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Post-Exposure Effects of Cefepime and Cefpirome on Isogenic *Escherichia coli* Hosts Producing Shv-Extended-Spectrum β-Lactamases

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Summary

Persistent suppression of bacterial growth after short antimicrobial exposure is called postantibiotic effect (PAE). By definition, there should be no subinhibitory concentrations of antimicrobial agent left when the postantibiotic effect starts. However, if subinhibitory concentrations are maintained after removing the antibiotic, the recovery period of the treated cultures is markedly prolonged. This is defined as postantibiotic-sub-MIC-effect (PA-SME). The aim of this study was to determine the PAE and PA-SME of cefpirome and cefepime on isogenic *Escherichia coli* strains producing SHV-2, SHV-5, and SHV-12 extended spectrum β-lactamases (ESBL) compared to A non-ESBL *E. coli* strain. It was hypothesized that the presence of an ESBL would hydrolyze the cephalosporin molecule before it exerted a toxic effect on the bacterial cell and thus shorten the duration of PA-SME. Cefpirome and cefepime had no PAE against ESBL producing *E. coli* or it was of a short duration and present only at high antibiotic concentrations, but exposure to subinhibitory concentration of those antibiotics in the PA (postantibiotic) phase resulted in a significant delay of regrowth. The effect was more pronounced with higher concentrations of antibiotics, and uninfluenced by the type of enzyme and the antibiotic. The present study shows that the presence of subinhibitory concentrations of cefepime and cefpirome in the medium after exposure to suprainhibitory concentrations results in a significant delay of regrowth of both ESBL-positive and -negative *E. coli* strains. The production of SHV-2, SHV-5 and SHV-12 ESBLs did not shorten the duration of the PA-SME.

Key words: Postantibiotic effect, postantibiotic-subMIC effect, extended-spectrum β-lactamases, cefepime, cefpirome.

INTRODUCTION

Persistent suppression of bacterial growth after short antimicrobial exposure is called postantibiotic effect (PAE). It is the lag phase or recovery period of bacterial growth after brief exposure to antibiotic [1]. β-lactam antibiotics have consistently shown PAEs against various Gram-positive cocci [2-5]. In contrast to Gram-positive cocci, there are marked differences in PAE caused by β-lactam antibiotics on Gram-negative bacteria [6-7]. PAE was not observed when *Escherichia coli* and *Pseudomonas aeruginosa* were exposed to β-lactam antibiotics or had it was of very short duration [8]. The only reported exception to the absence of PAE when Gram-negative bacteria and β-lactams are combined, are imipenem and ceftibuten [9-10].

The presence of PAE may be an important con-

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sideration in designing antibiotic dosage regimens. A long PAE provides the potential for administering the antimicrobial agent with longer intervals between the doses, beyond the time when antibiotic concentration falls below the MIC [11-12]. By definition, there should be no subinhibitory concentrations of antimicrobial agent left when the PAE starts. However, if subinhibitory concentrations are maintained after removing the antibiotic, the recovery period of the treated cultures is markedly prolonged. This is defined as postantibiotic-sub-MIC-effect (PA-SME) [10-12]. Cefepime and cefpirome are fourth generation cephalosporins, which were previously believed to be stable to ESBLs, but it is a well known fact now that they are hydrolyzed by some SHV-ESBLs, particularly in cases of hyperproduction [13].

The aim of this study was to determine the PAE and PA-SME of cefpirome and cefepime on isogenic *E. coli* strains producing SHV-2, SHV-5, and SHV-12 β-lactamases, compared to non-ESBL *E. coli*. We hypothesized that the presence of an ESBL would hydrolyze the cephalosporin molecule before it exerted a toxic effect on the bacterial cell and thus shorten the duration of PA-SME.

MATERIALS AND METHODS

Bacteria

The experiments were performed on three isogenic *E. coli* strains with previously characterized and sequenced β-lactamases which included [14-15]:

SHV-2 producing *E. coli* A 15 R^+ 3992,

SHV-5 producing *E. coli* A15 R^+ 4554,

SHV-12 producing *E. coli* A15 R^+ 586 and

non-ESBL *E. coli* A15R^- (ESBL-negative).

