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Effect of Inoculum Size and β-Lactamase Production on In Vitro Activity of New Cephalosporins Against *Haemophilus* Species

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Sixty-three strains of *Haemophilus* species, 38 of which were β-lactamase producers (37 *H. influenzae* type b, 1 *H. parainfluenzae*) and 25 of which were β-lactamase negative (20 *H. influenzae*, 5 *H. parainfluenzae*), were tested for susceptibility to cefoxitin, moxalactam (LY127935) (Lilly), cefsulodin (CGP 7174 E, Ciba), and cefoperazone (T 1551, Pfizer). Cefsulodin was relatively inactive at both low and high inocula. LY127935 and cefoperazone displayed inoculum-dependent bactericidal activity. Cefoxitin displayed little inoculum effect against β-lactamase-producing strains: 8 and 16 μg/ml killed at least 90% of those tested at 10^4 and 10^6 colony-forming units per ml, respectively.

It has been well established that inoculum size substantially affects the susceptibility of *Haemophilus influenzae* to all of the currently commercially available cephalosporins (6), as well as cefamandole (5, 10, 18, 24), cefoxitin (5), and cefaclor (2, 23). The purpose of this investigation was to examine the effects of inoculum size on the inhibitory and bactericidal activities of cefoxitin, moxalactam (LY127935), cefoperazone, and cefsulodin against β-lactamase-positive and -negative strains of *Haemophilus*.

MATERIALS AND METHODS

Organisms. Most of the 63 organisms studied were recent isolates from the Clinical Microbiology laboratories of the Mayo Clinic, Rochester, Minn. Twelve strains of *H. influenzae* type b were obtained from the Center for Disease Control, Atlanta, Ga., and another 16 were frozen reference strains of β-lactamase-positive *Haemophilus*. The sources of the isolates and their respective numbers were as follows: sterile body fluids (blood, cerebrospinal fluid), 31; oronasopharynx, 13; eye, 2; sputum, 8; and source unknown, 9.

Of the 38 β-lactamase-positive strains, 37 were *H. influenzae* type b and one was *H. parainfluenzae*. Of the 25 β-lactamase-negative strains, 12 were *H. influenzae* type b, 8 were nontypable *H. influenzae*, and 5 were *H. parainfluenzae*. Identification of strains was based on the taxonomic system described by Kilian (11). Serological typing was performed by the immunofluorescent-antibody technique. β-Lactamase activity was determined by a rapid slide technique (16). Reference strains of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* Mayo strain were used as controls when determining minimum inhibitory concentrations (MICs). All organisms were subcultured to solid media to check for purity before inoculation into broth. Isolates were stored in rabbit blood at −70°C.

Antimicrobial agents. The following antimicrobial agents were used: cefoxitin (Merck, Sharpe and Dohme, West Point, Pa.); cefsulodin, or CGP 7174 E (Ciba Pharmaceutical Co., Summit, N.J.); cefoperazone, or T 1551 (Pfizer Pharmaceuticals, New York, N.Y.); and moxalactam (LY127935; Lilly Research Laboratories, Indianapolis, Ind.). Antibiotics were solubilized in appropriate diluents (21), and stock solutions were either used immediately or stored frozen at −40°C.

Media. The following media were briefly evaluated for their suitability in this study: (i) Mueller-Hinton broth (MHB) supplemented with 10 μg of hemin and 10 μg of nicotinamide adenine dinucleotide (NAD) per ml (4); (ii) Schaedler broth supplemented with 5% Fildes extract (peptic digest of blood) (19); and (iii) MHB supplemented with 5% lysed horse blood and 2.5 μg of NAD per ml (12). Such an evaluation was deemed appropriate, because some investigators have encountered problems with poor growth and reproducibility of susceptibility studies with *Haemophilus* species (1, 3, 8, 9, 22).

The three media were evaluated by testing the susceptibility of a β-lactamase-positive and a β-lactamase-negative strain of *H. influenzae* to ampicillin and cefamandole in each medium. The specific criteria examined were growth characteristics, ease of interpretation of endpoints in both macro- and microdilution systems, and reproducibility of endpoints.

Susceptibility test. MICs were determined by a broth microdilution method. Antimicrobial dilutions were dispensed into plates with the MIC 2000 dispenser (Dynatech, Inc., Alexandria, Va.) and stored at −70°C. Isolates of *Haemophilus* were subcultured to fresh chocolate agar plates, grown overnight and then subcultured into two tubes containing 2 and 20 ml of Schaedler broth, respectively. The light inoculum was prepared by adjusting the turbidity of the inoculum in broth to match that of one-half of a McFarland barium sulfate standard (approximately 10^8 colony-forming units [CFU] per ml) and then diluting the adjusted inoculum 1:10 (21). The heavy inoculum was prepared by adjusting the turbidity of the inoculum in broth to match that of a no. 3 McFarland barium sulfate standard (approximately 9×10^9 CFU/ml). The two ad-

justed organism suspensions were then delivered into the microdilution trays with the MIC 2000 inoculator so that the final light and heavy inocula were 10^4 and 10^6 CFU/ml, respectively. Triplicate plate counts were performed to ensure that the actual colony counts correlated well with those predicted by adjusting turbidity. The microdilution plates were incubated for 24 h at 35°C in sealed bags, without CO_2. The MIC was defined as that concentration in which there was no visible growth; a slight opacity, shown to be due to spheroplast formation at the higher inoculum, was disregarded (13). Minimum bactericidal concentrations (MBCs) were determined by delivering 50-μl samples of broth from the last turbid well and the next five wells without visible growth onto a chocolate agar plate. These plates were incubated at 35°C for 36 h in 5% CO_2. The MBC was the least concentration of antibiotic which yielded no growth upon subculture.

