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Susceptibility of *Haemophilus influenzae* to Chloramphenicol and Eight Beta-Lactam Antibiotics

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We examined the minimal inhibitory concentrations and minimal bactericidal concentrations of chloramphenicol, ampicillin, ticarcillin, cefamandole, cefazolin, cefoxitin, cefotaxime, ceforanide, and moxalactam for 100 isolates of *Haemophilus influenzae*, 25 of which produced β-lactamase. Susceptibility was not influenced by the capsular characteristic of the organism. The mean minimal inhibitory concentrations of cefamandole, ticarcillin, and ampicillin for β-lactamase-producing strains were 3-, 120-, and 400-fold higher than their respective mean minimal inhibitory concentrations for β-lactamase-negative strains. No such difference was noted for the other antibiotics. We performed time-kill curve studies, using chloramphenicol, ampicillin, cefamandole, cefotaxime, and moxalactam with two concentrations of the antimicrobial agents (4 or 20 times the minimal inhibitory concentrations) and two inoculum sizes (10^4 or 10^6 colony-forming units per ml). The inoculum size had no appreciable effect on the rate of killing of β-lactamase-negative strains. The rates at which β-lactamase-producing strains were killed by chloramphenicol, cefotaxime, and moxalactam were not influenced by the inoculum size. Whereas cefamandole in high concentrations was able to kill at 10^6 colony-forming units/ml of inoculum, it had only a temporary inhibiting effect at low drug concentrations. Methicillin and the β-lactamase inhibitor CP-45,899 were able to neutralize the inactivation of cefamandole by a large inoculum of β-lactamase-producing *H. influenzae*.

Haemophilus influenzae is an important pathogen in infections occurring during the early childhood years. Encapsulated organisms (predominantly type b) are the most common cause of meningitis in children, and nontypable strains of the organism are responsible for a substantial portion of middle ear infections. The increasing role played by this organism in infections occurring in adults has been recognized in recent years. The emergence of ampicillin resistance among type b and nontypable strains of this organism resulted in the need for the use of other antimicrobial agents. Chloramphenicol is now included in the initial therapy of *H. influenzae* meningitis. Ticarcillin and carbenicillin have been reported to be active against β-lactamase-producing *H. influenzae* (22). Cefamandole, a new cephalosporin, has been shown to be active against ampicillin-susceptible and -resistant strains of *H. influenzae* (11). The investigational β-lactam antibiotics moxalactam, ceforanide, and cefotaxime have been reported to be very active against many gram-negative bacteria, including *H. influenzae* (1, 2, 15).

In the present study, we compared the in vitro activity of chloramphenicol and eight β-lactam antibiotics (ampicillin, ticarcillin, cefazolin, cefoxitin, cefamandole, ceforanide, moxalactam, and cefotaxime) against *H. influenzae*. We also studied the effect of inoculum size on the rate of bacterial killing for several of the antimicrobial agents.

MATERIALS AND METHODS

Organisms. A total of 100 strains were randomly chosen from clinical isolates at the Children's Hospital of Michigan. The isolation, identification, and serotyping of *H. influenzae* were done according to standard bacteriological techniques (14). Beta-lactamase production was determined by the acidometric technique of Escamilla (6) or by the use of Betalactam reagent disks (Marion Scientific Corp., Kansas City, Mo.). Of the 100 strains, 75 were type b (18 β-lactamase producers) and 25 were nontypable (7 β-lactamase producers). All organisms were stored lyophilized.

Antibiotics. The antibiotic powders were laboratory standards of assayed potency and were gifts from the following pharmaceutical companies: Beecham Laboratories (ticarcillin), Bristol Laboratories (ampicillin and ceforanide), Eli Lilly & Co. (cefazolin, cefamandole lithium, and moxalactam), Hoechst-Roussel Pharmaceuticals (cefotaxime), Merck Sharp & Dohme (cefoxitin), and Parke, Davis & Co. (chloramphenicol).

