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Noncompromised Penicillin-Resistant Pneumococcal Pneumonia CBA/J Mouse Model and Comparative Efficacies of Antibiotics in This Model

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The present study confirms that CBA/J mice are susceptible to several clinical isolates of *Streptococcus pneumoniae*, including four of five penicillin-susceptible and all five penicillin-resistant strains tested, thus providing the first noncompromised animal model for penicillin-resistant *S. pneumoniae* pneumonia. In this model, doses of penicillin G of 0.6 mg/kg of body weight given six times at 1-h intervals produced effective pulmonary clearance of a penicillin-susceptible strain (penicillin G MIC, 0.015 μg/ml), while doses of 40 mg/kg given six times at 1-h intervals were required to clear a penicillin-resistant strain (penicillin G MIC, 1 μg/ml). Imipenem (MIC, 0.25 μg/ml) was the most active antibiotic tested against the penicillin-resistant strain, with a calculated dose of 0.42 mg/kg given six times at 1-h intervals, resulting in a 2-log decrease in the number of pulmonary bacteria. Comparable effects were seen with vancomycin (MIC, 0.5 μg/ml), cefotaxime (MIC, 0.5 μg/ml), and penicillin G at doses of 3.3, 5.5, and 31.0 mg/kg given six times at 1-h intervals, respectively. The pharmacokinetic profile of vancomycin in infected lungs was superior to those of the other antibiotics, especially in regard to the elimination half-life (215.4 min for vancomycin versus 15.0, 14.5, and 14.5 min for penicillin G, cefotaxime, and imipenem, respectively). Both imipenem and vancomycin allowed 90% survival when 40-mg/kg doses were administered twice a day beginning 5 days after infection. Survival rates with penicillin G (160-mg/kg doses) and cefotaxime (40-mg/kg doses) were 40 and 30%, respectively, while no saline-treated mice survived. The present study shows that the CBA/J mouse pneumonia model may be useful for evaluating antibiotic efficacies against penicillin-resistant pneumococcal pneumonia in immunocompetent individuals. Our data suggest that imipenem and vancomycin may be the most active agents against penicillin-resistant *S. pneumoniae* pneumonia.

Streptococcus pneumoniae continues to be the leading cause of bacterial pneumonia, and it is accompanied by significant morbidity and mortality (2, 18). Although this organism was at first exquisitely susceptible to penicillin, in the past two decades, and particularly in the last few years, an alarming worldwide increase in the number of strains resistant to both penicillin and other antimicrobial agents has been seen (1, 7, 26). Clinical treatment failures in patients with infections caused by penicillin-resistant *S. pneumoniae* have prompted investigators to reexamine the in vitro and in vivo therapeutic efficacies of antibiotics (4, 12, 19).

Animal models of disease whose features closely resemble those of the human disease are needed to find the optimal antimicrobial treatment. Several investigators have recently reported an inability to infect healthy mice with penicillin-resistant *S. pneumoniae* strains and have accordingly used immunocompromised mice to evaluate the therapeutic efficacies of antibiotics against this organism (3, 20). In those studies, mice were kept leukopenic through cyclophosphamide administration and were challenged with a relatively high number of organisms.

Since penicillin-resistant *S. pneumoniae* pneumonia frequently presents as community-acquired pneumonia in immunocompetent humans (11, 28), animal models not requiring immunosuppressive drugs may provide a clearer pathogenic understanding of this disease and a more precise estimate of the therapeutic efficacies of various antimicrobial agents. We have recently found that CBA/J mice, but not CBA/N, C3H/HeN, C3H/HeJ, C57BL/6, or ICR mice, are susceptible to intranasal challenge with penicillin-resistant *S. pneumoniae* TUM19 isolates. Those experiments demonstrated fatal pneumonia in noncompromised CBA/J mice, with the number of bacteria in the lungs gradually increasing from 10^4 CFU per animal at day 1 to 10^5 CFU per animal at day 3 and 10^7 CFU per animal at day 5 (27).

