ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1994, p. 1953–1958
0066-4804/94/$04.00+0

Vol. 38, No. 9

In Vivo Efficacy of a Broad-Spectrum Cephalosporin, Ceftriaxone, against Penicillin-Susceptible and -Resistant Strains of *Streptococcus pneumoniae* in a Mouse Pneumonia Model

PIERRE MOINE,[1]* ÉRIC VALLÉE,[1,2] ESTHER AZOULAY-DUPUIS,[1] PHILIPPE BOURGET,[3] JEAN-PIERRE BÉDOS,[1] JACQUELINE BAUCHET,[1] AND JEAN-JACQUES POCIDALO[1]

Institut National de la Santé et de la Recherche Médicale Unité 13, 75944 Paris,[1] Service de Microbiologie, Centre Hospitalier Universitaire Xavier Bichat-Claude Bernard, 75877 Paris,[2] and Laboratoire de Pharmacologie et de Toxicologie, Hôpital Antoine Béclère, 92141 Clamart,[3] France

Received 7 September 1993/Returned for modification 16 January 1994/Accepted 27 June 1994

The increasing emergence of penicillin-resistant (P^r) strains of *Streptococcus pneumoniae* could pose a therapeutic problem in the next few years. Ceftriaxone (CRO), a broad-spectrum cephalosporin, exhibits a smaller increase in MICs against P^r *S. pneumoniae* strains than amoxicillin (AMO) (usually referred as to the "gold standard" therapy for pneumococcal infections). Therefore, we compared their respective efficacies in a leukopenic Swiss mouse model of pneumococcal pneumonia. Infection was induced with two serotype 19 strains: a penicillin-susceptible (P^s) strain (MICs of <0.01 for penicillin, 0.03 for AMO, and 0.03 for CRO) and a P^r strain (MICs of 4 for penicillin, 2 for AMO, and 0.5 for CRO). Untreated mice died within 2 or 3 days. Against the P^s strain, the minimal protective dose (two subcutaneous injections at 12-h intervals for 3 days) for both CRO and AMO was 5 mg/kg of body weight (87% survivors). Ten-fold-increased doses of CRO (50 mg/kg) gave similar protection (75% survivors) against the P^r strain, whereas 20- and 40-fold-increased doses of AMO protected 0 and 34% of the animals, respectively, against the P^s strain. CRO had a marked and prolonged antibacterial effect in the lungs (2.7-log-unit reduction of CFU in 24 h after a single 50-mg/kg injection) against the P^r strain in comparison with AMO. A standard dosage of 50 mg of CRO per kg in mice resulted in peak levels in serum and protein binding comparable to those observed with 1 g given intravenously in humans. This dosage remained effective against a highly P^r *S. pneumoniae* strain in this model. The microbiological activity and pharmacodynamic and pharmacokinetic properties of CRO (time during which concentrations exceed the MIC for the test pathogen [ΔtMIC], ≥8 h; and peak/MIC ratio, >90 for free active drug) accounted for its efficacy relative to AMO (50 mg/kg: ΔtMIC, <2 h; peak/MIC ratio, <25) against the highly P^r *S. pneumoniae* strain used in this study.

Ceftriaxone (CRO) is a cephalosporin characterized by a very broad spectrum of activity (1, 6, 8, 10) and especially by an extraordinarily long elimination half-life in serum, which is an unusual pharmacological property for this class of antibiotics (5, 8, 30, 33). It has been established that in both healthy subjects and most patients, the mean half-life of CRO in serum is approximately 8 h (8, 9, 33). CRO exhibits a broad spectrum of antimicrobial activity, including common respiratory pathogens (e.g., *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and members of the family *Enterobacteriaceae*) (1, 6, 8, 10, 11, 15).

S. pneumoniae remains the most frequently isolated organism in community-acquired pneumonia (22, 28). Pneumococci retained a uniformly high degree of susceptibility to penicillin (MICs, 0.005 to 0.01 mg/ml) throughout the first 2 to 2.5 decades of the antibiotic era. However, after this latent period and particularly during the 1980s, penicillin-resistant (P^r) strains have been reported, and the frequency has increased in virtually every part of the world (2). In some countries, the incidence of resistant bacteria isolated from clinical specimens rose to extremely high levels (50 to 70% in Spain and Hungary) (2, 13, 23). Increasing numbers of resistant and multiresistant pneumococcal strains appeared in parallel with their increase in geographical spread (25).