SHV-2 β-lactamase is a cefotaximase according to the substrate profile while SHV-5 and SHV-12 β-lactamases are ceftazidimases and elevate the MICs of ceftazidime and aztreonam more than those of cefotaxime and ceftriaxone. Both types of enzymes increase the MICs of cefpirome and cefepime although they were still below the resistance breakpoint for the strains used in this study. The SHV-2 producer had a slightly higher MIC of cefpirome than SHV-5 and SHV-12 producers.

Minimum inhibitory concentrations (MICs)

MICs of cefepime, cefpirome and cefotaxime were determined in duplicate by the broth microdilution method according to the NCCLS guidelines. The standard inoculum of 5 x 10^5 CFU/ml was used [16]. The tests were run in triplicate on different occasions.

Antibiotic powders: cefotaxime - Belupo, Zagreb, Croatia; cefpirome - Hoechst AG, Frankfurt, Germany; and cefepime - Bristol-Myers Squibb, Zagreb, Croatia; were supplied by the respective manufacturers.

Time- kill experiments

Time-kill experiments were carried out by exposing test cultures to cefepime, cefpirome and cefotaxime and by establishing bacterial numbers by viable counting. An overnight broth culture was diluted to 10^5 CFU/ml and exposed to 0.1, 1 and 10 x MIC. An unexposed control was run in parallel. Bacterial counts at times 0, 2, 4, 6, 8 and 24 h were determined by viable counting [17]. The samples were diluted by a factor of 10^{-3} and 10^{-6} and 100 µl of undiluted cultures and each dilution was seeded on Mueller-Hinton (MH) agar for CFU determination. The samples were spread on the whole agar surface to avoid the antibiotic carryover.

Determination of postantibiotic- effect (PAE)

Inocula of 10^5 to 10^6 CFU/ml were exposed to 0.1, 1 or 10 x MIC of cefepime, cefpirome, or cefotaxime for 2 h, after which antibiotic was eliminated by 1:1000 dilution in the test medium. Regrowth of these and a control culture was followed by viable counting. Samples were withdrawn at time 0 (when the antibiotic was added), after 2 h (before and after dilution) and at 4, 5, 6, 7, 8, 9 and 24 h and diluted with PBS (phosphate buffered saline). The dilutions and the original cultures were seeded on the MH agar plates and counted for determination of CFU. If there were less that 10 colonies on MH agar plate, which happened in some cases after exposure to 10 x MIC, the experiment was repeated using an alternative method to remove the antibiotic. The cultures were spun in the centrifuge for 10 min at 14,000 rpm, the pellet was washed twice and resuspended in PBS to yield an inoculum of approximately 10^3 to 10^4 CFU/ml. The PAE was defined according to the following formula: PAE=T-C, where T is the time required for the viable counts of the antibiotic-exposed cultures to increase by 1 $\log_{10}$ above the counts observed immediately after dilution, and C is the corresponding time for the unexposed cultures [12]. The experiments were run in triplicate on different occasions.

Determination of postantibiotic-subMIC-effect (PA-SME)

Inocula of 10^6 to 10^7 CFU/ml were exposed to 10 x MIC of cefepime, cefpirome, or cefotaxime for 2 h, after which the cultures were diluted to achieve subMIC concentrations of 0.12, 0.25, 0.5 and 0.9 x MIC. Regrowth was followed by viable counting. Every hour the aliquots of the cultures were taken,

diluted, and the amount of 100 µl was spread on the MH agar. Unexposed controls were run in parallel. The control sample was diluted to reach an inoculum similar to the cultures exposed to antibiotics. If there were fewer than 10 colonies on the surface of the MH agar, the method was modified to concentrate the bacteria. The cultures in the PA phase were spun in the centrifuge, washed with saline and re-exposed to 0.12, 0.25, 0.5 and 0.9 x MIC. Samples were made and the number of viable bacteria determined as described previously. The experiments were run in triplicate on different occasions. The effect of sub-MICs on bacteria in PA phase (PA-SME) was defined as follows: PA-SME= T_{PA}-C, where T_{PA} is the time taken for the cultures previously exposed to antibiotics and then exposed to different sub-MICs to increase by 1 $\log_{10}$ above the counts observed immediately after dilution step, and C is the corresponding time for the control cultures [12].

