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Molecular Basis of the Efficacy of Cefaclor against *Haemophilus influenzae*

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Cefaclor sustained its inhibitory activity against a β-lactamase-producing strain of *Haemophilus influenzae*. Although a relatively high permeability coefficient was calculated for ampicillin compared with that calculated for cefaclor, the resulting periplasmic concentration of cefaclor was 5.7 times that of ampicillin. The efficacy of cefaclor may be due to its higher β-lactamase resistance, which allows it to achieve a greater periplasmic concentration and adequate binding to crucial penicillin-binding proteins.

Haemophilus influenzae is a common bacterial pathogen in children and immunocompromised adults (1, 10). Ampicillin and chloramphenicol were recommended for the treatment of *H. influenzae* infections. However, the emergence of ampicillin- and chloramphenicol-resistant strains over the past few years (26), the concern over the toxicity of chloramphenicol, and the difficulties in monitoring concentrations of chloramphenicol in the sera of young children (31) have prompted a search for alternative antibacterial agents.

An important alternative to the antibiotics mentioned above for the treatment of *H. influenzae* infections is the use of cephalosporins such as cefaclor. This antibiotic is less susceptible than ampicillin to the action of the commonly found TEM-1 β-lactamase in *H. influenzae* (8, 11, 15, 27) and demonstrates good antibacterial activity against such β-lactamase-producing strains (12, 13, 17, 19, 35). A study (16) reported a reduced activity of cefaclor against β-lactamase-producing *H. influenzae*, a discrepancy that could be due to specific test media, inoculum size, and the known instability of cefaclor in test medium (17, 18).

Cefaclor has been used clinically for about 12 years, and despite its extensive use worldwide, *H. influenzae* isolates remain highly susceptible to the drug (12, 35). This is in contrast to the high prevalence of ampicillin resistance which is now observed in 15 to 50% of *H. influenzae* clinical strains (6, 7, 26). The molecular basis underlying the efficacy of cefaclor has never been systematically studied.

In this report, we present data that enhance the molecular understanding of cefaclor's inhibitory effect on *H. influenzae*. The use of a whole-cell system permitted us to clearly record and characterize the antibiotic binding to cell targets of β-lactams (the penicillin-binding proteins [PBPs]) in bacteria that had kept their outer membrane integrity and cryptic β-lactamase.

The test organisms were *H. influenzae* Rd and its isogenic TEM-1 β-lactamase-producing counterpart Rd/TEM. The origin and construction of these strains have been described elsewhere (30). Cells were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 15 μg of both hemin (Eastman Kodak, Rochester, N.Y.) and NAD (Sigma Chemical Co., St. Louis, Mo.) per ml. The strains were maintained on chocolate agar plates (Quélab Laboratories, Montreal, Quebec, Canada). MICs were determined by a broth microdilution technique (14) in brain heart infusion broth supplemented with 15 μg of hemin and NAD per ml. Test organisms were adjusted to a final inoculum of 10^6 CFU/ml. Following 18 h of incubation at 37°C, the MICs were read as the lowest dilution of antibiotic allowing no visible growth. Ampicillin was from Ayerst Laboratories (Montreal, Quebec, Canada), cefaclor was from Eli Lilly & Company (Indianapolis, Ind.), and cephaloridine and cephalothin were from Sigma.

The procedure for binding radiolabeled penicillin was described previously (9, 23). Radiolabeling of PBPs was performed by a modification of the method of Spratt (34), as described by Preston et al. (32), by using ^{125}I-penicillin V as the labeled β-lactam (4). The *p*-(trimethylstannyl)penicillin V was kindly provided by Larry C. Blaszczak (Lilly Research Laboratories) and was iodinated by using Na^{125}I (specific activity, 16 to 17 mCi/μg of iodine) from Amersham Canada Ltd. (Oakville, Ontario, Canada). Cells in phosphate-buffered saline were incubated with 10 μg of clavulanic acid (a generous gift from SmithKline Beecham Pharmaceuticals, Surrey, United Kingdom) per ml for 10 min prior to the addition of 20 μg of ^{125}I-penicillin V (37.3 Ci/mmol) for 30 min at room temperature.