Susceptibility testing. Minimal inhibitory con-

centrations (MICs) were determined by the broth dilution method. Mueller-Hinton broth supplemented with hemin (10 μg/ml) and nicotinamide adenine dinucleotide (10 μg/ml) as described by Brinkley and Huber (5) was used. Twofold dilutions of the antibiotics (ranging from 0.03 to 64 μg/ml) were made in the broth. Log-phase cultures of the isolates were added to each tube. The resulting final inoculum was approximately 5×10^4 colony-forming units (CFU)/ml. After 18 h of incubation 0.1-ml samples of broth from tubes showing no visible growth were subcultured to chocolate agar plates. The lowest concentration of antibiotic yielding less than five colonies (99.9% killing) was regarded as the minimal bactericidal concentration (MBC).

Killing rates. The rates at which either small (10^4 CFU/ml) or large (10^6 CFU/ml) inocula of a β-lactamase-negative (β-lac$^-$) strain and a β-lactamase-producing (β-lac$^+$) strain of *H. influenzae* were killed by five antimicrobial agents were determined. The antibiotics and the two concentrations at which they were tested are shown in Table 1. For each antibiotic, other than ampicillin, the low concentration represented 4 times the MIC, and the high concentration represented 20 times its MIC for the two strains. For ampicillin these concentrations were 4 and 20 times the MIC for the β-lac$^-$ strain. Samples of broth were removed at 1, 2, 4, 8, and 24 h, and 0.1-ml samples of serial dilutions of the broth were plated on chocolate agar plates. Time-kill curves were plotted after determining the numbers of viable organisms. The rates at which cefamandole (4 μg/ml) killed 10^6-CFU/ml inocula of six additional β-lac$^+$ strains were also determined.

Bactericidal rate of cefamandole in the presence of β-lactamase inhibitors. Methicillin or CP-45,899 (penicillanic acid, 1,1-dioxide) were used as inhibitors of β-lactamase. CP-45,899 was a gift from J. A. Retsema (Pfizer Inc., Groton, Conn.). The MICs of methicillin and CP-45,899 for a β-lac$^+$ strain of *H. influenzae* were determined. The bactericidal rates of cefamandole in low concentrations (4 μg/ml) against a 10^6-CFU/ml inoculum were determined with cefamandole alone and in combination with a subinhibitory concentration of methicillin of CP-45,899.

RESULTS

There was no difference in the antimicrobial susceptibility of type b and nontypable strains of *H. influenzae*, and they were combined for analysis.

The cumulative percentage of strains inhibited by seven antibiotics is shown in Fig. 1. Moxalactam and cefotaxime were the most active drugs, inhibiting all isolates at concentrations of 0.12 μg/ml or less. All isolates were inhibited by 2 μg or less of cefamandole and chloramphenicol per ml. The mean MICs (μg/ml) of these drugs for the 100 strains were: moxalactam (0.04), cefotaxime (0.05), cefamandole (0.5), chloramphenicol (1.0), cefoxitin (2.1), cefazolin (3.0), and ceforanide (4.1).

The susceptibility of *H. influenzae* to moxalactam, cefotaxime, chloramphenicol, cefoxitin, cefazolin, and ceforanide was not affected by β-lactamase production. In contrast the MICs of ampicillin, ticarcillin, and cefamandole were higher for β-lac$^+$ strains than for β-lac$^-$ strains (Fig. 2). These three antimicrobial agents inhibited all β-lac$^-$ strains at concentrations of 1 μg/ml or less. Ampicillin and ticarcillin inhibited β-lac$^+$ organisms at much higher concentrations with no overlap between β-lac$^-$ and β-lac$^+$ strains. Whereas cefamandole MICs were higher for β-lac$^+$ strains than for β-lac$^-$ strains, these values overlapped. The mean MICs of ampicillin, ticarcillin, and cefamandole for β-lac$^-$ strains were 0.16, 0.2, and 0.35 μg/ml, and for β-lac$^+$ strains they were 62.2, 24.5, and 1.0 μg/ml, respectively.

In all cases the MBC was either the same as or within one dilution of the MIC.

