

## Studies of the Killing Kinetics of Benzylpenicillin, Cefuroxime, Azithromycin, and Sparfloxacin on Bacteria in the Postantibiotic Phase

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Most antibiotics are known to be incapable of killing nongrowing or slowly growing bacteria with few exceptions. Bacterial cell division is inhibited during the postantibiotic phase (PA phase) after short exposure to antibiotics. Only scarce and conflicting data are available concerning the ability of antibiotics to kill bacteria in the PA phase. The aim of the present study was to investigate the killing effect of four different antibiotics on bacteria in the PA phase. A postantibiotic effect (PAE) was induced by exposing *Streptococcus pyogenes* and *Haemophilus influenzae* to 10× MICs of benzylpenicillin, cefuroxime, sparfloxacin, and azithromycin. The bacteria were thereafter reexposed to a 10× MIC of the same antibiotic used for the induction of the PAE at the beginning of and after 2 and 4 h in the PA phase. Due to a very long PAE, the bacteria in PA phase induced by azithromycin were also exposed to 10× MICs after 6 and 8 h. A previously unexposed culture exposed to a 10× MIC was used as a control. The results seem to be dependent on both the antibiotic used and the bacterial species. The antibiotics exhibiting a fork bactericidal action gave significantly reduced killing of the bacteria in PA phase (cefuroxime with *S. pyogenes*,  $P < 0.01$ , and sparfloxacin with *H. influenzae*,  $P < 0.001$ ), which was restored at 4 h for cefuroxime with *S. pyogenes*. There was a tendency to restoration of the bactericidal activity also with sparfloxacin and *H. influenzae*, but there was still a significant difference in killing between the control and the test bacteria in PA phase at 4 h. However, in the combinations with a lesser bactericidal effect (benzylpenicillin with *S. pyogenes* and sparfloxacin with *S. pyogenes*), there was no difference in killing between the control and the test bacteria in PA phase. Azithromycin induced long PAEs in both *S. pyogenes* and *H. influenzae* and exhibited a slower bactericidal action on both the control and the bacteria in PA phase especially at the end of the PAE, when the killing was almost bacteriostatic. Our findings in this study support the concept that a long interval (>12 h) between doses of azithromycin, restoring full bactericidal action, may be beneficial to optimize efficacy of this drug but is not necessary for the other antibiotics evaluated, since the bactericidal effect seems to be restored already at 4 h.

In the early studies of the mechanism of the effects of penicillin, it was demonstrated that penicillin had mainly a bacteriostatic effect on slowly growing bacteria (4, 14, 15, 29). The failure to kill slowly or nongrowing bacteria is a characteristic common to most antibiotics and has been named phenotypic tolerance (2, 5, 23-26). Another pharmacodynamic factor described early in the antibiotic era was the persistent suppression of bacterial growth after the active drug had disappeared (1, 8, 21). This so-called postantibiotic effect (PAE) is today well studied both in vitro and in vivo. The PAE is dependent on the type of antibiotic used, the concentration of the drug, and also on the bacterial species studied (3, 6, 10, 12, 18). Since bacteria in the postantibiotic phase (PA phase) seem to multiply slowly, it has been suggested that the ideal dosing interval of antibiotics would be the sum of the time that the concentration stayed above the MBC, the duration of the PAE, and the time required for the bacteria to enter a sensitive logarithmic phase (9). However, there are only scarce and conflicting data available concerning the ability of antibiotics to kill bacteria in the PA phase (10, 13, 19, 20, 27). The aim of the present study was to investigate four different antibiotics and their killing activity for bacteria in the PA phase.

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### MATERIALS AND METHODS

**Antibiotics.** The antibiotics investigated in the study were benzylpenicillin (Astra Läkemedel, Södertälje, Sweden), cefuroxime (Glaxo AB, Göteborg, Sweden), azithromycin (Pfizer AB, Täby, Sweden), and sparfloxacin (Rhône-Poulenc, Helsingborg, Sweden). The antibiotics were obtained as dry powder with known potency. Benzylpenicillin was diluted in distilled water, cefuroxime was diluted in phosphate-buffered saline (pH 7.2), azithromycin was diluted in methanol, and sparfloxacin was diluted in 0.1% sodium hydroxide.

