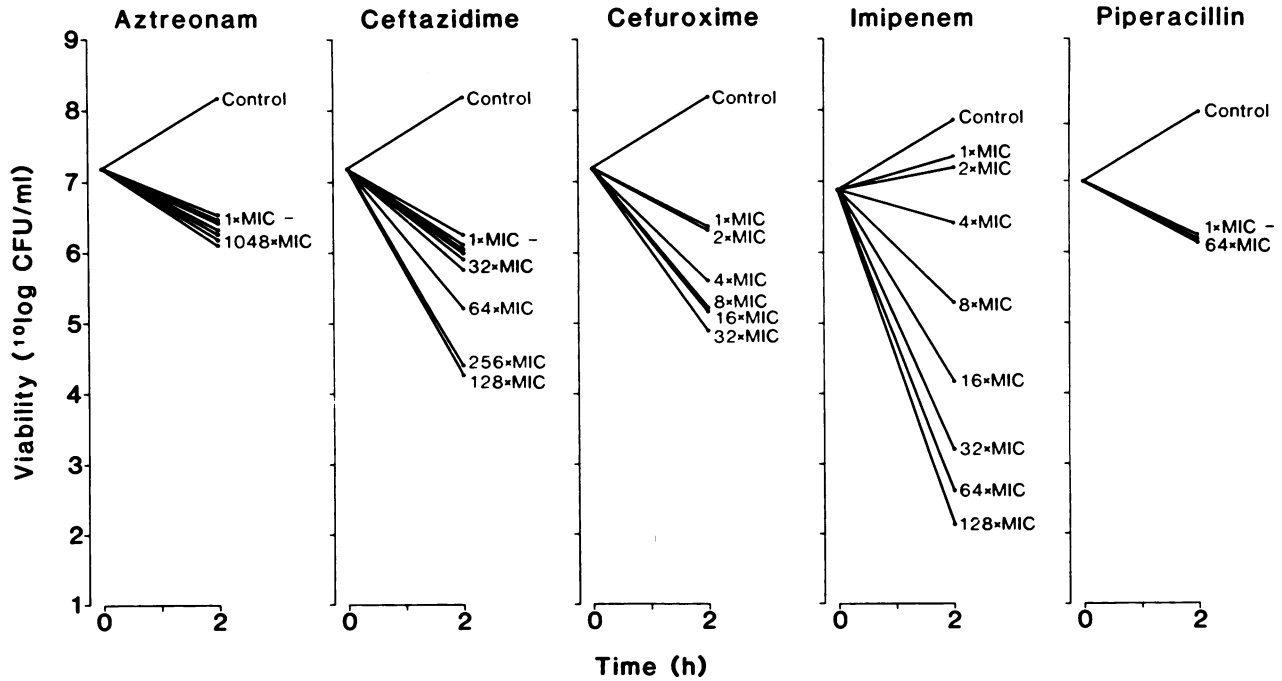

FIG. 1. Bactericidal effects of aztreonam, ceftazidime, cefuroxime, imipenem, and piperacillin on *E. coli* ATCC 25922 at different concentrations (expressed as multiples of the MIC).

Effect of residual antibiotic. When studying PAE, it is important to ascertain that the dilution after drug exposure is sufficient to prevent any potential residual drug from affecting bacterial growth.

The effects of the residual concentrations of aztreonam on regrowth of *E. coli* ATCC 25922 after 10^{-3} and 10^{-4} dilutions of bacterial cultures exposed to 2 to 32 μg of aztreonam per ml for 2 h are shown in Table 1. The regrowth was monitored by the bioluminescence assay of bacterial ATP. After a 10^{-3} dilution of the culture exposed to 32 μg of aztreonam per ml, there was 0.032 μg (1/2 MIC) of aztreonam per ml left in the culture. After a 10^{-4} dilution, the residual aztreonam concentration was 0.0032 μg/ml (1/20 MIC). The bacterial growth was suppressed 7.13 and 0.95 h, respectively, after 10^{-3} and 10^{-4} dilutions, i.e., a more than seven-times-longer suppression of growth in cultures with 1/2 MIC compared with 1/20 MIC residual antibiotic concentrations (Table 1). Residual concentrations of 1/32 to 1/4 MIC also prolonged the suppression of growth compared with cultures with residual concentrations of 1/320 to 1/40 MIC (Table 1).

