Mecillinam-Ampicillin Synergism in Experimental Enterobacteriaceae Meningitis

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The in vitro activities of mecillinam, a new ß-amidinopenicillin, and ampicillin, alone and in combination, against an Escherichia coli strain and a Klebsiella pneumoniae strain were compared, and these results were correlated with their respective activities in vivo in experimental meningitis. The mecillinam-ampicillin combination was synergistic in vitro against both strains when tested by a modified checkerboard technique (bacteriostatic synergy). However when quantitative bactericidal synergy studies were made, the relative bactericidal rate of the combination was more rapid than that of either drug alone ("bactericidal synergy") against the Escherichia coli isolate only. In a rabbit model of Enterobacteriaceae meningitis, in vivo bactericidal activity correlated with results obtained in vitro. Both drugs were administered by continuous intravenous infusion for 8 h. Serum and cerebrospinal fluid antibiotic levels were similar to those achieved in humans. Cerebrospinal fluid bacterial concentrations (colony-forming units [CFU] per milliliter) were quantitatively titrated at 2-h intervals. Both drugs, alone or the combination, were ineffective against the K. pneumoniae strain in vivo (change in titer <1 log in 8 h). In contrast, the combination produced a markedly enhanced bactericidal effect against the E. coli strain (mean ± standard deviation, decrease of log₁₀ CFU per milliliter of 3.65 ± 1.02) compared with those of ampicillin alone (decrease of log₁₀ CFU per milliliter of 0.07 ± 0.8) and mecillinam alone (decrease of log₁₀ CFU per milliliter of 1.6 ± 0.05) (P < 0.001). When bactericidal synergism can be demonstrated for mecillinam-ampicillin in vitro in a case of gram-negative-bacillary meningitis this combination may be useful in the therapy of the illness.

Gram-negative-bacillary meningitis is an uncommon but devastating form of central nervous system infection. Most cases of neonatal or post-neurosurgical meningitis are now caused by the Enterobacteriaceae or Pseudomonas (7, 9, 11). The mortality rate and frequency of neurological sequelae remain high (2, 17). Depressed host defenses, pathogenicity of the infecting organisms, foreign body placement, or numerous surgical procedures may all contribute to the poor therapeutic results (12, 13). In addition, antimicrobial therapy is difficult to administer and often ineffective. Most infecting strains are now resistant to ampicillin (12), and chloramphenicol has only a bacteriostatic effect. Since antibiotic and complement-mediated opsonic activity are absent in the cerebrospinal fluid (CSF) in bacterial meningitis (M. S. Simberkoff, N. H. Molderover, and J. J. Rahal, Abstr. Clin. Res. 27: 356A, 1979), bacteriostatic antibiotics may be ineffective in this disease. The use of chloramphenicol is limited further by the immature liver function of neonates and concerns with hematological toxicity. Results with either drug alone have been erratic (8, 11). The aminoglycosides are now considered the drugs of choice for gram-negative-bacillary meningitis, but their low therapeutic index and poor penetration into the CSF have necessitated direct instillation of these drugs into the CSF by either the lumbar or the ventricular route. The high rate of ventriculitis in these infections, especially in neonatal meningitis (3, 9), and the inability of gentamicin to reach ventricular sanctuaries of bacteria after intralumbar administration (8) have favored the intraventricular route. This mode of therapy notwithstanding, the mortality and complication rates remain high (13).

Mecillinam is a new ß-amidinopenicillin with a unique mode of action (6, 18) and a wide in vitro spectrum of activity, especially against the Enterobacteriaceae (4, 10, 19). In addition, this agent displays synergy at very low concentrations with other beta-lactam antibiotics against members of this family in vitro (1, 6, 16, 26). The two most commonly isolated species in gram-negative-bacillary meningitis, Escherichia coli and Klebsiella pneumoniae, are usually suscep-
tible to mecillinam (4) and synergistically inhibited by a mecillinam-beta-lactam combination (1, 16, 19). Since mecillinam may, like ampicillin, cross the inflamed meninges well, synergism might be demonstrable in vivo and cure achieved with systemic therapy alone and avoidance of toxic aminoglycosides. We tested this hypothesis in experimental models of Enterobacteriaceae meningitis.

