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Assessment of Two Penicillins plus β-Lactamase Inhibitors versus Cefotaxime in Treatment of Murine *Klebsiella pneumoniae* Infections

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The in vivo efficacies of piperacillin, piperacillin plus tazobactam, ticarcillin, ticarcillin plus clavulanic acid, piperacillin plus clavulanic acid, and cefotaxime were compared in a mouse model of pneumonia induced by the SHV-1 β-lactamase-producer *Klebsiella pneumoniae*. Each antibiotic was injected either once intraperitoneally at 24 h postinfection or at repeated times during 24 h. The efficacies of the drugs and therapeutic protocols were assessed by counting viable bacteria recovered from the lungs of mice sacrificed at selected times. No emergence of β-lactam-resistant organisms was detected. Ticarcillin at 300 mg/kg was ineffective. Repeated injections of piperacillin at 300 mg/kg, either alone or in combination with tazobactam (8:1), led to a significant decrease in bacterial counts, but this was followed by bacterial regrowth. The pharmacokinetic analysis demonstrated that this short-lasting antibacterial effect was not due to a failure of piperacillin and/or tazobactam to penetrate the lungs. The combinations of ticarcillin at 300 mg/kg plus clavulanic acid (15:1) and piperacillin at 300 mg/kg plus tazobactam (4:1) were proven to be effective in that they decreased the bacterial burden in the lungs from 10^5 to $<10^3$ CFU. This dose effect of tazobactam can be explained by its dose-dependent penetration in the lungs. Cefotaxime at 100 mg/kg and the combination of piperacillin (slightly hydrolyzed by SHV-1) at 300 mg/kg plus clavulanic acid (15:1) led to the best efficacy. Both of these treatments induced a decrease in bacterial counts of nearly 4 $\log_{10}$ units. The survival rates correlated with the quantitative measurements of in vivo bacterial killing. These experimental results obtained from the restricted animal model used here may help in the design of further protocols for clinical trials.

Klebsiella pneumoniae produces SHV-1 penicillinase, which hydrolyzes amino- and carboxypenicillins and slightly hydrolyzes ureidopenicillins (6). However, the phenotype can be interpreted as resistant to amoxicillin and ticarcillin and susceptible to piperacillin on the basis of empirical breakpoints (16). In fact, clinical failures in the treatment of infections due to *K. pneumoniae* with piperacillin have been reported (14). Therefore, we have categorized these strains as having an intermediate (in fact, uncertain) degree of resistance to piperacillin by using "comparative and interpretative reading" (20, 21).

Several factors make it difficult to evaluate the efficacy of an antimicrobial agent on the basis of clinical reports, including the severity of the infection, the immune status of the host, interactions with other drugs, and the criteria chosen for assessing success or failure. Therefore, experimental therapeutic effects in animal models can help in the interpretation of the efficacy of a drug. In the present study we used a reproducible mouse model mimicking natural pulmonary infection to compare the in vivo efficacies of piperacillin, piperacillin plus tazobactam, ticarcillin plus clavulanic acid, piperacillin plus clavulanic acid, and cefotaxime against a *K. pneumoniae* human isolate.

MATERIALS AND METHODS

Bacterial strain. *K. pneumoniae* DAR, serovar K2, received from V. Vernet, (Bacteriology Department, University Hospital, Reims, France) harbors plasmid-associated virulence factors, i.e., aerobactin and a mucoid phenotype (5, 15, 26). This strain was grown on tryptocasein-soy agar (TCS) (Diagnostics Pasteur, Marnes-la-Coquette, France) overnight at 37°C from stock cultures frozen at −70°C in 30% glycerol. It produced the SHV-1 β-lactamase (pI 7.7). Using the disk diffusion method and the recommendations of the National Committee for Clinical Laboratory Standards (16), we found that the strain was resistant to amoxicillin and ticarcillin and susceptible to piperacillin.

Antibiotics. The following five β-lactams with known potencies were kindly supplied by the manufacturers: piperacillin and tazobactam (Léderlé, Oullins, France), ticarcillin and clavulanic acid (Beecham, Paris, France), and cefotaxime (Roussel, Romainville, France).