Broad-spectrum cephalosporins such as CRO showed better in vitro activity than penicillin against resistant strains (18, 20, 29, 35), which suggested their potential usefulness in the treatment of P^r *S. pneumoniae* infections. We therefore compared the efficacy of CRO with that of amoxicillin (AMO), usually referred as to the "gold standard" therapy for *S. pneumoniae* infections (21), in mouse models of acute pneumonia induced by penicillin-susceptible (P^s) and P^r *S. pneumoniae* strains.

MATERIALS AND METHODS

Animals. Female Swiss mice (body weight, 20 to 22 g) were obtained from Iffa-Credo Laboratories, France.

Challenge organisms. We failed to induce pneumonia with any P^r strain in immunocompetent mice. All of these strains belonged to serotypes 6, 9, 14, 19, and 23, which are naturally avirulent for mice independently of their isolation sites in humans (4, 7). Thus, pneumonia was induced in leukopenic Swiss mice with two serotype 19 strains: a P^s strain originally isolated from a blood culture (strain P52181, provided by Hôpital Bichat, Paris, France) and a highly P^r strain (MIC = 4 μg/ml) originally isolated from middle ear fluid (strain P15986, provided by the Centre de Référence du Pneumocoque [P. Gelin], Créteil, France).

* Corresponding author. Mailing address: INSERM U 13, 190 Bd. Mac Donald, 75944 Paris Cedex 19, France. Phone: 40-35-36-44. Fax: 40-36-16-99.

TABLE 1. Microbiological data for *S. pneumoniae* challenge strains and study drugs

Study drug	MIC/MBC (μg/ml)[a]	
	P52181	P15986
Penicillin	0.03/0.03	4/8
AMO	0.03/0.03	2/4
CRO	0.03/0.03	0.5/1

[a] Determined by macrodilution in nonsupplemented Mueller-Hinton broth with an initial inoculum of 10^6 CFU/ml.

Antibiotics. The drugs used in this study were CRO (Roche Laboratories, Neuilly-sur-Seine, France) and AMO sodium salt (Beecham Laboratories, Paris, France). The antibiotics were reconstituted according to the instructions on the package insert and diluted in sterile water to the desired concentrations.

In vitro studies. MICs and MBCs were determined in Mueller-Hinton infusion broth (Diagnostic Pasteur, Marnes-la-Coquette, France) by means of the tube dilution method (26). Each tube contained twofold dilutions of antibiotic and a final bacterial density of 10^6 CFU/ml. After aerobic incubation for 18 h at 37°C, the MIC was defined as the lowest concentration of antibiotic at which no turbidity was visible to the naked eye. The MBC was determined by plating 0.01-ml aliquots from tubes with no visible growth onto Columbia agar supplemented with 5% sheep blood (Bio-Mérieux, Lyon, France). The plates were incubated overnight at 37°C, and the MBC was defined as the lowest concentration of antibiotic killing ≥99.9% of the original inoculum.

Leukocyte depletion in mice. We induced sustained leukopenia in Swiss mice by daily intraperitoneal injections (150 mg/kg of body weight) of cyclophosphamide (Endoxan; Sarget Laboratories, Mérignac, France) starting 3 days before infection. Counts of circulating leukocytes per cubic millimeter of blood were reduced from about 7,000 to 880 on the day of infection. Under these conditions, mice became more susceptible to the two poorly virulent P^s and P^r serotype 19 strains.

Experimental pneumococcal pneumonia in mice. Animals were infected by intratracheal instillation via the mouth as described in detail elsewhere (3). Briefly, animals were anesthetized intraperitoneally with 0.2 ml of 0.65% sodium pentobarbital and were suspended by the upper incisors. The trachea was cannulated via the mouth with a blunt needle, and 50 μl of bacterial suspension (10^7 CFU of P^s and P^r *S. pneumoniae* per mouse) was instilled. Leukopenic mice developed acute pneumonia and died within 2 or 3 days. All the animals quickly became bacteremic. Bacterial counts exceeded 10^8 CFU per lung for the infected mice at the time of their death (≥10^6 CFU/ml of blood).