The purposes of this study were: (i) to determine whether ampicillin and mecillinam act synergistically against representative Enterobacteriaceae meningeal pathogens in vitro, (ii) to assess the penetration of ampicillin and mecillinam into the infected CSF in animals with Enterobacteriaceae meningitis, and (iii) to compare the capacities of ampicillin alone, mecillinam alone, and the combination of these two agents to eradicate bacteria from the CSF.

MATERIALS AND METHODS

In vitro studies. The strain of E. coli used in these studies, K; antigen positive, was isolated from the CSF and blood of a 3-day-old premature infant at the University of Virginia Hospital, Charlottesville, in 1975. The strain of K. pneumoniae (HE71), provided through the courtesy of Hoffman-La Roche, Inc. (Nutley, N.J.), was obtained from an infant with meningitis. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for both strains were determined by a twofold broth dilution microtiter method. An overnight culture of the test strain in tryptic soy broth (Difco Laboratories, Detroit, Mich.), was diluted to ensure a final inoculum of 10^8 colony-forming units (CFU) per ml in NIH broth. The composition of NIH broth is (in grams per liter): yeast extract (Difco), 5; casein hydrolysate (Trypticase; BBL Microbiology Systems, Cockeysville, Md.), 15; dextrose, 1; NaCl, 2.5, and L-cysteine, 0.05. The osmolality and conductivity of NIH broth are low, and this medium is preferred for in vitro broth susceptibility tests with mecillinam (15), since other media produce more variable results. Twofold dilutions of each drug were carried out, and the microtiter plates were incubated for 18 h at 37°C. The MIC was defined as the lowest concentration of antibiotic which inhibited visible turbidity after this procedure. The MBC was defined as the lowest concentration of antibiotic which produced sterile cultures when the 18-h wells were subcultured onto blood agar plates and incubated for a further 24 h at 37°C. MICs for both drugs were also determined for a 10^-1 inoculum of the test organisms deposited onto Mueller-Hinton agar; in addition, MICs for mecillinam were measured on NIH agar. Unlike broth dilution, MIC results are comparable for mecillinam when determined by both of these agar dilution procedures (data on file, Hoffman-La Roche). Results were read after incubation at 37°C for 18 h. Multiple dilutions of ampicillin and mecillinam were also incorporated into Mueller-Hinton and NIH agars at 10:1 and 20:1 ratios, and the tests were repeated (modified checkerboard technique). By this technique, synergy is defined as at least a fourfold reduction in the MICs for both drugs.

Quantitative bactericidal studies were also performed in NIH broth. A dilution of an overnight culture of the test strain to give an inoculum of 10^6 CFU/ml was added to NIH broth in 25-ml cotton-stoppered flasks. When Klebsiella was the indicator, ampicillin (5 μg/ml), mecillinam (5 or 1 μg/ml), and various combinations of the two drugs were added. The combinations kept the ampicillin concentration constant (5 μg/ml) but varied the mecillinam concentration to achieve ratios of 10:1, 5:1, 3:1, 2:1, 1:1, and 1:5. When E. coli was the test species, the following concentrations were used (in micrograms per milliliter): ampicillin, 0.5; mecillinam, 0.1; or ampicillin, 0.5, plus mecillinam, 0.1. All flasks were incubated at 37°C in 5% CO₂ (or ambient air, with similar results) and continuously shaken. Samples were removed at 4, 8, 24, and 48 h and quantitatively cultured on tryptic soy agar (Difco) pour plates. These quantitative bactericidal studies were run in duplicate with essentially identical results. The criterion for synergism was a >2-log reduction in bacterial concentration at any of the time points sampled, when compared with the most active agent alone.