In the present study, we confirmed the validity of our CBA/J mouse model of penicillin-resistant *S. pneumoniae* pneumonia by examining the virulences of several clinical isolates of penicillin-susceptible and penicillin-resistant strains in ICR and CBA/J mice. We also evaluated the pharmacokinetics and therapeutic efficacies (pulmonary clearance and survival) of penicillin G, cefotaxime, imipenem, and vancomycin in this pneumonia model.

MATERIALS AND METHODS

Challenge organisms. Ten strains of *S. pneumoniae* isolated at Toho University School of Medicine were used in the present study. These strains were isolated from different patients on dates ranging from 1993 to 1995, inclusive, with no two strains representing strains from the same outbreak. Strains 39 and 40 were mucoid type, whereas the other pneumococci showed rough colonies with various degrees of alpha-hemolytic activity. All strains were frozen at −80°C in skim milk until they were used.

Animals. Five-week-old male CBA/J mice (body weight range, 16 to 20 g) and ICR mice (body weight range, 18 to 22 g) were obtained from Charles River Japan (Kanagawa, Japan).

Antimicrobial agents. Penicillin G, cefotaxime, imipenem, and vancomycin were obtained from Meiji Seika (Tokyo, Japan), Hoechst Japan (Tokyo, Japan),

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TABLE 1. Penicillin G MICs and capsular types of *S. pneumoniae* strains tested

Penicillin susceptibility and strain	Colony type	Penicillin G MIC (μg/ml)	Capsular type
Susceptible			
10	Nonmucoid	0.015	11
11	Nonmucoid	0.015	19
39	Mucoid	0.03	3
40	Mucoid	0.03	3
TUH3	Nonmucoid	0.015	29, 35, 42[a]
Resistant			
691	Nonmucoid	2	19
741	Nonmucoid	1	19
861	Nonmucoid	2	19
909	Nonmucoid	1	19
TUM19	Nonmucoid	2	19

[a] Strain TUH3 reacted with the three types of anticapsular antibody indicated.

Banyu Pharmaceutical Co. (Tokyo, Japan), and Shionogi & Co. (Osaka, Japan), respectively. Imipenem was mixed with cilastatin (Banyu) at a ratio of 1:1 and was used in an in vivo pneumonia model.

Antimicrobial susceptibility test. The MICs of the antibiotics were determined by the broth dilution method with Mueller-Hinton broth (Difco, Detroit, Mich.) supplemented with 5% lysed horse blood. After adding an inoculum of 5.0×10^4 CFU to each well, microtiter plates were incubated at 35°C for 18 h. The MICs were defined as the lowest concentration of antimicrobial agent that did not allow visible growth of the *S. pneumoniae* strains. After determination of the MIC, 10 μl of suspension from each well was plated onto Mueller-Hinton agar (Difco) supplemented with 5% lysed horse blood, and the plates were incubated for 24 h at 35°C to determine the MBC. The MBCs were defined as the lowest antibiotic concentration that eliminated 99.9% of the original numbers of CFU (22).

Capsular typing. The capsular types of the *S. pneumoniae* strains were determined as described previously (14) by the Quellung reaction with capsule type-specific antisera (Statens Seruminstitut, Copenhagen, Denmark).

Pneumonia model. Bacteria were inoculated into Todd-Hewitt broth (Difco) supplemented with 30% horse serum, and the mixture was incubated at 37°C until the culture was turbid to the naked eye (typically 5 to 6 h). This culture of organisms in the exponential growth phase was suspended in 0.9% saline to the desired concentration (confirmed by plating serial 10-fold dilutions onto 5% blood agar and incubating the resulting plates for 18 h at 37°C). Mice were anesthetized lightly by intramuscular injection of a mixture of 6 mg of ketamine (Sankyo Pharmaceutical, Tokyo, Japan) per kg of body weight and 1 mg of xylazine (Bayer Japan, Tokyo, Japan) per kg, after which each mouse was intranasally challenged with approximately 10^5 logarithmic-phase organisms.

