Inoculum Effect of β-Lactam Antibiotics on Enterobacteriaceae

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Seven β-lactam antibiotics were studied for both their antimicrobial activity and the degree to which they produced inoculum effect on Escherichia coli, Klebsiella pneumoniae, and Salmonella typhimurium. Aztreonam, cefoperazone, and ceftazidime were poorly bactericidal, caused marked bacterial filamentation, and exhibited a large inoculum effect on E. coli, K. pneumoniae, and S. typhimurium. Cefotaxime and ceftriaxone were more rapidly bactericidal, caused only a moderate amount of filamentous forms, and exhibited a modest inoculum effect, while cefoxitin and imipenem both were rapidly bactericidal and exhibited only a minimal-to-no-inoculum effect. The inoculum effect did not correlate with drug stability during incubation with the bacteria.

Inoculum effect on these species of the family Enterobacteriaceae appears to be a manifestation of increase in optical density secondary to the development of filamentous bacterial forms with an increase in bacterial mass during exposure to antibiotics which are not rapidly bactericidal. These observations have a clear significance for the susceptibility testing of beta-lactam antibiotics when turbidity is used as a parameter to determine presence of bacterial growth.

Although the clinical importance of the inoculum effect of antibiotics is as yet unclear, it is of major importance in laboratory susceptibility testing (2, 4). The inoculum effect, or the dependence of susceptibility results on the inoculum size, has been shown to be particularly large and dramatic for Pseudomonas aeruginosa with the β-lactam antibiotics (4, 5). In that setting, those β-lactam antibiotics which did not rapidly kill the organisms but caused the organisms to form filamentous structures were shown to have large inoculum effects (5). The explanation for the presence and the extent of the inoculum effect is not known for the β-lactam antibiotics with members of the family Enterobacteriaceae, which are generally regarded as very susceptible to these antibiotics (1).

The following experiments were designed to determine the relationship between inoculum effect and the morphological changes induced in the gram-negative rods by the β-lactam antibiotics. We examined the ability of a variety of beta-lactam antibiotics to kill bacteria, to induce filamentous forms, and to exhibit an inoculum effect on Escherichia coli, Klebsiella pneumoniae, and Salmonella typhimurium.

**MATERIALS AND METHODS**

Isolates and antibiotics. One clinical isolate each of E. coli, K. pneumoniae, and Salmonella typhimurium was studied. These organisms were identified by the API system (Analytab Products, Plainview, N.Y.), conventional biochemical studies, and serological agglutination (salmonella). The isolates of E. coli and Salmonella typhimurium had typical susceptibility patterns and were susceptible to ampicillin, and K. pneumoniae was susceptible to cephalothin. All laboratory antibiotic test powders were obtained from their respective distributors and used according to the suggestions of the manufacturers. The antibiotics tested included: aztreonam, E. R. Squibb & Sons, Princeton, N.J.; cefotaxime, Hoechst-Roussel Pharmedicals Inc., Somerville, N.J.; cefoperazone, Roerig-Pfizer, Inc., New York, N.Y.; ceftazidime, Glaxo, Inc., Research Triangle Park, N.C.; ceftriaxone, Hoffmann-La Roche Inc., Nutley, N.J.; chloramphenicol, Parke, Davis & Co., Detroit, Mich.; gentamicin, Schering Corp., Bloomfield, N.J.; and imipenem, Merck Sharp & Dohme, Rahway, N.J.

Inoculum effect. The degree of the inoculum effect of each antibiotic on the three isolates was tested by both microbroth (7) and macrobroth (7) dilution with inocula of 5 x 10^5 and 5 x 10^6 CFU/ml. Tests were performed in calcium- and magnesium-supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.). The final volume in each test well was 0.1 ml and in each tube was 1.0 ml. At the lower inoculum, growth was defined as the detection of any turbidity or bacterial button after an 18-h incubation at 35°C. At the higher inoculum, growth was defined as any increase in turbidity over that of the original inoculum in medium containing 10% Formalin. Visual readings of the MICs presented little difficulty according to these definitions, which were also used in an earlier study (5). The degree of inoculum effect was determined by the ratio of the MIC obtained at the higher inoculum to the MIC obtained at the standard inoculum of 5 x 10^5 CFU/ml. All inoculum sizes were confirmed by quantitative subcultures.

Killing kinetics. Determinations of the bactericidal activity of the antibiotics were performed with divalent cation-supplemented Mueller-Hinton broth. All antibiotics were used at 4 x MIC which was obtained by using inoculums of 5 x 10^5 CFU/ml. Subcultures or dilutions of subcultures (0.1 ml) were streaked on blood agar surfaces at 0, 3, 6, and 24 h of incubation, and the viable colonies were determined after an overnight incubation of the agar plates.

