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Development of Experimental Pneumonia by Infection with Penicillin-Insensitive *Streptococcus pneumoniae* in Guinea Pigs and Their Treatment with Amoxicillin, Cefotaxime, and Meropenem

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Acute respiratory infection with penicillin-insensitive *Streptococcus pneumoniae* (MIC and MBC, 1 and 2 μg/ml, respectively) was established in guinea pigs. Intratracheal instillation of 0.5 ml of an overnight culture of *S. pneumoniae* concentrated 25 times (approximately 3×10^9 CFU) induced a bacteremic and fatal pneumonia in >85% of untreated animals within 46 h, with a mean ± standard deviation bacterial count of 8.83 ± 1.11 $\log_{10}$ CFU in lung homogenates. This model was used to evaluate the efficacies of two doses each of amoxicillin, cefotaxime, and meropenem given 1 h after bacterial inoculation. The antibiotics were given at 8-h intervals for up to a total of four injections. The dose of 50 mg of any antibiotic per kg of body weight gave 66.6% survival, compared with 5.05% survival for untreated control animals ($P < 0.001$). A dose of 200 mg/kg gave a survival rate of 77.8% for meropenem and 83.3% for amoxicillin and cefotaxime, while survival for untreated controls was 11.1% ($P < 0.001$). Although antibiotic treatment decreased mortality compared with that in untreated controls, the antibiotics contributed to a high early (less than 9 h after bacterial inoculation) mortality, being 53.5% compared with only 6.06% for the untreated controls ($P < 0.001$). Quantitative cultures of the lungs of animals that died during the 46-h observation period or that were killed after this time showed a significant reduction in the numbers of organisms among treated animals compared with numbers among the control animals ($P < 0.001$). The described model is an appropriate system for evaluating antibiotic efficacy in invasive pulmonary infection caused by penicillin-insensitive *S. pneumoniae*.

Streptococcus pneumoniae is the most common cause of community-acquired bacterial pneumonia, and penicillin-insensitive (MIC ≥ 0.1 μg/ml) strains are increasingly involved in this severe disease (1). At present up to 35% of pneumococcal pneumonia in some areas is produced by penicillin-insensitive pneumococci (18), with mortality reaching up to 38% in some series (17, 18). Consequently, there is a need for infection models to study not only the in vivo efficacies of some antibiotics but also to determine the pathophysiological aspects of pneumococcal pneumonia. Animal models of pneumonia caused by penicillin-insensitive *S. pneumoniae* are difficult to develop. It is known that some serotypes are not pathogenic for mice, animals which are usually used in most experimental studies (3), and also, penicillin-insensitive pneumococci seem less pathogenic for most rodents used in such studies (2, 16, 21). Guinea pigs have been widely used to test antibiotics against pneumonia caused by different organisms (6, 8, 14, 24), but to the best of our knowledge, they have not been used to test antibiotics against pneumonia caused by penicillin-insensitive *S. pneumoniae.*

The aim of this investigation was to develop an animal model of pneumonia caused by penicillin-insensitive *S. pneumoniae* in nonneutropenic guinea pigs in which the activity of meropenem, an antibiotic usually hydrolyzed by the renal dehydropeptidase I (DHP-I) of most rodents, could be evaluated (7, 10, 23).

MATERIALS AND METHODS

Bacteria. A strain of *S. pneumoniae* type 9 originally isolated from the blood of a bacteremic patient was used for these studies.

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Antibiotics. The antibiotics used for in vitro studies were amoxicillin trihydrate (SmithKline Beecham Pharmaceuticals, Worthing, England), cefotaxime (Sigma Chemical Co., St. Louis, Mo.), and meropenem (Zeneca Pharmaceuticals, Macclesfield, United Kingdom). For in vivo (therapeutic) use, commercial vials (Clamoxyl; SmithKline Beecham Pharmaceuticals, Toledo, Spain; Claforan; Roussel Ibérica, S.A. Laboratories, Madrid, Spain; and meropenem; Zeneca Pharmaceuticals) were reconstituted in nonpyrogenic sterile distilled water to the desired concentrations.

