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Resistance to Amikacin and Isepamicin in Rabbits with Experimental Endocarditis of an *aac(6′)-Ib*-Bearing Strain of *Klebsiella pneumoniae* Susceptible In Vitro

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The effect of production of the aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Ib] in *Klebsiella pneumoniae* on the outcome of amikacin and isepamicin treatment of rabbits with experimental endocarditis was assessed. Isogenic high-level (Hi) and low-level (Lo) AAC(6′)-Ib-producing transconjugants (T) were constructed from clinical isolates with plasmid-borne resistance determinants. The MICs of amikacin and isepamicin, their bactericidal effects, and AAC(6′)-Ib production appeared to be well correlated among the clinical isolates and the transconjugants. The susceptibility data determined in vitro, with MICs (in micrograms per milliliter) of amikacin and isepamicin for LoT and HiT of 4 and 0.5 and 32 and 8, respectively, were, however, not predictive of the in vivo efficacies of the drugs. While amikacin and isepamicin caused reductions in bacterial densities ($\log_{10}$ CFU per gram of cardiac vegetation) of 5.1 and 4.8 of the fully susceptible recipient strain (MICs of amikacin and isepamicin, 0.5 and 0.25, respectively), the reductions in density of both LoT and HiT caused by the two drugs (2.7 and 2.4 and 2.9 and 2.2, respectively) were only marginally significant, if at all. There was no significant difference ($P > 0.05$) when the reductions in density of LoT and HiT by either drug were compared or when the efficacies of the two drugs in reducing the density of any strain [non-AAC(6′)-producing, LoT, or HiT] were compared ($P > 0.5$). It is concluded that AAC(6′)-Ib in *K. pneumoniae*, even when produced at a low level and not conferring resistance to amikacin and isepamicin in vitro, compromises the efficacies of both drugs in vivo and possibly does so beyond the experimental model studied here.

Klebsiella pneumoniae, a gram-negative bacterium of the family *Enterobacteriaceae*, is among the most frequently reported pathogens responsible for nosocomial infections. In this family, it is also one of the species that frequently acquire resistance to aminoglycosides. This resistance is primarily due to antibiotic modification by enzymes belonging to one of three classes, i.e., *N*-acetyltransferases, *O*-nucleotidyltransferases, and *O*-phosphotransferases, and aminoglycoside-resistant strains generally emerge after acquisition of plasmid-borne genes encoding such enzymes (7). Among these, aminoglycoside 6′-*N*-acetyltransferases of type Ib [AAC(6′)-Ib], which are widespread among gram-negative bacteria (17, 19, 24), are capable of modifying the clinically important aminoglycosides tobramycin, netilmicin, and amikacin (7, 20). Large surveys of aminoglycoside-resistant members of the family *Enterobacteriaceae*, including *K. pneumoniae*, have shown that over 50% of them may carry an *acc(6′)-Ib* gene (17). However, the gene is not efficiently expressed in all strains, and when it is, the levels of resistance may vary (21). In vitro-determined resistance profiles of strains carrying and expressing an *aac(6′)-Ib* gene typically include amikacin and sometimes isepamicin (20, 21). Isepamicin, a derivative of gentamicin B, has an activity comparable to that of amikacin against non-AAC(6′)-I-producing strains (15, 23), but its in vitro activity against strains producing this enzyme has been reported to be somewhat superior to that of amikacin (11).

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It was the aim of this study to determine whether the therapeutic responses to amikacin and isepamicin, as evaluated with a rabbit model of *K. pneumoniae* endocarditis, were influenced by the level of expression of an *aac(6′)-Ib* gene in the causative strain or whether they were conditioned merely by the presence or absence of the gene.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Three strains of *K. pneumoniae*, KPn191, KPn491, and KPn1391, were collected in France during a national survey of aminoglycoside resistance in clinical isolates of the family *Enterobacteriaceae* (17). The strains were resistant to multiple aminoglycosides (Table 1). A derivative of KPn491 which had lost resistance to all aminoglycosides was obtained spontaneously and called KPn491-1. A rifampin-resistant mutant of KPn491-1 was selected on agar containing 1 mg of rifampin per ml. This mutant, KPn491-1Rif, was used as a recipient for conjugation (14) with the three clinical KPn isolates. Transconjugants were selected on 4 μg of amikacin per ml and called Tc191, Tc491, and Tc1391 (Table 1).