Pulmonary clearance and survival studies. To evaluate the effects of the antibiotics on the numbers of bacteria in the lungs, the indicated doses of antibiotics were administered subcutaneously six times at 1-h intervals beginning 48 or 96 h after infection. Animals ($n = 5$ for each group) were sacrificed 2 h after administration of the final antibiotic dose. The lungs were then removed and homogenized in saline, and 0.1 ml of serial 10-fold dilutions of the homogenates were plated onto blood agar to obtain counts of viable bacteria. The results were expressed as the mean ± standard deviation log CFU per lung. 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. To compare the efficacies of the various antibiotics on survival, the indicated doses of antibiotics were administered subcutaneously to groups of 10 animals twice a day for 6 days beginning 5 days after infection. The survival rates were recorded daily for 16 to 18 days after infection.

Preparation of lungs for histopathological examination. The lungs of mice infected with strain 741 were removed 48 h after infection. The organs were fixed in 10% formalin, embedded in paraffin wax, sectioned, and then stained with hematoxylin-eosin for histological examination or with Gram stain for the identification of bacteria.

Pharmacokinetic studies. The pharmacokinetics of penicillin G, cefotaxime, imipenem-cilastatin, and vancomycin in groups of three CBA/J mice were determined 48 h after infection with *S. pneumoniae* 741. Serum and lungs were collected at 5, 15, 30, 60, and 120 min after subcutaneous administration of a 40-mg/kg antibiotic dose. Antibiotic concentrations in serum and lung homogenates were determined by the agar well diffusion method by using *Bacillus subtilis* ATCC 12432 as the bioassay reference strain.

Statistical analysis. The Mann-Whitney U test was used to compare the bacterial numbers in the lungs; *P* values of 0.05 or less were considered statistically significant.

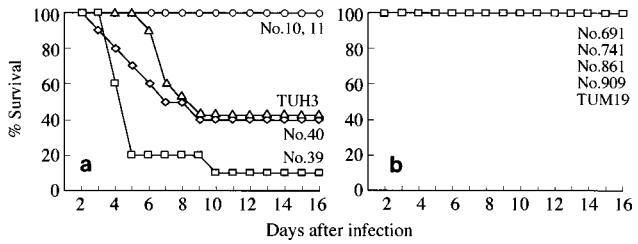

FIG. 1. Survival in ICR mice ($n = 10$) following infection with penicillin-susceptible (a) and penicillin-resistant (b) *S. pneumoniae* strains.

RESULTS

Antibiotic susceptibility and capsular type. The capsular types of and corresponding penicillin G MICs for 10 *S. pneumoniae* strains are given in Table 1. As indicated, strain TUH3 reacted with three different types of anticapsular antibody.

In the present study, strains 10, 11, 39, 40, and TUH3 were identified as penicillin susceptible, whereas strains 691, 741, 861, 909, and TUM19 were identified as penicillin resistant. The MICs of cefotaxime, imipenem, and vancomycin for strain 741 were 0.5, 0.25, and 0.5 μg/ml, respectively, whereas the corresponding MICs for strain 11 were 0.125, ≤0.06, and 0.5 μg/ml, respectively.

The MBCs of penicillin G, cefotaxime, and imipenem for strain 741 were 2, 1, and 0.125 μg/ml, respectively, whereas those for strain 11 were 0.03, 0.125, and ≤0.06 μg/ml, respectively. Thus, neither strain 741 nor strain 11 was tolerant to these cell wall-active antibiotics. Furthermore, no strains included in the present study were tolerant to penicillin G.