Animals. Female Hartley strain guinea pigs (weight, 350 to 400 g) were obtained from Charles River (Iffa-Credo, Lyon, France). Animals were housed in regulation cages and were given free access to food and water.

In vitro studies. MICs and MBCs were determined by the broth microdilution method in cation-supplemented Mueller-Hinton broth (Oxoid, Basingstoke, United Kingdom) to which 5% lysed sheep blood was added. Each well contained a twofold dilution of antibiotic and a final bacterial concentration of 5×10^4 CFU per well. The plates were incubated at 37°C for 18 h, and the MIC was defined as the lowest concentration of antibiotic at which no growth was visible to the naked eye. For MBC determination, the whole content of each well (100 μl) with no visible growth was plated onto Trypticase soy agar with 5% sheep blood (Becton Dickinson, Mayland, France), and the plate was incubated overnight at 37°C. MICs and MBCs were also determined by the broth macrodilution method (22) with two different inoculum sizes (2.5×10^5 and 2.5×10^7 CFU/ml). The lowest drug concentration that killed ≥99.9% of the inoculum was defined as the MBC. The mean of five separate determinations was used to calculate the MIC and MBC of each antibiotic.

Development of pneumonia model. The animals were infected by the method of Pennington and Ehrie (19). In short, after the anterior part of the neck was shaved, each animal was anesthetized with atropine (0.05 mg/kg of body weight; B. Braun Medical, S.A., Jaén, Spain), ketamine (70 mg/kg; Ketolar; Parke Davis, Barcelona, Spain), and xylazine (5 mg/kg; Rompun; Bayer, Leverkusen, Germany) by subcutaneous injection, and each guinea pig's trachea was exposed by making a vertical midline incision. A 0.5-ml volume of inoculum prepared as indicated later was instilled intratracheally with a syringe with a 25-gauge needle. Following inoculation, the incision was closed with stainless-steel wound clips, and the animal was gently shaken for 15 s to help distribute the inoculum into the lungs, after which the animal was kept in a semisitting position until it awakened.

To prepare the inoculum, an overnight culture of *S. pneumoniae* was grown in Todd-Hewitt broth (Oxoid) and was frozen in 1-ml aliquots at −70°C. For each experiment, a 2-ml volume of a thawed suspension was used to seed 50 ml of fresh Todd-Hewitt broth, and the mixture was incubated overnight at 35°C in a 5% CO_2 atmosphere. The cultures were centrifuged (C.411, E4; Jouan, Paris, France) at 2,000 × *g* for 20 min and were resuspended in fresh Todd-Hewitt broth to obtain concentrations 1, 5, 10, and 25 times higher than the concentra-

TABLE 1. Influence of inoculum concentration on outcome from *S. pneumoniae* pneumonia

Group	No. of animals surviving at 46 h/total no. of animals (%)	Bacterial titer (log_{10} CFU/lung [mean ± SD])	
		Died[a]	Killed[b]
1 (1×)[c]	5/5 (100)		<2.0
2 (5×)	5/6 (83.3)	6.94	4.11 ± 1.26
3 (10×)	3/6 (50)	8.79 ± 0.70	5.62 ± 1.18
4 (25×)[d]	1/10 (10)	8.23 ± 1	6.23

[a] Spontaneous death before 46 h postinoculation.
[b] Survivors, killed at 46 h postinoculation.
[c] Inoculum concentration of 1×, approximately 5×10^8 CFU/ml.
[d] Inoculum concentration of 25×, approximately 6×10^9 CFU/ml.

tion of the uncentrifuged culture. Up to a total of 27 guinea pigs were instilled with 0.5 ml of each bacterial concentration intratracheally.

The animals that were alive at 46 h were sacrificed with a 1.0-ml intraperitoneal injection of sodium pentobarbital (Nembutal; Abbott Laboratories, Chicago, Ill.). For all animals killed as well as those that died spontaneously before 46 h, both lungs were aseptically removed and washed in phosphate-buffered saline to remove contaminating blood; the lungs were then homogenized with glass tissue grinders. The homogenates were serially diluted in Todd-Hewitt broth, 100-μl volumes were plated in duplicate onto 5% blood agar, and the plates were incubated at 35°C for 24 h in a 5% CO_2 atmosphere to determine the number of viable *S. pneumoniae* organisms present. The lower limit of detection was 2.0 log_{10} CFU per lung, which corresponded to the weakest dilution of tissue homogenates that avoided significant drug carryover with the control inoculum. Cumulative survival rates and the clearance of *S. pneumoniae* from the lungs were compared.