All strains were grown in Mueller-Hinton (MH) broth or on MH agar (Sanofi Diagnostics Pasteur) and, where appropriate, in the presence of amikacin (4 μg/ml).

Susceptibility testing: disk diffusion, determination of MICs, and kill curves. Standard disk diffusion assays were carried out to determine resistance patterns and to infer the underlying resistance mechanism (16, 21). Aminoglycoside resistance patterns were determined with a set of 12 disks (kindly provided by Schering-Plough) containing amikacin, apramicin, 5′-episisomicin, 2′-*N*-ethylnetilmicin, 6′-*N*-ethylnetilmicin, fortimycin, gentamicin, isepamicin, kanamycin, netilmicin, neomycin, and tobramycin. We screened for the production of extended-spectrum β-lactamases (ESBL) by the juxtaposition of disks (Sanofi Diagnostics Pasteur) containing cefotaxime, ceftriaxone, and amoxicillin-clavulanic acid. MICs were determined on agar plates containing serially twofold-diluted antibiotics. Plates, inoculated with a Steers-type multiprong device and ca. 10^4 CFU per spot, were read after incubation for 18 h at 37°C.

Kill curves were established with bacteria growing in liquid medium as previously described (12) with inocula of 10^6 and 10^8 CFU/ml. The latter concentration was chosen to approximate in vitro the bacterial concentrations usually observed in cardiac vegetations. The concentrations of the antibiotics tested, amikacin and isepamicin, were 32, 16, and 4 μg/ml and were chosen in order to mimic in rabbits the antibiotic levels usually achieved in human serum. At 0, 2,

TABLE 1. MICs of various aminoglycosides in MH agar at an inoculum of 6 $\log_{10}$ CFU/ml for clinical isolates of *K. pneumoniae* and derivatives obtained in vitro

Strain	MIC (μg/ml)[a]					
	Ami	Ise	Gen	Net	Kan	Tob
Kpn491-1Rif	0.5	0.25	<0.12	0.12	0.25	0.12
Kpn491	8	0.5	32	32	64	16
Tc491	4	0.5	32	32	32	8
Kpn1391	16	1	128	128	128	32
Tc1391	8	1	64	64	128	32
Kpn191	32	8	128	128	>512	128
Tc191	32	8	128	128	>512	32

[a] Ami, amikacin; Ise, isepamicin; Gen, gentamicin; Net, netilmicin, Kan, kanamycin; Tob, tobramycin.

4, 6, and 24 h after inoculation of bacteria into the antibiotic-containing broth, cultures were serially 10-fold diluted, samples of 0.1 ml were spread on MH agar plates and incubated at 37°C, and colonies were counted after 18 h.

Immunoblot analysis. Acetyltransferase production in the clinical strains and transconjugants was monitored by immunoblot analysis, after electrophoresis of crude bacterial extracts on a sodium dodecyl sulfate-containing polyacrylamide gel, with anti-AAC(6′)-Ib antibodies as described previously (24).

Virulence testing in mice. The virulence of the *K. pneumoniae* strains was tested in 7-week-old male OF1 mice (5) weighing 35 to 40 g. Overnight MH broth cultures were serially 10-fold diluted in the same medium to yield bacterial concentrations ranging from 10^4 to 10^8 CFU/ml. Groups of five mice were injected intraperitoneally with 0.5 ml of each dilution, with a total of 25 mice per strain. The animals were observed for 7 days, and the number of survivors was scored for each dilution.