In vitro susceptibility testing. The β-lactams were tested alone or in combination with β-lactamase inhibitors. Initially the ratios were those used clinically, i.e., 15:1 for ticarcillin-clavulanic acid and 8:1 for piperacillin-tazobactam. For the latter combination, another ratio, 4:1, was also used. A new combination, piperacillin-clavulanate (15:1) was also tested.

The MICs were determined by the macrodilution method in Mueller-Hinton broth (7). The final antibiotic concentrations ranged from 0.12 to 128 μg/ml. The inocula, prepared from an overnight Mueller-Hinton broth culture at 37°C and adjusted by dilution in phosphate-buffered saline (PBS), were 5×10^5 and 5×10^7 CFU/ml. The MBCs were determined by the macrodilution method in Mueller-Hinton broth with the same inocula used for determinations of MICs (24). The bacterial counts were determined after 3, 6, and 18 h at 37°C.

Escherichia coli ATCC 25922 was used as a quality control for each antibiotic in each experiment.

Experimental infection of mice. Pathogen-free female BALB/c mice aged 5 weeks and weighing 20 ± 2 g at the time of the experiment (CERJ, Le Genest St Isles, France) were held in groups of six in filter-topped cages with free access to sterile food and water in a biosafety containment facility.

Bacterial inocula were prepared from an 18-h TCS broth culture at 37°C and diluted in PBS buffer. For preliminary experiments, the inoculum of 2×10^3 CFU per mouse was chosen to ensure both a reproducible and fatal pulmonary infection and a survival longer than 48 h and shorter than 96 h for all of the animals. Mice anesthesized by a short exposure to ether vapors received this bacterial inoculum in a volume of 50 μl intranasally.

Assays of pharmacokinetics in infected mice. The doses of antibiotics were similar to those previously reported for mouse models (1–3, 8, 9). The goal of the

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dosage choices was to achieve peak serum drug levels (or, more exactly, early concentrations) in mice that were very similar to those achieved in humans.

Pharmacokinetics in serum and/or tissues of mice are known for all of the β-lactams used in the present work (1–3, 8, 9) except the combination of piperacillin and tazobactam. Therefore, concentrations of piperacillin and tazobactam in both sera and lungs of infected mice were determined, since the pharmacokinetics of tazobactam and tazobactam plus piperacillin in mice have not been documented. Five mice were sacrificed by an overdose of sodium pentobarbital (Sanofi Santé Animale, Libourne, France) at 5, 15, 30, and 60 min after a series of intraperitoneal injections, over 24 h, of piperacillin-tazobactam combinations at ratios of 8:1 and 4:1. Blood samples collected by cardiac puncture were centrifuged at 3,000 × *g* for 5 min. Serum specimens were kept frozen at −80°C until used. The lungs were dissected from the bronchia and dried to avoid blood contamination. They were homogenized in liquid nitrogen (Spex grinder 6700 Freezer/Mill; Spex Industries Inc., Edison, N.J.). The sedimented tissue was resuspended in 500 μl of sterile water by gentle shaking and centrifuged at 3,000 × *g* for 5 min. The supernatant was used for the assay. Hemoglobin in the supernatant was measured for the evaluation of residual blood contamination (18).

Piperacillin and tazobactam concentrations in both sera and lungs were measured by high-performance liquid chromatography as previously described (10).

Antibiotic concentrations were derived from a regression curve obtained from standard solutions ($r = 0.997$ and 0.998 for piperacillin and tazobactam, respectively); the limit of detection was 1 μg/ml.

Establishment of therapy. In the first series of experiments, the antibiotics were administered by a single intraperitoneal injection at 24 h postinfection, when pulmonary infection was established and proved by histologic results; this time point corresponds to the mid-log phase of *K. pneumoniae* in vivo growth. The following drugs were injected at the indicated doses: cefotaxime, 100 mg/kg of body weight; piperacillin, 300 mg/kg (alone and with tazobactam at 37.5 mg/kg [ratio, 8:1]); and ticarcillin, 300 mg/kg (alone and with clavulanic acid at 20 mg/kg [ratio, 15:1]). The second series of experiments was the same except that (i) two new combinations of drugs were also injected (piperacillin at 300 mg/kg plus tazobactam at 75 mg/kg [ratio, 4:1] and piperacillin at 300 mg/kg plus clavulanic acid at 20 mg/kg [ratio, 15:1]) and (ii) the antibiotic injections were repeated over 24 h (piperacillin and ticarcillin alone or with inhibitors were administered every 3 h, and cefotaxime was administered every 6 h).