The effects of residual concentrations of ceftazidime, cefuroxime, imipenem, and piperacillin on growth of *E. coli* ATCC 25922 after 10^{-3} and 10^{-4} dilutions were also studied. The bacteria were exposed to up to 64 μg of each agent per ml for 2 h. The residual antibiotic concentrations after 10^{-3} dilutions for ceftazidime, cefuroxime, imipenem, and piperacillin, expressed as multiples of the MIC, were 1/4, 1/64, 1/4, and 1/32, respectively. The suppression of growth in these cultures was not longer than in cultures diluted 10^{-4}.

Killing curves. The initial killing of *E. coli* ATCC 25922 caused by aztreonam, ceftazidime, cefuroxime, imipenem, and piperacillin after 2-h exposures is shown in Fig. 1.

The five antibiotics have different bactericidal potencies. Aztreonam and piperacillin showed a weak, non-dose-dependent bactericidal effect. Ceftazidime, cefuroxime, and imipenem showed dose-dependent, strong bactericidal effects.

PAE. The PAEs of the antibiotics on *E. coli* ATCC 25922 after 2-h exposures were calculated from regrowth curves monitored by bioluminescence. The PAEs at different concentrations, expressed as multiples of the MIC, are shown in Fig. 2.

Imipenem had the longest dose-dependent PAE of the antibiotics tested. Ceftazidime and cefuroxime also produced long dose-dependent PAEs, while aztreonam and piperacillin produced short, non-dose-dependent PAEs.

The PAEs for *E. coli* ATCC 25922 were as follows (at eight times the MIC): aztreonam, 0.52 h; ceftazidime, 0.61 h; cefuroxime, 0.9 h; imipenem, 2.0 h; and piperacillin, 0.75 h. At 32 times the MIC, the PAEs were as follows: aztreonam, 0.64 h; ceftazidime, 1.2 h; cefuroxime, 2.6 h; imipenem, 4.5 h; and piperacillin, 0.78 h. After exposure to 32 times the MIC of imipenem, regrowth did not occur in two of four experiments.

Correlation between PAE morphology and initial killing. In Table 2, the dominating morphology is shown together with the corresponding PAE and initial killing expressed as a percentage of surviving bacteria in the initial inoculum. Aztreonam and piperacillin caused predominantly filament formation at all the investigated concentrations. Cefuroxime and ceftazidime produced spheroplasts at high concentrations. Imipenem produced spheroplasts regardless of the concentration.

DISCUSSION

Studies of PAE performed by viable counts on gram-negative bacteria exposed to β-lactam antibiotics have not