Quantitative determinations of optical density. During the killing kinetic experiments, contents of tubes containing a high inoculum of bacteria were subjected to optical density measurements at 0, 3, 6, and 24 h of incubation. Optical density measurements were made in a Gilford 1200 visible/UV spectrophotometer (Leeds & Northrup, Inc., North Wales, Penn.) with the narrowest possible slit width setting and at 580 nm and with a 1-cm cell pathway.

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TABLE 1. Inoculum effect of β-lactam antibiotics on E. coli, Salmonella typhimurium, and K. pneumoniae

<table>
<thead>
<tr>
<th>Inocula (CFU/ml)</th>
<th>Aztreonam</th>
<th>Ceftazidine</th>
<th>Cefoperazone</th>
<th>Ceftriaxone</th>
<th>Cefotaxime</th>
<th>Imipenem</th>
<th>Cefoxitin</th>
<th>Gentamicin</th>
<th>Chloramphenicol</th>
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<tbody>
<tr>
<td></td>
<td>MIC ratio</td>
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<td>E. coli</td>
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<tr>
<td>5 × 10⁴</td>
<td>0.5</td>
<td>&gt;128</td>
<td>0.12</td>
<td>0.25</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
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<tr>
<td>5 × 10⁵</td>
<td>&gt;64</td>
<td>64</td>
<td>8</td>
<td>8</td>
<td>0.5</td>
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<td>Salmonella</td>
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<td>typhimurium</td>
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<tr>
<td>5 × 10⁴</td>
<td>0.25</td>
<td>256</td>
<td>0.5</td>
<td>1.0</td>
<td>0.06</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
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<tr>
<td>5 × 10⁵</td>
<td>64</td>
<td>16</td>
<td>32</td>
<td>32</td>
<td>0.5</td>
<td>8</td>
<td>2</td>
<td>4</td>
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<tr>
<td>K. pneumoniae</td>
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<td>0.25</td>
<td>4</td>
<td>1</td>
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*All MICs are measured in micrograms per milliliter. The degree of inoculum effect was determined by the MIC ratio which is the ratio of the MIC determined at the higher inoculum to the MIC obtained at the standard inoculum of 5 × 10⁵ CFU/ml.

**Morphologic transformations.** The morphology of the bacteria was examined at 0, 3, 6, and 24 h of antibiotic exposure by microscopic examinations of the Gram stains.

Quantitation of antibiotics. Antibiotic levels were assayed in tubes of macrobroth dilution to determine whether antibiotic destruction was responsible for the inoculum effect. With each antibiotic, only tubes containing E. coli had antibiotic concentrations determined since the extents and patterns of inoculum effect for K. pneumoniae and Salmonella typhimurium were similar to those of E. coli. Tubes inoculated with 5 × 10⁵ CFU/ml and containing antibiotic concentrations midway between the MIC determined by an inoculum of 5 × 10⁵ CFU/ml and the apparent MIC with 5 × 10⁷ CFU/ml were chosen for antibiotic-level determinations.

The antibiotic levels were determined by bioassay. E. coli ATCC 25922 was used as the seed organism in Mueller-Hinton agar (Difco). Since antibiotic levels to be measured were sometimes not much higher than the MIC of the seed organism, the assay mixtures often had to be concentrated prior to testing. Portions (1 ml) of the assay mixtures or antibiotic standards were filtered through 0.22-μm pore-size filters (Millipore Corp., Bedford, Mass.) to remove the organisms. The filtrates were lyophilized to dryness and dissolved in 0.1 ml of distilled water (10-fold concentration). The resultant solutions were then bioassayed. Filtrates of E. coli grown in Mueller-Hinton broth containing no antibiotics and antibiotic solutions of the same concentrations as those added to the macrobroth dilution tubes were included as controls in the assay.

RESULTS

Degree of inoculum effect. The inocula used were reproducible, and quantitative subcultures showed a maximum of 20% variation from that of the desired densities, i.e., a range of 4.0 × 10⁵ to 6.0 × 10⁵ or 4.0 × 10⁷ to 6.0 × 10⁷ CFU/ml. The MICs at both inoculum sizes derived from macrobroth dilution are shown in Table 1. The results of microbroth dilution were within 1 dilution of these results. The increase in the MIC with the higher inoculum over that of the standard inoculum of 5 × 10⁵ CFU/ml was consistently the largest for aztreonam. The broad-spectrum cephalosporins, cefotaxime, ceftriaxone, cefazidime, and cefoperazone showed a wide range of inoculum effects. Cefotaxime had the lowest MIC ratio of the broad-spectrum cephalosporins (ratio of 8), while ceftriaxone had a ratio of up to 16. Cefazidime and cefoperazone had the highest ratios of ≥32.