Experimental endocarditis in rabbits. Experiments were performed with transconjugants Tc491 and Tc191 and with the isogenic strain KPn491-1Rif as a control. Aortic endocarditis was induced in female New Zealand White rabbits (weight range, 2.2 to 2.8 kg) according to the method of Perlman and Freedman (18) as previously described (4, 13). On day 1, rabbits were anesthetized with ketamine hydrochloride (15 mg/kg intramuscularly [i.m.]) and a polyethylene catheter, left in place for the duration of the experiment, was inserted through the right carotid artery into the left ventricle. Twenty-four hours after catheter insertion, 10^8 CFU of each strain (mean, 4.75×10^8; range, 2×10^8 to 7×10^8 per ml), in 1 ml of 0.9% NaCl, was injected into a marginal ear vein. For each bacterial strain, eight rabbits were randomly assigned to one of three groups: (i) untreated controls, (ii) rabbits treated with amikacin, and (iii) rabbits treated with isepamicin, each at 15 mg/kg i.m. once a day, from day 5 to 8. On day 9, the animals were killed by intracardiac injection of phenobarbital, 24 h after the last injection of antibiotic. The hearts were removed and inspected to confirm vegetative endocarditis. All vegetations from each rabbit were excised, pooled, weighed, and rinsed in sterile saline solution. Vegetations were homogenized in 0.5 ml of the same solution, and 0.1-ml portions were quantitatively subcultured on MH agar plates at 37°C for 24 h. Vegetations were considered sterile when no colony was detected during subculture of the undiluted homogenate.

The results of the colony counts were expressed as $\log_{10}$ CFU per gram of vegetation and, for each group, as the mean ± the standard deviation. The sensitivity limit of this technique was 2 $\log_{10}$ CFU/g. For calculation of the means, sterile vegetations were considered to contain a maximum of 1 CFU. Results were expressed as the reductions of $\log_{10}$ CFU per gram compared with the mean of the untreated controls.

The persistence of the resistance genes in the transconjugants Tc491 and Tc191 was verified by testing for the aminoglycoside resistance patterns of the colonies isolated at the end of therapy.

Testing for uniformly non-enzyme-dependent, aminoglycoside-resistant variants. The resistance phenotypes of bacteria subcultured from among those growing after 24 h in the time-kill experiments and those persisting in the cardiac vegetations and differing in cultural morphology (microcolonies) and appearance (transparency) were tested by the disk diffusion method in order to detect uniformly aminoglycoside-resistant variants.

Antibiotic assays. The concentrations of amikacin and isepamicin in sera were measured, by a fluorescence polarization immunoassay (8) with a TDX automaton (Abbott, Abbott Park, Ill.) immediately before (trough) and 1 h after (peak) the final i.m. injection, the times to the maximum concentration of the drug in serum previously reported being 1 ± 0.5 h for isepamicin and 0.9 ± 0.3 h for amikacin (13). Residual antibiotic levels were determined in serum samples collected immediately before killing of the animals. The sensitivity limits were 0.09 μg/ml for amikacin and 0.4 μg/ml for isepamicin.

Statistical analyses. Bacterial counts in vegetations, expressed as means ± standard deviations, were compared by an analysis of variance for multiple comparisons (F test) (10). A P value of <0.05 was considered significant. When this significance threshold was reached, a pairwise group comparison was made.

RESULTS

Aminoglycoside susceptibility and AAC(6′)-Ib production. The MICs of several aminoglycosides for the clinical isolates of *K. pneumoniae* and the corresponding transconjugants are shown in Table 1. The resistance profiles were in agreement with those observed in the antibiotic diffusion assay using 12 aminoglycosides (data not shown). They indicated the production of two aminoglycoside acetyltransferases, an AAC(6′)-I, responsible for decreased susceptibilities to amikacin, tobramycin, netilmicin, and sometimes isepamicin, and an AAC(3), entailing additional resistance to gentamicin. The presence of an *aac(6′)-Ib* and an *aac(3)-II* [formerly *aac(3)-V*] gene in each of the three clinical strains was verified by DNA-DNA hybridization (data not shown). AAC(6′)-Ib production was also revealed by immunoblotting (Fig. 1). Since the resistance profiles of the transconjugants were practically identical to those of the respective donor strains, we inferred that in each case both resistance markers were carried by a conjugative plasmid. According to generally accepted interpretative criteria, the AAC(6′)-Ib production did not result in resistance of strains KPn491, KPn1391, and KPn191 to isepamicin and of strain KPn491 to amikacin, with MICs of 0.5, 1, 8, and 8 μg/ml, respectively. Strain KPn1391 was in the intermediate category with respect to amikacin (16 μg/ml), while KPn191 was fully resistant (32 μg/ml) (Table 1). The MICs of the two drugs (just as of the remaining aminoglycosides tested) for the transconjugants were practically the same as for the respective clinical isolates, with transconjugants Tc491 and Tc1391 being slightly more susceptible (Table 1). Levels of AAC(6′)-Ib production paralleled the MICs quantitatively in that they were highest in Kpn191 and Tc191, intermediate in KPn1391 and Tc1391, and lowest in KPn491 and Tc491 (Fig. 1).