**Bacterial strains.** All four antibiotics were tested against *Streptococcus pyogenes* M12, NTCC (National Type Culture Collection) P 1800, and a randomly selected non-β-lactamase-producing clinical strain of *Haemophilus influenzae* (7002) obtained from the Department of Clinical Microbiology, Uppsala University, Uppsala, Sweden. *S. pyogenes* was grown in Todd-Hewitt broth for 6 h at 37°C in 5% CO<sub>2</sub> in air, resulting in approximately 10<sup>9</sup> CFU/ml, and *H. influenzae* was grown in PDM broth (Progressive Diagnostics Manufactures; Biodisk, Solna, Sweden) supplemented with 30 mg of hemin and 1% IsoVitalX for 6 h at 37°C in 5% CO<sub>2</sub> in air, resulting in the same inoculum.

**Determination of MICs.** The MICs for the investigated strains were determined in fluid media by a macrodilution technique in triplicate on different occasions according to National Committee for Clinical Laboratory Standards. Twofold serial dilutions of the antibiotics were added to the broth and inoculated with approximately 10<sup>5</sup> CFU of the test strain per ml. The MIC was read after 24 h and was defined as the lowest concentration of antibiotic allowing no visible growth.

**Determination of the PAE.** The PAEs were studied in triplicate on different occasions for all antibiotic-bacterial combinations except for those for which no PAE was detected, which were studied once. After an incubation of 6 h, the strains were diluted 10<sup>-1</sup> in fresh broth and exposed to 10× MICs for 2 h. To eliminate the antibiotics, the cultures were washed twice (three times for sparfloxacin-*H. influenzae*) by centrifugation for 10 min at 1,400 × g, and depending on the rate of killing during the antibiotic exposure, *S. pyogenes* was diluted 1:100

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TABLE 1. Killing in the PA phase<sup>a</sup>

Antibiotic and organism	PAE (h)	Decrease in log <sub>10</sub> CFU over 4 h					
		Control	0 h	2 h	4 h	6 h	8 h
<b>Benzylpenicillin</b>							
<i>S. pyogenes</i>	3.3 (2.2–4.7)	1.8 (1.2–2.3)	1.2 (1.0–1.5)	1.9 (1.6–2.3)	1.8 (1.2–2.3)		
<i>H. influenzae</i> <sup>b</sup>	–1.2	2.2	2.3	2.1	1.0		
<b>Cefuroxime</b>							
<i>S. pyogenes</i>	3.1 (2.7–3.7)	3.7 (3.2–4.0)	1.1 <sup>c</sup> (0.9–1.3)	2.0 <sup>d</sup> (1.7–2.5)	3.2 (2.8–3.5)		
<i>H. influenzae</i> <sup>b</sup>	–0.6	2.4	2.8	2.7	1.6		
<b>Sparfloxacin</b>							
<i>S. pyogenes</i>	2.1 (1.7–2.3)	2.4 (2.0–2.6)	2.0 (1.7–2.2)	1.9 (1.8–2.0)	2.6 (2.4–3.0)		
<i>H. influenzae</i>	3.8 (3.7–3.9)	4.1 (4.0–4.2)	2.1 <sup>c</sup> (2.0–2.2)	2.6 <sup>c</sup> (2.5–2.8)	3.0 <sup>d</sup> (2.8–3.2)		
<b>Azithromycin</b>							
<i>S. pyogenes</i> <sup>e</sup>	5.2 (4.8–5.3)	1.3 (1.0–1.8)	2.5 <sup>d</sup> (2.4–2.5)	0.7 <sup>d</sup> (0.3–1.0)	0.5 <sup>d</sup> (0.0–0.7)	0.3 <sup>d</sup> (0.2–0.4)	0.3 <sup>d</sup> (0.3–0.3)
<i>H. influenzae</i> <sup>e</sup>	7.8 (7.5–8.2)	1.8 (1.5–2.2)	2.9 (1.8–3.8)	1.5 (1.2–1.9)	1.3 (0.8–1.8)	0.6 <sup>d</sup> (0.5–0.7)	0.5 <sup>d</sup> (0.5–0.5)

<sup>a</sup> Results are shown as means (ranges) of three experiments unless otherwise noted.

