Effects of Erythromycin in Combination with Penicillin, Ampicillin, or Gentamicin on the Growth of *Listeria monocytogenes*

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Since the optimal antimicrobial therapy for infections caused by *Listeria monocytogenes*, particularly in patients allergic to penicillin, is uncertain, we investigated the in vitro effects of erythromycin, alone and in combination with other antibiotics, on listeriae. Seven strains of listeriae were inhibited but not killed by erythromycin, penicillin G, or ampicillin when tested by a microtiter broth dilution method. Susceptibility to gentamicin decreased when tryptose phosphate broth was substituted for Mueller-Hinton broth, but was independent of their calcium and magnesium concentrations. Quantitative killing studies performed with erythromycin combined with either penicillin G or ampicillin yielded antagonism for all strains, in contrast to microtiter checkerboard determinations, which did not indicate antagonism in all instances. Antagonism occurred with strains in both the stationary and log phases of growth and was slightly reversed by a 120-min preincubation of the listeriae with penicillin before the addition of erythromycin. Erythromycin and gentamicin were antagonistic in quantitative killing studies. Based on these in vitro findings, we conclude that the addition of gentamicin to erythromycin offers no advantage in the treatment of listeriosis in the penicillin-allergic patient.

There is a need to optimize the antibiotic therapy of infections due to *Listeria monocytogenes* since this species has a predilection for causing life-threatening illness in the infirm, the neonate, and the immunosuppressed (2, 7, 14). Furthermore, *L. monocytogenes* is inhibited in growth but not killed by easily achievable concentrations of those antibiotics to which it is most susceptible (12, 21).

Erythromycin on a weight basis has the greatest inhibitory activity against listeriae and is the most commonly suggested therapeutic alternative for the penicillin-allergic patient. Recommendations for the therapy of serious listeriosis have included antibiotic combinations, such as penicillin combined with gentamicin and erythromycin combined with penicillin (7, 14). In addition, erythromycin is often combined with other antimicrobial agents given to immunosuppressed patients because of its minor side effects and its activity against major respiratory pathogens (3). The effects of potentially useful antibi-otic combinations which include erythromycin have not been evaluated for their interactions with listeriae.

Therefore, we investigated the in vitro effects on *L. monocytogenes* of erythromycin when combined with penicillin G, ampicillin, or gentamicin, to evaluate the potential of combination therapy of listeriosis in the penicillin-allergic patient.

**MATERIAL AND METHODS**

**Bacterial strains.** The seven strains of *L. monocytogenes* were isolated from either blood or cerebrospinal fluid submitted to the Clinical Microbiology Laboratory, Strong Memorial Hospital, Rochester, N.Y. Confirmation of the identity and serotyping were performed by the Centers for Disease Control. Listeriae were stored at 4°C on Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar (TSA) slants. In preparation for each experiment, listeriae were incubated at 37°C in Trypticase soy broth (TSA) for 16 to 20 h. The overnight growth was washed twice in phosphate-buffered saline and adjusted to the appropriate concentration with optical density readings, using a Bausch & Lomb Spectronic 710 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). After dilution in Hanks balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, N.Y.), the actual number of colony-forming units was determined by
duplicate plating on TSA, 24 h of incubation at 37°C, and counting of visible colonies.

**Antibiotic solutions.** Penicillin G and ampicillin were obtained from Eli Lilly & Co. (Indianapolis, Ind.), and gentamicin was obtained from Schering Corp. (Kenilworth, N.J.); stock solutions were prepared in sterile distilled water. Erythromycin (Eli Lilly & Co.) was first dissolved in 1 ml of ethanol, then diluted to stock concentration as above. All solutions were stored at −70°C and thawed on the day of use, and any residual was discarded.

**Media.** Mueller-Hinton broth (MHB), TSB, and TSA were obtained from Difco Laboratories (Detroit, Mich.). Tryptose phosphate broth (TPB) was prepared by a standard procedure (19).