Determination of the effects of subMICs (SME)

The cultures were exposed to 0.12, 0.25, 0.5 and 0.9 x MIC of the same antibiotics used for induction of PAE and PA-SME and incubated for 24 hours. Samples were withdrawn and the number of viable bacteria determined as described above.

The effect of sub-MICs (subMIC effect -SME) on bacteria not pre-exposed to antibiotics was defined as follows: SME= Ts-C, where Ts is the time taken for the cultures exposed only to sub-MICs to increase by 1 $\log_{10}$ above the counts observed at the beginning of the experiment, and C is the corresponding time for the unexposed cultures [12].

RESULTS

Minimum inhibitory concentrations (MICs)

MICs were as follows: *E. coli* A15R$^+$4554 (SHV-5) - cefotaxime - 32 mg/L, cefepime - 16 mg/L, and cefpirome - 8 mg/L; *E. coli* A15R$^+$3992 (SHV-2) - cefotaxime - 32 mg/L, cefepime and cefpirome - 16 mg/L: *E. coli* A15R$^+$ 586 (SHV-12) - cefotaxime - 8 mg/L, cefepime - 16 mg/L and cefpirome - 2 mg/L; and *E. coli* A15R$^-$ cefotaxime - 0.06 mg/L, cefepime - 0.06 mg/L and cefpirome - 0.03 mg/L.

Time- kill experiments
E. coli A15R+ 4554-SHV-5

Cefotaxime: After exposure to 0.1 x MIC of cefotaxime the growth was slightly slower compared to unexposed control. At 1 x MIC there was a decrease in viable counts of 1 log10 after 2 h, and 2 log10 after 4 h. A regrowth was detected after 24 h. At 10 x MIC a decrease of 2 log10 occurred after 2 h, and 3 log10 after 4 h. After 8 h there were no surviving bacteria and no regrowth was found after 24 h. The results are shown in *Table 1*.

Cefpirome: The exposure to 0.1 x MIC of cefpirome resulted in a growth delay in comparison with the unexposed control. At 1 x MIC a decrease in viable counts of 1 log10 was found after 2 h, and 2 log10 after 4 h. An increase in viable counts of 1 log10 compared to the initial inoculum was detected after 24 h. At 10 x MIC there was a 2 log10 decrease after 2 h, and 3 log10 after 4. No surviving bacteria were found after 8 h. The regrowth was not detectable after 24 h.

Cefepime: At the concentration of 0.1 x MIC cefepime produced a delay in growth kinetics. At 1 x MIC it produced a 3 log10 decrease of viable counts after 2 h, and 4 log10 after 4 h with a moderate regrowth after 24 h. At 10 x MIC a decrease of 2 log10 was found after 2 and 4 h and complete killing occurred after 6 h. The results are shown in *Table 1*.

E. coli A15R+ 3992-SHV-2

Cefotaxime: At the concentration of 0.1 x MIC cefotaxime exerted a moderate delay in growth but no bactericidal activity was observed. Exposure to 1 x MIC resulted in 2 log10 decrease of viable counts after 2 h, and 3 log 10 after 4, 6 and 8 h. A slight regrowth was demonstrated after 24 h. The concentration of 10 x MIC produced a 2 log10 decrease after 2 h and 3 log10 after 4 h. No bacteria were detectable after 8 and 24 h.

Cefpirome: At 0.1 x MIC of cefpirome the strain grew slower than the control culture but no reduction in CFU/ml was found. At 1 x MIC the reduction in viable counts of 2 log10 was detected after 2 h and 3 log10 after 6 h with regrowth after 24 h. At 10 x MIC a complete killing occurred after 8 h.

Cefepime: At 0.1 x MIC the bacterial counts increased for 1 log10 after 6 h, while in unexposed control it happened after 3 h. The concentration of 1 x MIC reduced the viable counts by 2 log10 after 2 and 4 h and by 3 log10 after 6 h. At 10 x MIC the culture was sterilized after 4 h.

