The post-exposure response of Enterobacteriaceae to ceftibuten

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The responses of ten isolates of Enterobacteriaceae to ceftibuten exposure were monitored by measuring several parameters. Post-antibiotic effect (PAE), control-related effective regrowth time (CERT) and post-antibiotic sub-MIC effect (PA-SME) were determined by bacterial enumeration carried out either by impedance in combination with viable counting (IMP/VC) or by impedance in combination with bioluminescence (IMP/BIO). Kill curves were carried out by bioluminescence, viable counting and direct microscopy and post-exposure morphology was established. Ceftibuten primarily provoked filamentation. Over 24 h, kill of up to 3.6 log_{10} was evident by viable counting and direct microscopy at and above the MIC. Minimal kill, of up to 0.26 log_{10}, was shown by bioluminescence. PAE was found to be method dependent, with statistical differences established by Student’s t-test. PAE values of up to 0.48 h and 1.47 h (by IMP/BIO and IMP/VC respectively) were not concentration dependent above 1 × MIC. CERT values were not method dependent, with values of up to 1.71 h also showing a lack of concentration dependence above 1 × MIC. PA-SME may reflect the situation in vivo more accurately than either PAE or CERT. In PAE and CERT studies the antibiotic is eliminated almost immediately, whereas in vivo there is gradual decrease in antibiotic levels. These persisting levels are reflected more accurately by PA-SME. Compared with PAE and CERT, significantly longer values, of up to 7.27 h, were obtained by PA-SME, although this parameter was also found to be method dependent. The results of the PA-SME studies, which may be the most clinically relevant pharmacodynamic parameter, confirm the appropriateness of the current once- or twice-daily dosing schedules despite the lack of PAE.

Introduction

Pharmacodynamics is broadly described as the study of the interaction of antimicrobial agents with microorganisms. More specifically, this area relates the concentration of an antimicrobial agent over time to the antimicrobial effect occurring at the site of infection and is thus increasingly being applied to the design of antibiotic dosing regimens.

This study is an investigation of the following pharmacodynamic parameters: post-antibiotic effect (PAE), post-antibiotic sub-MIC effect (PA-SME) and control-related effective regrowth time (CERT). These parameters were studied in relation to morphology and kill induced in ten strains of Enterobacteriaceae by ceftibuten. PAE measures the delayed regrowth of bacteria after antibiotic exposure.¹ The emphasis placed on PAE is questionable as, increasingly, it is recognized that this parameter is dependent on the methods used to quantify bacterial cells. CERT, which is a measure of both PAE and kill, is independent of these methods. It has, therefore, been suggested that less emphasis should be placed on PAE data and that CERT should be used in future pharmacodynamic studies.² So far, this suggestion has not found favour, relatively few CERT studies having appeared in the literature. PA-SME is also considered by some to be a more important pharmacodynamic measurement than PAE, reflecting more closely the in-vivo state of slowly declining antibiotic concentrations with long periods of persistently sub-inhibitory concentrations.

If PAE is to remain pre-eminent in the realm of pharmacodynamics, careful consideration must be given to the methodology employed in enumerating bacterial cells. Methods which have been employed in the past include the ‘reference’ viable counting method,¹ spectrophotometry,² electronic particle counting,³ direct microscopy,⁴ bioluminescence assay of bacterial adenosine triphosphate (ATP)⁵,⁶ and impedance monitoring.⁷,⁸

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Traditional methods of bacterial enumeration employ viable counting, which relies on the growth of colony-forming units on a solid agar surface. The suitability of this method has been questioned when enumerating aberrant morphological forms induced by antibiotic exposure, as viable counting underestimates numbers of aberrant morphological forms, leading to an underestimation of the PAE. Previously, we have used the alternative techniques of the bioluminescence assay of ATP and impedance monitoring. We have found no significant differences in PAE measured by viable counting alone and in combination with impedance (IMP/VC) and also no significant differences by bioluminescence alone, and in combination with impedance (IMP/BIOL). We have found significant differences in PAE measured by IMP/VC and IMP/BIOL.