The labeling reactions were stopped by collecting the cell pellets by centrifugation and boiling the samples for 5 min in electrophoretic loading buffer consisting of 2.5% glycerol, 5% 2-mercaptoethanol, and 1% sodium dodecyl sulfate (SDS) in 1 M Tris (pH 6.8). Proteins were separated by electrophoresis on SDS-polyacrylamide gels by using the system of Laemmli and Favre (20) with 6% stacking and 10% separating gels and were stained with Coomassie blue. PBP profiles were visualized by autoradiography after the dried gels were exposed to Kodak X-OMAT AR5 films for 1 to 7 days at −20°C.

In PBP competition experiments, the cells (Rd and Rd/TEM) were prelabeled with various concentrations of a competing unlabeled β-lactam (ampicillin or cefaclor) for 10 min prior to the addition of clavulanic acid and ^{125}I-penicillin V as described above. The concentration of the competing β-lactam that blocked at least 50% of the subsequent binding of the radiolabeled penicillin to a particular PBP (I_{50}) was determined by scanning the PBP profiles obtained on the X-ray film with a Bio-Rad model 620 video densitometer. The I_{50}s were calculated with linear plots ($r \geq 0.95$) of the logarithm of the relative percentage of inhibition of the binding of ^{125}I-penicillin to PBPs versus the logarithm of the

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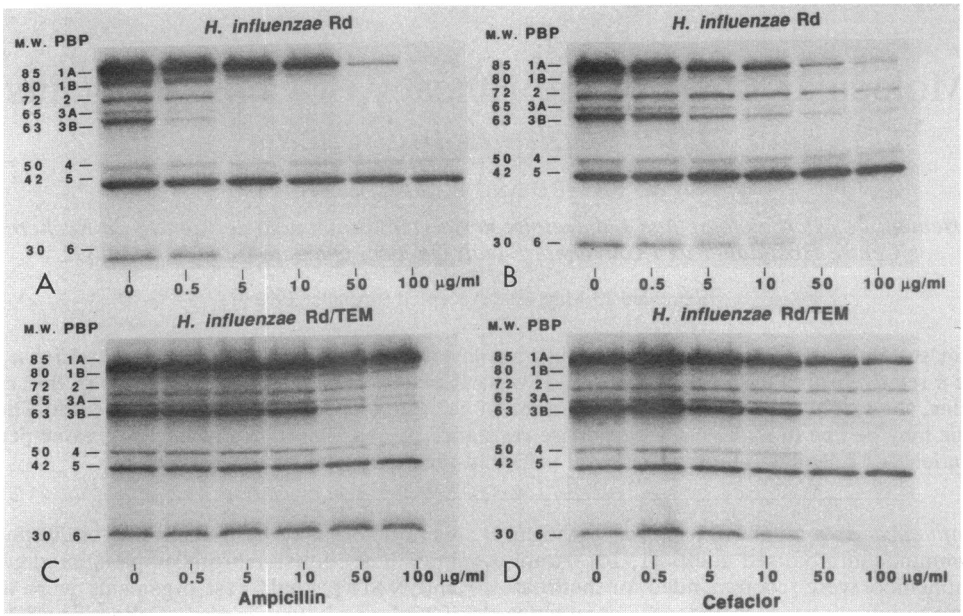

FIG. 1. PBP profiles of *H. influenzae* cells. Cells were labeled with ^{125}I-penicillin V, and proteins were separated by electrophoresis on SDS-polyacrylamide gels before autoradiography. (A and C) PBP profiles obtained with strains Rd and Rd/TEM in PBP competition experiments with unlabeled ampicillin at the indicated concentrations. (B and D) PBP profiles obtained with strains Rd and Rd/TEM in PBP competition experiments with unlabeled cefaclor at the indicated concentrations. The PBPs of *H. influenzae* Rd are identified on the left of each autoradiograph on the basis of the numbering system of Parr and Bryan (30).

concentration of β-lactams. Especially in experiments with strain Rd/TEM, the use of clavulanic acid in all assays was necessary to prevent hydrolysis of the labeled penicillin V, which is necessary for the detection of the unsaturated PBPs. A concentration of 10 μg of clavulanate per ml was chosen because it completely inhibited the β-lactamase activity in whole-cell samples, as tested by the addition of nitrocefin (Oxoid Canada, Nepean, Ontario, Canada) in preliminary tests. We also made sure that the concentration of clavulanic acid used in the PBP assays did not alter the binding of ^{125}I-penicillin V or the PBP profiles of strains Rd and Rd/TEM on autoradiographs.