The rates at which a 10^6-CFU/ml inoculum of a β-lac$^-$ strain of *H. influenzae* type b was killed by high concentrations (20 times MIC) of five antimicrobial agents are shown in Fig. 3. Chloramphenicol was the most rapidly bactericidal agent, killing all the inoculated bacteria within

TABLE 1. *Antibiotics and concentrations used in the bactericidal rate studies*

Antibiotic	Concn (μg/ml)	
	Low	High
Ampicillin	1.0	5.0
Cefamandole	4.0	20.0
Cefotaxime	0.25	1.25
Chloramphenicol	4.0	20.0
Moxalactam	0.25	1.25

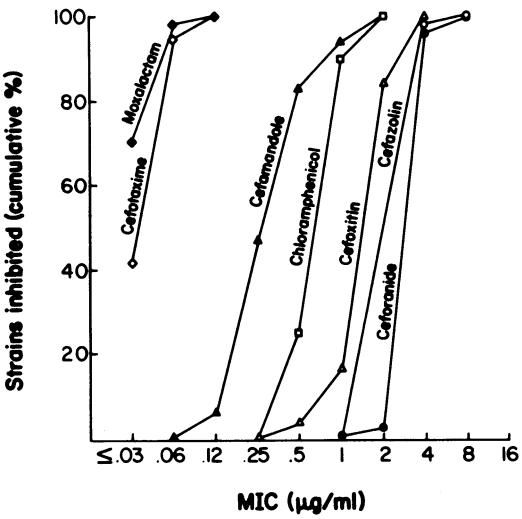

FIG. 1. *Cumulative percentage of H. influenzae strains (25 β-lac$^+$, 75 β-lac$^-$) inhibited by seven antibiotics.*

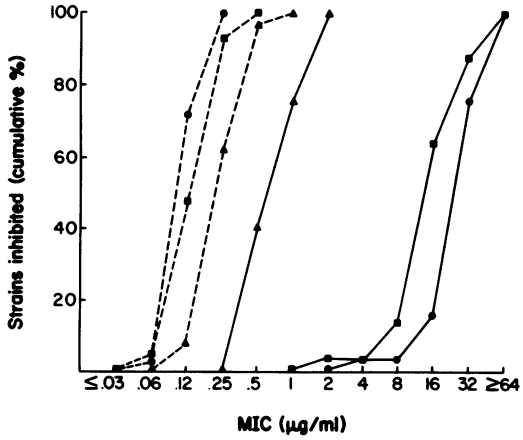

FIG. 2. *Cumulative percentage of β-lac⁻ H. influenzae strains (----) and β-lac⁺ strains (——) inhibited by ampicillin (●), ticarcillin (■), and cefamandole (▲).*

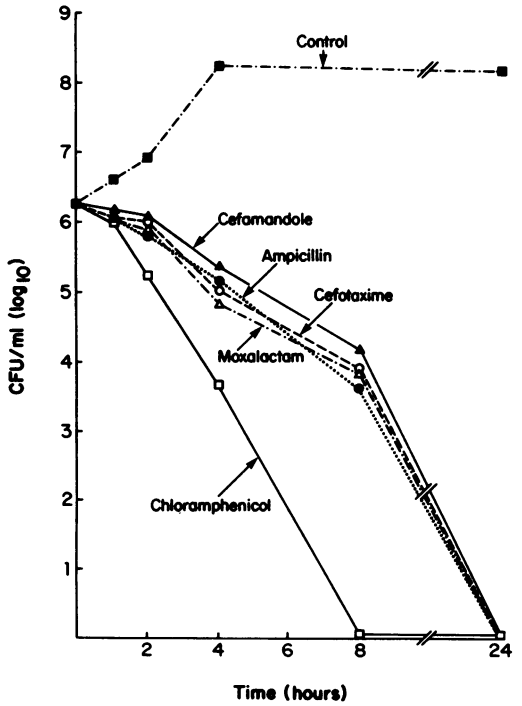

FIG. 3. *Rates of killing of H. influenzae by chloramphenicol (20 μg/ml), moxalactam (1.25 μg/ml), cefotaxime (1.25 μg/ml), ampicillin (5 μg/ml), and cefamandole (20 μg/ml).*

8 h. The other four drugs were comparable to one another in their rates of killing with no viable bacteria remaining at 24 h.