Materials and methods

Bacteria, antibiotics and culture media

The bacteria used in this study were a mixture of control strains and recent clinical isolates sensitive to ceftibuten and are listed in Table I. Strains CTX 224, CTX 603 and BLR 02 are multiply resistant, exhibiting extended spectrum β-lactamase (ESBL) activity due to an SHV type enzyme. I 112 and T 767 are clinical isolates which we have studied in the past. Mueller-Hinton broth was used throughout the study. Ceftibuten was provided by Schering-Plough Ltd (Welwyn Garden City, U K).

Table I. MIC values for test isolates

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (mg/L) for inoculum of 5 × 10⁵ cfu/mL</th>
<th>MIC (mg/L) for inoculum of 1 × 10⁸ cfu/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 25922</td>
<td>0.5</td>
<td>32</td>
</tr>
<tr>
<td>NCTC 10418</td>
<td>0.125</td>
<td>32</td>
</tr>
<tr>
<td>NCTC 8879</td>
<td>0.125</td>
<td>32</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 11228</td>
<td>0.016</td>
<td>16</td>
</tr>
<tr>
<td>NCTC 9633</td>
<td>0.125</td>
<td>16</td>
</tr>
<tr>
<td>I 112</td>
<td>0.125</td>
<td>&gt;64</td>
</tr>
<tr>
<td>T 767</td>
<td>0.06</td>
<td>32</td>
</tr>
<tr>
<td>CTX 224</td>
<td>4.0</td>
<td>32</td>
</tr>
<tr>
<td>CTX 603</td>
<td>8.0</td>
<td>32</td>
</tr>
<tr>
<td>Enterobacter agglomerans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLR 02</td>
<td>8.0</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

Determination of MICs

MICs were determined by the microdilution method according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines. The standard inoculum of 5 × 10⁵ cfu/mL was used in addition to an inoculum of 1 × 10⁸ cfu/mL.

Kill curves

Kill curves were carried out in duplicate by exposing test cultures to ceftibuten and establishing bacterial numbers by three methods. An overnight broth was diluted 1:100 and exposed to 0.1, 1 and 10 × MIC. A unexposed control was obtained by diluting an overnight broth 1:1000. Counts at times 0, 1, 2, 3, 4, 5, 6 and 24 h were determined by bioluminescence and viable counting. Counts by direct microscopy were determined up to 6 h after exposure to 0, 1, 10 and 100 × MIC.

Determination of PAE and CERT

Inocula of c. 10⁷ cfu/mL were exposed to ceftibuten (0.1-100 × MIC) for 2 h, after which antibiotic was eliminated by 1:1000 dilution in test medium. Regrowth of these and a control culture was followed by impedance (eight replicates) in combination with viable counting and bioluminescence (both of which were carried out in duplicate). PAE was calculated as the difference in time between antibiotic-exposed and unexposed cultures to reach 10⁷ cfu/mL (allowing for differences in their respective inocula). CERT was calculated as the difference in time after antibiotic exposure and elimination for test and control cultures to grow to their pre-exposure inocula plus 1 log

Determination of PA-SME

The primary method used is based on that of Odenholt-Tornqvist et al. and was carried out on selected test isolates as detailed in Table II. A n inoculum of approximately 10⁷ cfu/mL was exposed to 10 × MIC ceftibuten for 2 h, after which the cultures were diluted to achieve sub-MIC concentrations of 0.1, 0.2 and 0.3 × MIC. After the 2 h exposure, bioassays were performed to confirm the ceftibuten concentration. Regrowth was followed by impedance (eight replicates) in combination with viable counting (carried out in duplicate). Unexposed controls were run in parallel and PA-SME was calculated in the same way as PAE. In addition, as a comparison, test cultures were exposed to 100 × MIC for 2 h, followed by 1:1000 dilution, leaving 0.1 × MIC remaining in the cultures. The growth of these cultures was followed by IMP/VC and IMP/BIOL and the PA-SME calculated in the same manner as PAE.
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Determination of predominant morphological forms

Inocula of $10^7$ cfu/mL were exposed to ceftibuten (0.1–100 $\times$ MIC) for 2 h, after which the cultures were observed by interference contrast microscopy and the predominant morphological forms present were recorded.