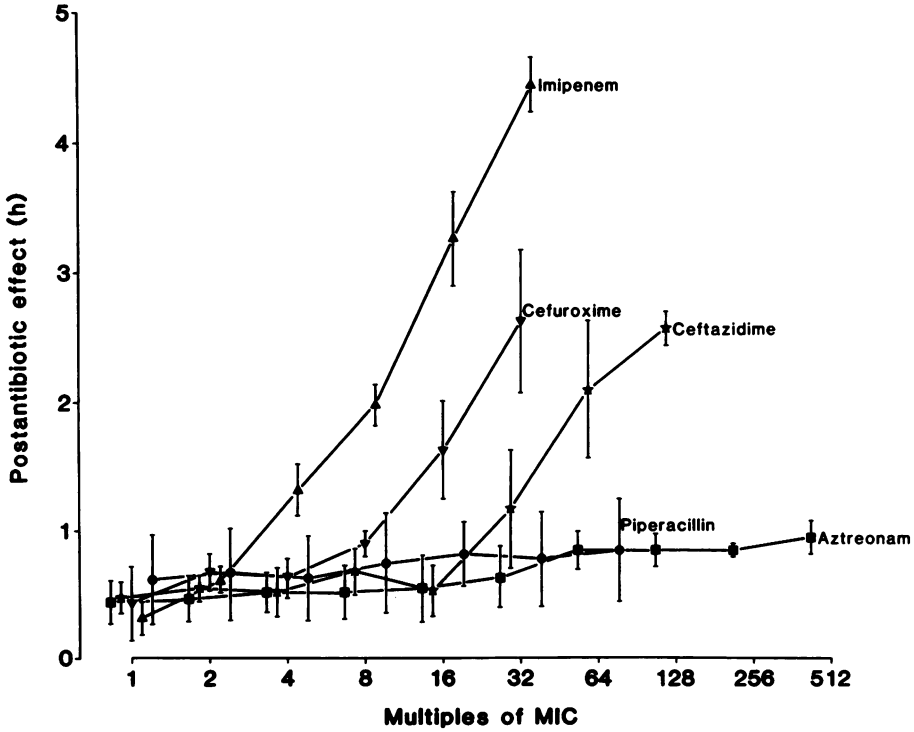

FIG. 2. PAEs of aztreonam, ceftazidime, cefuroxime, imipenem, and piperacillin on *E. coli* ATCC 25922 as determined by bioluminescence at different concentrations (expressed as multiples of the MIC). Mean values of the pooled results of three experiments are shown. Vertical bars denote standard deviations.

TABLE 2. Correlation between concentration of drug, PAE, initial killing, and morphology

Drug and concn of drug (μg/ml [×MIC])	PAE (h) (mean ± SD)	Initial killing (%)	Predominant morphology[a]
Aztreonam			
0.062 (1)	0.44 ± 0.17	90	Filaments
0.25 (4)	0.52 ± 0.15	88	Filaments
1 (16)	0.55 ± 0.26	75	Filaments
4 (64)	0.85 ± 0.15	75	Filaments
16 (256)	0.85 ± 0.05	86	Filaments
32[b] (512)	0.95 ± 0.13	83	Filaments
Ceftazidime			
0.25 (1)	0.48 ± 0.12	0	Filaments
1.0 (4)	0.52 ± 0.19	0	Filaments
4 (16)	0.53 ± 0.20	60	Filaments
16 (64)	2.10 ± 0.53	99	Spheroplasts
32[b] (128)	2.57 ± 0.13	99.9	Spheroplasts
Cefuroxime			
4 (1)	0.43 ± 0.29	87.5	Filaments
16[b] (4)	0.63 ± 0.15	98.6	Filaments
32 (8)	0.9 ± 0.10	99	Filaments
64 (16)	1.63 ± 0.38	99.5	Spheroplasts
128 (32)	2.63 ± 0.55	99.7	Spheroplasts
Imipenem			
0.25 (1)	0.32 ± 0.13	0	Spheroplasts
1 (4)	1.32 ± 0.20	80	Spheroplasts
4 (16)	3.26 ± 0.36	99.9	Spheroplasts
8[b] (32)	4.45 ± 0.21	99.9	Spheroplasts
Piperacillin			
2 (1)	0.62 ± 0.35	0	Filaments
8 (4)	0.63 ± 0.33	75	Filaments
32[b] (16)	0.82 ± 0.25	50	Filaments
64 (32)	0.78 ± 0.37	50	Filaments
128 (64)	0.85 ± 0.40	67	Filaments

[a] Morphology of more than two-thirds of the total cells.
[b] Highest clinically achievable concentration in serum 2 h after increased sepsis dose given intravenously.

demonstrated any PAE, except in the case of imipenem (3, 5, 10, 13). Craig and Gudmunsson studied the same *E. coli* ATCC strain as we did in our experiments, but they used the viable count method (3). Negative PAEs were obtained at low concentrations of drugs in these studies. This phenomenon is probably due to filament formation caused by β-lactam antibiotics (3). These filaments may contain a biomass corresponding to more than 20 bacteria. After drug removal, filaments start to divide in ordinarily shaped gram-negative bacteria. This causes a faster increase in CFU in the exposed cultures than in the unexposed cultures (3, 15).

One filament, which might contain 20 bacteria, is measured as one CFU when assessed by viable count. If the biomass (bacterial ATP) is measured with bioluminescence, this filament has a biomass of 20 bacteria. This means that the bacterial growth monitored by bioluminescence will not accelerate due to the reseptation of the filaments, while growth monitored by viable count gives an artifact showing an accelerated rate of proliferation.