E. coli A15R+ 586-SHV-12

The bactericidal activity of all three antibiotics was similar to SHV-5 and SHV-12 producers. The rate of bactericidal activity depended on the antibiotic concentration. No significant difference in the killing kinetics among the three cephalosporins was observed. At the highest antibiotic concentration (10x MIC) the most rapid killing was expressed with cefepime and the slowest with cefpirome. When the strain was combined with cefpirome there were still surviving bacteria after 24 h upon exposure to 10 x MIC.

TABLE 1 - *Killing kinetic of three cephalosporins at various concentrations against isogenic Escherichia coli strains producing SHV extended-spectrum β-lactamases.*

CFU/ml												
***E. coli* A15 R$^+$ 4554- SHV-5**												
	Cefotaxime				**Cefpirome**				**Cefepime**			
Time	Control	0.1xMIC	1xMIC	10xMIC	Control	0.1xMIC	1xMIC	10xMIC	Control	0.1xMIC	1xMIC	10xMIC
0	3.2x10^4	9x10^4	9.5x10^4	7.4x10^4	1.2x10^4	2x10^4	4.5x10^4	3.9x10^4	4.3x10^5	3.7x10^5	2.9x10^5	8.6x10^4
2	9.6x10^4	9.7x10^4	2.2x10^3	130	8.7x10^4	5.3x10^4	2.1x10^3	120	9.1x10^5	4.9x10^5	120	19
4	7.8x10^5	3.6x10^5	880	12	5.6x10^5	8.8x10^4	240	18	7.6x10^6	9.2x10^5	26	82
6	8.8x10^6	4.2x10^6	150	1	2.2x10^6	4.4x10^5	210	5	8.2x10^7	5.6x10^6	0	0
8	5.3x10^7	9.8x10^6	360	0	3.7x10^7	6.5x10^6	1.9x10^3	0	3.9x10^8	2.7x10^6	5	0
24	7.4x10^9	2.8x10^8	3.6x10^6	0	4.4x10^9	2.5x10^9	5.5x10^5	0	9.3x10^9	1.7x10^9	4.8x10^5	0
***E. coli* A15 R$^+$ 3992- SHV-2**												
	Cefotaxime				**Cefpirome**				**Cefepime**			
Time	Control	0.1xMIC	1xMIC	10xMIC	Control	0.1xMIC	1xMIC	10xMIC	Control	0.1xMIC	1xMIC	10xMIC
0	2.8x10^5	9.2x10^4	5.6x10^5	4.2x10^5	3.4x10^5	5x10^5	6.8x10^5	2x10^5	9.2x10^4	7.6x10^4	5x10^4	6.4x10^4
2	8.9x10^5	1.8x10^5	3.3x10^3	2.8x10^3	9.8x10^5	3.6x10^5	2.3x10^3	3.6x10^2	6.7x10^5	9.2x10^4	7.8x10^2	45