<sup>b</sup> One experiment.

<sup>c</sup>  $P < 0.001$ .

<sup>d</sup>  $P < 0.01$ .

<sup>e</sup> Five experiments.

(benzylpenicillin, sparfloxacin, and azithromycin) or 1:50 (cefuroxime) in Todd-Hewitt broth. The strain of *H. influenzae* was diluted 1:10 in PDM broth (benzylpenicillin, cefuroxime, and azithromycin) or used undiluted (sparfloxacin). The controls were similarly washed and diluted 1:1,000 in order to reach approximately the same inoculum as the exposed strains. The cultures were then reincubated at 37°C and samples were drawn at –2 h, at 0 h (before and after washing) and then hourly up to 7 h, at 9 h, and at 12 h. The samples were, if necessary, diluted in phosphate-buffered saline, and three dilutions of each sample were then spread on blood agar plates, incubated at 37°C in 5% CO<sub>2</sub> in air for 24 h, and counted. Only plates with 10 to 500 colonies were counted in the study. The PAE was defined according to the work of Craig and Gudmundsson (6) as  $PAE = T - C$ , where  $T$  is the time required for the viable counts of the exposed bacteria to increase by one log<sub>10</sub> above the counts observed immediately after washing and  $C$  is the corresponding time for the unexposed controls.

**Determination of killing kinetics of antibiotics against bacteria in PA phase.** To study the ability of the antibiotics to kill bacteria in the PA phase, the 10× MIC of the same antibiotic used for the induction of the PA phase was added to the preexposed bacteria immediately after washing and dilution (0 h) and after 2 and 4 h in the PA phase. Due to long-duration PAEs induced by azithromycin, the bacteria in PA phase were also exposed after 6 and 8 h. A control culture not previously exposed to the antibiotic was used after washing and dilution (1:1,000) at time zero. Samples were then drawn at –2 h, 0 h, hourly up to 7 h, at 9 h, and at 12 h; diluted; and counted as described above. The bacteria exposed to azithromycin at 6 and 8 h were also drawn hourly up to 10 and 12 h, respectively. The killing rate was calculated as the decrease in log<sub>10</sub> CFU during 4 h after the exposure to the antibiotic. Most experiments were performed in triplicate with the exception of the experiments with azithromycin, which were performed five times. Since no PAE was seen with the β-lactam antibiotics and gram-negative bacteria, these experiments were performed only once.

**Statistics.** The Student  $t$  test for unpaired samples was used to compare the PAEs and the killing in PA phase.

## RESULTS

**MICs.** The MICs of benzylpenicillin and cefuroxime were 0.016 mg/liter against *S. pyogenes* and 0.5 and 2 mg/liter, respectively, against *H. influenzae*. Sparfloxacin had a MIC of 0.25 mg/liter against *S. pyogenes* and 0.03 mg/liter against *H. influenzae*. The corresponding values for azithromycin were 0.1 and 1 mg/liter, respectively.

**The PAEs.** Azithromycin produced significantly longer PAEs against *S. pyogenes* compared to those of sparfloxacin and cefuroxime ( $P < 0.01$ ). The difference between azithromycin and benzylpenicillin did not reach significance ( $P = 0.06$ ), which was probably due to one discordant experiment with benzylpenicillin. Also, against *H. influenzae* azithromycin produced a significantly longer PAE compared to that of spar-

floxacin ( $P < 0.001$ ). As expected, no PAE was noted for the β-lactam antibiotics against *H. influenzae* (Table 1).

**The killing kinetics of antibiotics for bacteria in the PA phase.** There was significantly slower killing of *H. influenzae* in the PA phase when the test bacteria were reexposed to sparfloxacin (Fig. 1) compared to the controls both in the initial phase and at 2 and 4 h, respectively ( $P < 0.01$ ). There was a tendency for restoration of the bactericidal activity at the end of the PA phase (3 log<sub>10</sub> CFU killing versus 4.1 log<sub>10</sub> CFU for the controls). Another antibiotic that exhibited an early bactericidal effect was cefuroxime against *S. pyogenes*. Also here, significantly reduced killing was noted in the PA phase at time zero and 2 h. The PAE duration was in this combination shorter compared to that of sparfloxacin and *H. influenzae*, which probably explains the restoration of the bactericidal effect at 4 h. However, in the combinations with a slower bactericidal activity and short PAEs (benzylpenicillin-*S. pyogenes* [Fig. 2] and sparfloxacin-*S. pyogenes*), there was no difference in killing between the control and the test bacteria in PA phase. Azithromycin induced long PAEs against both *S. pyogenes* and *H. influenzae* and exhibited a delayed bactericidal