At low concentrations (4 times MIC) chloramphenicol killed a 10^6-CFU/ml inoculum in 24 h, whereas the other four drugs reduced the number of viable bacteria to between 10 and 100 CFU/ml at 24 h and killed all by 48 h.

When the rates of killing of a β-lac⁺ strain of *H. influenzae* by the same five antimicrobial agents were studied, ampicillin was found to have no activity other than a temporary inhibition of bacterial multiplication. The rates at which 10^6-CFU/ml inocula of the β-lac⁺ strain were killed by high (20 times MIC) concentrations of the other four drugs were similar to the rates at which comparable inocula of the β-lac⁻ strain were killed. Chloramphenicol, moxalactam, and cefotaxime killed the β-lac⁺ strain nearly as well in low concentrations as in high concentrations, with no viable bacteria remaining at 24 h. However, the bactericidal activity of cefamandole against a 10^6-CFU/ml inoculum was influenced by the drug concentration, as shown in Fig. 4. Both concentrations reduced the number of viable bacteria to approximately 3.5×10^3 CFU/ml at the end of 8 h. At 24 h the higher concentration of cefamandole completely killed the bacteria, whereas with the lower concentration of cefamandole, the number of viable organisms increased and reached control values at the end of 48 h. Similar patterns were ob-

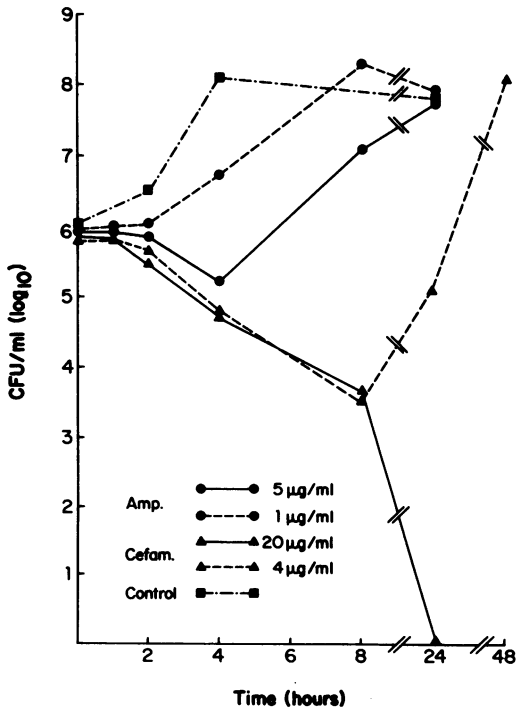

FIG. 4. *Rates of killing of H. influenzae by ampicillin (amp) and cefamandole (cefam) at different concentrations. The MIC of ampicillin for the test strain was 32 μg/ml and that of cefamandole was 1 μg/ml.*

served when the activity of cefamandole (4 μg/ml) was tested against 10^6-CFU/ml inocula of six other strains of β-lac$^+$ *H. influenzae*. When the cefamandole MICs of the survivors were determined with 10^4-CFU/ml inocula, the MICs were unchanged, suggesting that incubation with cefamandole did not result in the selection of resistant mutants.

Low and high concentrations of chloramphenicol, moxalactam, cefotaxime, and cefamandole were equally effective against 10^4-CFU/ml inocula of the β-lac$^+$ strain, killing the entire inoculum within 24 h.

In an attempt to determine the mechanism by which a large inoculum of β-lac$^+$ *H. influenzae* escapes the effect of a low concentration of cefamandole, we studied the effect of β-lactamase inhibitors such as methicillin or CP-45,899. The MIC of methicillin for the test strain was 25 μg/ml. The effects of methicillin (5 μg/ml) and cefamandole (4 μg/ml) individually and in combination against a β-lac$^+$ *H. influenzae* type b are shown in Fig. 5. In subinhibitory concentrations methicillin alone had no activity. Cefamandole alone reduced the number of viable organisms during the first 8 h; however, this was followed by an "escape" with active growth of the organism. The combination of the two drugs was able to kill the inoculum completely in 24 h. A similar experiment was carried out using CP-45,899 instead of methicillin. The MIC of CP-45,899 for the test strain was 62.5 μg/ml. When the effect of CP-45,899 (2.5 μg/ml) and that of cefamandole (4 μg/ml) were studied separately and in combination, the resulting time-kill curves were nearly identical to the ones illustrated in Fig. 5.

