Sodium Clavulanate Potentiation of Cephalosporin Activity Against Clinical Isolates of Cephalothin-Resistant
*Klebsiella pneumoniae*

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Plasmid-carrying *Klebsiella pneumoniae* clinical isolates with agar dilution minimum inhibitory concentrations (MIC) of 32 μg/ml or greater were tested for in vitro potentiation of cephalothin activity by clavulanic acid (BRL-14151), an inhibitor of beta-lactamases. The addition of 10 μg of clavulanate per ml caused greater than a 500-fold reduction in geometric mean cephalothin agar dilution MIC, with lesser but significant reductions resulting from clavulanate concentrations of 5 or 1 μg/ml. Clavulanate-potentiated reduction of cephalothin MICs in broth against resistant *Klebsiella* were comparable to reduction in agar dilution MICs as a rule. However, a low concentration (1 μg/ml) of clavulanate produced cephalothin MICs in broth several-fold higher than by the agar dilution method. Modest cephalothin-potentiating effects of clavulanate on cephalothin-susceptible strains and on cefoxitin against cephalothin-resistant *Klebsiella* strongly suggested that the major effect of clavulanate was beta-lactamase inhibition.

Cephalosporins remain the beta-lactam antibiotics of choice for *Klebsiella pneumoniae* infections (11). However, high-level natural resistance of a minority of strains and ease of induction of resistance in the presence of subinhibitory concentrations of cephalosporins in vitro led to the prediction that cephalosporin-resistant *Klebsiella* would likely become prevalent (1). Resistance among *Klebsiella* to beta-lactam antibiotics, which has become increasingly prevalent among clinical isolates of gram-negative bacilli (2), results largely from beta-lactamase. Recent hospital-associated cephalosporin-resistant *Klebsiella* species have often possessed TEM-like or other beta-lactamases which inactivate cephalosporins as well as penicillins (7). Since 1974 our hospital has been experiencing an outbreak of infections with *Klebsiella* usually resistant to all antimicrobial agents except tobramycin, amikacin, and polymyxin B (10) (Fig. 1). These plasmid-carrying strains have cephalothin agar dilution minimal inhibitory concentrations (MICs) of ≥32 μg/ml, presumably mainly due to beta-lactamase activity. Consequently, clavulanic acid, a potent inhibitor of beta-lactamases (8), was examined for in vitro potentiation of cephalothin against our cephalothin-resistant *Klebsiella*.

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**MATERIALS AND METHODS**

**Bacteria.** Ten multidrug-resistant *K. pneumoniae* isolates from clinical specimens were lyophilized. These were of serotypes 2, 21, and 22, which were the prevalent resistant serotypes in our hospital. Serotyping was performed by the Enteric Section of the Enterobiology Laboratory, Center for Disease Control, Atlanta, Ga. Lyophilized cultures were reconstituted and plated on Mueller-Hinton agar where they were maintained at 4°C for periods up to 1 month during antimicrobial testing. No change in antimicrobial agent susceptibility occurred during storage or testing. Ten randomly selected cephalothin-susceptible *Klebsiella* of several serotypes were obtained from clinical isolates from our hospital and stored and tested in a similar fashion.

**Antimicrobial agents.** Clavulanic acid as sodium salt was supplied lyophilized by Beecham Laboratories, Bristol, Tenn. It was reconstituted in 0.1 N phosphate-buffered saline, pH 7.4, and subsequently frozen in 1,000-μg/ml aliquots, which were frozen at –70°C until used. Cephalothin diagnostic powder was supplied by Lilly Research Laboratories, Indianapolis, Ind. Cefoxitin diagnostic powder was supplied by Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.

**Agar dilution MICs.** The International Collaborative Study method (12) for agar dilution susceptibility testing was performed using Mueller-Hinton agar. The method was modified only in that the inoculum from overnight growth was carefully adjusted to yield 10⁶ colony-forming units (CFU) when applied to the surface of agar plates spotted with a 0.001-ml calibrated loop. Agar dilution inhibitory concentrations...
were defined by the antimicrobial agent concentration inhibiting visible growth at 37°C after 24 h.

Broth dilution susceptibility testing. International Collaborative Study methods were also employed for a tube broth dilution system using Mueller-Hinton broth (12). The adjusted inoculum was approximately 10⁶ CFU per ml. MICs in broth were determined by the concentration of antimicrobial agent inhibiting visible growth.