Survival studies. Therapy was initiated 3 h after bacterial challenge. A total of six subcutaneous (s.c.) injections were administered at 12-h intervals. Treatment schedules are presented in the figures, with the results. Fifteen animals per treatment group were used, and in each experiment the animals were infected simultaneously. Experiments were repeated at least twice. The observation period was 14 days. Death rates were recorded daily, and cumulative survival rates were compared.

Bactericidal activity in vivo. The study drugs were assessed for their ability to eradicate bacteria in the lungs. Drug administration was initiated 3 h after infection and consisted of a single dose of AMO and CRO. Various doses (5, 10, 50, and 100 mg/kg of body weight) of each drug, depending on the infective strain, were administered in 0.5 ml of sterile water. Controls received the same volume of isotonic saline. The total CFU recovered from whole-lung homogenates was determined at 1, 3, 6, 8, and 24 h after one injection. Mice were killed by CO_2 asphyxiation and were exsanguinated by cardiac puncture (the blood was used for cultures). The lungs were removed and homogenized in 2 ml of saline. Serial 10-fold dilutions of the homogenates were plated onto Columbia agar (0.1 ml/9-cm-diameter plate). Results are expressed as the mean log CFU per lung ± standard deviation for groups of three mice. The lower limit of detection was 2 log CFU per lung, which corresponded to the weakest dilution of tissue homogenates (10^{-1}) that avoided significant drug carryover with control inocula.

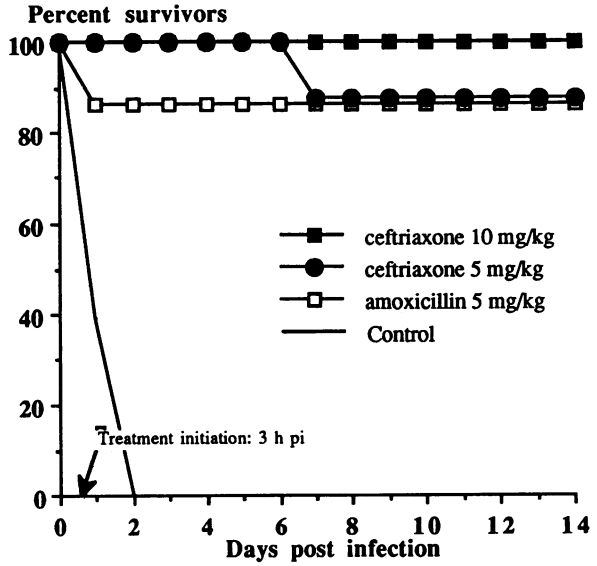

FIG. 1. Cumulative survival of treated and control leukopenic Swiss mice after intratracheal challenge with 10^7 CFU of the P^s *S. pneumoniae* strain (P52181). As of 3 h postinfection (pi), mice received six s.c. administrations of the indicated amounts of drug at 12-h intervals.

Pharmacokinetic studies. The pharmacokinetic profiles of AMO and CRO were examined in parallel in infected Swiss mice.

(i) Bioassay. Concentrations in lungs and sera were determined after single s.c. doses of 50 mg of AMO and CRO per kg. In infected mice, the antibiotic was given 3 h after challenge with strain P15986. At 0.5, 1, 2, 4, 6, 8, 10, and 24 h following drug administration, three animals per group were killed with CO_2 and exsanguinated by intracardiac puncture. Blood samples were centrifuged to isolate serum, pooled, and frozen at −80°C until assay. Lungs were harvested from exsanguinated mice, washed in sterile sodium chloride solution, and frozen. On the day of assay, organs were weighed, pooled, and homogenized in phosphate buffer (pH = 6.8). Homogenates were centrifuged and the supernatants were used for assay. AMO concentrations were determined by the agar well diffusion method, using *Sarcina lutea* ATCC 9341 as the bioassay organism and Antibiotic Medium 1 (Difco Laboratories, Detroit, Mich.) as the growth medium. CRO concentrations were determined by the agar well diffusion method, using *Escherichia coli* ATCC 39118 as the bioassay organism and Antibiotic Medium 1 (Difco Laboratories) as the growth medium. Standard curves were determined with solutions of AMO and