The time period between two injections of an antibiotic was based on the bacterial killing curve for the lungs after a single injection of this drug. This is the time to reach the nadir of the curve before the in vivo bacterial regrowth (see Fig. 2).

The time of treatment was determined from the mortality rate of the mice: this is the shortest time taken to switch from the death of all untreated control mice between 72 and 96 h to the survival of all mice treated by the efficient control drug. In our study, the preliminary experiments showed that this time was 24 h with cefotaxime.

Assessment of infection and therapy. In each experiment, at 3 and 24 h postinfection, six untreated mice were sacrificed by an overdose of sodium pentobarbital to check the colonization of the lungs by the bacterial inoculum before the initiation of therapy. Thereafter, six animals from each group of mice, treated or untreated, were sacrificed at each time point, i.e., at 27, 30, 48, and 72 h postinfection for the first series of experiments and at 36 h (12 h after the initiation of therapy), 48 h (the end of therapy), and 51 and 72 h postinfection for the second series of experiments. The lungs were dissected from the bronchia and then homogenized in 1 ml of PBS buffer and serially 10-fold diluted. At all time points 100-μl aliquots were plated on TCS agar, and the CFU were counted after 24 h of incubation at 37°C. At the same times, blood samples were collected by cardiac puncture and incubated on TCS in the same way. Additionally, 20 mice per experiment and per group (untreated and treated with different drugs) were kept for determination of survival rates at 72, 96, and 168 h postinfection. Each experiment was carried out in duplicate.

Pathology. Three mice were killed at 24 h postinfection and injected intratracheally with a 30% formalin solution. The lungs were embedded in paraffin. Serial sections were stained with both May-Grumvald-Giemsa and hematein-eosin-saffron solutions and examined microscopically.

Detection of antibiotic-resistant mutants. To determine whether the bacteria recovered from the lungs at 72 h postinfection might be in vivo-selected resistant mutants, lung homogenates were plated onto antibiotic concentration gradients (from 0 to 500 μg/ml in agar) (25). Moreover, the MICs of the antibiotics used were determined with a sample of these bacteria.

Detection of carryover effect. *K. pneumoniae* DAR was seeded into TCS agar, and then aliquots of lung homogenates from mice sacrificed at 51 h postinfection (3 h after the end of the 24-h treatment) were placed into 100-μl wells bored into the agar. The plates were incubated for 26 h at 37°C.

Statistical methods for CFU counts. The time was considered as a nonordered series of time points. A two-way analysis of variance was carried out with the SAS program, using the factors treatment and time of treatment. Results for each time point were determined six times, i.e., from six mice at each time point in each experiment. In this way we obtained the *F* value and the residual variance. With this type of experiment, it is possible to look for an interaction between time and treatment. Thus, the means are compared in pairs at each time point by the Student *t* test, using the residual variance.

TABLE 1. In vitro activities of penicillins plus β-lactamase inhibitors versus cefotaxime against *K. pneumoniae* DAR

Drug	MIC[a] (μg/ml) with the following inoculum (CFU/ml):		MBC[a] (μg/ml) with the following inoculum (CFU/ml):	
	5×10^5	5×10^7	5×10^5	5×10^7
Ticarcillin	>128			
Ticarcillin-clavulanic acid (15:1)	2	4	4	8
Piperacillin	8	32	32	64
Piperacillin-tazobactam (8:1)	4	8	8	16
Piperacillin-tazobactam (4:1)	2	4	4	8
Piperacillin-clavulanic acid (15:1)	1	2	2	4
Cefotaxime	0.25	0.5	0.5	0.5
Clavulanic acid	>128			
Tazobactam	>128			

[a] After 18 h.

The significance of the differences between the mortality rates of the different groups was evaluated by using the χ^2 test with the Yates correction.

RESULTS

In vitro susceptibility testing. The results of in vitro susceptibility tests are summarized in Table 1. From the MICs and MBCs, cefotaxime, piperacillin, and combinations of penicillins with inhibitors were interpreted as active, and ticarcillin was inactive. An inoculum effect was observed only with piperacillin.