Treatment regimen. The response to two doses of each antibiotic was studied in 144 guinea pigs previously instilled intratracheally with 0.5 ml of an overnight broth culture of *S. pneumoniae* concentrated 25 times. Each experiment used 24 animals, which were divided into one control group (6 animals) and three treatment groups (6 animals each by antibiotic and dosage). Each antibiotic was tested at two doses (50 and 200 mg/kg per dose) and was administered intramuscularly (i.m.) at 1, 9, 17, and 25 h after inoculation. The animals in the control group received nonpyrogenic sterile distilled water in the same way. Each experiment was repeated three times on the same schedule for each dose of antibiotic.

Evaluation of therapy. The number of guinea pigs that survived during the treatment period was recorded at 8-h intervals for up to 46 h. Animals alive at 46 h were sacrificed, and both lungs as well as those of the animals that died spontaneously before this time were processed, and the results were evaluated as described above.

Pharmacokinetic studies. Amoxicillin, cefotaxime, and meropenem were administered by the i.m. route to healthy animals at dosages of 50 and 200 mg/kg. Groups of five to six animals each were used. Blood was obtained by cardiac puncture at 0.5, 1, 2, 4, and 8 h after drug administration. Antibiotic concentrations were determined by microbiological assay with *Micrococcus luteus* ATCC 9341 and *Escherichia coli* ATCC 25922, as required. The concentration of antibiotic in the samples was derived from a standard solution prepared in pooled guinea pig serum. Assay variability for individual samples was <10%. Pharmacokinetic analyses were performed by routine graphical methods (9), and the area under the serum concentration-time curve (AUC) was calculated by using the trapezoidal rule.

Statistical analysis. Survival data at 46 h were compared by Yates' chi-square test with continuity correction. Bacterial titers in lung tissue for control and each antibiotic group were expressed as the arithmetic mean ± standard deviation (SD) log_{10} CFU per lung. The results were analyzed by the Student *t* test. When colony counts were below the limit of detection, the values for those samples were placed at the limit of detection (2.0 log_{10} CFU per lung); however, this results in possible underestimates of the levels of statistical significance.

RESULTS

In vitro studies. The respective mean MICs and MBCs for the test strain were as follows: amoxicillin 1 and 2 μg/ml; cefotaxime, 1 and 2 μg/ml; and meropenem, 0.5 and 1 μg/ml. No differences in results were observed by using the broth micro- or macro-dilution method. The organism did not show an inoculum effect, so the MICs and MBCs obtained with two different inoculum sizes were identical.

Animal pneumonia model. Table 1 presents the results obtained after intratracheal instillation of the four inoculum concentrations: Inoculation of 0.5 ml of the lowest inoculum (1×, which corresponded to approximately 2.5×10^8 CFU) did not produce mortality, the bacteria were eliminated by the guinea pigs, with no bacteria detectable at 46 h postinoculation, and the morphology of the lungs was apparently unaffected or less than 10% of the lung surface was involved. With increasing inoculum concentrations, the mortality rates, the area of lung surface involved, and the bacterial titers in the lungs were progressively higher. In all 13 animals that died before 46 h postinoculation, a bilateral hemorrhagic pneumonia was found and titers of 8.26 ± 1.0 log_{10} CFU of *S. pneumoniae* per lung were obtained. Cultures of blood obtained from seven animals at different intervals after the intratracheal instillation of 0.5 ml of *S. pneumoniae* concentrated 25 times were positive for growth of this organism.