In contrast, chloramphenicol, gentamicin, imipenem, and cefoxitin had a minimal-to-no-inoculum effect.

Killing kinetics. The rate of bacterial killing by each antibiotic differed, and most significantly, this rate was unaffected by the inoculum size (i.e., the log decreases achieved at various points in time were the same at both inocula of 5 × 10⁵ and 5 × 10⁷ CFU/ml). Beta-lactam antibiotics having the largest degrees of inoculum effect also demonstrated the slowest killing rates (Table 1 and Fig. 1, 2 and 3). Imipenem and cefoxitin had the smallest inoculum effects for the three Enterobacteriaceae species; these two antibiotics were also the most rapidly bactericidal. In general, for each antibiotic, the killing rates did not vary among the three bacterial species examined (Fig. 1, 2, and 3).

For comparison, the rates of killing by chloramphenicol and gentamicin for these organisms were also included. Although chloramphenicol showed no detectable bactericidal activity, it demonstrated no inoculum effect. Gentamicin was found to be the most rapidly bactericidal antibiotic, and it had no observable inoculum effect (Fig. 1, 2, and 3).

Optical densities and morphologic transformations. Aztreonam caused the greatest degree of filamentous formation for the bacteria tested, did not significantly change the viable colony counts, and produced the highest optical densities (Fig. 1, 2, and 3). The morphological changes of the bacteria are illustrated in Fig. 4. Cefazidime and cefoperazone also induced the appearance of highly filamentous forms, reduced the colony counts relatively little as compared with the other antibiotics, and led to significant optical density increases during incubation. Cefotaxime and ceftriaxone also caused filamentous forms, although to a lesser degree, but also decreased the colony counts by at least 2 logs and produced only a small increase in observed optical density. Cefoxitin produced only a minimal amount of filamentous forms and was able to rapidly reduce the number of viable bacteria, resulting in no increase in optical density. Imipenem caused bacterial balloon forms, rapid bacterial death, and only a minimal increase in optical density. For a comparison, chloramphenicol caused no observable morphological changes and resulted in no change in optical density or any reduction in the viability of the bacterial population, while gentamicin was rapidly bactericidal and produced no elongated forms or increase in optical density.

Antibiotic levels. Levels of antibiotics in macrobroth dilu-
INOCULUM EFFECT OF β-LACTAMS ON ENTEROBACTERIACEAE

FIG. 1. Turbidity change (A) and killing kinetics (B) of β-lactam antibiotics for $5 \times 10^7$ CFU of *E. coli* per ml. The antibiotic designations are AZ (aztreonam), CHL (chloramphenicol), CP (cefoperazone), CTR (ceftriaxone), CTX (cefotaxime), CTZ (ceftazidime), FOX (cefoxitin), GENT (gentamicin), and IP (imipenem).

FIG. 2. Turbidity change (A) and killing kinetics (B) of β-lactam antibiotics for $5 \times 10^7$ CFU of *K. pneumoniae* per ml.

DISCUSSION

The dependence of susceptibility results on inoculum size may occur with many groups of antibiotics for a wide variety of organisms. The tubes containing $5 \times 10^7$ CFU/ml were determined. Filtrates from the tubes containing only culture media and *E. coli* showed no inhibitory activity in the bioassay for the seed organism. The tubes chosen for bioassay were those with the β-lactam antibiotics at concentrations greater than that which inhibited growth at inocula of $5 \times 10^5$ CFU/ml but not as much as needed to inhibit the increase in turbidity of inocula of $5 \times 10^7$ CFU/ml. The measured antibiotic levels of these tubes after an 18-h incubation with the organisms are expressed as a percentage of the initial concentration: aztreonam, 91%; ceftazidime, 43%; cefoperazone, 28%; ceftriaxone, 95%; cefotaxime, 78%; imipenem, 43%; cefoxitin, 85%; gentamicin, 101%; and chloramphenicol, 97%. A parallel experiment to determine antibiotic levels in the test wells of microtiter plates in microbroth dilution was not possible since the levels were too low to be measured by bioassay and the well contents were too small to provide an adequate volume to be concentrated prior to bioassay.
of organisms. With chloramphenicol and gentamicin, this phenomenon is minimal or nonexistent, whereas with certain β-lactam antibiotics, this effect is of major importance (Table 1).