Penicillin-susceptible and -resistant pneumonia in ICR and CBA/J mice. Three of five strains of penicillin-susceptible *S. pneumoniae* caused lethal pneumonia in ICR mice, whereas no deaths were observed in ICR mice infected with any of the five strains of penicillin-resistant pneumococci tested (Fig. 1). In CBA/J mice, on the other hand, all five strains of penicillin-resistant *S. pneumoniae* caused death, as did four of five strains of penicillin-susceptible pneumococci (Fig. 2). Death was generally more rapid following infection with penicillin-susceptible strains than with penicillin-resistant strains and, for a given strain, was more rapid in CBA/J mice than in ICR mice.

These results indicate that CBA/J mice are generally susceptible to pneumococci, including both penicillin-resistant and penicillin-susceptible strains. They thus provide a nonimmunocompromised animal model for community-acquired penicillin-resistant *S. pneumoniae* pneumonia.

Lung pathology of mice infected with a penicillin-resistant strain. Figure 3a shows typical lobar involvement in the lungs of mice infected with strain 741. In the affected areas, microscopic examination indicated that alveolar spaces were packed

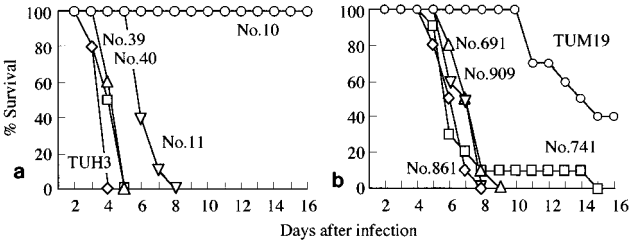

FIG. 2. Survival in CBA/J mice ($n = 10$) following infection with penicillin-susceptible (a) and penicillin-resistant (b) *S. pneumoniae* strains.