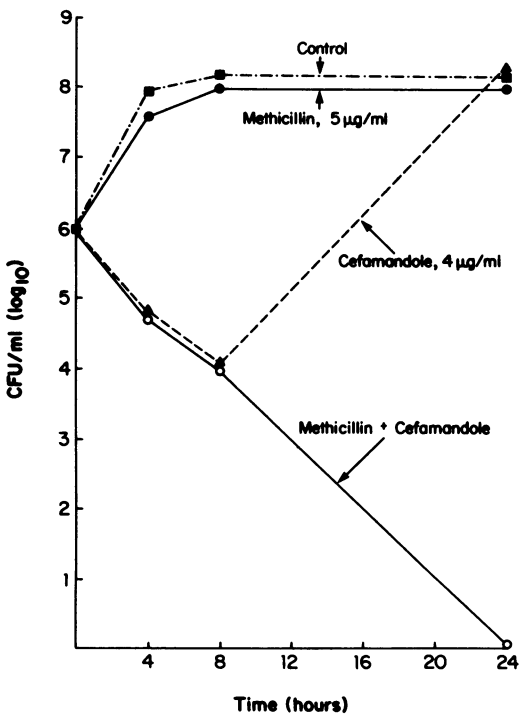

FIG. 5. *Effects of cefamandole and methicillin, independently and in combination, on the viability of a β-lac$^+$ strain. The individual MICs for this strain were: cefamandole, 1.0 μg/ml; methicillin, 25 μg/ml.*

DISCUSSION

The emergence of ampicillin resistance among *H. influenzae* has prompted an evaluation of the activity of many alternative antimicrobial agents against this important pathogen (1, 2, 11, 15). Chloramphenicol still remains effective against β-lac$^+$ and β-lac$^-$ strains, although isolates resistant to chloramphenicol (with or without concomitant resistance to ampicillin) have been encountered recently (13). Carbenicillin and ticarcillin have been found to be effective against some strains of β-lac$^+$ *H. influenzae* (22); however, the MICs of these two drugs are many-fold higher for β-lac$^+$ strains than for β-lac$^-$ strains (11, 15). Cefamandole is reported to be active against β-lac$^+$ strains when tested by the agar dilution technique, inhibiting such strains at concentrations of 2 μg/ml or less (11), and has been suggested as a possible alternative in the treatment of infections caused by β-lac$^+$ *H. influenzae*. Rylander et al. observed that for some organisms, the MIC of cefamandole, as determined by the broth dilution technique, is substantially higher than the MIC determined by agar dilution (18). Although Bergeron et al. observed no difference between cefamandole MICs for β-lac$^+$ *H. influenzae* in broth and agar, they noted that cefamandole was much less active against β-lac$^+$ than against β-lac$^-$ strains (4). Others have noted that the MBCs of cefamandole for β-lac$^+$ isolates are 4 to 8 times higher than the MICs (9, 23).

Ceforanide, cefotaxime, and moxalactam are among the many β-lactam antimicrobial agents under evaluation. The spectrum of activity of ceforanide is similar to that of the available cephalosporins (1); however, it is effective against *H. influenzae* in clinically achievable concentrations. The long half-life of ceforanide permits intramuscular administration of the drug every 12 h (A. S. Dajani, M. C. Thirumoorthi, R. Bawdon, J. Buckley, D. R. Van Harken, and M. Pfeffer, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 647, 1980). Moxalactam and cefotaxime are very active against most gram-negative organisms, including *H. influenzae* (2, 15).