Aztreonam had the lowest MIC for the tested *E. coli* strain. As we used dilution to eliminate the antibiotics, there is a great risk for residual antibiotic effects after dilution. As shown in Table 1, 10^{-3} dilution leads to much longer growth suppression at 256 and 512 MICs when 1/4 and 1/2 MIC were left after the dilution, compared with a 10^{-4} dilution in which the residual antibiotics were 1/20 or 1/40 MIC. The residual antibiotic concentrations observed in this study resemble the subinhibitory concentrations to which bacteria are normally exposed in vivo.

Penicillin-binding protein 3 (PBP 3) is essential for septum formation, and β-lactam antibiotics with high affinities for this protein cause filament formation (8). Aztreonam and piperacillin have their major affinity for PBP 3, and in this study they mainly produced filaments. Concomitantly with the production of filaments, we observed short, non-dose-dependent, but always positive PAEs for aztreonam and piperacillin.

Ceftazidime has its primary affinity for PBP 3 at low concentrations (14). We found short PAEs and mainly filaments for ceftazidime at up to 16 times the MIC. At higher concentrations of the drug, it also has affinities for PBP 1a and PBP 1b (14). At 64 times the MIC and at higher concentrations, we observed PAEs of at least 2 h and a morphology of predominantly spheroplasts. Cefuroxime resembles ceftazidime, with long PAEs and spheroplasts produced at high concentrations, but in contrast to ceftazidime, these concentrations are not clinically achievable for cefuroxime. Imipenem has its greatest affinity for PBP 2 (8) and its lowest affinity for PBP 3 (5, 8). Preferential binding to PBP 2 is considered to account for a distinctive morphologic effect, the direct production of spheroplasts (8). We could confirm these reports concerning morphology. At the MIC of imipenem we found only spheroplasts. PAEs longer than 1 h were recorded at a concentration as low as 4 MICs of imipenem. Imipenem showed the strongest bactericidal effect of the β-lactam antibiotics tested, and we observed no regrowth after exposure to 32 and 64 MICs in two and one experiments, respectively, of a total of four performed at each concentration.

The decrease in intracellular ATP was not as pronounced as the decrease in viability after 2 h of exposure to imipenem, ceftazidime, and cefuroxime. This may be due to the fact that the bactericidal effect assayed by viable count may be overestimated because of the inability of bacteria containing intracellular antibiotics to form colonies on agar plates (7, 13). With the bioluminescence method this problem is avoided, because intracellular ATP is extracted and measured directly. Another explanation may be that the bioluminescence assay measures the ATP in intact spheroplasts, while the reversion of spheroplasts to bacterial cells does not occur in quantitative numbers on agar plates, giving a rapid decrease in viability (CFU). It has been shown that in a hypertonic broth (342 mosM/liter) intracellular bacterial ATP remained normal during spheroplast formation, while viability decreased (6). The ATP assay thus indicated an approximation of the density of cells estimated in light microscopy, while viability studies reported a lower cell density (6). When using a broth with lower osmolality (50 mosM/liter), no spheroplast formation occurred, and a close relation between viability and intracellular ATP was observed (6).

The osmolality in our experiments was 323 mosM/liter (supplemented Mueller-Hinton broth). This is an osmolality which in broth protects the lysis of the spheroplasts and could be a part of the explanation of the differences we saw between the initial decrease in viability and intracellular ATP. Furthermore, this may explain why the bioluminescence method yielded longer PAEs for imipenem with *P.*

aeruginosa than did the viable count method in a comparison of the two methods (13).

In conclusion, the long PAEs for β-lactam antibiotics on *E. coli* were seen simultaneously with a potent initial killing. Concomitantly, the bacteria predominantly form spheroplasts. The bioluminescence method, in contrast to the viable count, is not jeopardized by filament formation. This makes it possible to study PAE for β-lactam antibiotics on gram-negative bacteria with bioluminescence.

β-Lactam antibiotic induction of filaments coincides with short PAEs and weak initial killing. The exact mechanisms for PAE in the case of β-lactam antibiotics and gram-negative bacteria are not known, but it may be the result of spheroplast formation. The PAE would then represent the time it takes for the spheroplasts to resynthesize normal cell walls and to resume cell division and logarithmic growth.

The clinical implication of long PAEs is the possibility of increasing intervals between administrations of antibiotics.

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