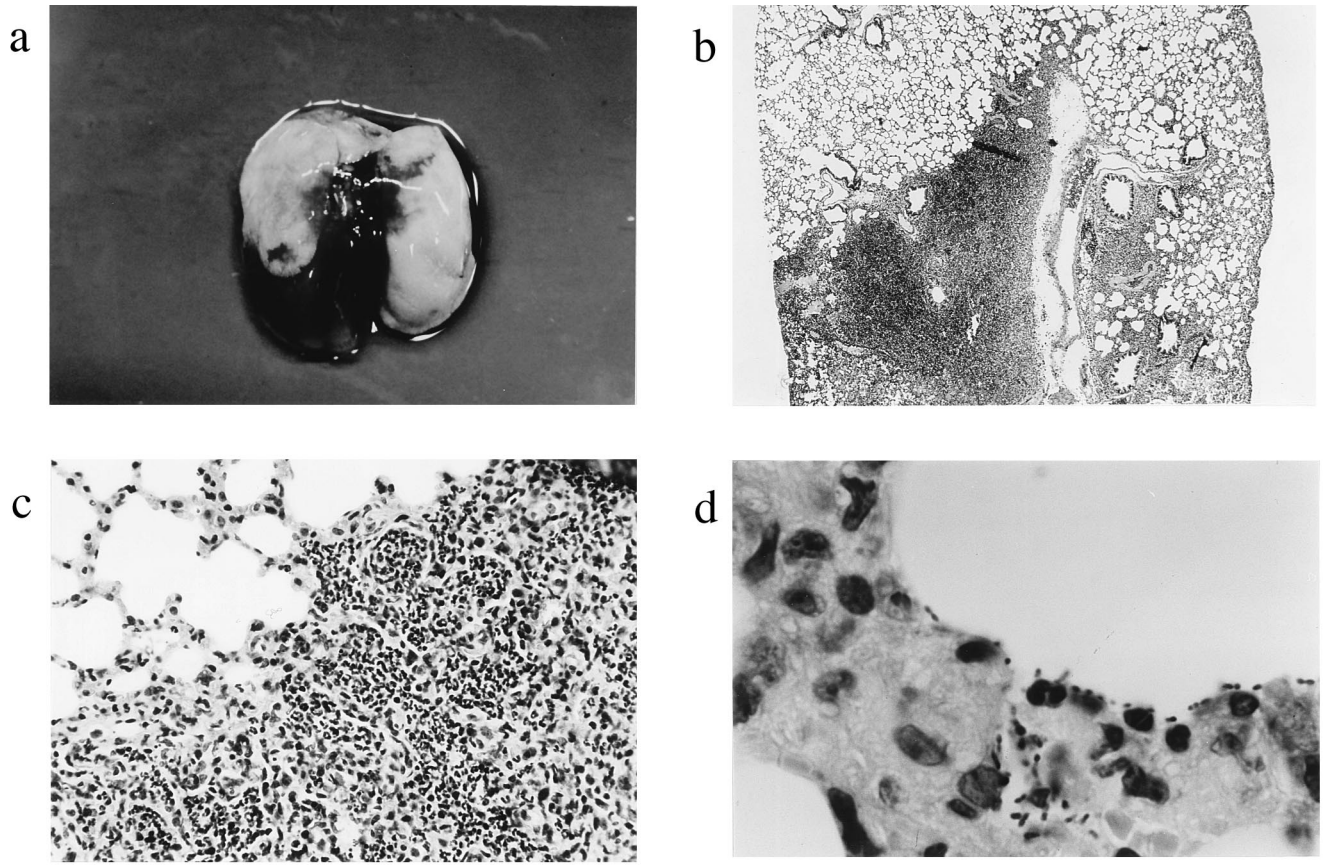

FIG. 3. Pathology of lungs of CBA/J mice infected with strain 741. (a) Representative lobar involvement (48 h after infection). (b and c) Microscopic examination showing alveolar spaces packed with inflammatory cells, mainly neutrophils (hematoxylin and eosin stain). (d) cluster of gram-positive cocci adhering to alveolar lining.

with large numbers of inflammatory cells, mainly neutrophils; thickening of the alveolar septum was also observed (Fig. 3b and c). Gram staining revealed clusters of gram-positive cocci adhering to the alveolar lining (Fig. 3d).

Pulmonary clearance efficacy of penicillin G against penicillin-susceptible and -resistant organisms. Since Fig. 2 suggested that the virulences of strains 11 and 741 were similar, these strains were selected for use in comparative studies of the therapeutic efficacy of penicillin G. In pneumonia caused by strain 11, penicillin G at doses of 0.6 mg/kg or greater given six times at 1-h intervals reduced the number of bacteria in the lungs from 6.4 to less than 3 log CFU per animal (Fig. 4) ($P < 0.05$). In contrast, when mice were infected with strain 741, at least 40 mg of penicillin G per kg given six times at 1-h intervals was required to induce a significant decrease in the number of bacteria in the lungs. From these results, we calculated the penicillin G doses required for a 2-log decrease in bacterial numbers to be 30.2 mg/kg for pneumonia caused by strain 741 and 0.41 mg/kg for pneumonia caused by strain 11. The ratio of penicillin G doses required to achieve this in vivo therapeutic result (73.7) is thus similar to the ratio of MICs for the two strains (1/0.015; 66.7). In other words, the dose required for pulmonary clearance of the organism is proportional to the MIC.

Comparative antibiotic efficacies in clearance of penicillin-resistant organisms. Penicillin G, cefotaxime, imipenem, and vancomycin were tested for their abilities to reduce the numbers of pulmonary bacteria in mice with pneumonia caused by penicillin-resistant strain 741. Imipenem proved to be the most active, with a dose as low as 0.6 mg/kg being adequate to reduce the number of pulmonary bacteria from 7.6 to 5.0 log CFU per animal (Fig. 5). The doses of imipenem, cefotaxime, vancomycin, and penicillin G required for a 2-log decrease in lungs were calculated to be 0.42, 5.5, 3.3, and 31.0 mg/kg, respectively. Unlike the other antibiotics, vancomycin produced a decrease in bacterial numbers that was dose proportional throughout the tested range.