The three clinical strains were in addition resistant or showed reduced susceptibilities to cefotaxime and ceftriaxone and were susceptible to the protective effect of clavulanic acid (data not shown), indicating the production of an ESBL. The corresponding β-lactamase genes were cotransferred to Tc491 and Tc1391 but not Tc191 (data not shown).

In vitro activities of amikacin and isepamicin against transconjugants and in vitro-selected uniformly aminoglyside-resistant variants. The activities of amikacin and isepamicin were tested against the three isogenic transconjugants Tc191, Tc491, and Tc1391 and its isogenic, susceptible recipient KPn491-1Rif, as well as against uniformly aminoglycoside-resistant variants isolated at the end of the time-kill experiments (24 h). The activity of isepamicin was somewhat greater and faster than that of amikacin against the susceptible recipient strain (Fig. 2A and B). This relationship was maintained in transconjugant Tc491 with decreasing susceptibility (Fig. 2C

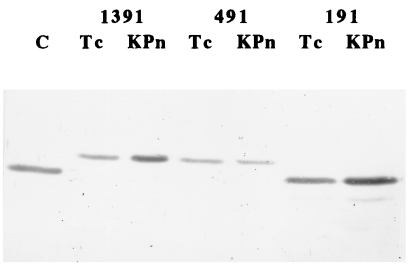

FIG. 1. Immunoblot of AAC(6′)-Ib preparations from the clinical *K. pneumoniae* isolates (KPn) and the corresponding KPn491-1Rif transconjugants (Tc). Lane C, *E. coli*(pAZ505) producing the reference AAC(6′)-Ib [formerly called AAC(6′)-4 (7, 25)] (24).