Pharmacokinetics. The blood concentration in the lungs never exceeded 3% (vol/vol). Thus, calculations of tissue drug concentrations were made directly from results for lung homogenates. The pharmacokinetic characteristics of piperacillin in mice infected with *K. pneumoniae*, especially its short half-life in serum (about 15 min), were in accordance with previously reported data for mice (1). The early concentrations of piperacillin in sera and lungs were found to be >10 times higher than its MBC for *K. pneumoniae* DAR. The early concentrations of piperacillin and tazobactam measured in the sera and tissues of the mice (Fig. 1) were about the same as those found in humans at approximately the same time following injection of the usual doses (23, 27). The concentrations of these drugs in the lungs remained high after 1 h: the means ± standard errors of the means (Student *t* test) were 29 ± 7 μg/g for tazobactam at 37.5 mg/kg, 175 ± 94 μg/g for tazobactam at 75 mg/kg, and 220 ± 102 μg/g for piperacillin, with a 5% risk of error. Moreover, whereas the concentrations of tazobactam in the sera were nearly proportional to its dosage regimens, its concentrations in pulmonary tissue increased much more, i.e., two to three times more, during the first 60 min (Fig. 1).

Antibiotic efficacy against experimental pulmonary infection. The efficacies of the drugs were quantified by comparing the bacterial counts in the blood (data not shown) and in the lungs of treated and untreated mice at each time point. *K. pneumoniae* DAR was highly virulent for mice at a dose of as little as 2×10^3 CFU, which we had selected for intranasal insertion: it reproducibly induced pulmonary infection and secondary bacteremia in all of the experiments. As shown in Fig. 2 and 3, reproducible in vivo growth curves were obtained for the lungs, with an increase of nearly 4 $\log_{10}$ units at 48 h and 100% mortality before 96 h postinfection.

In the first series of experiments performed with a single drug injection, ticarcillin was ineffective, as suggested by in

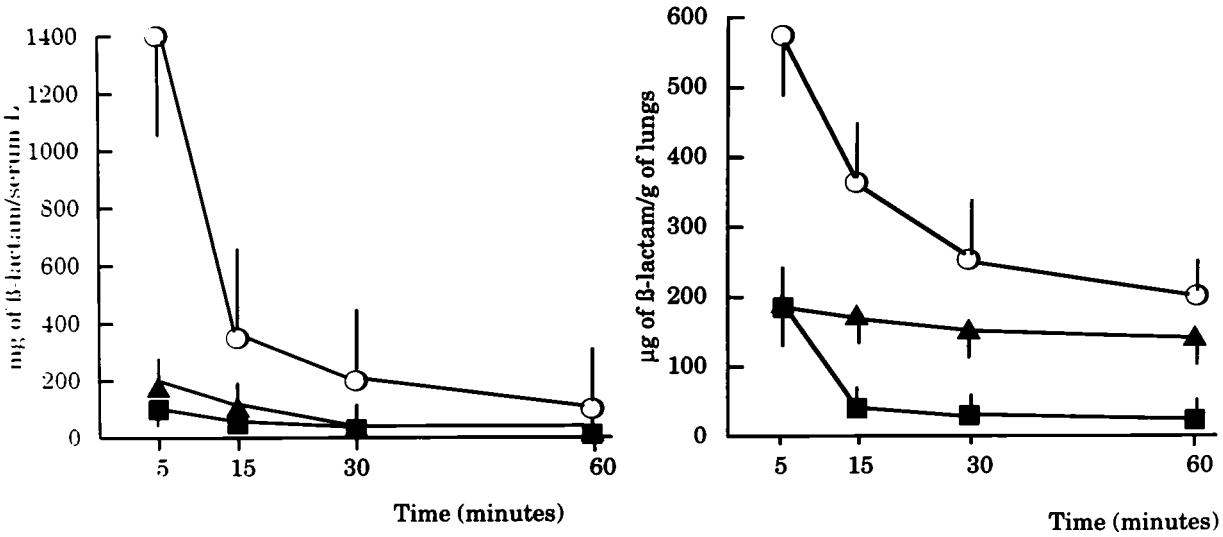

FIG. 1. Piperacillin and tazobactam concentrations in sera and pulmonary tissues from infected mice. Time zero represents the time of the last of the repeated intraperitoneal injections over 24 h of piperacillin at 300 mg/kg alone (○) or with tazobactam at 37.5 mg/kg (ratio, 8:1) (■) or 75 mg/kg (ratio, 4:1) (▲). Each point represents the geometric mean of drug concentrations in the sera or lungs of six mice. The vertical bars indicate half of the confidence interval of the mean (standard error of the mean with a 5% risk of error; Student *t* test).