Pharmacokinetic studies. Figure 6 shows the pharmacoki-

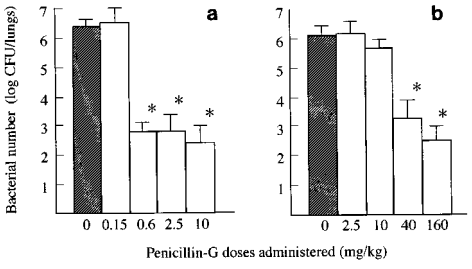

FIG. 4. Efficacies of penicillin G in clearance of penicillin-susceptible and penicillin-resistant pneumococcal pneumonia. (a) Strain 11; (b) strain 741. The indicated doses of penicillin G (given six times at 1-h intervals) were administered subcutaneously beginning 48 h after infection, and the numbers of bacteria in the lungs were determined 2 h after the last injection of penicillin G ($n = 5$). *, $P < 0.05$ compared with control.

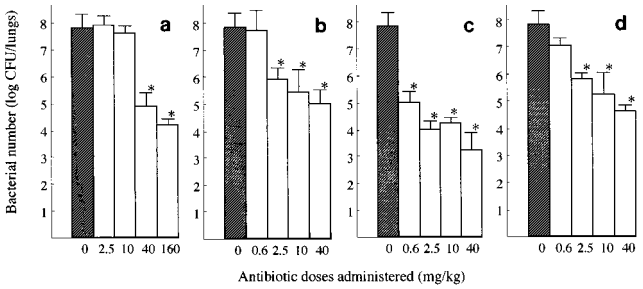

FIG. 5. Efficacies of penicillin G (a), cefotaxime (b), imipenem (c), and vancomycin (d) on the numbers of bacteria in the lungs of mice infected with *S. pneumoniae* 741. The indicated doses of antibiotics were administered (six times at 1-h interval) to mice ($n = 5$) beginning 4 days after infection with strain 741, and the numbers of bacteria in the lungs were determined 2 h after the last injection of antibiotics. *, $P < 0.05$ compared with control.

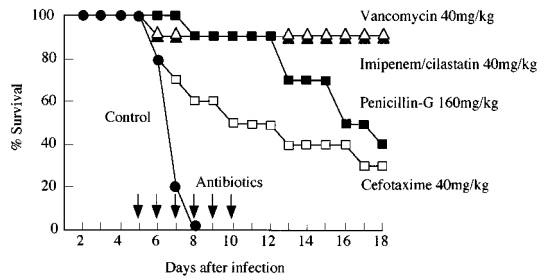

FIG. 7. Therapeutic efficacies of penicillin G, cefotaxime, imipenem-cilastatin, and vancomycin on survival following infection of CBA/J mice with penicillin-resistant strain 741. The indicated doses of antibiotics were administered subcutaneously twice a day for 6 days beginning 5 days after infection ($n = 10$).

netic profiles of the various antibiotics in the sera and lungs of mice infected with *S. pneumoniae* 741. Following subcutaneous administration of 40-mg/kg doses, the peak concentrations of penicillin G, cefotaxime, imipenem, and vancomycin in the lungs were 10.5 μg/g at 5 min, 7.3 μg/g at 5 min, 24.3 μg/g at 15 min, and 35.2 μg/g at 15 min, respectively. The corresponding elimination half-times of these antibiotics were 15.0, 14.5, 14.5, and 215.4 min in the lungs and 8.5, 14.3, 12.1, and 32.2 min in sera. The elimination half-life of vancomycin in the lungs was clearly longer than that in serum. The times above the MICs of these antibiotics in the lungs of infected mice were 42.6, >60, >60, and >120 min, respectively. Thus, vancomycin's pharmacokinetics in infected lungs were superior to those of penicillin G, cefotaxime, and imipenem in these respects.