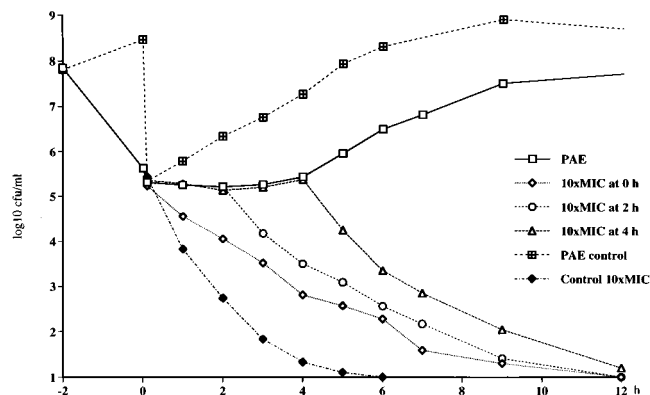


FIG. 1. The killing effect of sparfloxacin on *H. influenzae* in the PA phase. Results are shown as mean CFU from three experiments.

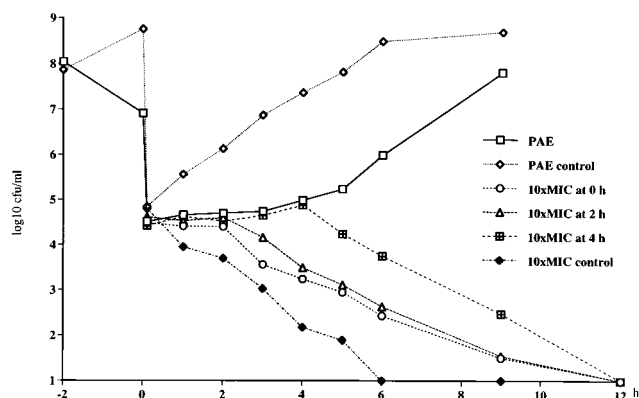


FIG. 2. The killing effect of benzylpenicillin on *S. pyogenes* in the PA phase. Results are shown as mean CFU from three experiments.

effect on both the control and the test bacteria in PA phase, especially at the end of the PAE, when the killing was almost bacteriostatic (Fig. 3). The fastest killing was noted for both the bacterial species in the initial PA phase, which was significant for *S. pyogenes*. As expected, benzylpenicillin and cefuroxime did not induce a PAE against *H. influenzae*, and no differences in killing of the controls and the test bacteria earlier exposed to the antibiotics were seen (Table 1).

## DISCUSSION

Antibiotic tolerance was first described in 1970, when a mutant of *S. pneumoniae* that underwent a very slow loss of viability after exposure to penicillin, despite its growth rate and susceptibility to penicillin being identical to those of the parent strain, was described. It was later shown that this mutant produced a nonfunctional autolytic enzyme (22). This genotypic tolerance appears to be different from the tolerance seen when bacteria in a nongrowing state demonstrate an ability to resist the action of a wide variety of antibiotics. The first description of this so-called phenotypic tolerance was made by Wood and Smith, who studied serial histologic sections of consolidated lungs from rats with pneumococcal pneumonia. They showed that in zones with rapidly growing pneumococci, the bacteria were lysed after exposure to penicillin, while in zones in which the growth was slow or absent, the bacteria did not respond to the action of penicillin (29). Eagle also reported for streptococcal infection in mice that penicillin had an effect only on