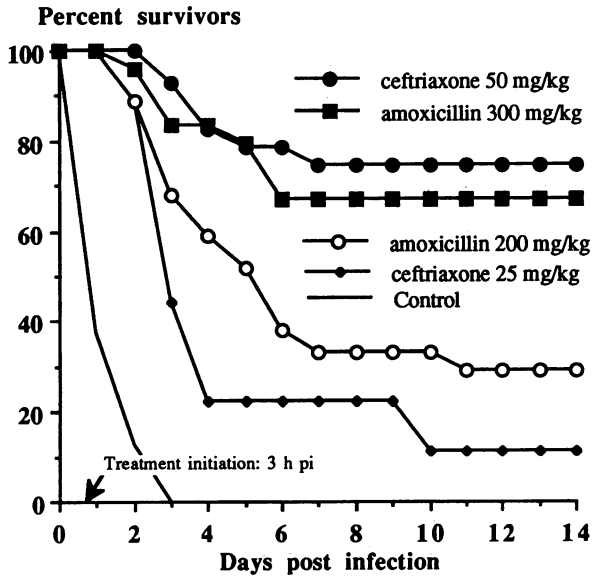

FIG. 2. Cumulative survival of treated and control leukopenic Swiss mice after intratracheal challenge with 10^7 CFU of the P^r *S. pneumoniae* strain (P15986). As of 3 h postinfection (pi) mice received six s.c. administrations of the indicated amounts of drug at 12-h intervals.

CRO in phosphate buffer for serum and tissue in order to evaluate the active fractions of the drugs. Results were expressed as micrograms per milliliter of blood or per gram of lung tissue. The standard curve was linear from 0.125 to 32 μg/ml. The sensitivity of the assay was about 0.1 μg/ml of sample, and the coefficients of between- and within-day variations for replicates (n = 5) were ≤7.5% at 0.5, 1, 7.5, and 20 μg/ml.

(ii) HPLC. CRO samples were also assayed by high-performance liquid chromatography (HPLC) combined with UV spectrophotometric detection at 274 nm. The HPLC technique (ion pair reversed-phase chromatography) complied with the analytical recommendations of Trautmann and Haefelfinger (34). The method used a test specimen of 100 μl of serum, and its lower limit of sensitivity was 0.5 μg/ml. The accuracy of the assay method, which was studied at target values of 1.0, 75, and 250 mg/liter (serum loaded with CRO in vitro), gave coefficients of between- and within-day variations of ≤2.7% for total CRO. The linear range of the assay was between 0 and 300 mg/liter. Under the analysis conditions, the retention times for CRO and the internal standard were 2.1 and 2.8 min, respectively. All samples were assayed in duplicate. Control runs were performed regularly.

(iii) Protein binding. The extent of CRO binding to plasma proteins was determined with an ultrafiltration system. The Ultrafree CL device (UFC4LG25; Millipore, Saint-Quentin-en-Yvelines, France) was used (27). In brief, 700 μl of pooled plasma samples from six mice was placed in the filter cup (upper part of the device), which is separated from the filtrate cup by an ultrafiltration membrane. Ultrafiltration was obtained under nitrogen pressure (4 kg/cm^2). The device was kept under these conditions for 2 h (at 37°C). In the ultrafiltrate (filtrate cup), the concentrations of unbound CRO were determined by the method described above. The accuracy of the assay method, which was studied at target values of 0.5, 7.5, and 20 mg/liter, gave coefficients of between- and within-day variations of ≤8.5% for unbound CRO.

