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 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 the bacterial species. 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 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 Lakemedel, Södertälje, Sweden), cefuroxime (Glaxo AB, Göteborg, Sweden), azithromycin (Pfizer AB, Täby, Sweden), and sparfloxacin (Rhône-Poulec, 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 1880, 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% CO2 in air, resulting in approximately 106 CFU/ml, and H. influenzae was grown in PDM broth (Progressive Diagnostics Manufactures; Biódisk, Solna, Sweden) supplemented with 30 mg of hemin and 1% IsoVitalexX for 6 h at 37°C in 5% CO2 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 105 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 10-1 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.
(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% CO₂ 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 log₁₀ above the counts observed immediately after washing and C is the corresponding time for the unexposed controls.

### Table 1. Killing in the PA phase

<table>
<thead>
<tr>
<th>Antibiotic and organism</th>
<th>PAE (h)</th>
<th>Control 0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylpenicillin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>3.3 (2.2–4.7)</td>
<td>1.8 (1.2–2.3)</td>
<td>1.2 (1.0–1.5)</td>
<td>1.9 (1.6–2.3)</td>
<td>1.8 (1.2–2.3)</td>
<td></td>
</tr>
<tr>
<td>H. influenzae</td>
<td>-1.2</td>
<td>2.2</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>3.1 (2.7–3.7)</td>
<td>3.7 (3.2–4.0)</td>
<td>1.1 (0.9–1.3)</td>
<td>2.0 (1.7–2.5)</td>
<td>3.2 (2.8–3.5)</td>
<td></td>
</tr>
<tr>
<td>H. influenzae</td>
<td>0.6</td>
<td>2.4</td>
<td>2.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>2.1 (1.7–2.3)</td>
<td>2.4 (2.0–2.6)</td>
<td>2.0 (1.7–2.2)</td>
<td>1.9 (1.8–2.0)</td>
<td>2.6 (2.4–3.0)</td>
<td></td>
</tr>
<tr>
<td>H. influenzae</td>
<td>3.8 (3.7–3.9)</td>
<td>4.1 (4.0–4.2)</td>
<td>2.1 (2.0–2.2)</td>
<td>2.6 (2.5–2.8)</td>
<td>3.0 (2.8–3.2)</td>
<td></td>
</tr>
<tr>
<td>Azithromycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>5.2 (4.8–5.3)</td>
<td>1.3 (1.0–1.8)</td>
<td>2.5 (2.4–2.5)</td>
<td>0.7 (0.3–1.0)</td>
<td>0.5 (0.0–0.7)</td>
<td>0.3 (0.2–0.4)</td>
</tr>
<tr>
<td>H. influenzae</td>
<td>7.8 (7.5–8.2)</td>
<td>1.8 (1.5–2.2)</td>
<td>2.9 (1.8–3.8)</td>
<td>1.5 (1.2–1.9)</td>
<td>1.3 (0.8–1.8)</td>
<td>0.6 (0.5–0.7)</td>
</tr>
</tbody>
</table>

* Results are shown as means (ranges) of three experiments unless otherwise noted.
* One experiment.
* P < 0.001.
* P < 0.01.
* Five experiments.

### 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 sparfloxacin (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 log₁₀ CFU killing versus 4.1 log₁₀ 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 effect.

![FIG. 1. The killing effect of sparfloxacin on H. influenzae in the PA phase. Results are shown as mean CFU from three experiments.](http://aac.asm.org/Downloaded from http://aac.asm.org)
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).
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 (β-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 10^6 CFU/ml. In the combinations ce-
furoxime-S. pyogenes and sparfloxacin-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 sparfloxacin-H. influen-
zae, even if there was still significantly reduced killing com-
pared to those of sparfloxacin and benzylpenicillin against S. pyogenes after the PAE has ended. However, with a relatively longer PAE and less bactericidal activity, less difference in killing against cells in PA phase from that with control organ-
isms is seen. Azithromycin behaved differently. The drug not only induced the longest PAE but also had the least bacteri-
cidal 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 PAE 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 pa-
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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