Timed growth curve studies ("killing curves"). Mueller-Hinton broth cultures were inoculated with 10⁵ CFU of Klebsiella. Cultures were initiated without antimicrobial agents or containing either cephalothin (10 μg/ml), clavulanate (1 or 10 μg/ml), or a mixture of 10 μg of cephalothin and 1 or 10 μg of clavulanate per ml. Cultures were incubated at 37°C and were sampled at various times up to 24 h for counts of viable bacteria. CFU of surviving bacteria were enumerated by counting visible colonies of bacteria in Mueller-Hinton agar pour plates incubated for 24 h at 37°C.

Beta-lactamase production. Beta-lactamase activity was confirmed by adding growing bacteria or supernatant fluids from pressure-disrupted (Aminco laboratory press, 15,000 lb/inch²) bacteria to a 500-μg/ml solution of the chromogenic cephalosporin 87/312 provided by Glaxo Laboratories, Greenford, Middlesex, England (5). Rapid change in color from straw yellow to cherry red indicating lysis of the cephalosporin beta-lactam ring characterized our cephalothin-resistant isolates compared with a very gradual change for susceptible strains.

RESULTS

Klebsiella from our clinical isolates belonged to three serotypes—2 (four isolates), 21 (three isolates), and 22 (three isolates). Beta-lactamase (cephalosporinase) existed to a much greater degree in the resistant than susceptible Klebsiella isolates, as indicated by rapid hydrolysis of the chromogenic cephalosporin 87/312.

The degree of resistance to cephalothin is indicated in Fig. 2, depicting the cumulative percentage of the 10 isolates susceptible to either cephalothin or cephalothin plus clavulanate. None of the 10 isolates was inhibited in the agar dilution assay by less than 64 μg of cephalothin alone, and two isolates required 1,000 μg/ml for inhibition. The geometric mean agar dilution MIC of cephalothin was 376 μg/ml. Clavulanate by itself had a mean agar dilution MIC of 28 μg/ml against these Klebsiella, whereas concentrations of clavulanate far below that level potentiated cephalothin activity. In a mixture containing 1 μg of clavulanate, no isolate required more than 8 μg of cephalothin per ml for inhibition or, in the presence of 5 or 10 μg of clavulanate, more than 4 μg of cephalothin per ml. The geometric mean concentration of cephalothin inhibiting these resistant Klebsiella in a mixture containing 10 μg of clavulanate per ml was only 0.7 μg/ml.

Results of agar dilution susceptibility testing of 10 randomly selected cephalothin-susceptible isolates from our hospital are indicated in Fig. 3. These 10 isolates were of 10 different serotypes—2, 5, 9, 14, 16, 17, 20, 21, 27, and 63. None of these susceptible isolates required greater than 16 μg of cephalothin per ml for inhibition. Clavulanate susceptibility was virtually the same for these as the resistant isolates. However, cephalothin-potentiating activity of clavulanate was much less for susceptible than resistant isolates. In the presence of a mixture containing 1 to 10 μg of clavulanate per ml, 8 μg of cephalothin was still required for inhibition of all susceptible strains.

Table 1 summarizes clavulanate potentiation of cephalothin inhibition of clinical isolates of resistant Klebsiella. The results in agar dilution assays were compared to results using similar concentrations of the beta-lactam antimicrobial agents in broth dilution assays. An inoculum of 10⁶ CFU was used for both agar dilution and broth dilution tests. Results by the two methods were comparable except that in broth lower concentrations of clavulanate (especially 1 μg/ml) resulted in disparately higher requirements of cephalothin for Klebsiella growth in-
FIG. 2. Potentiation of cephalothin activity against multidrug-resistant Klebsiella by sodium clavulanate. The vertical axis depicts the cumulative percentage of strains (isolates) inhibited. The horizontal axis indicates the agar dilution MIC against Klebsiella in the presence of the designated concentration of clavulanate.

FIG. 3. Minor potentiating effect of clavulanate on cephalothin’s activity against cephalothin-susceptible Klebsiella. The vertical axis indicates the cumulative percentage of strains (isolates) inhibited by the concentrations of cephalothin agar dilution MIC in the horizontal axis. Three concentrations of clavulanate are indicated.

Inhibition than in agar. Incorporation of the standard $10^4$ International Collaborative Study inoculum in the agar dilution tests (data not shown) resulted in even lower cephalothin agar dilution MICs. With the lower inoculum, 0.29 μg of cephalothin per ml was inhibitory in the presence of 5 μg of clavulanate as compared with 1.5 μg of cephalothin per ml with the $10^6$ CFU inoculum. Disparity between agar and broth dilution assays did not occur with susceptible *Klebsiella* isolates.