vitro data (Table 1). All of the other antibiotics and their combinations with inhibitors were effective: they reduced the CFU in the lungs by at least 1 $\log_{10}$ unit within 3 h (Fig. 2). Cefotaxime was the most effective, reducing the bacterial burden by >3 $\log_{10}$ units within 6 h. The bacterial clearance from the blood in mice treated with these agents (data not shown) correlated with data obtained for the lungs. However, no drug was able to fully eliminate *K. pneumoniae*; single injections had only a transient effect, and regrowth was observed from 3 h postherapy with all the drugs except cefotaxime (regrowth

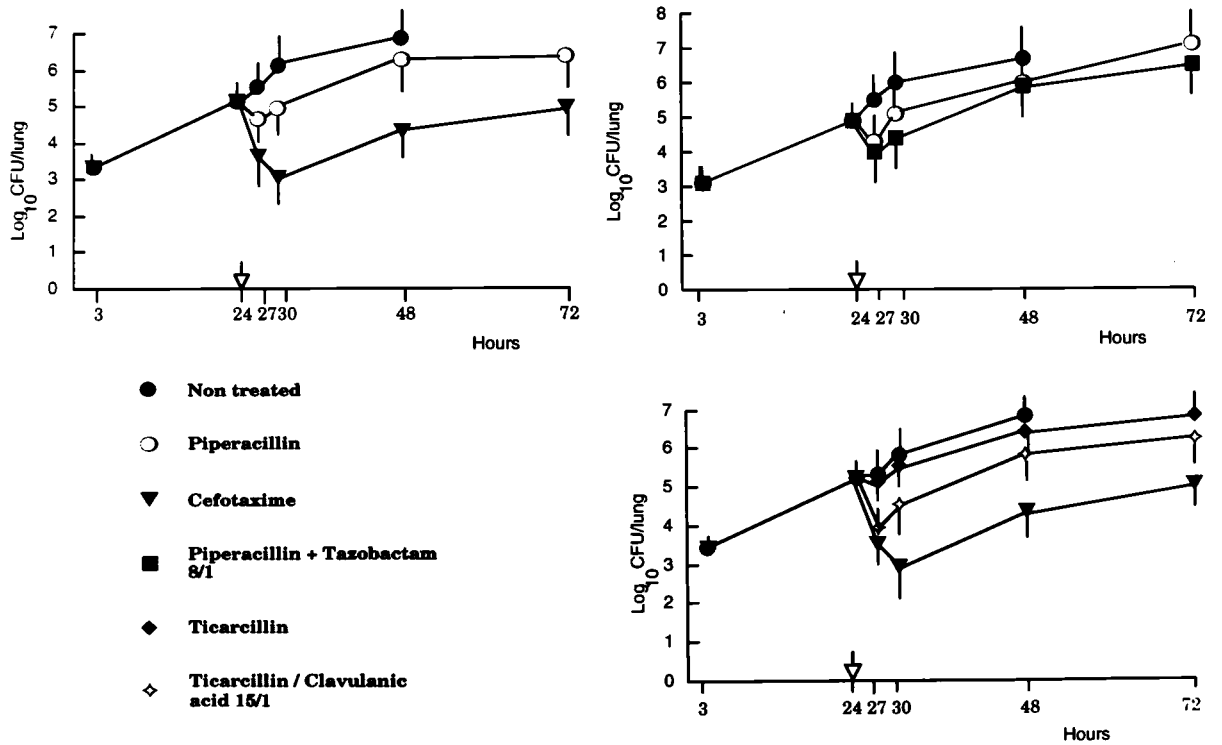

FIG. 2. Effect of one antibiotic injection on *K. pneumoniae* infections in mice. The arrows on the horizontal axes represent the times postinfection of antibiotic injections. The growth kinetics of the strain in the lungs of mice infected intranasally with a standardized inoculum (2×10^3 CFU) were measured. Each point represents the geometric mean of bacterial counts after homogenization of the lungs from six mice that were either treated or not (controls). The vertical bars indicate half of the confidence interval of the mean (standard error of the mean [Student *t* test] with a 5% risk of error). The antibiotic doses, expressed in milligrams per kilogram, were as follows: cefotaxime, 100; piperacillin, 300 (alone or with tazobactam, 37.5 [ratio, 8:1]); and ticarcillin, 300 (alone or with clavulanic acid, 20 [ratio, 15:1]).