(iv) Analysis. Pharmacokinetic analyses were performed by routine graphical methods, and parameters were estimated by standard methods (17). $t_{1/2\beta}$ is the terminal-elimination half-life at β phase calculated by using linear least-square regression for the log linear terminal-elimination phase; ΔtMIC is the time during which concentrations exceed the MIC for the test pathogen (P15986); and AUC_{0-24} is the area under the concentration-time curve from 0 to 24 h, calculated by the trapezoidal rule.

Statistical analysis. Survival rate data were analyzed by unpaired t test. Analysis of variance (repeated measures) was performed to compare CFU values. P values of 0.05 or less were considered statistically significant.

RESULTS

In vitro data. Against the P^s strain, penicillin, AMO, and CRO showed identical low MICs and MBCs. However, against the P^r strain, AMO was four times less active than CRO (Table 1).

Therapeutic efficacy in experimental pneumonia. With our treatment schedule (treatment initiated 3 h after infection and consisting of 6 s.c. injections at 12-h intervals), both AMO and CRO treatments at 5 mg/kg were associated with an 87% survival rate ($P < 0.001$ versus untreated control group [0%]) (Fig. 1). A 100% survival rate was achieved with CRO at 10 mg/kg.

With the P^r strain, results were as follows. A 10-fold-increased dose of CRO (50 mg/kg) gave protection (75% survivors) (P = 0.22) similar to that against the P^s strain, whereas 20- and 40-fold-increased doses of AMO protected 0 and 34% of the animals, respectively (Fig. 2). Treatment with

TABLE 2. Clearance of *S. pneumoniae* from lungs and blood of Swiss mice infected with P^s strain P52181 following a single s.c. injection of study drugs

Time (h)	Value after treatment with:							
	Nothing (control)		AMO, 5 mg/kg		CRO, 5 mg/kg		CRO, 10 mg/kg	
	Lung[a]	Blood[b]	Lung	Blood	Lung	Blood	Lung	Blood
1	5.33 ± 0.35	1/3	4.58 ± 0.51	0/3	4.95 ± 0.33	0/3	4.33 ± 0.58[c]	0/3
3	5.74 ± 1.04	1/3	3.97 ± 0.45[c]	1/3	4.38 ± 0.38[c]	0/3	3.42 ± 0.39[c]	0/3
6			3.69 ± 0.81	0/3	3.54 ± 1.20	0/3	2.43 ± 0.93	0/3
8	5.50 ± 0.71	3/3	4.26 ± 0.24	0/3	3.95 ± 0.49	0/3	3.00 ± 1.41[c]	0/3
24			4.87 ± 0.55	2/3	3.95 ± 0.91	2/3	3.26 ± 0.83	1/3

[a] $\log_{10}$ CFU per milliliter of lung homogenate. Values are means ± standard deviations (n = 3).
[b] Number of animals with positive blood cultures/total number of animals.
[c] Value lower than for the control group ($P < 0.03$).

TABLE 3. Clearance of *S. pneumoniae* from lungs and blood of Swiss mice infected with P^r strain P15986 following a single s.c. injection of study drugs

Time (h)	Value after treatment with:							
	Nothing (control)		AMO, 100 mg/kg		CRO, 50 mg/kg		CRO, 100 mg/kg	
	Lung[a]	Blood[b]	Lung	Blood	Lung	Blood	Lung	Blood
1	7.1 ± 0.22	1/3	6.82 ± 0.04	0/3	6.77 ± 0.67	0/3	7.05 ± 0.19	0/3
3	7.63 ± 0.06	1/3	6.69 ± 0.21[c]	2/3	6.39 ± 0.27[c]	2/3	6.31 ± 0.36[c]	1/3
6			6.77 ± 0.24	1/3	6.29 ± 0.13[d]	2/3	5.51 ± 0.22[d,e]	0/3
8	7.42 ± 0.4	3/3	7.35 ± 0.08	2/3	5.41 ± 0.46[c,d]	1/3	5.26 ± 0.44[c,d]	1/3
24	7.74 ± 0.37	3/3	7.35 ± 0.32	3/3	4.13 ± 0.98[c,d]	3/3	4.35 ± 0.58[c,d]	3/3

[a] $\log_{10}$ CFU per milliliter of lung homogenate. Values are means ± standard deviations (n = 3).
[b] Number of animals with positive blood cultures/total number of animals.
[c] Value lower than for the control group ($P < 0.002$ to 0.0001).
[d] Lower than value for AMO at 100 mg/kg ($P < 0.03$ to 0.0001).
[e] Lower than value for CRO at 50 mg/kg ($P = 0.003$).