Animal model. The test organisms were grown overnight in tryptic soy broth, centrifuged at 3,000 rpm for 15 min, suspended in saline, and diluted to ensure a final inoculum of 10^7 CFU. Meningitis was produced in 2- to 3-kg New Zealand white rabbits as described previously (21, 24). A dental acrylic helmet was attached to each animal's skull, and the rabbit was suspended in a stereotaxic frame. A Quincke spinal needle (25 gauge by 3 inches [ca. 9.5 cm]) was inserted into the cisterna magna, and 0.3 ml of clear CSF was withdrawn. The test organisms, in a volume of 0.25 ml (10^7 CFU), were then injected intracisternally. The needle was withdrawn, and the animal was returned to its cage. Eighteen hours later, all animals had meningitis as manifested by fever greater than 40°C, neurological signs (seizures, opisthotonus), CSF leukocyte counts of 0.8 × 10^5 to 24 × 10^5/μl (mean, 4.07 × 10^5/μl; greater than 95% polymorphonuclear leukocytes), and titers of 10^6 to 10^8 CFU/ml of CSF.

Light anesthesia was induced by intravenous administration of 30 mg of pentobarbital (Barber Veterinary Supply, Richmond, Va.). Polyethylene catheters (Intramedic 7420; Becton, Dickinson & Co., Parsippany, N.J.) were inserted into the femoral artery and vein. Each animal was then repositioned in the stereotaxic frame with the spinal needle reinserted in the cisterna magna. An initial CSF sample (0.3 ml) was obtained for bacterial titer and leukocyte count. The treatment periods encompassed 8 h. Both drugs were dissolved in 34 ml of 0.9% NaCl and delivered through the femoral vein catheter via constant intravenous infusion by a Sage syringe infusion pump (model 351). The dosage used was 30 mg/kg per h for both agents. A loading dose, representing 20% of the total 8-h dose, was administered intravenously immediately before starting the infusion. The animals were kept lightly anesthetized with less than 10 mg of pentobarbital per kg per h. Fourteen animals received no antibiotics and served as controls.
Arterial blood samples (3 ml) and simultaneous CSF samples (0.2 ml) were collected at 0, 2, 4, 6, and 8 h of therapy. The CSF samples were cultured quantitatively with serial 10-fold dilutions in 0.9% NaCl and tryptic soy agar pour plates. The remainder of the CSF sample and the serum samples were kept at −70°C until antibiotic assays were done (within 2 weeks). This period of storage did not affect the assay results.

**Antibiotic assays.** Antibiotic concentrations were determined with agar well diffusion techniques. A *Bacillus subtilis* (control no. 640925; Difco) spore suspension (0.2 ml) was added to 100 ml of antibiotic medium no. 1 (adjusted to pH 6.0), poured into plates, and used for ampicillin level determinations. An ampicillin-resistant *E. coli* (Leo Ha2, kindly provided by Hoffman-La Roche) was used as the indicator strain for determination of mecillinam concentrations. This strain was maintained on slants of antibiotic medium no. 1 containing 100 μg of ampicillin per ml and transferred at weekly intervals. A fresh slant was inoculated the day before the assay and incubated at 34°C for 16 to 18 h. The growth was washed from the slants with 3 to 5 ml of sterile saline and adjusted to 90% turbidity at 650 nm on a Gould spectrophotometer. This adjusted suspension (20 ml) was added to 1,000 ml of antibiotic medium no. 1 and poured into plates. All assays were done in triplicate in 150-mm-diameter plates with an agar depth of 5 mm. Standard curves were constructed with known concentrations of each drug diluted in normal rabbit serum, saline, or a phosphate buffer, pH 7.2 (mecillinam only). This buffer contains (in grams per liter): KH2PO4, 2.73; K2HPO4, 8.32. No zone of inhibition was observed with either serum or CSF from untreated, infected rabbits in these assay systems. CSF standards were diluted in normal saline after zone sizes were found to be equivalent with dilution in normal saline, normal-rabbit CSF, or infected-rabbit CSF.