4	6.4x10^6	6.5x10^5	9.2x10^2	10^2	6.7x10^6	9,5x10^5	10^3	5.2x10^2	4.3x10^6	2.9x10^5	3.6x10^2	0
6	1.7x10^7	2.8x10^6	2.3x10^2	3	4.9x10^6	7.7x10^6	2.2x10^2	14	5.8x10^7	9.6x10^5	92	0
8	7.7x10^7	1.2x10^7	1.6x10^2	0	1.6x10^8	5.6x10^7	1.7x10^3	0	1.4x10^8	5.9x10^6	1.4x10^2	0
24	6.6x10^9	2.8x10^9	6,4x10^4	0	2.9x10^9	8.6x10^9	5.5x10^6	0	2.6x10^9	8.3x10^8	4.2x10^6	0
***E. coli* A15R$^+$ 586-SHV-12**												
	Cefotaxime				**Cefpirome**				**Cefepime**			
Time	Control	0.1xMIC	1xMIC	10xMIC	Control	0.1xMIC	1xMIC	10xMIC	Control	0.1xMIC	1xMIC	10xMIC
0	1.5x10^5	3.1x10^5	9.5x10^4	1.2x10^5	2.5x10^5	1.4x10^5	10^5	3.9x10^5	1.2x10^5	2.5x10^5	9x10^4	9.6x10^4
2	6.5x10^5	1.4x10^5	10^4	14	1.3x10^6	3x10^5	2.3x10^3	72	7.5x10^5	1.9x10^5	3.2x10^2	95
4	3.7x10^6	5.5x10^5	5.9x10^2	9	8.6x10^6	7.8x10^5	1.9x10^2	67	3.5x10^6	7.4x10^5	10^2	19
6	6.5x10^7	3.4x10^6	10^2	10	9.9x10^7	1.3x10^7	2.2x10^2	95	2.9x10^7	8.4x10^6	3.9x10^2	0
8	6.5x10^8	1.5x10^7	3.7x10^2	0	4.4x10^8	2.2x10^7	7.5x10^2	88	9.6x10^7	5.9x10^7	8.4x10^2	0
24	3.5x10^9	4.1x10^9	3.8x10^5	0	6.5x10^9	1.2x10^9	4.3x10^7	4	6.7x10^9	3.3x10^9	4.2x10^7	0
***E. coli* A15R$^-$**												
	Cefotaxime				**Cefpirome**				**Cefepime**			
Time	Control	0.1xMIC	1xMIC	10xMIC	Control	0.1xMIC	1xMIC	10xMIC	Control	0.1xMIC	1xMIC	10xMIC
0	7.2x10^5	6.4x10^5	4.6x10^5	8.3x10^5	2.3x10^6	9.6x10^5	3.3x10^6	1.5x10^6	8.7x10^5	9.6x10^5	7.6x10^5	1.2x10^6
2	5.1x10^6	8.2x10^5	2.4x10^3	1.6x10^2	1.5x10^7	3.9x10^6	2.2x10^5	2.4x10^2	5.5x10^6	1.3x10^6	4.2x10^3	28
4	3.5x10^7	4.8x10^6	9.8x10^2	75	8.6x10^7	7.4x10^6	10^5	10	4.4x10^7	6.9x10^6	1.6x10^3	0
6	1.5x10^8	2.2x10^7	1.3x10^2	15	3.6x10^8	6.4x10^7	4.1x10^5	23	8.8x10^7	4.3x10^7	450	0
8	4.6x10^9	1.7x10^7	3x10^2	1	7.2x10^8	2.6x10^8	5.9x10^5	1	1.3x10^8	8.4x10^7	940	0
24	8.5x10^9	5.3x10^9	2.4x10^6	0	4.5x10^9	6.3x10^9	1.4x10^{07}	0	9.4x10^9	9.5x10^8	3.7x10^5	0