In outer membrane permeability studies, the rate of diffusion of cefaclor, cephaloridine, and cephalothin across the outer membrane of whole bacterial cells and the kinetic parameters (K_m and V_{max}) of the β-lactamase from disrupted cells were measured essentially by the method of Zimmermann and Rosselet (38), which allows calculation of the antibiotic periplasmic concentration. For ampicillin, data were obtained by using the iodometric method described by Coulton et al. (11). Permeability coefficients for antibiotics at 100 μM were calculated exactly as described by Nikaido et al. (29) and represented the averages of three different experiments.

Eight *H. influenzae* Rd PBPs were labeled with ^{125}I-penicillin V under our experimental conditions. This PBP profile was identical to those obtained in a previous study with ^{35}S-penicillin G in the absence of clavulanic acid in the assay (23). PBPs 1A, 3B, and 5 were the most strongly labeled, as judged by the autoradiograph and the X-ray scan obtained by densitometry. The binding of ampicillin and cefaclor to the PBPs of *H. influenzae* Rd was demonstrated by a competition assay (Fig. 1A and B). The high-affinity targets of both antibiotics were PBPs 3A and 3B, since the binding of ^{125}I-penicillin V to PBPs was efficiently blocked and reduced by low concentrations of ampicillin or cefaclor. Both antibiotics also showed good binding to PBPs 1A and 1B, secondary to PBP 2 in the case of ampicillin. With the β-lactamase-negative strain Rd, the I_{50} of ampicillin to PBP 3B was very low (0.1 μg/ml) and correlated with the antibiotic MIC (0.25 μg/ml) (Table 1). There was a similar correlation with the I_{50} of cefaclor to PBP 3B and its MIC (2.6 and 2 to 8 μg/ml, respectively). In the β-lactamase-producing strain, the situation changed dramatically (Fig. 1C and D and Table 1). Cefaclor sustained its efficacy (MIC, 2 to 8 μg/ml), with an observed I_{50} to PBP 3B of 5.12 μg/ml, whereas ampicillin (MIC, 32 to 64 μg/ml) showed an apparent reduction in its binding to all PBP targets, presumably because of inactivation of the drug by the cryptic β-lactamase. In both cases, the correlation between the MIC and the I_{50} to PBP 3B was maintained (Table 1). Also, only cefaclor seemed sufficiently resistant to the action of β-lactamase to retain PBP 1A binding in strain Rd/TEM, in contrast to the result for ampicillin (Table 1) (apparent ampicillin I_{50}, >100 μg/ml).

The calculated permeability coefficients of β-lactams across the outer membrane of whole bacterial cells and the kinetic parameters (K_m and V_{max}) of the β-lactamase from

TABLE 1. Antibiotic MICs and I_{50}s for *H. influenzae* strains and PBPs

Strain	Antibiotic	MIC (μg/ml)	I_{50} (μg/ml)	
			PBP 1A	PBP 3B
Rd	Ampicillin	0.25-0.25	8.98	0.10
	Cefaclor	2-8	24.6	2.64
Rd/TEM	Ampicillin	32-64	>100	46.3
	Cefaclor	2-8	67.5	5.12

TABLE 2. Kinetic parameters of β-lactamase activity (K_m and V_{max}) from *H. influenzae* Rd/TEM determined for the hydrolysis of β-lactam antibiotics and β-lactam outer membrane permeability

Antibiotic	K_m (μM)	V_{max} (nmol/min/mg)	Permeability coefficient (cm/s [10^{-4}])[a]	β-Lactam concn in periplasm (μM)[a]
Cephaloridine	696.3	833.3 (100)[b]	33.4	104.1
Cefaclor	380.3	64.2 (7.7)	3.10	94.6
Cephalothin	350.3	72.5 (8.7)	0.28	56.1
Ampicillin	84.9	760.8 (91.3)	18.9	16.6

[a] Calculated from the equation of Zimmermann and Rosselet (38) exactly as described by Nikaido et al. (29).