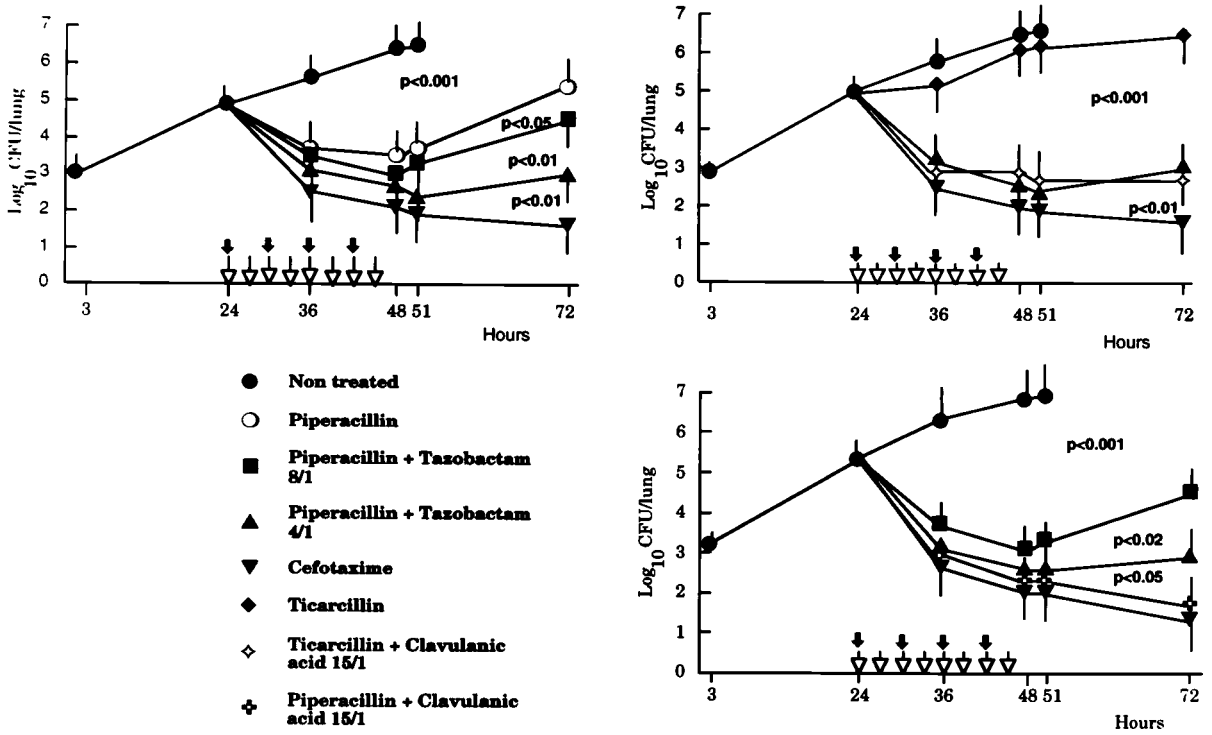

FIG. 3. Effect of repeated antibiotic injections during 24 h on *K. pneumoniae* infections in mice. The arrows on the horizontal axes represent the times postinfection of antibiotic injections (open arrow for piperacillin or ticarcillin; solid arrow for cefotaxime). The growth kinetics of the strain in the lungs of mice infected intranasally with a standardized inoculum (2×10^3 CFU) were measured. Each point represents the geometric mean of bacterial counts after homogenization of the lungs from six mice that were either treated or not (controls). The vertical bars indicate half of the confidence interval of the mean (standard error of the mean [Student *t* test] with a 5% risk of error). The antibiotic doses, expressed in milligrams per kilogram, were as follows: cefotaxime, 100; ticarcillin, 300 (alone or with clavulanic acid, 20 [ratio 15:1]); and piperacillin, 300 (alone or with tazobactam, 37.5 [ratio, 8:1] and 75 [ratio, 4:1] or clavulanic acid, 20 [ratio, 15:1]).

from 6 h). The mortality rates for the drugs were equivalent (six of six mice died between 72 and 96 h).