The potentiating effect of clavulanate upon activity of a beta-lactamase-resistant cephalosporin compound, cefoxitin, against resistant...
Klebsiella isolates is depicted in Fig. 4. The clavulanate-potentiating effect on cefoxitin is illustrated in the same figure for comparative purposes. Clavulanate failed to potentiate appreciably cefoxitin activity. A 10-μg/ml amount of clavulanate resulted in a 537-fold reduction (376 μg reduced to 0.7 μg/ml) in the mean agar dilution MIC of cefoxitin against Klebsiella. The same concentration of clavulanate afforded only a 2.5-fold decrease in the cefoxitin agar dilution MIC (11.3 μg reduced to 4.6 μg/ml). Against susceptible (non-beta-lactamase-producing) isolates 10 μg of clavulanate caused the same type of low-order reduction in cefoxitin agar dilution MIC (9.5 μg reduced to 3.8 μg/ml).

Time growth curve studies (killing curves) confirmed that clavulanate’s action with cephalothin was lethal to Klebsiella in broth cultures. Figure 5 depicts such a killing curve with strain no. 10 (serotype 22), which was the most susceptible of our isolates to the mixture of cefoxitin and clavulanate (broth dilution cefoxitin MIC in the presence of 1 μg of clavulanate per ml = 4 μg/ml). Greater than 4 log decrease in the number of surviving Klebsiella occurred in the presence of 10 μg of cephalothin per ml with the addition of either 1 μg or 10 μg of clavulanate per ml—a clear bactericidal effect.

A killing curve employing another resistant strain (no. 5, serotype 21), which had more typical susceptibilities by broth dilution to the mixture of beta-lactam agents (cephalothin MIC in

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* Concentration of added sodium clavulanate.

b Geometric mean concentration (micrograms per milliliter) of cephalothin (± clavulanate) inhibiting K. pneumoniae growth. Numbers in parentheses indicate range of inhibitory concentrations.

FIG. 4. Comparison of cefoxitin and cefoxitin potentiation by clavulanate. The percentage of cephalothin-resistant strains (isolates) inhibited is indicated on the vertical axis. The inhibitory concentrations (agar dilution MICs) of the cephalosporin compounds with and without addition of 10 μg of clavulanate per ml are indicated on the horizontal scale.
FIG. 5. Growth inhibition (killing) curves depicting the number of viable cephalothin-resistant *Klebsiella* in broth cultures containing the denoted concentrations of cephalothin or clavulanate alone or in combination. The vertical axis depicts the number of viable bacteria (CFU) at the periods of incubation denoted on the horizontal axis. The numerals beside the antibiotics indicate their concentrations (micrograms per milliliter).

broth of 128 μg/ml in the presence of 1 μg of clavulanate per ml), is illustrated in Fig. 6. Although the inoculum was killed by 10 μg of cephalothin plus 10 μg of clavulanate, only a temporary reduction in the number of *Klebsiella* followed by regrowth to control concentrations of organisms was achieved by 10 μg of cephalothin and 1 μg of clavulanate.

**DISCUSSION**

*K. pneumoniae* infections may be associated with significant morbidity and mortality. Cephalosporins have become valued therapeutic agents in *Klebsiella* infections due to their marked intrinsic activity against *Klebsiella* and their safety and ease of administration. *Klebsi-
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**Fig. 6.** Growth inhibition curve experiment similar to that in Fig. 5 except that an isolate (strain no. 5) was used which has a higher cephalothin broth dilution MIC in the presence of clavulanate. The numerals beside the antibiotics indicate their concentrations (micrograms per milliliter). Regrowth to control values occurred in the presence of 10 μg of cephalothin and 1 μg of clavulanate per ml.

_Ella_ strains with high-level cephalosporin resistance have now emerged in a number of hospitals in Europe and the United States (7, 10). These fully virulent strains owe their resistance mainly to acquisition of plasmids coding for cephalosporin-degrading beta-lactamases (7, 10). Presently, approximately 60% of _Klebsiella_ clinical isolates in our hospital have cephalothin agar dilution MIC ≥32 μg/ml, causing us to examine alternative therapeutic regimens. Sodium clavulanate (BRL-14151), a relatively stable salt of clavulanic acid, is effective against a variety of gram-positive and gram-negative beta-lactamases (8). Clavulanate, itself a beta-lactam compound, potentiates ampicillin to a degree which might be clinically effective against certain _Klebsiella_.