RESULTS

All three media evaluated readily supported growth of *H. influenzae* type b and gave similar and reproducible MICs for ampicillin and cefamandole. Schaedler broth with Fildes extract and MHB with NAD and hemin provided clearer endpoints than could be obtained in MHB with lysed horse blood and NAD. MHB with NAD and hemin, however, failed to yield adequate growth in the microdilution system of 30 strains of unencapsulated and nontypable strains of *H. influenzae*. For these reasons and because of its ease of preparation, Schaedler broth with Fildes extract was used during the remainder of the study.

The results of testing the 63 strains of *Haemophilus* are shown in the accompanying tables. The cumulative percentage of 25 strains of β-lactamase-negative strains of *Haemophilus* inhibited (MIC) and killed (MBC) for each antimicrobial agent at the two different inoculum sizes is shown in Table 1. At the generally recommended inoculum, 10^4 CFU/ml (20), cefsulodin was relatively inactive; cefoperazone and LY127935 were the most active, followed closely by cefoxitin. LY127935 and cefoperazone displayed inoculum-dependent bactericidal effects; however, even against an inoculum of 10^6 CFU/ml, both of these compounds killed ≥80% of strains at 2 μg/ml and ≥90% at 8 μg/ml. Cefoxitin demonstrated no appreciable inoculum effect and was bactericidal to all β-lactamase-negative strains at a concentration of 16 μg/ml.

The effects of inoculum size and β-lactamase production on the susceptibility of 38 strains of *Haemophilus* are shown in Table 2. At an inoculum of 10^4 CFU/ml, LY127935 and cefoperazone were the most inhibitory and bactericidal of the compounds tested. Both compounds displayed substantial inoculum-dependent effects on both inhibitory and bactericidal activity;

TABLE 1. *Cumulative percentage of 25 β-lactamase-negative Haemophilus strains inhibited or killed (MIC/MBC)*

Antibiotic	Inoculum (CFU/ml)	% Strains inhibited/killed at increasing concn (μg/ml):										
		0.03	0.06	0.125	0.25	0.5	1.0	2	4	8	16	32
Cefoxitin	10^4			4/0	12/8	12/8	20/12	72/32	100/88	100/100		
	10^6				8/4	12/4	12/4	68/12	100/72	100/88	100/100	
LY127935	10^4	48/16	84/44	92/80	96/84	96/88	100/100					
	10^6	20/4	64/12	64/24	76/28	80/36	84/64	92/80	100/84	100/92	100/96	100/100
Cefsulodin	10^4							4/0	8/8	8/8	16/12	72/16
	10^6							4/0	8/4	12/4	12/8	52/8
Cefoperazone[a]	10^4	75/50	100/83	100/92	100/100							
	10^6	50/0	75/17	83/42	83/42	83/50	92/67	100/83	100/92	100/100		

[a] Only 12 strains tested.

TABLE 2. *Cumulative percentage of 38 β-lactamase-positive Haemophilus strains inhibited or killed (MIC/MBC)*

Antibiotic	Inoculum (CFU/ml)	% Strains inhibited/killed at increasing concn (μg/ml)									
		0.03	0.06	0.125	0.25	0.5	1.0	2	4	8	16	32
Cefoxitin	10^4					3/0	16/3	79/40	100/79	100/97	100/100	
	10^6						3/0	66/18	100/61	100/84	100/92	
LY127935	10^4	58/47	95/76	100/92	100/95	100/97	100/100					/100
	10^6	34/3	71/26	79/42	87/42	90/58	97/71	97/81	97/89	100/95	/95	55/11
Cefsulodin	10^4										3/0	37/0
	10^6											
Cefoperazone[a]	10^4	60/20	90/40	90/60	100/60	100/60	100/80	100/100				
	10^6				10/0	30/0	50/0	60/10	60/20	70/20	100/30	

[a] Only 10 strains tested.

however, LY127935 was bactericidal at a concentration of 8 μg/ml against all but one strain at the higher inoculum. Comparable bactericidal activity but without inoculum effect was displayed by cefoxitin.

DISCUSSION

Under all experimental conditions, LY127935 was the most inhibitory and bactericidal compound against all 63 strains of *Haemophilus*. Cefoperazone had similar activity under standard conditions, but showed marked inoculum-dependent decreases in inhibitory and bactericidal activity when tested against β-lactamase-producing organisms. Although somewhat less active at low concentrations with standard inocula than LY127935 and cefoperazone, cefoxitin was unaffected by high inocula of β-lactamase-producing strains of *Haemophilus*, corroborating data previously published by Eickhoff and Ehret (5).

In their evaluations of LY127935 and of cefoperazone, Neu et al. (14, 15) reported that 90% of *H. influenzae* tested at 10^5 CFU/ml were inhibited by 1.6 and 0.8 μg of these compounds per ml, respectively. It is not clear in either study how many β-lactamase-producing strains were tested. Moreover, they performed no tests of the bactericidal activity of the two compounds against *Haemophilus*.

The inoculum-dependent formation of spheroplasts (3, 13, 17) and the problems posed by the medium (1, 4, 8, 9, 19, 22) make it difficult to arrive at uniformly agreed upon endpoints and at a suitable definition for susceptibility of *Haemophilus* to β-lactam antibiotics; however, the known numbers of bacteria in the cerebrospinal fluid of patients with *H. influenzae* meningitis (7) suggest the unrealistic nature and doubtful clinical significance of determining the inhibitory activity of β-lactam antibiotics against 10^4 CFU of *H. influenzae* per ml. The purported activity of cephalosporins against *Haemophilus* must, therefore, be interpreted cautiously unless results include the inhibitory and bactericidal activities of such compounds against high inocula of organisms.

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