Results

The MIC values for the test strains against ceftibuten can be found in Table I; all fall within the sensitive range, given that ceftibuten has a breakpoint value of 8 mg/L. Although still active against the ESBL producers, ceftibuten is markedly less active against these three isolates. In common with other filament-inducing antibiotics in its class, ceftibuten showed a marked inoculum effect, with significant increases in MIC at an inoculum of approximately $10^8$ cfu/mL. The elevations in MICs which resulted ranged from 11 to two dilution steps.

Figure 1 presents mean kill-curve data for all ten test isolates. By viable counting (Figure 1a) the cultures exposed to 0 and 0.1 $\times$ MIC showed no kill although growth was slower in the culture exposed to 0.1 $\times$ MIC. The 100 $\times$ MIC culture showed kill over the 24 h test period whereas all of the other cultures demonstrated kill over 6 h followed by concentration-dependent regrowth during the 6–24 h period. Direct microscopy (Figure 1c) gave similar results, with similar kill patterns after exposure to 1, 10 and 100 $\times$ MIC over the 6 h test period. Kill curves by bioluminescence (Figure 1b) gave similar patterns for the 0 and 0.1 $\times$ MIC exposed cultures, but not for the cultures exposed to ceftibuten concentrations of 1 $\times$ MIC or above. During the first 2 h no kill was demonstrated: in fact, growth of over 1 log$_{10}$ was observed.

Over the range of test organisms, after 2 h of exposure to ceftibuten at 0.1 to 100 $\times$ MIC, mean differences in bacterial numbers by viable counting and bioluminescence are presented in Figure 2. These differences in counts were concentration dependent up to 4 $\times$ MIC, after which a plateau effect was demonstrated.

Table III presents mean PAE and CERT values as well as the predominant morphological responses exhibited by each culture in response to ceftibuten. Using Student’s t-test, the 15 mean PAE values determined by IMP/VC were compared with those determined by IMP/BIOL. The differences in PAE values were found to be highly significant ($t = 8.67$). By contrast, using the t-test, the mean CERT values determined by IMP/VC and IMP/BIOL were found not to be significantly different ($t = 1.61$).

Figure 3 illustrates how PAE, CERT and differences in counts by bioluminescence and viable counting vary with the predominant morphological form present. There is a consistent trend whereby the greatest values were measured in cultures made up of filaments and the smallest values were measured in cultures made up of bacilli/long bacilli.

Table II presents the PAE-SME values obtained for the five strains tested. PAE-SME values are significantly longer than PAE values and are concentration dependent. PAE-SME values obtained after exposure to 100 $\times$ MIC followed by 0.1 $\times$ MIC were determined for all ten isolates by both IMP/VC and IMP/BIOL. By the former the mean value was 1.10 h (range –0.33 to 7.17) and by the latter 1.94 h (range 0.30 to 7.27). The ten PAE-SME values by these two methods are significantly different ($t = 4.79$).

Discussion

This study found that, after exposing the test Enterobacteriaceae to ceftibuten, most of the cultures were made up of filamentous forms (Table III) as a result of
preferential binding to penicillin binding protein 3 (PBP3) and also to PBPs 1a and 1b. Braga & Piatti also found that after exposing Escherichia coli isolates to ceftibuten some of the filaments bore polar or spherical enlargements, although these were rarer than filaments. In the current study filaments with spherical enlargements were almost as common as filaments amongst the Klebsiella pneumoniae isolates tested. To date no PBP profiles have been published for K. pneumoniae isolates, but it seems likely that ceftibuten binds to either PBP1 or

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**Figure 1.** Mean kill-curve data. Counts were determined by (a) viable counting, (b) bioluminescence, and (c) direct microscopy.

- Control
- 0.1 x MIC
- 1 x MIC
- 10 x MIC
- 100 x MIC
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PBP2 in addition to PBP3 in order to induce the spherical enlargements.