The explanations for the observed inoculum effect have not yet been clearly defined. One proposed explanation is that an organism may be less susceptible when present in large numbers because the combined production of β-lactamases (2, 8) or alternatively, because in a larger bacterial population, an occasional resistant variant would emerge which would shift the susceptibility results towards that of the more resistant (6). The evidence to support the former explanation is found with β-lactam antibiotics for Staphylococcus aureus (3). However, neither of these two proposed mechanisms can be the major cause of inoculum effect in the gram-negative rods. Many of the β-lactam antibiotics which exhibit an inoculum effect are β-lactamase stable, i.e., aztreonam, cefotaxime, cefazidime, and ceftriaxone. When measured, these β-lactam antibiotics were found to be present in their original potency despite luxurious growth of P. aeruginosa in the test tube (4). Independent of factors causing resistance of the gram-negative bacteria to the β-lactam antibiotics, the inoculum effect of the β-lactam antibiotics on a susceptible strain of P. aeruginosa has been shown to be due to an increase in bacterial mass in the presence of the antibiotic and in the absence of any marked bactericidal effect by the antibiotic (4).

The same situation appears to exist for the β-lactam antibiotics tested here for the three Enterobacteriaceae species. Loss of activity during an overnight incubation of a high inoculum of organisms as compared with a standard inoculum can account for at most 75% (ceftoperazone) or at most a two-tube increase in MIC (fourfold). With other antibiotics, such as ceftriaxone, antibiotic loss was minimal (5%) during a coincubation with 5 × 10^7 CFU of E. coli per ml. However, the inoculum effect for this antibiotic was a 16-fold MIC increase (0.06 μg/ml for an inoculum of 5 × 10^7 CFU/ml and 1.0 μg/ml for an inoculum of 5 × 10^7 CFU/ml).

The order of increasing bactericidal activity for the β-lactam antibiotics at 4 × MIC for the bacteria was aztreonam (the least), followed by cefoperazone and ceftazidime, then ceftriaxone and cefotaxime, and finally, imipenem and cefoxitin. The order of β-lactam antibiotics ranked by the degree of inoculum effect was the reverse order listed and correlated with the amount of filamentous forms produced in the bacteria.

While the major cause of inoculum effect for the β-lactam antibiotics on members of the family Enterobacteriaceae appears to be the same as that for these antibiotics on P. aeruginosa, some differences in the activity of individual antibiotics were noted. Ceftazidime, which had good bactericidal activity for P. aeruginosa in an earlier study (5), had only a minimal amount of bactericidal activity for the three Enterobacteriaceae species studied. Imipenem, in contrast, showed good bactericidal activity for both P. aeruginosa and the Enterobacteriaceae species and caused balloon formation of the latter organisms that resulted in cell death. Various β-lactam antibiotics apparently can exhibit different degrees of morphological changes with different bacterial species and can cause different degrees of inoculum effect and bactericidal activity.

In summary, the inoculum effect for the species studied was the result of visually noticeable increases in optical density resulting from the induction filamentous bacterial forms coupled with little reduction in the number of viable organisms. The increases in turbidity were most noticeable when the initial inoculum was high and the bacterial suspension was already visibly turbid. The inoculum effect was found to be especially large when the antibiotic caused a large degree of filamentous forms without any change in the number of viable bacteria even when the antibiotic concentration was increased. Chloramphenicol, although not bactericidal, demonstrated no inoculum effect as it did not induce filamentous forms, while gentamicin was bactericidal, failed to cause filamentous forms, and produced no inoculum effect. Both a lack of bacterial killing and the presence of induced filamentous forms are undoubtedly interrelated for the β-lactam antibiotics, but at what concen-

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**FIG. 3.** Turbidity change (A) and killing kinetics (B) of β-lactam antibiotics for 5 × 10^7 CFU of Salmonella typhimurium per ml.
INOCULUM EFFECT OF β-LACTAMS ON ENTEROBACTERIACEAE

FIG. 4. Gram stain morphology of Escherichia coli exposed to aztreonam (A), ceftazidime (B), cefoperazone (C), cefotaxime (D), imipenem (E), cefoxitin (F), and chloramphenicol (G).

Bacterial killing is achieved relative to the induction of filamentous forms would determine the degree of inoculum effect. Although the implications of inoculum effect on the effectiveness of these antibiotics in therapy are unknown, our observations have clear implications on susceptibility testing of these antibiotics for the gram-negative rods. When an inoculum of slightly greater than $5 \times 10^3$ CFU/ml is used, detectable turbidity or the presence of a bacterial button may occur in tubes or wells containing antibiotic concentrations which did not permit an increase in CFU per milliliter but did permit an increase in bacterial mass. Such turbidity may be interpreted by the laboratory as growth or resistance. At a lower inoculum, $10^4$ or $10^5$ CFU/ml, a similar extent of bacterial mass increase per organism would not result in visually detectable turbidity. Laboratory personnel should be aware of this alternate cause of turbidity with the β-lactam antibiotics, aside from the usual cause of bacterial multiplication. Our results emphasize the importance of carefully adjusting the inoculum to that presently recommended for macrobroth or microbroth dilution clinical susceptibility testing to avoid the presence of turbidity due to only bacterial morphologic alterations.

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LITERATURE CITED