AMO at 300 mg/kg was associated with 67% survival. For both antibiotics, the survival rate did not significantly improve with larger doses.

Bacterial clearance from lungs. Results with the P^s strain are presented in Table 2. Three hours after a single-dose injection (AMO at 5 mg/kg; CRO at 5 and 10 mg/kg), the intrapulmonary bacterial decrease was significant with either compound relative to the control value ($P < 0.03$).

Results with the P^r strain were as follows (Table 3). Three hours after a single s.c. injection of CRO (50 and 100 mg/kg) or AMO (100 mg/kg), bacterial counts in the lungs were significantly lower than those in controls. However, CRO had a prolonged antibacterial effect compared with that of AMO, as shown by a 2.7-log-unit reduction in CFU 24 h after a single administration. In contrast, pulmonary bacterial counts in the AMO-treated group remained unchanged over the 8-h period following a single administration.

Serum and lung pharmacokinetics. After a single s.c. injection of 50 mg/kg, CRO showed the same peak concentration in serum but with a larger serum AUC than AMO (97 versus 61 μg · h/ml) (Table 4). The elimination half-life in serum was longer for CRO than AMO (1.41 versus 0.41 h). Similarly, CRO showed higher peak levels in the lung (36.7 μg/ml), a longer pulmonary half-life (1.12 h), and a larger serum AUC (79 μg · h/ml) than AMO (15 μg/ml, 0.79 h, and 15 μg · h/ml, respectively). Within the range of the observed total concentrations in serum (0.6 to 94.5 μg/ml), the protein binding of CRO decreases with increasing drug concentration from 96.4 to 85.6% (Fig. 3).

DISCUSSION

S. pneumoniae is the most frequent causative pathogen of community-acquired pneumonia (22, 28). The increasing prevalence of antimicrobial resistance in strains from invasive infections has severe implications for future antimicrobial strategies against pneumococci. Alternatives to penicillin therapy must be considered, especially for the management of pneumonia caused by highly resistant strains (29). Treatment of pneumococcal infections must take into account the susceptibility patterns of the strains in the community. CRO showed relatively good activity against resistant strains, despite the association between a high penicillin resistance level and higher MICs of this agent (18, 20, 29, 35). For such isolates, the MICs of CRO were nevertheless two to eight times lower than the respective MICs of penicillin (20). Thus, these increased MICs remain far below levels of this agent in serum (30).

Our data from a mouse model of pneumonia showed that CRO had greater efficacy than AMO against a highly P^r *S. pneumoniae* strain (penicillin MIC = 4 μg/ml). At a dose of 50 mg/kg, CRO showed a sustained bactericidal activity in relation to levels in serum and tissues that remained far higher than the MIC for the challenge strain (CRO MIC, 0.5 μg/ml). A sixfold-higher dose of AMO was required to achieve the same efficacy. In addition, CRO had longer half-lives in serum and lungs and a greater AUC than AMO. The lower MICs and MBCs and more favorable pharmacokinetics of CRO than those of AMO were the determinant factors involving efficacy against highly resistant pneumococci in vivo. The ΔtMIC appeared to be a major pharmacodynamic parameter correlating with efficacy in this model. There is considerable controversy over the relevance of drug-protein binding in terms of drug efficacy (12, 24, 32). Protein binding has long been recognized as an important factor in determining antimicrobial activity (31, 36). Results of the present study showed that the high rate of protein binding of CRO, relative to that usually reported for AMO, did not disadvantage its activity in vivo. On the contrary, the prolonged ΔtMIC suggested that the protein-bound fraction of the drug may serve as a reservoir and contribute to the therapeutic efficacy of this agent.