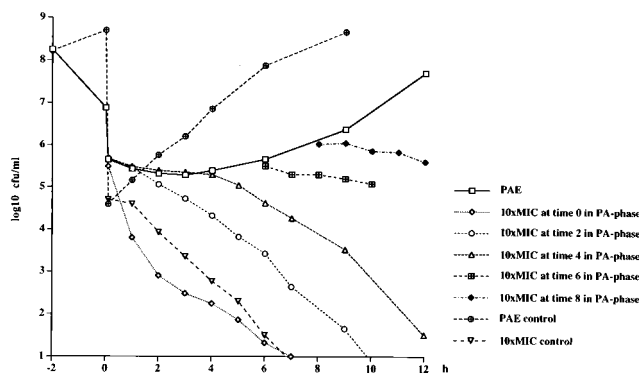


FIG. 3. The killing effect of azithromycin on *H. influenzae* in the PA phase. Results are shown as mean CFU from three experiments.

actively growing bacteria (7). In 1951, Jawetz et al. suggested that ongoing protein synthesis was critical to the activity of penicillin. They also showed that tolerance appeared when the synthesis of specific but unidentified proteins was disturbed (16). Phenotypic tolerance was later described in detail by Tuomanen, who studied the phenomenon in rabbits and showed a lack of response to penicillin on slowly growing pneumococci in cerebrospinal fluid (23). She also showed that the time between the addition of an antibiotic and the onset of bacterial lysis was directly proportional to the rate of bacterial growth prior to antibiotic exposure (25). In addition, it was also shown that the lytic activity of antibiotics was a function of the duration of starvation prior to the exposure to the antibiotic (5, 23, 24).

The PAE refers to the time period after complete removal of an antibiotic, during which there is delayed regrowth of the bacteria. This phenomenon was first described by Bigger in 1944 (1) and further studied by Parker and Marsh (21), but it was only many years thereafter that the PAE was recognized as an important pharmacodynamic parameter (6, 10, 12, 18). Bacteria in the initial PA phase seem to be nonmultiplying because of the fact that there is no DNA synthesis as measured by thymidine uptake (11, 19). Gerber and Craig showed that pneumococci brought into PA phase by erythromycin were relatively resistant to the action of ampicillin (10). Two years later, Vogelman et al. reported that *Escherichia coli* and *Klebsiella pneumoniae* in the PA phase induced by rifampin or erythromycin were less susceptible to aminoglycosides,  $\beta$ -lactams, and trimethoprim. The reduced rate of killing closely paralleled the duration of the PAE. However, killing of *Staphylococcus aureus* was only slightly reduced even with a PAE of 4 h. They concluded that bacteria in the PA phase are less susceptible to the bactericidal effect of antibiotics but that the degree of inhibition is dependent on both the organism and the antibiotic used for the killing in the PA phase (27). Gudmundsson et al. extended this study to include different strains of *E. coli*, *K. pneumoniae*, and *S. aureus*. The results were fairly similar to those of the first study. They found in most instances that the inhibition of the bactericidal action of the second antibiotic increased with longer PAE periods. However, they also found no significant decrease in bactericidal activity when *S. aureus* brought into PA phase with erythromycin was challenged with gentamicin and nafcillin as the second dose (13).

In contrast to these studies, in which one class of antibiotics had been used for the induction of the PAE and another had been used for exposure during the PA phase, we previously investigated the ability of benzylpenicillin to kill *S. pyogenes* in the PA phase when the same antibiotic was used both for the induction of the PAE and as the bactericidal agent in the PA phase. In these experiments, we found no difference in bactericidal activity from that for an unexposed control (19). This was later also shown in vivo for rabbits with implanted tissue cages (20). In the present study, when *S. pyogenes* was exposed to benzylpenicillin during the PA phase, the bactericidal effect was only slightly delayed at time zero and the rate of killing did not significantly differ from that for the controls. This confirms our previous findings that the bactericidal mechanism is still operating during the PA phase in this combination. Similar results were obtained with sparfloxacin and *S. pyogenes*, for which no difference in killing between the controls and the test bacteria in PA phase was seen. This is in accordance with the findings of Gudmundsson et al. (13), in which they emphasized that the shorter the PAE, the less the difference in bactericidal activity between that against cells in PA phase and that for control organisms. The relatively long PAE of benzylpenicillin and *S. pyogenes* determined in the present study was due to one