Comparative antibiotic efficacies on survival. Control mice died between 6 and 8 days after infection (Fig. 7), while the survival rates of mice treated with penicillin G (160 mg/kg) and cefotaxime (40 mg/kg) were 40 and 30%, respectively, at 18 days after infection. In contrast, imipenem (40 mg/kg) and vancomycin (40 mg/kg) both provided 18-day survival rates of 90%.

DISCUSSION

Moine et al. (20) failed to induce penicillin-resistant pneumococcal pneumonia in immunocompetent mice and therefore used leukopenic mice as a model for evaluating the efficacy of ceftriaxone. Since leukopenic mice developed acute pneumonia and died within 2 or 3 days after the intratracheal instillation of 10^7 CFU of organism per mouse, therapy was initiated 3 h after bacterial challenge. Azoulay-Dupuis et al. (3) also used leukopenic mice when they investigated sparfloxacin's therapeutic efficacy in mice with pneumonia.

Our pneumonia model with CBA/J mice is quite different. Without any immunosuppressive drugs, CBA/J mice intranasally challenged with a relatively low number of organisms develop pneumonia in 2 to 4 days, and a majority die within 5 to 10 days following infection. Moreover, the present study has confirmed that CBA/J mice are generally susceptible to clinical isolates of penicillin-resistant *S. pneumoniae*. Since the CBA/J mouse pneumonia model appears to resemble human community-acquired penicillin-resistant *S. pneumoniae* pneumonia, this model provided us with an opportunity to investigate antibiotic efficacies and the pathogenesis of this pneumonia in immunocompetent individuals.

Several investigators have suggested that penicillin is even useful for the treatment of penicillin-resistant *S. pneumoniae* infection. For example, Barry et al. (5) reported that in a gerbil model of acute otitis media, infections caused by penicillin-susceptible, penicillin-resistant, and highly penicillin-resistant pneumococci were cured by two injections of 2.5, 10, and 25 mg of amoxicillin per kg, respectively. Similarly, in a leukopenic mouse pneumonia model, increasing the dose of amoxicillin from 5 to 50 mg/kg improved survival rates following infection with penicillin-resistant pneumococci (3). Clinically, the experience of Pallares et al. (23) with 24 adults with penicillin-resistant pneumonia led them to conclude that this disorder may respond to intravenous high-dose penicillin therapy if the MICs are ≤2 g/ml.

The improved therapeutic efficacy that we found with an increasing penicillin G dosage was consistent with those found in the previous studies. It seems surprising, however, that the dose of penicillin G required to obtain equal therapeutic responses was at least 70-fold higher in penicillin-resistant than in penicillin-susceptible pneumococcal pneumonia. However, the ratio of these effective penicillin G doses is similar to the ratio of MICs for the two strains. Our results are consistent, moreover, with those of Knudsen et al. (16) in a mouse peritonitis model, in which a highly significant correlation between log MIC and log 50% effective dose was found.

As alternatives to penicillin, the efficacies of antimicrobial agents such as cephalosporins (6, 20, 24), newer quinolones (3, 17), imipenem (4, 19), and vancomycin (12, 13, 19) have been investigated in in vitro and in vivo models. For example, Doit et al. (9) have reported that the MICs of imipenem (0.12 to 0.25 μg/ml) were lower than those of penicillin G (2 μg/ml) for 15 strains of penicillin-resistant pneumococci; furthermore, imipenem was the most active drug tested in in vitro killing systems. Likewise, in a rabbit model of meningitis caused by penicillin-resistant pneumococci, a single dose of penicillin (50