The results of susceptibility testing of *Pseudomonas aeruginosa* to gentamicin vary with cation concentrations in the media (17). To detect any similar effect on *Listeria* susceptibility to gentamicin, MHB and TPB were supplemented with calcium and magnesium chloride (MHB-S and TPB-S) to yield final concentrations of approximately 70 mg of calcium per liter and 20 mg of magnesium per liter. The pH of media ranged between 7.38 and 7.41 both before and after cation supplementation.

**Determination of MIC and MBC.** The minimal inhibitory concentration (MIC) of each antibiotic was determined in quadruplicate by the use of a microtiter broth dilution method. Penicillin, ampicillin, or erythromycin were serially diluted in TPB to leave 0.1 ml in the wells of a covered microtiter plate (Falcon Plastics, Oxnard, Calif.). Gentamicin dilutions were similarly performed, utilizing TPB, TPB-S, MHB, and MHB-S. Wells were inoculated with approximately 1.5 × 10^8 listeriae in 0.0015 ml of phosphate-buffered saline with the use of an automated device (MIC-2000 Inoculator, Dynatech Labs, Alexandria, Va.). Control wells consisted of media alone and inoculated media without antibiotic. After 18 h of incubation at 37°C, we determined the MIC (defined as the lowest concentration of antibiotic which prevented visible growth).

With a calibrated loop, 0.01 ml was removed from each well with no visible growth, streaked on blood agar, and incubated for 24 h at 37°C, after which the number of colony-forming units was enumerated. The minimal bactericidal concentration (MBC) was defined as the lowest concentration of antibiotic which resulted in the killing of ≥99.9% of the initial inoculum.

**Checkerboard method for the evaluation of antibiotic interactions.** Serial dilutions in TPB of penicillin alone, ampicillin alone, erythromycin alone, penicillin combined with erythromycin, and ampicillin combined with erythromycin were prepared in microtiter plates with a final volume of 0.01 ml per well. Microtiter wells were inoculated with approximately 1.5 × 10^3 listeriae as described above. The plates were covered with plastic film and incubated at 37°C for 18 h, and each well was examined for visible growth. The fractional inhibitory concentrations (FIC) were determined for the most inhibitory combinations, and the FIC index was calculated (5). The FIC for each antibiotic is the ratio of the MIC necessary for inhibition of growth with the antibiotic in combination to the MIC necessary for inhibition with the antibiotic alone.

The FIC index is the sum of the lowest FIC for each antibiotic. If the FIC index was ≤0.5, the antibiotic combination effect was considered to be synergistic; if the FIC index was >1.0, then the combination was considered antagonistic; and if the FIC index was 1.0, then the combination was considered indeterminate (or additive) in effect.

**Quantitative killing curve method of the evaluation of antibiotic interactions.** Kinetic killing studies were performed by a modification of a standard method (12). Cells prepared as above were added to TPB in 125-ml Erlenmeyer flasks to yield a final concentration of 1 × 10^7 listeriae per ml. Antibiotics were added to give the desired concentrations in a final volume of 20 ml, and the flasks were incubated in a 37°C shaking water bath. The final antibiotic concentrations were 5 μg/ml for erythromycin, 10 μg/ml for penicillin G and ampicillin, and 0.5 μg/ml for gentamicin. Samples of 0.5 ml were removed at 0, 4, and 24 h and serially diluted in HBSS, and portions were spread in duplicate on TSA. After overnight incubation at 37°C, the number of colony-forming units was determined. A synergistic effect was defined as a ≥100-fold increase in killing at 24 h and an antagonistic effect was defined as a ≤100-fold decrease in killing by the antibiotic combination as compared with the effect of each antibiotic alone.

The effect of growth phase on the results obtained with penicillin or ampicillin in combination with erythromycin was evaluated by modifying the initial steps of the procedure. Starting with a concentration of 1 × 10^6 listeriae per ml, the flasks were incubated for 195 min at 37°C in the shaking water bath. At this time, the cells were in logarithmic growth, antibiotics were added, and the experiments were completed as previously described. Preliminary experiments demonstrated that 195 min of preincubation resulted in growth to a concentration of approximately 1 × 10^7 listeriae per ml.

The effect of the timing and the sequence of addition of each antibiotic was evaluated by performing experiments to include bacteria preincubated with penicillin or ampicillin for 0, 30, 60, and 120 min before adding erythromycin.