In the second series of experiments, which were carried out with repeated injections over 24 h, the efficacies of all of the drugs except ticarcillin were confirmed (Fig. 3): the in vivo bacterial killing curves obtained with these drugs were significantly different from those measured for bacteria in the lungs of untreated controls ($P < 0.001$ at 36, 48, and 51 h postinfection). The bacterial killing curves obtained with piperacillin alone and with piperacillin plus tazobactam (8:1) were parallel and close to each other; they differed only at 72 h postinfection ($P < 0.05$). With both of these treatments, an initial decrease in CFU of at least 1.5 $\log_{10}$ units followed by regrowth at 48 h postinfection was observed. The bacterial killing curves obtained with piperacillin plus tazobactam (4:1) or ticarcillin plus clavulanic acid (15:1) intersected, and these combinations reduced the bacterial count by >2 $\log_{10}$ units. The decrease of >3 $\log_{10}$ units in the bacterial counts was stable with cefotaxime or with piperacillin plus clavulanic acid (15:1). These last two bacterial killing curves differed significantly from the preceding ones at 72 h ($P < 0.05$); they did not differ from each other.

These results were corroborated by the survival rates for the mice (Fig. 4). There were three groups of treatments that had significantly different results at several time points ($P < 0.01$): (i) ineffective (ticarcillin), (ii) uncertain (piperacillin alone and piperacillin plus tazobactam [8:1] [different from each other at 96 h but not at 168 h]), and (iii) efficient (all the other agents).

Histologic results. At 24 h postinfection, pleurisy and alveolitis foci were detected; the pleureae and foci were filled with

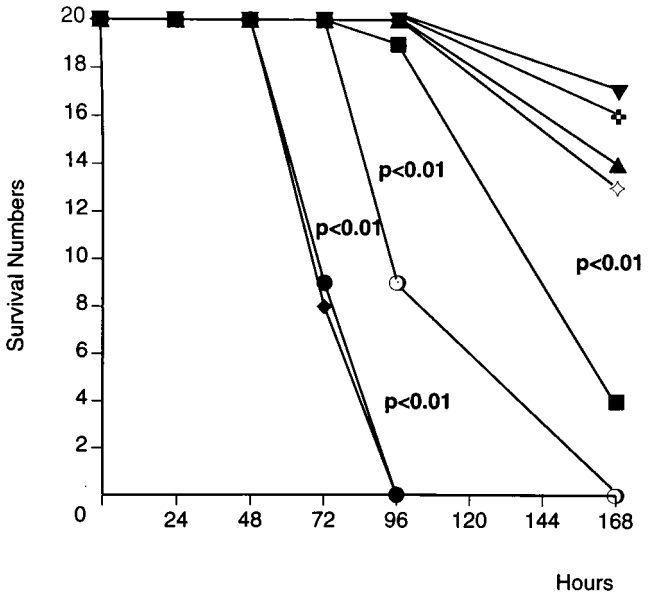

FIG. 4. Survival of mice after intranasal challenge with *K. pneumoniae*. Antibiotics were administered repeatedly during 24 h. The horizontal axis represents the time postinfection. The symbols and antibiotic doses are as described in Fig. 3 and its legend. Cumulative survival rates were established by counting the number of survivors daily.

altered polymorphonuclear neutrophils and encapsulated gram-negative bacteria. Moreover, gross inflammatory lesions were macroscopically visible in the lungs of all mice by 48 h.

Detection of antibiotic-resistant mutants. No antibiotic resistant mutants that might have been selected by the therapy were detected after the unsuccessful treatments in the first and second series of experiments.

Antibiotic concentrations in the lungs. No bacteriostatic effect of the lung homogenates was observed. Moreover, the numbers of CFU were multiples of 10 in the homogenate and serial 10-fold dilutions of each bacterial count. Therefore, we can conclude that the remaining concentrations of antibiotic in tissues did not have much of an influence on our results.