Analysis of data. The penetration of either drug into the CSF was calculated as: percent penetration = (CSF concentration/serum concentration) × 100. All statistical analysis was done on unaired data with Student’s *t* test.

**RESULTS**

**In vitro studies.** The NIH agar dilution MICs and MBCs for ampicillin, mecillinam, and various combinations of the two agents against the two strains used in the in vivo experiments are presented in Table 1. The MIC was always slightly higher for either drug when the NIH broth dilution procedure was compared with Mueller-Hinton or NIH agar dilution. Although the MICs obtained against the *K. pneumoniae* strain were much higher than levels achievable within the infected CSF (see Table 2), marked synergism was observed with the modified checkerboard technique for the ampicillin-mecillinam combination, and the combination produced inhibition at concentrations similar to those achievable in CSF (Table 1); 1.25 μg of ampicillin per ml combined with 0.125 μg of mecillinam per ml inhibited growth of the strain. However, the two drugs in various ratios and concentrations did not produce a more rapid bactericidal effect against *K. pneumoniae* than either drug alone when quantitative bactericidal studies were performed in NIH broth. In these studies, the bacterial concentrations (CFU per milliliter) at all time points did not differ significantly from those of controls when ampicillin (5 μg/ml), mecillinam (5 μg/ml), or various combinations (ampicillin/mecillinam in ratios of 1:5, 1:1, 2:1, 3:1, 5:1, and 10:1) were added to the flasks.

The MBCs obtained in NIH broth for the *E. coli* strain were 2 and 4 μg/ml for mecillinam and ampicillin, respectively, levels which are potentially achievable within infected CSF in vivo. In contrast to results with the *K. pneumoniae* strain, where only inhibitory synergism was demonstrable, an enhanced bactericidal effect ("bactericidal synergism") was observed for this *E. coli* strain in time-kill experiments in NIH broth (Fig. 1). The mean initial bacterial titer was log10 6.457 ± 0.023 (standard deviation) CFU/ml. Ampicillin (0.5 μg/ml) did not reduce bacterial titers when compared with control flasks at 4 and 24 h of incubation, and the final titer was essentially the same as the initial titer (log10 6.301 ± 0.069 CFU/ml). Mecillinam (0.1 μg/ml) reduced bacterial titers by approximately 4 logs at 24 h, but titers increased over 2 logs between 24 and 48 h (Fig. 1). In contrast, the combination of mecillinam and ampicillin achieved rapid bactericidal activity, with complete sterilization of the flasks in 24 h. Thus, the combination demonstrated synergism against both strains by checkerboard technique but produced an enhanced bactericidal effect only for the *E. coli* isolate.

**Penetration into CSF.** At the dosage used, serum and CSF antibiotic concentrations were similar for both drugs and did not differ significantly between the two types of meningitis.

**Table 1. In vitro activities of ampicillin, mecillinam, and various combinations against *K. pneumoniae* and *E. coli***

<table>
<thead>
<tr>
<th>Antibiotic(s)</th>
<th><em>K. pneumoniae</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (μg/ml)</td>
<td>MBC (μg/ml)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Mecillinam</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Ampicillin/mecillinam</td>
<td>10:1</td>
<td>1.25/0.125</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>2.50/0.125</td>
</tr>
</tbody>
</table>
Therefore, the concentrations (Table 2) are depicted for both drugs regardless of the infecting strain. Serum antibiotic concentrations attained steady-state levels by 2 h of constant intravenous infusion (the first time point sampled) and remained stable for 8 h. The mean (± standard deviation) serum levels were 22.6 ± 9.2 µg/ml for mecillinam and 22.4 ± 4.6 µg/ml for ampicillin, respectively. These values are similar to those found in humans after appropriate parenteral doses of each drug (12, 14, 27). The CSF levels reached steady states by 2 h and were reproducible between animals (Table 2). The mean (± standard deviation) CSF concentrations were 0.8 ± 0.5 and 2.6 ± 1.4 µg/ml for ampicillin and mecillinam, respectively. Despite virtually identical serum levels, mecillinam, achieved higher CSF levels than did ampicillin. The percent penetration, defined as the CSF concentration expressed as a percentage of the concurrent serum concentration, was nearly three times higher for mecillinam when compared with ampicillin (P < 0.01).