**RESULTS**

**Antibiotic susceptibilities and media effects.** Table 1 lists the *Listeria* strains, their serotypes, and the MICs and MBCs of penicillin G, ampicillin, and erythromycin as determined in TPB. For all strains but one, the MIC of penicillin G and ampicillin was 0.24 μg/ml; the exception had MICs of 0.12 μg/ml. The MBCs for penicillin G...
TABLE 2. MICs and MBCs of gentamicin as determined in TPB and MHB

<table>
<thead>
<tr>
<th>L. monocytogenes strain</th>
<th>MIC/MBC in TPB (µg/ml)</th>
<th>MIC/MBC in MHB (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.25/2.5</td>
<td>0.156/2.5</td>
</tr>
<tr>
<td>2</td>
<td>1.25/5.0</td>
<td>0.625/1.25</td>
</tr>
<tr>
<td>3</td>
<td>1.25/5.0</td>
<td>0.312/1.25</td>
</tr>
<tr>
<td>4</td>
<td>1.25/2.5</td>
<td>0.312/1.25</td>
</tr>
<tr>
<td>5</td>
<td>2.5/5.0</td>
<td>0.156/0.625</td>
</tr>
<tr>
<td>6</td>
<td>2.5/10.0</td>
<td>0.312/1.25</td>
</tr>
<tr>
<td>7</td>
<td>1.25/2.5</td>
<td>0.312/2.5</td>
</tr>
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* Results did not differ from those shown when TPB-S and MHB-S were used.
* Unsupplemented TPB was determined to contain 23 mg of calcium per liter and 16 mg of magnesium per liter.
* Unsupplemented MHB was determined to contain 18 mg of calcium per liter and 3 mg of magnesium per liter.

Ranges from 7.8 to 62.5 µg/ml; those for ampicillin ranged from 15.6 to 125.0 µg/ml. Penicillin and ampicillin were equal in their inhibitory and bactericidal activities against these seven Listeria isolates. The activity of erythromycin was similar for six of seven strains which had an MIC of 0.12 µg/ml and an MBC of 15.6 µg/ml for two strains and 62.5 µg/ml for the five other strains.

The MIC and MBC of gentamicin for these same seven Listeria strains are listed in Table 2, as determined in MHB and in TPB. In MHB the MICs were between 0.156 and 0.625 µg/ml, whereas those determined in TPB were consistently 2 to 16 times higher. The MBCs were less disparate between the two media but, when different, were two to eight times higher in TPB (strains 2 to 6). The use of TPB-S and MHB-S yielded results which were, in all instances, within 1 dilution of those determined in the respective unsupplemented media.

Erythromycin combined with a penicillin. The effects of combining erythromycin with a penicillin on the growth of L. monocytogenes 1 through 6 were initially evaluated with the checkerboard method. Table 3 lists the FIC index for these antibiotic combinations. Erythromycin plus penicillin G was antagonistic for two isolates, indeterminant for three, and synergistic in one instance. However, erythromycin plus ampicillin was antagonistic for three isolates, indeterminant for three, and synergistic for none. For L. monocytogenes 2, 5, and 6, the effect of erythromycin combined with penicillin G was different from the effect of erythromycin combined with ampicillin.

The effect on Listeria growth of erythromycin combined with a penicillin also was evaluated by the quantitative killing curve method. Erythromycin (5 µg/ml) in combination with either penicillin G or ampicillin (10 µg/ml) antagonized the bactericidal activity of the penicillin for all seven strains. Figure 1 depicts, as representative, the killing curve of L. monocytogenes 2, the strain with the most diverse checkerboard results. Results for all strains were unchanged when bacteria in the log phase of growth were studied.

To investigate the importance of timing and the sequence of combining the antibiotics on the observed antagonism, we preincubated the lis-
Erythromycin plus penicillin. Shown are the results for *L. monocytogenes* 2; see Fig. 1 for the effects of antibiotics added simultaneously.