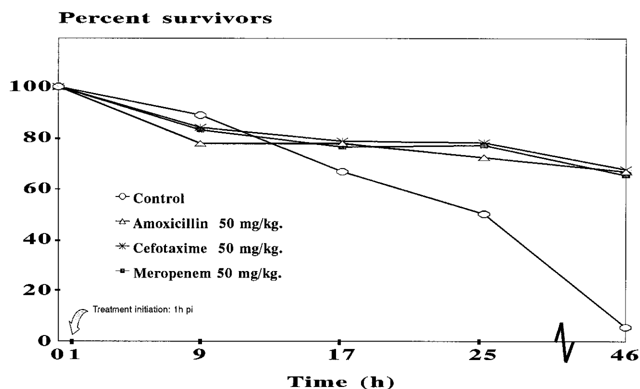

FIG. 1. Cumulative survival of treated and control guinea pigs after intratracheal challenge with 3×10^9 CFU of *S. pneumoniae*. As of 1 h postinfection (pi), guinea pigs received four i.m. administrations of the indicated amounts of drugs at 8-h intervals.

Efficacy of therapy. (i) Therapeutic efficacy in experimental pneumonia. When an overnight broth culture of *S. pneumoniae* concentrated 25 times (approximately 3×10^9 CFU), which results in a high mortality rate, was used in the pneumonia model, the responses to 50 and 200 mg (per kg per dose) of standard antibiotics with moderate in vitro activities against this organism were studied. Figures 1 and 2 present the survival rates observed at each interval. With this treatment schedule, each antibiotic at 50 mg/kg per dose (Fig. 1) was associated with a 66.6% survival rate, while the survival rate among un-

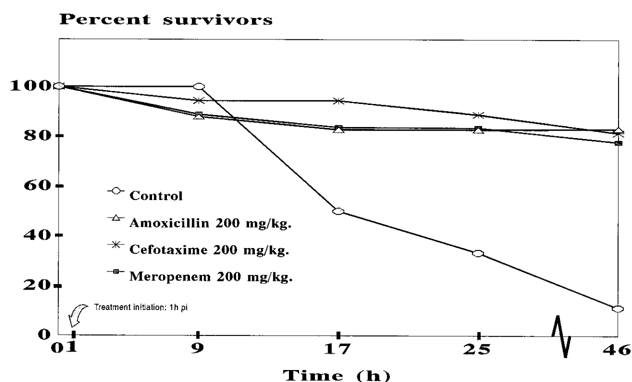

FIG. 2. Cumulative survival of treated and control guinea pigs after intratracheal challenge with 3×10^9 CFU of *S. pneumoniae*. As of 1 h postinfection (pi), guinea pigs received four i.m. administrations of the indicated amounts of drugs at 8-h intervals.

TABLE 2. Clearance of *S. pneumoniae* from lungs of infected guinea pigs treated with different antibiotic regimens

Group	Dose (mg/kg)	Bacterial titer (log_{10} CFU/lung [mean ± SD])		
		Died[a]	Killed[b]	Total
Control		8.83 ± 1.11 (33)[c]	6.95 ± 2.10 (3)	8.67 ± 1.33 (36)
Amoxicillin	50	4.64 ± 2.04[d] (6)	<2.0 (12)	2.88 ± 1.71[d,e] (18)
Amoxicillin	200	5.30 ± 2.33[d] (3)	2.15 ± 0.58[d] (15)	2.68 ± 1.60[d,e] (18)
Cefotaxime	50	4.15 ± 1.67[d] (6)	<2.0 (12)	2.71 ± 1.40[d,e] (18)
Cefotaxime	200	2.53 ± 0.39 (3)	2.13 ± 0.50[d] (15)	2.20 ± 0.51[d,e] (18)
Meropenem	50	3.73 ± 2.08[d] (6)	<2.0 (12)	2.57 ± 1.45[d,e] (18)
Meropenem	200	3.60 ± 1.17[d] (4)	<2.0 (14)	2.35 ± 0.86[d,e] (18)

[a] Spontaneous death before 46 h postinoculation.
[b] Survivors, killed at 46 h postinoculation.
[c] Values in parentheses are numbers of animals.
[d] Bacterial titers of <2.0 log_{10} CFU per lung were considered to be 2.0 for mean ± SD calculations.
[e] $P < 0.001$ compared with control group.