E. coli A15R$^-$

The killing rate of *E. coli* A15R$^-$ was also concentration dependent. As with other strains, cefepime displayed the strongest and most rapid bactericidal activity. When cefepime was combined with the strain at the highest concentration, no viable bacterial were found after 4 h. The results are shown in *Table 1*.

PAE,PA-SME and SME

The results are shown in *Table 2* which shows that cefepime, cefpirome and cefotaxime had a short PAE or it was not demonstrated except at high antibiotic concentrations (10 x MIC) against both ESBL-producing and non-ESBL *E. coli*, but exposure to subinhibitory concentrations of those antibiotics in the PA (postantibiotic) phase resulted

TABLE 2 - *Postantibiotic-effect (PAE), postantibiotic- subMIC-effect (PA-SME) and subMIC -effect (SME) of three cephalosporins on isogenic Escherichia coli strains producing SHV- extended-spectrum β-lactamases. Mean values of three experiments and the data range are shown.*

Antibiotic-bacteria	PAE (h)			SME (h)				PA-SME (h)			
combination	0.1x MIC	1 x MIC	10 x MIC	0.12 x MIC	0.25 x MIC	0.5 x MIC	0.9 x MIC	0.12 x MIC	0.25 x MIC	0.5 x MIC	0.9 x MIC
***E. coli* A 5R⁺ 4554- SHV-5**											
cefotaxime	0.09 (-019-0.25)	0.13 (-0.09-0.32)	0.64 (0.42-1.08)	1.8 (0.95-2.32)	2.96 (2.65-3.35)	6.2->7	>7[1] -NR[2]	1.74 (1.25-2.17)	3.17 (2.75-3.67)	4 ->7[1]	4.7->7[1]
cefpirome	0.04 (-0.38-0.44)	0.31 (0.11-0.54)	0.85 (0.67-1.02)	1.81 (0.85-2.55)	2.66 (1.8-3.4)	4.80 to ->7[1]	>7[1]	2.91 (1.81-4.7	4.31 (3.64-4.9)	>7[1] -NR[2]	>7[1] - NR[2]
cefepime	-0.006 (-0.25-0.28)	0.26 (0.15-0.45)	0.65 (0.5-0.96)	1.31 (1.05-1.45)	3.32 (2.8-3.67)	5.72 (5.27-6.25)	>7[1]-NR[2]	2.3 (1.3-3.15)	3.59 (3.17-4)	>7[1] - NR[2]	>7[1] - NR[2]
***E.coli* A15R⁺ 3992-SHV-2**											
cefotaxime	0.14 (-0.32-0.10)	0.76 (0.6-0.9)	0.88 (0.75-1.10)	2.61 (2.15-3.2)	3.58 (2.35-5.2)	6.55 to >7[1]	>7[1]	3.17 - NR[2]	4.1 (2.7-4.95)	NR[2]	NR[2]
cefpirome	0.10 (-0.07-0.4)	0.71 (0.29-1.05)	0.88 (0.69-1.05)	2.26 (1.75-2.55)	3.26 (2.65-3.75)	6.15 to>7[1]	>7 [1]- NR[2]	3.54 (2.73-4.25)	4.5 (3.52-5)	>7[1]- NR[2]	>7[1] - NR[2]
cefepime	-0.09 (-0.35-0.24)	0.41 (0.35-0.5)	0.79 (0.59-1.09)	2.71 (2.15-3.3)	4.21 (3.03-6.1)	6.15->7[1]	>7[1]- NR[2]	3.48 (3.1-4.15)	4.2 (3.95-4.6)	>7[1] - NR[2]	>7[1] - NR[2]
***E.coli* A15R⁺ 586-SHV-12**											
cefotaxime	-0-02 (-0.25-0.33)	0.7 (0.55-0.8)	0.96 (0.53-1.6)	2.6 (2.39-2.75)	3.53 (3.25-4.05)	5.21 (3.25-4.05)	>7[1]	4.11 (3.04-4.75)	4.47- >7[1]	>7[1] - NR[2]	NR[2]
cefpirome	0.28 (0.2-0.35)	0.82 (0.33-1.4)	1.29 (0.97-1.57)	2.37 (1.89-2.67)	3.56 (3.09-3.89)	5.83 to >7[1]	>7[1]	3.32 (2.33-3.85)	4.4 (3.94-4.7)	>7-NR[2]	NR[2]
cefepime	0.27 (-0-01-0.75)	0.33 (0-0.55)	0.58 (0.1-1)	2.48 (2.37-2.55)	3.84 (3.55-4.09)	5.18 to >7[1]	5.7->7[1]	3.64 (3.07-4.55)	5.83 (4.64-6.85)	NR[2]	NR[2]
***E.coli* A15R⁻**											
ESBL⁻ cefotaxime	-0.02 (-0.25-0.47)	0.65 (0.15-1.05)	0.93 (0.85-1.3)	1.83 (1.6-2.21)	2.91 (2.85-3.35)	5.51 (4.81-6.68)	>7[1]	3.12 (1.75-4)	4.32 (3.4-4.8)	4.90 ->7[1]	NR[2]
cefpirome	0.24 (-0.1-0.57)	0.27 (0.03-0.75)	0.81 (0.23-1.55)	2.24 (2-2.48)	3.36 (3.24-3.59)	5.16->7[1]	6.65->7[1]	3.16 (2.2-4.33)	4.23->7[1]	3.95 - NR[2]	NR[2]
cefepime	0.06 (-0-05-0.15)	0.66 (0.1-1.55)	1.07 (0.83-1.25)	1.97 (1.82-2.25)	3.27 (2.97-3.5)	4.99 (4.32-6.22)	>7[1]	2.7 (1.9-3.38)	3.84 (3.1-4.27)	3.45->7	NR[2]