**DISCUSSION**

We determined in vitro that erythromycin antagonizes the inhibitory effects of penicillin G and ampicillin on the growth of *L. monocytogenes*. These results are in keeping with the scheme for assessing antimicrobial combinations originally proposed by Jawetz and Gunnison (8) and as recently reviewed by Rahal (16). This scheme predicts that the combination of the bacteriostatic agent erythromycin with the bactericidal penicillins is potentially antagonistic. This is felt, in part, to be due to the inhibition of *Listeria* growth by erythromycin, which effectively inhibits the killing capacity, or with listeriae the inhibitory capacity, of the penicillins on the dormant survivors.

To investigate this possibility, we performed microtiter susceptibilities and quantitative killing curve determinations with listeriae in both the stationary and the log phase of growth. The effect of the penicillins is maximal when the bacteria are actively multiplying and undergoing cell wall synthesis (9, 22). Kim and Anthony have determined that the MBCs of penicillin for group B streptococci and of methicillin for *Staphylococcus aureus* are significantly decreased when log-phase cells are used (9). We were unable, however, to show any differences in the MIC, the MBC, or the antagonistic effects of the antibiotic combinations when listeriae were in the log phase of growth. The importance of timing and the sequence of addition of the antibiotics on the observed antagonism was also investigated. We determined that reversal of the antagonistic effect of erythromycin was not evident after 1 h of penicillin preincubation and was only partially evident after 2 h of penicillin preincubation. This contrasts with the previously reported observation that the antagonism by
chloramphenicol of the bactericidal activity of penicillin for the pneumococci is mostly reversed after 1 h of prior penicillin exposure (20). We chose concentrations of penicillin and ampicillin which were inhibitory but not bactericidal for listeriae (Fig. 1); this may partially account for the difficulty found in overcoming the antagonistic effects of erythromycin by the use of log-phase cells or by preincubation with the penicillins.

Quantitative killing curves documented erythromycin antagonism of the listericidal activity of penicillin G and ampicillin with all strains. In general, the checkerboard determinations yielded similar results (Table 3). However, for L. monocytogenes the FIC index indicated synergism between erythromycin and penicillin G, and yet antagonism was indicated between erythromycin and ampicillin (Table 3). The problems inherent in the checkerboard methodology and its interpretation, as well as the potential for discrepancy between this method and the killing curve method, have been recently reviewed (10, 11, 15).

The MICs and MBCs of erythromycin, penicillin G, ampicillin, and gentamicin for our seven test strains were comparable to those previously reported (1, 6, 12, 21). We documented, however, that the susceptibility of listeriae to gentamicin decreased considerably when the enriched medium TPB was substituted for MHB (Table 2). TPB supported listeria growth better than did MHB, an observation which probably accounts for the higher inhibitory and bactericidal concentrations determined in TPB. We further determined that susceptibility to gentamicin was independent of calcium and magnesium concentrations in both media. Although the in vitro susceptibility of P. aeruginosa to gentamicin has been found to be dependent on the cation concentrations in the medium (17), our findings are in keeping with the general lack of this dependency for gram-positive species. Wiggins et al. (21) also have noted the poor growth of listeriae in MHB and noted that growth was not enhanced by one lot of MHB with physiological cation concentrations. Although these investigators found that the endpoints of growth inhibition were uninterpretable, we noted distinct growth endpoints in MHB, but our results with this medium markedly overestimated Listeria susceptibility to gentamicin.

We found that erythromycin antagonized the listericidal action of gentamicin. Antagonism also has been observed when tetracycline was combined with gentamicin and incubation was extended to 48 h (6). In contrast, prior studies of a penicillin in combination with gentamicin have demonstrated synergism against listeriae, both in vitro and in vivo (1, 4, 6, 12, 13, 21).

Our in vitro findings cannot be directly extrapolated to the clinical setting. The combination of erythromycin plus a penicillin has been reported to be therapeutically successful in the treatment of Listeria infections, although it is of interest that one patient apparently developed Listeria meningitis during intravenous therapy with this combination (18). Based on our results, however, there is no apparent advantage in the addition of an aminoglycoside to erythromycin for the treatment of the penicillin-allergic patient with Listeria infection.

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LITERATURE CITED