treated control animals was 5.05% ($P < 0.001$), both at 46 h postinoculation. The survival rate for animals treated with 200 mg of amoxicillin and cefotaxime per kg per dose (Fig. 2) was 83.3%, and the survival rate for animals treated with meropenem was 77.8% while the survival rate for the control group was 11.1% ($P < 0.001$). These rates were determined at 46 h postinoculation. Higher doses of both antibiotics produced higher survival rates among the animals, but the differences were not statistically significant. When mortality was analyzed in relation to time, it was observed that for only 2 of the 33 control animals that died (6.06%), mortality occurred before 9 h postinoculation, while among 28 treated animals that died, up to 15 of them (53.5%) died during the first 9 h postinoculation ($P < 0.001$). If the animals that died during the first 9 h were disregarded in the therapeutic efficacy analysis, because these animals received just one dose, the survival rates would be 85.7 and 93.8% for amoxicillin, 80 and 88.2% for cefotaxime, and 80 and 87.5% for meropenem at doses of 50 and 200 mg/kg, respectively; the survival rates for the untreated animals were 6.2 to 11.1%. These results indicate that, for animals receiving two or more doses of any of the antibiotics administered, the survival rate was equal to or greater than 80%, compared with a survival rate of 6.2 to 11.1% for the untreated animals.

(ii) Bacterial clearance from lungs. Table 2 presents the number of colonies in the lungs of control and treated animals. By 46 h, 91.6% of untreated guinea pigs had succumbed, their lungs showed bilateral hemorrhagic pneumonia, and bacterial counts were 8.83 ± 1.11 log_{10} CFU per lung. The bacterial count in the three untreated animals killed at 46 h was 6.95 ± 2.1 log_{10} CFU per lung. The lungs of all except two of the animals that received any treatment and that were alive at 46 h postinoculation did not have *S. pneumoniae* at detectable limits ($n = 78$; <2 log_{10} CFU per lung); the bacterial counts in the two remaining animals, one treated with amoxicillin and the other treated with cefotaxime, both at 200 mg/kg, were 4.36 and 4.04 log_{10} CFU per lung, respectively. The mean ± SD bacterial counts in the treated animals that died spontaneously before 46 h postinoculation ($n = 28$) are presented in Table 2 according to each antibiotic and dose. The numbers of CFU per lung in this group of 28 animals were undetectable (<2 log_{10} CFU per lung) in 8 animals, and in the remaining 20 animals the numbers of colonies decreased in relation to time (fewer colonies were found in those animals that died later). When the entire numbers of CFU per lung were taken into account, the animals in any group receiving antibiotics had significantly fewer pneumococci than those in the control group ($P < 0.001$). Statistical differences were not found when these data for the animals treated with different antibiotics and doses were compared.

Pharmacokinetics of drugs in serum. The concentrations of the drugs in serum after the administration of a single i.m. dose of each antibiotic at 50 or 200 mg/kg as well as some pharmacodynamic parameters in relation to the susceptibility of the pathogen are presented in Table 3. The concentrations in serum 30 min after a single i.m. injection of 200 mg of any antibiotic per kg were 1.6 to 4.6 times higher than those after the administration of 50 mg/kg. These concentrations in serum and AUCs were higher for meropenem than for amoxicillin and cefotaxime. However, the better pharmacokinetic parameters for meropenem were not related to a better outcome.

DISCUSSION

Animal models of pneumonia caused by penicillin-insensitive *S. pneumoniae* have been difficult to develop because of the ability of a healthy animal to clear the infecting organism rapidly. For this reason, the induction of neutropenia with or

TABLE 3. Relation among some pharmacokinetic parameters in guinea pigs following administration of a single i.m. dose and antimicrobial susceptibility of *S. pneumoniae*

Drug and dose (mg/kg)	Concn in serum at 30 min (μg/ml)[a]	AUC (μg · h/ml)	Concn in serum at 30 min/MIC	AUC/MIC	Time above MIC (min)
Amoxicillin					
50	34.6 ± 5.5	47.0	34.6	47.0	156
200	157.3 ± 28.1	218.2	157.3	218.2	222
Cefotaxime					
50	85.3 ± 24.5	116.4	85.3	116.4	183
200	135.0 ± 6.5	250.0	135.0	250.0	360
Meropenem					
50	114.0 ± 23.9	166.4	228.0	332.8	225
200	496.0 ± 101.3	773.4	992.0	1,546.8	420