discordant experiment. Earlier experiments both in vitro and in vivo have reported a PAE of approximately 2 h in duration (6, 19, 20). Also in accordance with the results of Gudmundsson et al. (13), no difference between the controls and the bacteria previously exposed to antibiotics was demonstrated when no PAE was recorded ( $\beta$ -lactam antibiotics and gram-negative bacteria). The reduced killing seen at 4 h could be explained by the fact that at this time point, the bacteria had reached an inoculum of more than  $10^8$  CFU/ml. In the combinations cefuroxime-*S. pyogenes* and sparflloxacin-*H. influenzae*, longer PAEs were noted, as was also significantly reduced killing of the bacteria in the PA phase at time zero and 2 h. At 4 h, the bactericidal activity was restored with cefuroxime-*S. pyogenes*, and that tendency was also seen with sparflloxacin-*H. influenzae*, even if there was still significantly reduced killing compared to that with the controls. Both these combinations also had a very early bactericidal effect on the control organisms compared to those of sparflloxacin and benzylpenicillin against *S. pyogenes*, which might have influenced the results in that it would be easier to demonstrate a significant difference in killing if the bactericidal effect of the controls were pronounced. Azithromycin induced long PAEs against both *S. pyogenes* and *H. influenzae* and exhibited a delayed bactericidal effect on both the controls and the test bacteria in PA phase. The killing of the bacteria in PA phase was significantly reduced at all times with *S. pyogenes* and at 6 and 8 h with *H. influenzae*. The fastest killing, in contrast to all the other combinations, was noted for azithromycin against both bacterial species in the initial PA phase ( $P < 0.01$  and  $P < 0.02$ , respectively, compared to the controls). The reason for this is unknown, but it seems that azithromycin has an initial lag phase before the bactericidal activity is initiated in previously unexposed bacteria. That is, the killing is slower in the first 4 h compared to the next 4 h ( $1.3 \log_{10}$  CFU versus  $2.2 \log_{10}$  CFU for *S. pyogenes* and  $1.8$  versus  $>2.5 \log_{10}$  CFU for *H. influenzae*, respectively). In contrast, the bacteria at time zero in PA phase have already been exposed to the drug for 2 h during the induction of the PA phase and are then instantly reexposed to a  $10\times$  MIC for another 4 h.

The reason behind the differences among the classes of antibiotics in killing bacteria in the PA phase is unclear. The mechanism behind the PAE of  $\beta$ -lactam antibiotics has been suggested to be due to the time period necessary for resynthesis of new penicillin-binding proteins (6, 24, 30). However, the difference seen in our study between benzylpenicillin and cefuroxime could probably be explained by a greater bactericidal activity of cefuroxime. The mechanism behind the PAE of protein synthesis inhibitors is suggested to be caused by sublethal binding of the drug to the ribosomes with a subsequent disruption of protein synthesis (6). Normal RNA synthesis is probably required for the effect of these drugs, and with their long-lasting PAEs, it could be speculated that this protein synthesis requires a longer time to restore compared with that of the synthesis of new penicillin-binding proteins, which would explain the continuously decreased activity of azithromycin for bacteria in the PA phase and even after the PA phase had ended. This continuous reduced killing was not due to an inoculum effect, since there was no difference in the ability of azithromycin to kill bacteria with high or low inoculum (data not shown).

The results in this study have shown that in antibiotic-bacterial combinations with a relatively long PAE and a pronounced bactericidal effect, there is a reduced susceptibility to the action of antibiotics of the bacteria in the PA phase, which is restored after the PAE has ended. However, with a relatively shorter PAE and less bactericidal activity, less difference in

killing against cells in PA phase from that with control organisms is seen. Azithromycin behaved differently. The drug not only induced the longest PAE but also had the least bactericidal effect compared to the other antibiotics. A significantly reduced killing in the PA phase was noted at all times (except time zero) with *S. pyogenes* and at 6 and 8 h with *H. influenzae*. Even when the PA phase had ended at 8 h, the bactericidal activity was not restored. Craig and coworkers have shown in an animal model that the most important pharmacokinetic parameter correlating with efficacy for azithromycin is the ratio of area under the curve to the MIC, which allows long dosage intervals for these compounds (6, 17, 28). Our findings in this study support the concept that a certain interval between doses, restoring full bactericidal action, may be beneficial in optimizing the efficacy of this drug.

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