**Bacterial killing.** Bacterial killing in vivo was examined in the CSF of 22 rabbits with *Klebsiella* meningitis and 29 rabbits with *E. coli* meningitis. Measurements of CFU per milliliter were made in all rabbits before and 2, 4, 6, and 8 h after initiating antibiotic therapy. Seven untreated control animals in each group exhibited slight rises in bacterial titers after 8 h (Table 3); all died within 48 h of inoculation. The initial bacterial titers within the CSF did not differ significantly among the three treatment regimens in either type of meningitis (Table 3). These initial titers were usually between 10^4 and 10^6 CFU/ml of CSF. Bacterial killing was reproducible with each regimen despite slight variability in the initial bacterial titer.

None of the three regimens used produced a bactericidal effect in experimental *Klebsiella* meningitis (Table 3). Bacterial titers decreased more than 1 log in only one animal, and there were no significant differences between drug regimens with either agent alone or the combination. Thus, enhanced bactericidal activity was not demonstrable either in vivo or in vitro for this strain at the drug concentrations studied.

Ampicillin also failed to reduce bacterial titers when compared with untreated rabbits in *E. coli* meningitis (decrease of log_10 0.07 CFU/ml in 8 h) (Table 3). The bactericidal activity of mecillinam (decrease of log_10 1.59 CFU/ml in 8 h) was greater than that of ampicillin. This may reflect the lower MBC (2 µg/ml for mecillinam versus 4 µg/ml for ampicillin) and enhanced percent penetration of the former agent into the CSF. In contrast to *Klebsiella* meningitis, the bactericidal activity of the combination in *E. coli* meningitis (decrease of log_10 3.6 CFU/ml in 8 h) was far greater than that of either drug alone (P < 0.001). These results correlated with those of the in vitro studies.

**TABLE 2.** Antibiotic serum and CSF concentrations and penetrations into the CSF in experimental entero-bacteriaceae meningitis

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of animals</th>
<th>Serum concn (µg/ml)</th>
<th>CSF concn (µg/ml)</th>
<th>Penetration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mecillinam</td>
<td>14</td>
<td>22.6 ± 9.2</td>
<td>2.6 ± 1.4</td>
<td>93 ± 4.6</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>16</td>
<td>22.4 ± 4.6</td>
<td>0.8 ± 0.5</td>
<td>3.5 ± 1.5*</td>
</tr>
</tbody>
</table>

*P < 0.01
MECILLINAM-AMPICILLIN SYNERGISM IN MENINGITIS

DISCUSSION

In this study, the in vivo bactericidal activity of mecillinam was compared with those of ampicillin and the combination of mecillinam plus ampicillin in experimental Enterobacteriaceae meningitis due to K. pneumoniae or E. coli. Mecillinam penetrated the infected CSF in higher concentrations and was more active than ampicillin in experimental E. coli meningitis. When bactericidal synergy was evident in vitro, the combination exhibited enhanced bactericidal activity in vivo. In contrast, if only bacteriostatic synergy was present in vitro, the combination was no more effective in vivo than either drug alone. Since bactericidal and opsonic activities are absent in infected CSF (Simberkoff et al., Abstr. Clin. Res. 27:356A, 1979), bacterial meningitis represents an infection in an area of impaired host resistance. Thus, bactericidal agents or combinations may be necessary in this disease, as suggested in this study.