[1] time for the cultures to grow 1 $\log_{10}$ CFU extended the period of observation
[2]NR- no regrowth – an increase in CFU of 1 $\log_{10}$ was not detected after 24 h

in a significant delay of regrowth of all tested strains. Negative PAEs were observed at low antibiotic concentrations (0.1 x MIC). At this concentration the PAE lasted from -0.38 to 0.75 h. At 1 x MIC the duration of PAE ranged from -0.09 to 1.55 h and at 10 x MIC from 0.1 to 1.6 h, depending on the antibiotic-bacteria combination. The longest PAE was obtained with cefpirome against SHV-12 producer. The results did not seem to be much affected by the type of antibiotic or β-lactamase (*Table 2*). All three antibiotics had a strong initial killing effect at 10 x MIC, but it was not correlated with the duration of PAE or PA-SME. At 0.12 x MIC the duration of PA-SME ranged from 1.25 to 4.75 and at 0.25 x MIC from 2.75 to >7 h, depending on the antibiotic-bacteria combination. Exposure to 0.5 and 0.9 x MIC in the PA phase resulted in bacterial death in many experiments or the duration of PA-SME at this antibiotic concentration extended the period of observation (*Table 2*). No regrowth was observed in one experiment even at 0.12 x MIC when an SHV-2 producer was combined with cefotaxime. The duration of PA-SME was not shorter in ESBL-producing *E. coli* strains compared to non-ESBL and in many experiments it was even longer for ESBL-positive strains. The phenomenon was found to be concentration dependent (*Table 2*).

The SME at 0.12 x MIC lasted from 0.85 to 3.3 h, at 0.25 x MIC from 1.8 to 5.2 h and at 0.5 x MIC from 4.8 to more than 7 h. At 0.9 x MIC the time necessary for the cultures to grow to 1 $\log_{10}$ above the counts at the beginning of the experiment, extended the observation period (9 h) in most cases or there was no regrowth at all (*Table 2*). It was noticed that the SMEs obtained with SHV-2 and SHV-12 producers were in principle longer with all three antibiotics than those observed with SHV-5 and ESBL-negative strain. The duration of SME was shorter than PA-SME. The only exception found was when *E. coli* 4554-SHV-5 was combined with cefotaxime at 0.12 x MIC, 0.5 x MIC and 0.9 x MIC.

DISCUSSION

The present investigation showed that the presence of subinhibitory concentrations of cefepime and cefpirome in the medium after exposure to suprainhibitory concentrations resulted in a significant delay of regrowth of all *E. coli* strains independently of ESBL production. It turned out that the duration of PA-SME was not shorter in ESBL-producing *E. coli* compared to non-ESBL, as was hypothesized. ESBL-producing strains were more resistant to 4th generation cephalosporins as shown by their MICs, but that did not affect the duration of the PAE and PA-SME. Fourth generation cephalosporins are in principle hydrolyzed slowly by ESBLs and the hydrolysis rate depends on the amount of enzyme and the inoculum size. For that reason the strains with high level β-lactamase production were used in this study. Antibiotic concentrations of 10 x MIC produced a strong killing effect, especially cefepime and cefpirome, and for that reason a high inoculum of 10^7 cells/ml was required to abrogate the bactericidal effect and to demonstrate the PA-SME.