[b] Values in parentheses are the relative V_{max} expressed in percent.

disrupted Rd/TEM cells are reported in Table 2. The β-lactamase hydrolyzed ampicillin more rapidly than it did the cephalosporins tested on the basis of the calculated V_{max}. In a whole-cell permeability assay with antibiotics used at a concentration of 100 μM, a relatively high permeability coefficient was calculated for ampicillin compared with that for cefaclor. However, the resulting periplasmic concentration of cefaclor in the presence of the cryptic β-lactamase was 5.7 times that of ampicillin.

The efficacies of β-lactam antibiotics against gram-negative bacteria depend on the antibiotic concentration attainable in the periplasmic space, where the inner membrane-bound cell targets (PBPs) can be affected. This periplasmic concentration is determined by the rate of penetration of the antibiotic through the bacterial outer membrane, and in the case of β-lactamase-producing strains, the periplasmic concentration of a β-lactam is also limited by its rate of hydrolysis by cryptic β-lactamase (5, 28).

To define the molecular basis of the inhibitory activity of cefaclor against *H. influenzae* in the present study, we used a whole-cell system in which all factors that influence β-lactam activity concurrently interact. As reviewed and discussed before (2, 3), we recognize that the traditional technology used in the present study may have introduced some artifacts leading to imprecisions in the outer membrane permeability data and β-lactamase kinetic measurements: drug concentrations of 100 μM were used in the permeability assays and were well over and below the K_m values determined for ampicillin and cefaclor, respectively. However, our study system allowed us to clearly record and characterize the relative binding of cefaclor and ampicillin (used at different concentrations) to PBPs of bacteria that had kept their integrity (Fig. 1 and Table 1). Furthermore, the whole-cell PBP assays used in the study described here provided external β-lactam I_{50}s, which represented the concentration of β-lactam in the medium needed to bind efficiently to target proteins inside the bacteria after membrane permeation and β-lactamase confrontation.

The permeability of the *H. influenzae* outer membrane to β-lactams was extensively investigated by another group (11). Because of the relatively fast diffusion rate of all β-lactam antibiotics through the outer membrane barrier of *H. influenzae* (11), and because of the large exclusion limit of the *H. influenzae* porins (37), it is very likely that the β-lactam resistance of *H. influenzae* develops by acquisition of a plasmid-mediated β-lactamase and modification of PBPs. Accordingly, β-lactamase and ampicillin resistance is now found in 15 to 50% of strains in certain regions (6, 7, 26), and we (9) as well as other groups (25, 30, 33) have shown that PBP alterations as a mechanism of β-lactam resistance (21) can be found in some isolates. Inversely, the efficacy of a β-lactam against *H. influenzae* would be uniquely associated with its ability to bind to essential PBPs and its stability toward β-lactamase. A β-lactam that possesses these properties would certainly demonstrate good efficacy even against β-lactamase-producing *H. influenzae* strains.

The data presented here suggest that although ampicillin penetrates *H. influenzae* cells relatively faster than cefaclor does, the ability of cefaclor to resist the hydrolytic activity of β-lactamase allows this antibiotic to achieve a greater periplasmic concentration (Table 2) and adequate binding to crucial PBP targets (Fig. 1 and Table 1). Another interesting observation was that with both strains (Rd and Rd/TEM) and β-lactams (ampicillin and cefaclor), the correlation between the antibiotic MIC and the I_{50} to PBP 3B was striking (Table 1) and certainly suggests the primary importance of this *H. influenzae* PBP for normal cell functions. Our laboratory (23) and another group (24) have shown the essential role of this PBP in septal peptidoglycan synthesis and defined some of its properties (22, 23). We cannot exclude the fact that efficient inhibition of *H. influenzae* cells may be due to the combined inhibition of PBPs 1A and 3, which may result in the alteration of two types of peptidoglycan synthesis, as demonstrated previously (36) with a combination of antibiotics that independently target *Escherichia coli* PBPs 1 and 3. In such a case, the ability of cefaclor to bind both PBPs in the presence of a β-lactamase would certainly be a great advantage over an antibiotic like ampicillin, which could not bind sufficiently to PBP 1A in strain Rd/TEM (Fig. 1 and Table 1), presumably because of its greater susceptibility to β-lactamase and lower periplasmic concentration (Table 2).

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