Inactivation of β-Lactam Antibiotics by Legionella pneumophila

KWUNG P. FU AND HAROLD C. NEU*

Division of Infectious Diseases, Departments of Medicine and Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received for publication 13 August 1979

Beta-lactam-inactivating activity has been found in all sero-groups of Legionella pneumophila. The β-lactamase activity could be detected in intact cells and released by ethylenediaminetetraacetic acid treatment, indicating that it is located in the periplasmic space. The enzyme acted primarily as a cephalosporinase hydrolyzing cefamandole, cephalothin, cephaloridine, and also penicillin G and ampicillin. Cefoxitin and cefuroxime were not hydrolyzed. Clavulanic acid and CP-45,899, β-lactamase inhibitors, prevented the hydrolysis of cephalosporins and penicillins. The β-lactamase activity appears to be different from that found in Enterobacteriaceae and Pseudomonas.

The reported in vitro resistance of Legionella pneumophila to penicillins and cephalosporins (7, 8) as well as the clinical failure of these agents in the treatment of patients with Legionnaire's pneumonia (1) prompted us to evaluate the ability of this organism to destroy β-lactam antibiotics and to clarify whether the inactivating activity was due to the presence of a β-lactamase.

MATERIALS AND METHODS

Organisms and antibiotics. Isolates of L. pneumophila were provided by the Center for Disease Control, Atlanta, Ga. The following isolates were examined: Seattle, Vermont, Flint, Knoxville, Philadelphia, Pontiac, Bloomington, Togus, and Los Angeles.

Organisms were maintained on Mueller-Hinton agar plates supplemented with 0.04% cysteine and 0.025% ferric phosphate. The antibiotics studied were gifts from their respective manufacturers. Clavulanic acid was provided by Beecham Laboratories and CP-45,899, a penicillanic acid sulfone, was provided by Pfizer Inc.

Susceptibility tests. Susceptibility tests were performed in Mueller-Hinton broth containing 0.04% cysteine and 0.1% ferric phosphate. The inoculum used was 10^5 colony-forming units. This was prepared by making a suspension in broth of a 48-h growth removed from agar plates. Tubes were incubated at 35°C for 72 h in the presence of 5% CO₂.

Osmotic shock tests. Osmotic shock tests were prepared by the technique of Neu and Chou, using 0.1 g of bacteria to 10 ml of 1 mM ethylenediaminetetraacetic acid in 10 mM tri(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.0.

Enzyme preparation. Three-day-old cultures were removed from agar plates by means of a glass spatula, using potassium phosphate buffer (0.05 M, pH 7). The cells were washed twice in the same buffer and then suspended in buffer at a concentration of 10^8 cells per ml. The cells were sonically disrupted by a Branson Sonifier, using 30-s pulses for 3 min with the vessel cooled in an ice bath. All procedures were performed in a hood which incinerated the exhaust. The sonic extracts were centrifuged for 2 h at 4°C and 40,000 × g. The cellular debris was discarded, and the supernatant was dialyzed for 24 h at 4°C against the phosphate buffer. This material was used for the assays.

β-Lactamase assays. Intact organisms, material from osmotically shocked organisms, and the crude extract were tested for β-lactamase activity with the nitrocefin (5), microiodometric (3), and microbiological (2) assays. Destruction of penicillins and cephalosporins was determined by the agar well technique, using Staphylococcus aureus ATCC 25923 as the assay organism for penicillins and Sarcina lutea for cephalosporins; the exception was cefsulodin, which was assayed with Pseudomonas aeruginosa 9414, our collection, and cefotaxime was assayed with Escherichia coli 3989, our collection.

Determination of breakdown products. Intact cells or crude extracts were incubated with cefamandole or cephalothin for varying periods of time from 10 min to 3 h. The material was applied by spot application to Whatman no. 4 paper strips and subjected to chromatography for 5 h in a solvent system of methyl ethyl ketone saturated with water. The paper was air dried for 5 min and then applied to agar containing glass plates which had been inoculated with Bacillus subtilis spores. The strips were removed, and the plates were incubated at 37°C overnight. Standards of cephalothin, cefamandole, 3-hydroxycefamandole, and deacetylcephalothin were run in the same system.

RESULTS

The in vitro activities of a number of penicillins and cephalosporins against the Knoxville, Tenn., Philadelphia, Pa., and Pontiac, Mich.,
isolates of Legionella are given in Table 1. The minimum inhibitory concentrations of the penicillins are low, less than 1 \( \mu g/ml \), whereas those of cefalothin, cephaloridine, and cefamandole are 12.5 \( \mu g/ml \). In contrast, the minimum inhibitory concentration for cefoxitin was 0.1 \( \mu g/ml \) and that of cefuroxime was 0.2 \( \mu g/ml \).

Utilizing penicillin G or ampicillin as substrate and intact cells, growth medium, a supernatant of ethylenediaminetetraacetic acid cold-shocked cells, or a sonic extract as enzyme source, no \( \beta \)-lactamase activity could be found when the iodometric \( \beta \)-lactamase assay was used with penicillin G or ampicillin as substrate. Activity could be demonstrated with use of the chromogenic cephalexin assay nitrocefin for all of the isolates tested. Activity was cell bound and cryptic since it could be revealed by ethylenediaminetetraacetic acid treatment.

Use of a spectrophotometric assay with cephaloridine as substrate to determine hydrolysis of cephalexin proved to be an unsatisfactory means of analyzing hydrolysis. For this reason a microbiological assay was used. The optimal substrate for the enzyme was found to be cefamandole. If a unit of activity were defined as 1 \( \mu \)mol of cefamandole hydrolyzed at 37°C (pH 7.0) per h, a sonic preparation would contain only 1.69 U/mg of protein. The enzymatic activity of L. pneumophila (Knoxville) produced destruction of cephalexin other than cefamandole, namely, cephaloridine, cefalothin, cephalaxin, cephametide, and cephalaxin (Table 2). Cefoxitin, cefuroxime, cephaloglycin, and cef sulfotidin were not inactivated, but cefotaxime, which has been shown to be \( \beta \)-lactamase stable, underwent some inactivation.