A aberrant morphological forms, such as filaments, are clinically beneficial in that they undergo phagocytosis more easily than bacilli, but they are problematic in the laboratory. In common with other investigators, we have found that, after antibiotic exposure, different cell enumeration methods quantify aberrant morphological forms to varying degrees. This is exemplified in Figure 2, which shows the concentration-dependent nature of the differing abilities of viable counting and bioluminescence to quantify cells after ceftibuten exposure. Counts determined by bioluminescence were greater than those by viable counting, probably because although one filament may contain as many as 20 genomes, it will be represented by only 1 cfu by viable counting. Upon cell division one filament will split into separate cells, giving the impression of a rapid increase in cell numbers. In contrast, the decrease in counts by bioluminescence was not as pronounced as that by viable counting because levels of intracellular ATP were measured directly. Bioluminescence may better represent aberrant morphological forms than viable counting but it also has disadvantages. A constant ATP content of bacterial cells is assumed, but after antibiotic exposure, an enlarged bacillus may contain more ATP than a healthy bacillus. A lso, if a TP from intact dead cells is measured, counts determined by BIOL will be falsely inflated, giving the impression of less kill than has

Table III. Summary of mean (± s.e.m.) PAE, CERT values and predominant morphological forms

| E. coli (three strains) | IMP/BIO | IMP/V | IMP/BIO | IMP/V | Predominant morphological forms
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>10 x MIC</td>
<td>0.40 ± 0.31</td>
<td>-0.30 ± 0.20</td>
<td>0.75 ± 0.15</td>
<td>0.56 ± 0.44</td>
<td>F (3)</td>
</tr>
<tr>
<td>4 x MIC</td>
<td>0.27 ± 0.15</td>
<td>-0.44 ± 0.14</td>
<td>0.44 ± 0.35</td>
<td>0.45 ± 0.36</td>
<td>F (3)</td>
</tr>
<tr>
<td>1 x MIC</td>
<td>0.31 ± 0.31</td>
<td>-0.37 ± 0.25</td>
<td>0.42 ± 0.43</td>
<td>0.41 ± 0.44</td>
<td>F (3)</td>
</tr>
<tr>
<td>0.1 x MIC</td>
<td>0.04 ± 0.24</td>
<td>-0.28 ± 0.50</td>
<td>0.12 ± 0.40</td>
<td>0.12 ± 0.39</td>
<td>F (1), B (2)</td>
</tr>
<tr>
<td>K. pneumoniae (six strains)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 x MIC</td>
<td>0.68 ± 0.57</td>
<td>-0.34 ± 0.22</td>
<td>0.81 ± 0.47</td>
<td>0.81 ± 0.47</td>
<td>F (4), BF (1), B (1)</td>
</tr>
<tr>
<td>4 x MIC</td>
<td>0.64 ± 0.61</td>
<td>-0.50 ± 0.18</td>
<td>0.78 ± 0.45</td>
<td>0.78 ± 0.46</td>
<td>F (3), BF (2), B (1)</td>
</tr>
<tr>
<td>1 x MIC</td>
<td>0.60 ± 0.37</td>
<td>-0.45 ± 0.20</td>
<td>0.71 ± 0.31</td>
<td>0.71 ± 0.46</td>
<td>F (1), BF (4), B (1)</td>
</tr>
<tr>
<td>0.1 x MIC</td>
<td>-0.13 ± 1.01</td>
<td>-0.79 ± 0.41</td>
<td>0.17 ± 0.86</td>
<td>0.17 ± 1.04</td>
<td>F (1), BF (2), B (3)</td>
</tr>
<tr>
<td>E. agglomerans (one strain)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 x MIC</td>
<td>1.47</td>
<td>0.48</td>
<td>1.68</td>
<td>1.71</td>
<td>F</td>
</tr>
<tr>
<td>4 x MIC</td>
<td>1.47</td>
<td>0.33</td>
<td>1.78</td>
<td>1.71</td>
<td>F</td>
</tr>
<tr>
<td>1 x MIC</td>
<td>1.44</td>
<td>0.52</td>
<td>1.76</td>
<td>1.68</td>
<td>F</td>
</tr>
<tr>
<td>0.1 x MIC</td>
<td>0.14</td>
<td>0.18</td>
<td>-0.18</td>
<td>-0.21</td>
<td>LB</td>
</tr>
</tbody>
</table>

Key: B, bacilli; LB, long bacilli; F, filaments; BF, filaments with bulges.

Figure 2. Mean differences in counts determined by bioluminescence and viable counts.