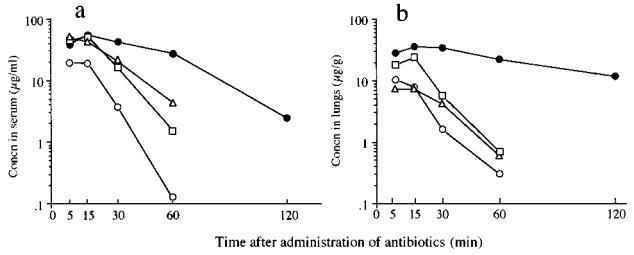

FIG. 6. Pharmacokinetics of penicillin G, cefotaxime, imipenem, and vancomycin in the sera (a) and lungs (b) of infected mice. ○, penicillin G (40 mg/kg); △, cefotaxime (40 mg/kg); □, imipenem-cilastatin (40 mg/kg); ●, vancomycin (40 mg/kg).

or 150 mg/kg) or ceftriaxone failed to lower the number of organisms in cerebrospinal fluid, whereas imipenem (24 mg/kg) or vancomycin (15 mg/kg) reduced the counts more than 2 log CFU/ml (19). As a further example, Catalan et al. (8) reported the failure of cefotaxime in the treatment of meningitis caused by relatively resistant pneumococci, but achieved a cure with vancomycin; they therefore recommended that cefotaxime should be used with caution to treat meningitis caused by *S. pneumoniae* strains for which MICs are $\geq$1 g/ml.

The present study showed that imipenem was the most active antibiotic tested in clearing penicillin-resistant pneumococci from the lungs. On the other hand, vancomycin was superior in several pharmacokinetic parameters not only to imipenem but also to cefotaxime and penicillin G. Probably reflecting these results, survival following the twice-daily administration of doses of imipenem (40 mg/kg) or vancomycin (40 mg/kg) was excellent, while survival was not as good when mice were treated with penicillin G (160 mg/kg) or cefotaxime (40 mg/kg).

In our survival study, penicillin G (160 mg/kg) was as effective as imipenem (40 mg/kg) or vancomycin (40 mg/kg) while therapy was continuing, but mice treated with penicillin G began dying about 2 to 3 days after therapy was stopped. At the end of this experiment (day 18), the lungs of the surviving mice showed contraction or scar formation, and pneumococci were still recovered from two of the four mice treated with penicillin G. In contrast, no organisms were recovered on day 18 from the lungs of mice treated with imipenem or vancomycin. These results suggest that penicillin G may be less effective than imipenem or vancomycin in clearing infection from the lungs.

Our results and those presented in previous reports strongly suggest that imipenem or vancomycin may be appropriate first-line antimicrobial treatment for pneumococcal pneumonia. These drugs should especially be considered for use in patients in whom infection with strains showing high-level penicillin resistance is suspected or in critically ill patients with systemic infections.

We must be very careful in considering the relevance of the results obtained with the mouse model described here to the treatment of human infections. There are substantial differences in pharmacokinetic parameters between mice and humans. In particular, the half-lives of penicillin, cefotaxime, imipenem, and vancomycin in human sera have been reported to be 112.0 (15), 108.0 (25), 54.0 (21), and 390.0 (10) min, respectively, compared with the half-lives of 8.5, 14.3, 12.1, and 32.2 min, respectively, that we found in mouse sera. We also found substantially longer half-lives of vancomycin in mouse lungs than in sera; antibiotic half-lives in human lungs have not been reported.

Despite these interspecies pharmacokinetic differences, in our mouse survival studies we used the usual twice-a-day dosage regimen used in humans. Whether this is the most appropriate regimen or whether different results might be obtained with approaches, such as frequent dosing or constant infusion, that address specific features of drug pharmacokinetics in mice requires further study.

In conclusion, penicillin-resistant *S. pneumoniae* pneumonia in CBA/J mice was proved to be a useful model for evaluating antibiotic efficacies in immunocompetent individuals. The present data indicate that imipenem and vancomycin are the most active agents against penicillin-resistant pneumococcal pneumonia. We believe that the CBA/J mouse model may contribute not only to the search for better antibiotic therapy but also to a better understanding of the pathogenesis of penicillin-resistant *S. pneumoniae* infections in humans.

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