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However, *Klebsiella* having plasmid-acquired beta-lactamase in addition to intrinsic beta-lactamase (9) may not respond optimally to ampicillin plus clavulunate. Ampicillin agar dilution MICs against our plasmid-containing *Klebsiella* strains were decreased much less by clavulunate than were cephalothin MICs (unpublished data), indicating especial potential for the clavulanate-cephalosporin combination against *Klebsiella* having acquired beta-lactamase.

The cephalothin-potentiating effect of clavulanate was striking (Fig. 2). Greater than 500-fold geometric mean reduction in cephalothin agar dilution MIC was achieved by addition of 10 μg of clavulanate per ml, a concentration which could be reasonably expected with oral clavulanate administration (P. Hunter, C. Reading, and D. A. Wittig, Program Abstr. 10th Internat. Cong. Chemother., Zurich, Switzerland, Abstr. no. 238, 1977). Such a striking reduction in MICs almost certainly resulted from inhibition of plasmid-acquired beta-lactamase. To test this supposition, we examined the clavulanate-cephalothin-potentiating effect against randomly selected cephalothin-susceptible *Klebsiella* isolates presumed to be free of plasmid-mediated beta-lactamase (Fig. 3). A minor degree of potentiation (sixfold reduction in agar dilution MIC) accrued from addition of 10 μg of clavulanate per ml. This lesser effect could have resulted either from inhibition of intrinsic (non-plasmid-mediated) *Klebsiella* beta-lactamase or might have resulted from the direct antimicrobial action of the beta-lactam, clavulanate.

Experiments with cefoxitin, a cephamycin compound which almost totally resists the action of a wide variety of gram-negative beta-lactamases (6), also indirectly supported the notion that clavulanate's major action was inhibition of beta-lactamase. The potentiating effect of clavulanate on cefoxitin (already beta-lactamase resistant) was small (only 2.4-fold in the presence of 10 μg of clavulanate per ml) and of the same order that 10 μg of clavulanate per ml effected with cephalothin on cephalothin-susceptible *Klebsiella* (4-fold). The latter apparently lacked plasmid-mediated beta-lactamase. Geometric mean agar dilution MICs for clavulanate against our cephalothin-resistant and -susceptible strains were 28 and 31 μg/ml, reflecting clavulanate's weak direct antimicrobial effect against *Klebsiella*.

The bactericidal effect of the cephalothin-clavulanate mixture was confirmed by time growth studies in which greater than 10,000-fold decrements in the number of viable *Klebsiella* regularly occurred in the presence of 10 μg of cephalothin plus 10 μg of clavulanate per ml. The majority of strains in broth containing 10 μg of cephalothin and 1 μg of clavulanate per ml, after temporary inhibition regrew to control values by 24 h. This suggested the presence of heterosusceptible populations of *Klebsiella* having a tendency to regrow in cultures containing marginal antibiotic concentrations. In vitro, resistance of *Klebsiella* to beta-lactam antibiotics including cefoxitin has been effected by serial passage in subinhibitory concentrations (3). Beta-lactamase induction could have resulted from subinhibitory concentrations of either or both of the beta-lactam agents. Alternatively, in broth, cephalothin could have been degraded to less active desacetyl cephalothin (13), contributing to regrowth. Under corresponding culture conditions, cephalothin biological activity diminished 50% in 24 h (4). Some degradation of low-concentration clavulanate also might have occurred over time. Similar considerations of antibiotic degradation and bacterial regrowth in broth could apply to discrepancies between broth and agar dilution MICs in the presence of a low concentration of clavulanate.

Clavulanate potentiation of cephalothin activity in vitro is of such a high degree that clinical efficacy in cephalothin-resistant *Klebsiella* infections might be anticipated. Such an approach could be particularly valuable in clinical situations where aminoglycosides are of limited efficacy such as in gram-negative bacillary pneumonias and in *Klebsiella* urinary tract infections occurring in patients with significantly compromised renal function. It is essential not to extrapolate the importance of *Klebsiella* regrowth in highly artificial killing-curve studies. However, it would seem prudent to adjust dosage levels and intervals in vivo to preclude such an environment in marginally perfused, infected tissues.

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**LITERATURE CITED**