In our mouse model, peak concentrations in serum obtained after an s.c. dose of 50 mg of CRO per kg are similar to those achieved in humans after a 1-g intravenous administration (133 to 151 μg/ml) (8, 30). Despite a shorter elimination phase ($t_{1/2\beta}$, 1.4 versus 7.6 h) (30) and a smaller serum AUC (97 versus 1,146 μg · h/ml) (32) in mice, the experimental level of efficacy of CRO was high and suggested that at least the same activity would be achieved in humans.

TABLE 4. Pharmacokinetic parameters of AMO and CRO in infected Swiss mice following a single s.c. dose of 50 mg/kg[a]

Drug	Site	C_{max} (μg/ml or μg/g)[b]	$t_{1/2\beta}$ (h)	ΔtMIC (h)[c]	AUC_{0-10} (μg · h/ml or μg · h/g)
AMO	Serum	47.0 ± 18.0	0.41	1– 2	60.8
	Lung	15.0 ± 9.0	0.79	1– 2	14.6
CRO	Serum	46.5 ± 0.5	1.41	8–10	96.6
	Lung	36.7 ± 7.9	1.12	6– 8	79.5

[a] Values are calculated from mean concentrations in serum and lung tissue samples taken at 0.5, 1, 2, 4, 6, 8, 10, and 24 h postdosing. AMO and CRO concentrations were determined by the agar well diffusion method.
[b] C_{max}, maximum concentration observed.
[c] Values calculated for the P^r strain (P15986; MICs = 2 for AMO and 0.5 for CRO).

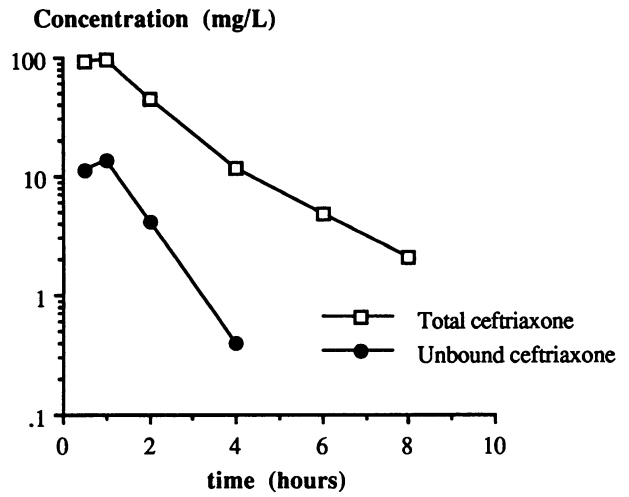

FIG. 3. Concentrations of CRO in serum following a single 50-mg/kg s.c. injection, as determined by HPLC.

The dosing interval was probably significant in producing a greater efficacy at a given dosage for AMO (19). On the other hand, the dosing interval probably had an impact on the relative efficacy at a given cumulative 24-h total dose for AMO. Since the activity of AMO against P^r strains could be strictly time dependent, the influence of dosing frequency on the cumulative dose-effect relationship of AMO remains to be demonstrated in this model.

Few authors have described clinical pneumococcal isolates (CFTR isolates) for which CRO MICs were unusually high (MIC > 2 mg/ml, while the MICs of penicillin were 0.3 to 0.6 mg/ml) (14, 16). However, it has been suggested that the CFTR isolates differed from previously examined P^r pneumococci and could represent another unique clone of beta-lactam-resistant pneumococci (14). Thus, these trains appeared unusual relative to other P^r isolates in regard to several properties. Indeed, they had an inversion of the relative MICs of penicillin compared with those of expanded-spectrum cephalosporins; unlike P^r strains, CFTR strains lysed quickly with penicillin; and they had patterns of penicillin-binding proteins which were atypical compared with those of P^r pneumococci (14). Although these CFTR strains are very rare, they warrant evaluation in a mouse pneumonia model.

In conclusion, CRO is effective in an immunocompromised-mouse model of pneumonia induced by a highly P^r strain of *S. pneumoniae*. These data suggest that a standard intravenous therapeutic regimen of CRO may be useful for treating severe community-acquired pneumonia.

ACKNOWLEDGMENTS

This work was supported by a grant from the Fondation pour la Recherche Médicale, Paris, France.

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