[a] Values are means ± SDs for five to six animals measured 30 min after administration for each antibiotic and dose.

without the addition of foreign bodies, like melted agar, have been used to induce pneumonia by these organisms (2, 16, 21). Neither neutropenia nor foreign bodies are close to the most common conditions found among patients with pneumococcal pneumonia. In addition, superinfections and drug interactions may occur when cytotoxic drugs are used. Furthermore, for therapeutic studies, most investigators have used mice or rats, animals which may be inadequate for studying the in vivo efficacies of some antibiotics, such as meropenem (10, 23), which is hydrolyzed by renal DHP-I, making it necessary to administer such an antibiotic in combination with cilastatin (25). Guinea pigs have the additional advantage, like human beings, of not significantly hydrolyzing meropenem (7, 10, 23), making it unnecessary to combine such an antibiotic with the DHP-I inhibitor.

Our study showed that by using a large pneumococcal inoculum directly instilled into the guinea pig's trachea, it is possible for guinea pigs to develop a serious pneumonia with a low early mortality and very high mortality at 46 h, allowing for treatment of the animals with different antibiotics and regimens. On the other hand, untreated animals died as a result of bilateral hemorrhagic pneumonia, with high numbers of organisms being present ($8.83 \pm 1.11 \log_{10}$ CFU per lung). Those few untreated animals that survived at 46 h showed, after sacrifice, fewer lung alterations and lower numbers of organisms, suggesting some kind of physiological clearance effect.

As far as therapeutic efficacy is concerned, our results also indicate that all antibiotics and doses were efficacious in diminishing the mortality caused by pneumococcal pneumonia, resulting in a high rate of survival among treated animals (between 66.6 and 83.3%) compared with the rate among the corresponding untreated controls (5.05 to 11.1%). Although higher doses of the antibiotics produced better therapeutic results, the differences were not statistically significant, and no antibiotic was superior to the others in terms of efficacy. Four doses were enough to significantly diminish the mortality caused by the penicillin-insensitive pneumococcal strain. The dosages administered to the guinea pigs (50 and 200 mg/kg every 8 h) resulted in serum drug concentrations similar to, or higher than in the case of a high dose of meropenem, those obtained in humans after the administration of pharmacological doses (4, 11, 13). When the therapeutic results are compared with the pharmacokinetic data, it can be seen that for all antibiotics and doses, the levels of drug in serum were higher than the corresponding MICs for at least 156 min, a favorable pharmacodynamic factor widely accepted for predicting the therapeutic efficacies of β-lactam antibiotics (5, 27). Of interest is that mortality among the treated animals occurred very early (53.5% before 9 h postinoculation), while only 6.06% of the untreated dead animals succumbed so early, with the difference being highly statistically significant. These results suggest that, as occurs in other experimental infections like meningitis (15, 26) or acute otitis media (12, 20), the bactericidal activity of β-lactam antibiotics may favor a greater inflammatory response because of the cell wall debris released after the antibiotic effect, which could contribute to the observed early mortality among treated animals.

In conclusion, our experimental model in nonneutropenic guinea pigs with a penicillin-insensitive *S. pneumoniae* strain is an adequate and reliable procedure for studying the in vivo efficacies of antibiotics. All antibiotics and doses administered in our experiments improved survival and decreased the number of bacteria in the lungs in comparison with those for untreated animals, and we found no statistically significant differences in the in vivo efficacies of the three β-lactams at the doses administered. High doses of benzylpenicillin have proved to be reasonable treatment for pneumonia caused by penicillin-insensitive pneumococci (18). The use of other antibiotics, such as amoxicillin, cefotaxime, or meropenem, could be justified for the empiric treatment of some bacterial pneumonias. Meropenem may have the additional advantage of its favorable pharmacokinetic properties, which could be of interest for treating pneumococcal meningitis or other pneumococcal infections if, regrettably, very high level β-lactam resistant strains emerged.

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