DISCUSSION

In the present study, we have used a reproducible mouse model to evaluate and compare antibiotic efficacies against the SHV-1 β-lactamase producer *K. pneumoniae.*

Although the treatments are intended to ensure the survival of infected hosts, the aim of this study was to compare bacterial counts rather than mortality rates. The in vivo bactericidal effect is actually more informative with regard to the efficacies of the drugs. Through previous work (2, 12, 19, 22), we gradually fixed the experimental parameters we have used here, i.e., (i) a strain with defined molecular characteristics of virulence, (ii) an inoculum which ensured a constant and exponential kinetics of infection and a fixed interval of time for the death of the animals, (iii) the initiation of therapy as the infection was irreversibly established and histologically confirmed, (iv) dosage regimens which achieve early concentrations in mouse serum and tissues similar to those found in humans, (v) a time of treatment which is the shortest for a change from the death of all of the control mice to the survival of all of the mice treated by the efficient drug (24 h with cefotaxime in our study), and (vi) periods of time between two injections of a drug which are equal to the time required to reach the nadir of the bacterial killing curve from a single dose of the drug before in vivo bacterial regrowth (for instance, 3 h for piperacillin and 6 h for cefotaxime [compare Fig. 2 and 3]). Thus, we have compared the efficacies of drugs independently of the specific lengths of their in vivo activities.

Statistically significant results regarding the bactericidal effects of different antibiotics or combinations could be achieved with this restricted experimental model, and the mortality rates confirmed the data obtained from measurement of the bacterial burden.

Our results show that the intranasal insertion of an inoculum of 2×10^3 CFU of a virulent strain of *K. pneumoniae* ensured a constant infection which mimicked the pathogenesis of pneumonia and bacteremia in humans. In the first series of therapeutic experiments, a single injection of each drug or combination provided different specific killing curves and made it possible to determine the times it took for the curves to reach their nadirs. In the second series of experiments, repeated doses given over 24 h allowed us to design therapeutic protocols leading to full recovery and presumably to extrapolate these results more easily for clinical trials.

The results obtained with cefotaxime showed that (i) its efficacy was outstanding and (ii) reinjections of this agent just after the nadir of the killing curve were advantageous. Whether this was due to the active residual concentrations in the deep pulmonary compartment and/or to a postantibiotic effect related to early immune response of the host remains to be determined. The pulmonary influx of polymorphonuclear leukocytes shown by the histologic results at 24 h points to such a mechanism.

The bacterial killing curve obtained with piperacillin illustrates the unquestionable effect of this drug, but the bacterial regrowth and the mortality rate corroborate reports of clinical failures (14). A prolonged treatment with piperacillin might lead to recovery, but clear-cut discriminating results were obtained here, which is what this experimental model is especially designed to do. In any case, the ratio between early concentrations in lungs and the MIC (or MBC) was higher with cefotaxime than with piperacillin in the dosages used, and above all, ureidopenicillin is slightly hydrolyzed by the SHV-1 enzyme. However the combination of piperacillin with tazobactam in the ratio used clinically (8:1) was only temporarily effective in this restricted experimental model.

Our pharmacokinetic assays demonstrated that these therapeutic results were not related to a failure of piperacillin and/or tazobactam to penetrate the lungs (Fig. 1). In mouse serum the pharmacokinetics of both drugs remained linear, and high early concentrations of each one were about the same as those observed in human studies (23, 27). In the lungs of mice, the penetration of tazobactam appeared to be dose dependent, and a much greater quantity of tazobactam coincided with an increased therapeutic efficacy. With this inhibitor, in vitro and in vivo dose effects were evident, as already reported (11, 17), and the combination in the ratio of 4:1 (300 mg of piperacillin per kg plus 75 mg of tazobactam per kg) led to a significant effect, as observed in a previous animal model (13). The combination in the ratio of 8:1 (300 mg of piperacillin per kg plus 37.5 mg of tazobactam per kg) was less effective (Fig. 3), probably because the concentration of β-lactamase inhibitor was insufficient to ensure full antimicrobial activity.

The effects of the two combinations, piperacillin-tazobactam (4:1) and ticarcillin-clavulanate (in the ratio used clinically [15:1]) were nearly the same in our study. Because these ratios are different and because carboxypenicillin is hydrolyzed to a greater extent than ureidopenicillin, clavulanic acid demonstrated a higher specific activity. Moreover, Bush et al. (4), recently demonstrated that tazobactam was 10 times less active than clavulanic acid as an inhibitor against the SHV-1 penicillinase, which could explain the experimental therapeutic results in our study.

From these results, the success of the combination of piperacillin and clavulanic acid is equivalent to that of cefotaxime in this restricted model of murine *K. pneumoniae* infection.

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