Figure 3. Predominant morphological forms versus mean differences in counts by bioluminescence and viable counts, mean PAE (IMP/BIO), and mean CERT (IMP/BIO).
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Figure 4. CERT values for three individual E. coli isolates: ATCC 25922 ( ■ ), NCTC 10418 ( ● ) and NCTC 8879 ( ○ ).

actually taken place. This may account for the difference between the kill curves determined by bioluminescence and those determined by viable counting and direct microscopy.

The concentration-dependent nature of the differences in post-exposure counts (Figure 2) was most marked up to $4 \times \text{MIC}$ after a plateau effect was seen. This correlates with the morphological responses shown by the test cultures in response to ceftibuten exposure. It was only above $4 \times \text{MIC}$ that all of the cultures showed a predominance of filaments (Table III). The largest differences in counts after ceftibuten exposure and the longest PAE and CERT values were found in cultures made up of filaments. PAE and CERT may represent the time required for filamentous forms to resynthesize new PBPs, or the time during which antibiotic which has accumulated in the periplasmic space continues to inhibit newly formed PBPs. They may also represent the time during which the cells regenerate active enzyme molecules after the bound antibiotic has dissociated from the target site. The rate of synthesis varies for different bacteria and this could account for corresponding variations in the duration of both the PAE and CERT.

Impedance monitoring brings with it the associated benefits of an automated system. The only disadvantage is that the initial enumeration of bacterial cells must be performed by an additional method and this value used as a baseline. PAE and CERT values determined by IMP/VC and IMP/BIOL are presented in Table III. Differing abilities of viable counting and bioluminescence to enumerate aberrant morphological forms translated into highly significant differences between PAE values determined by IMP/VC and IMP/BIOL (Student's t-test, $t = 8.67$). Negative PAE values determined by IMP/VC are clearly artefactual and a function of the methodology. In contrast there were no significant differences between the CERT values ($t = 1.61$). As discussed previously, viable counting of aberrant morphological forms probably yields falsely low counts, which leads to an inflation of the bactericidal activity and an underestimation of PAE when calculated by viable counting, both alone and in combination with impedance monitoring.

Different strains of the same species do not respond in the same way to a specific antibiotic as demonstrated by the large standard deviation values in Table III. To illustrate this further, the CERT values for the three isolates of E. coli are presented in Figure 4. Differences in these values are most marked at $100 \times \text{MIC}$ with a range of 0.25–4.11 h. As PAE is method dependent but CERT is not, CERT values would yield a more meaningful pharmacodynamic parameter to compare the results of different research groups.

PAE and CERT determinations in vitro cannot reflect the situation in vivo. In vitro the antibiotic is eliminated almost instantaneously whereas in vivo there is a much more gradual decrease in antibiotic levels depending on the elimination half-life of the antibiotic. Once levels drop below the MIC, sub-inhibitory levels persist and this is more accurately reflected by measuring the PA-SME rather than the PAE. Five of the ten test isolates were investigated for PA-SME by IMP/VC. Sub-MIC levels which persisted beyond the 2 h $10 \times \text{MIC}$ exposure significantly increased the time taken for the cultures to regrow such that the PA-SME values were significantly longer than the PAE values. The effect was concentration dependent. In vivo, ceftibuten reaches maximal plasma concentrations within 2 h of oral dosing (15 mg/L after a 400 mg dose) and persists at detectable levels for $>16$ h (0.4 mg/L). Thus, PA-SME values are likely to be clinically relevant for many pathogens and confirm the appropriateness of the current once or twice a day dosing schedules despite the lack of PAE.

In conclusion, concentration-dependent differences in post-antibiotic exposure counts by bioluminescence and viable counting were found after exposure of the test Enterobacteriaceae to ceftibuten. These may form a direct relationship with morphological forms present in each culture. PAE, PA-SME and kill were found to be method dependent whereas CERT was not. Generally neither PAE nor CERT was concentration dependent above $1 \times \text{MIC}$, although different strains of E. coli and K. pneumoniae responded to ceftibuten differently with respect to PAE and CERT. The results of the PA-SME studies, which may be the most clinically relevant pharmacodynamic parameter, confirm the appropriateness of the current once or twice a day dosing schedules despite the lack of PAE.

Acknowledgements

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Seventh Interscience Conference on Antimicrobial Agents and Chemotherapy, 28 September–1 October 1997, Toronto, Canada (Abstract A79).

References


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