The structure and unique properties of mecillinam, a 6β-amidinopenicillin, were first described by Lund and Tybring in 1972 (10). Mecillinam differs from other beta-lactam antibiotics in two major respects (i) greater in vitro activity against certain gram-negative bacteria than against gram-positive organisms, the reverse of most penicillins and cephalosporins, and (ii) effects on the morphology of the test organisms (6, 20). This agent is highly active in vitro against the majority of Enterobacteriaceae meningal pathogens grown in standardized susceptibility testing media (NIH broth).

The observed synergy with other beta-lactam antibiotics may be due to the unique mode of action of mecillinam. Large cells are produced when E. coli is exposed to mecillinam in vitro, in contrast to the filamentous forms observed when this species is exposed to other beta-lactam antibiotics (18, 22). Mecillinam demonstrates remarkable affinity for penicillin-binding protein 2 in the cytoplasmic membrane (22). Other beta-lactams bind to this site with very low affinity, and their primary sites of action are elsewhere. These distinct modes of action may explain why mecillinam has synergistic activity against the Enterobacteriaceae when used in combination with other beta-lactams.

E. coli and the Klebsiella-Enterobacter group are the most common causative pathogens in neonatal meningitis and post-neurosurgical meningitis in adults (11, 13, 20). Many strains of these bacteria (including ampicillin-resistant strains) are susceptible to mecillinam in vitro. These forms of meningitis do not respond well to therapy. The mortality rate exceeds 30%, and a majority of survivors develop neurological sequelae (13). Combinations of mecillinam with other beta-lactam antibiotics may offer some advantages. This agent is highly active against many Enterobacteriaceae strains, and its penetration across the inflamed meninges is equivalent to that of ampicillin in this experimental meningitis model (23). Similar mean CSF ampicillin levels, of 1.2 to 2.9 μg/ml, have been found in humans receiving standard parenteral regimens (25). In this study we have demonstrated that enhanced bactericidal activity can be obtained in vivo with a mecillinam-ampicillin combination only when this property is demonstrated in vitro by quantitative bactericidal synergy techniques. These results suggest that this combination may be effective when given by the parenteral route alone in gram-negative-bacillary meningitis. The dosages used in this study produced serum levels slightly lower than those usually observed in humans; therefore, higher dosages may result in increased bacterial killing in vivo without an increase in toxicity. This combination deserves further study in the treatment of gram-negative-bacillary meningitis.

LITERATURE CITED

1. Baltimore, R. S., J. O. Klein, C. Wilcox, and M. Finland. 1976. Synergy of mecillinam (FL1060) with

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**Table 3. Bacterial killing by antibiotics in experimental enterobacteriaceae meningitis**

<table>
<thead>
<tr>
<th>Infecting species</th>
<th>Antibiotic(s)</th>
<th>No. of animals</th>
<th>Bacterial titer (log10 CFU/ml) [mean ± standard deviation]</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. pneumoniae</td>
<td>None</td>
<td>7</td>
<td>4.676 ± 0.480 +0.067 ± 0.501</td>
</tr>
<tr>
<td></td>
<td>Mecillinam</td>
<td>7</td>
<td>4.603 ± 1.658 -0.636 ± 0.240</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>7</td>
<td>4.384 ± 1.432 -0.437 ± 0.450</td>
</tr>
<tr>
<td></td>
<td>MeciUlinam-</td>
<td>8</td>
<td>4.565 ± 1.206 -0.531 ± 0.206</td>
</tr>
<tr>
<td></td>
<td>ampicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>None</td>
<td>7</td>
<td>4.838 ± 1.017 +0.121 ± 0.540</td>
</tr>
<tr>
<td></td>
<td>Mecillinam</td>
<td>9</td>
<td>4.465 ± 1.026 -1.594 ± 0.536</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>11</td>
<td>4.419 ± 1.684 -0.075 ± 0.858 P&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Mecillinam-</td>
<td>9</td>
<td>4.461 ± 1.168 -3.635 ± 1.029 P&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>ampicillin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
penicillins and cephalosporins against Proteus and 456Klebsiella, with observations on combinations with other antibiotics and against other bacterial species. Antimicrob. Agents Chemother. 9:701-705.