Since this pattern of inactivation of \( \beta \)-lactam compounds did not conform to that of any \( \beta \)-lactamase thus far described (6), we determined the effect of two \( \beta \)-lactamase inhibitors on the hydrolytic activity of extracts of Legionella. Clavulanic acid (4) and CP-45,899, a penicillanic acid sulfone (3), have been shown to be effective inhibitors of certain types of \( \beta \)-lactamases. CP-45,899 inhibited the hydrolysis of ampicillin by Legionella with 35 to 40% reduction in hydrolysis over a 3-h period (Fig. 1). On the other

| Antibiotic       | Rate of inactivation* |
|------------------|----------------------|
| Penicillin G     | 24                   |
| Ampicillin       | 20                   |
| Cefalothin       | 30                   |
| Cephaloridine    | 26                   |
| Cephalexin       | 38                   |
| Cephametide      | 40                   |
| Cephaloglycin    | 0                    |
| Cefamandole      | 100                  |
| Cefoxitin        | 0                    |
| Cefuroxime       | 15                   |
| Cefotaxime       | 15                   |
| Cef sulfotidin   | 0                    |

* Expressed as percentage of inactivation of cefamandole (20 \( \mu g/ml \)) by 0.01 U of crude enzyme for 30 min at 37°C.

FIG. 1. Inhibition of the hydrolysis of (A) ampicillin and (B) penicillin by CP-45,899. Symbols: ●, enzyme; ▲, enzyme + 0.5 \( \mu g \) of CP-45,899 per ml; ○, control blank; △, heat enzyme.
hand, it inhibited 60% of the hydrolysis of penicillin. In Fig. 2 is shown the inhibition of cefamandole hydrolysis by clavulanic acid and CP-45,899. The inhibition is only partial at low concentrations of clavulanic acid but is increased to 60% inhibition when the concentration of clavulanic acid is increased fourfold. In contrast, CP-45,899 produced almost complete inhibition of the hydrolysis of cefamandole.

To determine that the \( \beta \)-lactam-inactivating activity was not the result of esterase activity, the products of an incubation of the \( L. \) pneumophila sonic extract with cephalothin and cefamandole were chromatographed (Fig. 3). Neither deacetylcephalothin nor 3-hydroxycefamandole was detected. After a 3-h incubation, all antibacterial activity was destroyed, as it was when the compounds were incubated with a control \( \beta \)-lactamase.

**DISCUSSION**

These studies confirm the observations of Thornsberry and Kirven (8), who suggested that \( L. \) pneumophila contained a \( \beta \)-lactamase. As is the case with the \( \beta \)-lactamases found in members of the \( Enterobacteriaceae \), the enzyme is cell bound at the surface of the cell in the periplasmic space (6). The enzyme does not conform to the character of most of the described plasmid or chromosomally mediated \( \beta \)-lactamases (5, 6). It appears to be primarily a cephalosporinase. The destruction of cefamandole, but not of cefuroxime, suggests that the enzyme is similar to enzymes found in \( Citrobacter \) and \( Enterobacter \). However, the partial hydrolysis of cefotaxime and the inhibition of the hydrolysis of penicillin and cephalosporin by clavulanic acid and CP-45,899 suggest that this enzyme belongs to a new class since we have earlier shown that \( \beta \)-lactamases of the so-called Richmond type 1 class are not effectively inhibited by these \( \beta \)-lactamase inhibitors (3, 4).

It is difficult to explain the lack of clinical activity of the penicillins against \( L. \) pneumophila since minimum inhibitory concentrations of 0.4 to 0.8 \( \mu \)g/ml are well below the concentrations
required to inhibit aerobic and anaerobic gram-negative bacilli. What role β-lactamase might play in the resistance of this organism is unclear, unless the enzyme degrades antibiotic carried into phagocytic cells with the organisms.

Efforts are underway to further characterize the β-lactamase of *Legionella* and to clarify its role in these organisms.

**LITERATURE CITED**

1. Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, and P. S. Brachman. 1977. Legionnaire’s disease. Description of an epidemic pneumonia. N. Engl. J. Med. 297:1189-1197.

2. Fu, K. P., and H. C. Neu. 1978. A comparative study of the activity of cefamandole and other cephalosporins and analysis of the β-lactamase stability and synergy of cefamandole with aminoglycosides. J. Infect. Dis. 137(Suppl.):38-48.

3. Fu, K. P., and H. C. Neu. 1979. Comparative inhibition of β-lactamases by novel β-lactam compounds. Antimicrob. Agents Chemother. 15:171-176.

4. Neu, H. C., and K. P. Fu. 1978. Clavulanic acid, a novel inhibitor of β-lactamases. Antimicrob. Agents Chemother. 14:650-655.

5. O’Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler. 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. 1:283-288.

6. Richmond, M. H., and R. B. Sykes. 1973. The β-lactamases of gram-negative bacteria and their possible physiological role. Adv. Microb. Physiol. 9:31-85.

7. Thornsberry, C., C. N. Baker, and L. A. Kirven. 1978. In vitro activity of antimicrobial agents on the Legionnaire’s disease bacterium. Antimicrob. Agents Chemother. 13:78-80.

8. Thornsberry, C., and L. A. Kirven. 1978. β-Lactamase of the Legionnaire’s bacterium. Curr. Microbiol. 1:51-54.