Another important consideration in the in vitro antimicrobial susceptibility testing, especially with beta-lactam antibiotics, is the effect of inoculum size on the MIC values. The MICs of ampicillin and carbenicillin for β-lac$^+$ strains of *H. influenzae* are substantially increased when the inoculum size is increased from 10^4 to 10^6 CFU/ml (21). The effect of inoculum size on the cefamandole MIC for β-lac$^+$ *H. influenzae* is less clear. Syriopoulou et al. (21) noted that MIC_{50} of cefamandole for β-lac$^+$ isolates increased from 0.75 μg/ml to 40 μg/ml when the inoculum was increased from 10^5 to 10^7 CFU/ml. Fleming and Fierer, using a sensitive nitrate-reduction method, found that both the MIC and the MBC of cefamandole were higher for β-lac$^+$ strains than for β-lac$^-$ strains, even with a 10^4-CFU/ml inoculum (9). Yourassowsky et al. (23) stated that the cefamandole MIC for such strains did not change when the inoculum was increased to 10^7 CFU/ml, although the MBC increased from 0.8 to 6.4 μg/ml. Some early reports suggested that cefamandole is resistant to hydrolysis by β-lactamases (11); however, the various β-lactamases have different levels of activity against cefamandole. Type IVc is the most active, type IIIa is less so, and types Ia and Id have little or no activity (17). *H. influenzae* is known to produce type IIIa (TEM-type) β-lactamase (20). We did not specifically study the effect of inoculum size on the MIC of cefamandole for β-lac$^+$ *H. influenzae*; however, even with a 10^4-CFU/ml inoculum, the mean cefamandole MIC was threefold higher for β-lac$^+$ strains than for β-lac$^-$ strains. Farrar and Gramling found a correlation between the susceptibility of different cephalosporins to hydrolysis by β-lactamase and the magnitude of the effect of inoculum size on the susceptibility of *Staphylococcus aureus* to these agents (7). Since the MICs of many antibiotics for *H. influenzae*, particularly for β-lac$^+$ strains, are influenced by the method and the inoculum size used, it may be desirable to employ the broth dilution technique and an inoculum of 10^6 or 10^7 CFU/ml in such studies.

That large inocula of β-lac$^+$ *H. influenzae* hydrolyze cefamandole can be inferred from the fact that partial killing of such inocula was followed by exponential growth even though the remaining organisms did not acquire any resistance. Inactivation of cefamandole by β-lac$^+$ strains has been noted by Yourassowsky et al. (23). The hydrolysis of cefamandole could be prevented by the inhibition of β-lactamase activity with CP-45,899 as shown in this report. This semisynthetic β-lactamase inhibitor has been shown to rapidly and potently inhibit the β-lactamases of a variety of organisms (16). In view of the effect of inoculum size on the susceptibility of β-lac$^+$ *H. influenzae*, care needs to be exercised in treating *H. influenzae* infections with this drug. In *H. influenzae* type b bacteremia, the number of circulating bacteria frequently exceed 10^4 CFU/ml (19), and it is common to find cerebrospinal fluid bacterial densities of 10^6 CFU/ml or greater in *H. influenzae* meningitis (8). Evolution of meningitis during or shortly after cefamandole therapy of *H. influenzae* infections has also been reported (P. Azimi and P. Chase, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 195, 1980).

Chloramphenicol, moxalactam, and cefotaxime were effective even against large inocula of β-lac$^+$ strains. Moxalactam and cefotaxime are effective at very low concentrations (2, 5, 15). They are also reported to diffuse well into cerebrospinal fluid (3, 12) and have been suggested as possible alternatives to chloramphenicol in the treatment of *H. influenzae* and other gram-negative bacterial meningitis. However, we have noted that the cerebrospinal diffusion of moxalactam is unpredictable in children with meningitis (M. C. Thirumoorthi, J. A. Buckley, R. E. Kauffman, and A. S. Dajani, Pediatr. Res. **15**: 623, 1981). Both of these drugs are still investigational. More controlled clinical trials are necessary before they can be recommended as drugs of choice in *H. influenzae* meningitis. Despite the occurrence of a rare strain of *H. influenzae* resistant to chloramphenicol, this drug still remains the most satisfactory agent for the treatment of β-lac$^+$ *H. influenzae* infections. Chloramphenicol is rapidly bactericidal against even large inocula and diffuses well into cerebrospinal fluid regardless of the degree of meningeal inflammation (10).

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