In vivo efficacy of fourth generation cephalosporins in the treatment of infections caused by ESBL-producing bacteria is still controversial. According to the NCCLS, β-lactam antibiotics, except for imipenem, are not recommended for the therapy of infections caused by ESBL-producing organisms independently of their MIC values [16], but some authorities suggest they might be suitable for treating infections due to such organisms if the MICs show susceptibility [18]. Cefepime demonstrated good activity in *in vivo* experiments in which animals were injected with ESBL-producing *Klebsiella. pneumoniae* strains [19]. According to the results of this investigation, therapeutic failures of new cephalosporins against infections caused by ESBL-producing organisms cannot be due to the lack of PA-SME. It is a well known fact that they can be degraded by some ESBLs and that their activity is compromised in the presence of a high inoculum of β-lactamase producing organisms, but the effect of the β-lactamase activity on the duration of the PA and PA-SME of the above mentioned antibiotics has not been previously investigated.

Negative PAEs were obtained in some cases at low concentrations of the antibiotics used in this study. The phenomenon is probably due to the filament formation caused by β-lactam antibiotics. These filaments may contain a biomass corresponding to more than 20 bacteria. After drug removal the filaments start to divide into ordinarily shaped Gram-negative bacteria [1]. Furthermore, in some experiments the PAE was shorter at higher antibiotic concentrations. This could be explained by the fact that viable counting is not such an accurate method as some of the new more sophisticated techniques like electronic counting [20] and bioluminescence assay of bacterial ATP [1]. This method usually yields shorter PAEs compared to other methods. In previous investigations it was found that PAEs were generally two- to three-fold longer with the ATP method than when viable counting was used [21]. The main drawback of viable counting is that it does not detect the aberrant morphology forms and thus underestimates the PAE.

The PAE and PA-SME in this study were con-

centration dependent and uninfluenced by the type of antibiotic or β-lactamase, contrary to some previous investigations in which no obvious dose dependence for PAE on *E. coli* in the range of cefotaxime concentrations was detected [22]. The subMIC concentrations in the PA phase were associated with a delay in replication or even bacterial death within 24 hours, depending on the antibiotic concentration. In some experiments it was not possible to determine the PA-SME with the concentrations of 0.5 and 0.9 x MIC because they were bactericidal in the PA phase. It was necessary to repeat the experiment with the higher initial inoculum or to centrifuge the culture in order to concentrate the bacterial cells. One possible explanation for that is the pronounced inoculum effect of β-lactam antibiotics against ESBL-producing *Enterobacteriaceae*. The non-PAE bacteria were less susceptible to the inhibitory effect of subinhibitory antibiotic concentrations. It seems that the previous exposure to suprainhibitory concentrations of the antibiotics slows down the growth kinetics of bacteria in the presence of subMIC concentrations. This has been confirmed by the fact that the SMEs had shorter duration than PA-SMEs. Contrary to that finding, a strong inhibition of bactericidal activity of β-lactam antibiotics during the PAE was observed in the previous investigations which is explained by the fact that cell multiplication is necessary for the β-lactam antibiotics to exert their antibacterial activity [23].

There was no correlation between the initial killing and the duration of the PAE, in contrast with earlier investigations [1].

The mechanisms behind the effects of subinhibitory concentrations of β-lactam antibiotics on bacteria in the PA phase were explained previously. When bacteria are exposed to suprainhibitory concentrations of an antibiotic, the drug binds covalently to the active sites of the PBPs. Synthesis of PBPs is known to continue during antibiotic treatment. When excess drug is removed and challenge with subinhibitory concentrations is repeated, most of the PBPs are still inactivated, and only a low drug concentration is needed to inhibit the newly produced PBP. This results in prolonged inhibition of cell multiplication until a critical number of free PBP is once more available. Thus, it seems that the PAE can be substantially prolonged *in vitro* when the bacteria are re-exposed to sub-MICs of β-lactam antibiotics [12].

PAE determinations *in vitro* cannot reflect the situation *in vivo*. *In vitro*, the antibiotic is eliminated instantaneously, whereas *in vivo* there is a much more gradual decrease in antibiotic concentration, depending on the elimination half-life, and that is more accurately reflected by PA-SME [10]. In humans treated with intermittent dosage schedules of antibiotics, suprainhibitory concentrations will always be followed by subinhibitory levels and for that reason PA-SME better reflects the pharmacodynamics of antibiotics *in vivo* [12].

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