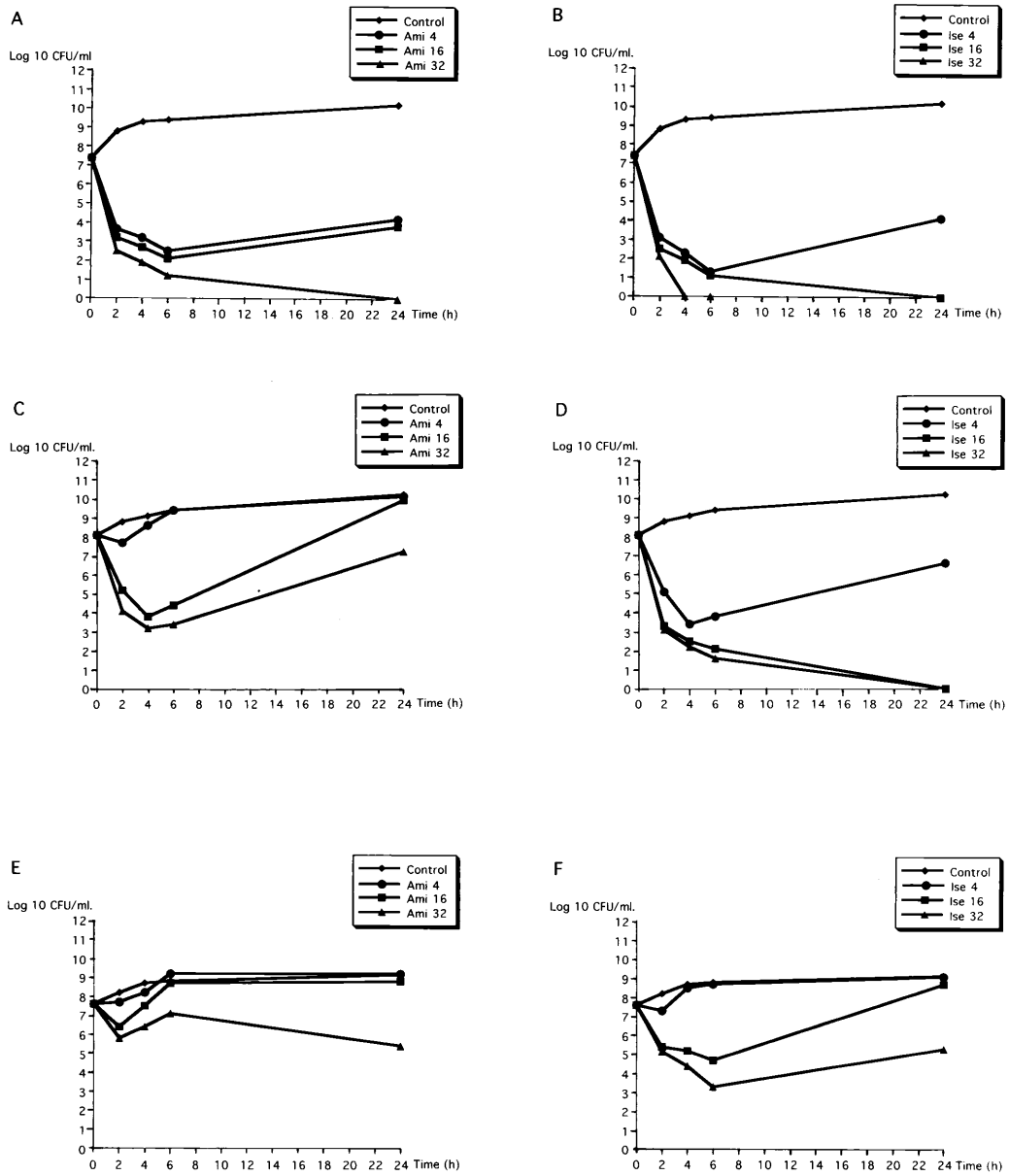

FIG. 2. In vitro kill kinetics with amikacin and isepamicin of the transconjugants producing low and high levels of AAC(6′)-Ib. (A) KPn491-Rif with amikacin; (B) KPn491-1Rif with isepamicin; (C) Tc491 with amikacin; (D) Tc491 with isepamicin; (E) Tc191 with amikacin; (F) Tc191 with isepamicin. The compounds, (Ami) and isepamicin (Ise) and the concentrations (in micrograms per milliliter) are indicated in the amikacin symbol keys.

and D), but the differences between the two drugs were less pronounced in their efficiencies in killing the most resistant transconjugant, Tc191 (Fig. 2E and F). The kill kinetics for Tc1391, like its susceptibilities to amikacin and isepamicin (Table 1) and its AAC(6′)-Ib production (Fig. 1), were intermediate to those observed for Tc491 and Tc191 (data not shown).

Uniformly aminoglycoside-resistant variants appeared after 24 h of contact with both antibiotics and grew as small grayish colonies. Neither amikacin nor isepamicin had any activity against these strains (data not shown).

Virulence of the transconjugants in mice. The three transconjugants and the plasmid-free recipient KPn491-1Rif were virulent in mice but not to the same degree. While the recipient, at the highest concentration used, killed 100% of the mice within 1 day, 80 to 100% of the mice injected with either transconjugant survived after 1 day and 40 to 60% survived after 1 week. Only a comparative analysis using plasmids with the *aac(6′)-Ib* genes rendered nonfunctional would allow a conclusion as to whether this decrease in virulence was related to the resistance gene or another plasmid function.

Experimental endocarditis. The responses to amikacin and isepamicin of the low- and high-level AAC(6′)-Ib producers Tc491 and Tc191, respectively, were tested with a rabbit endocarditis model. Both transconjugants induced practically the same bacterial densities in cardiac vegetations of untreated control animals, which were, however, somewhat lower (ca. 2 $\log_{10}$) than the densities induced by the fully susceptible, plasmid-free recipient KPn491-1Rif (Table 2). The possibility of a link between the induction of higher densities by the plasmid-

TABLE 2. Bacterial densities, in cardiac vegetations, of AAC(6′)-Ib-producing and -nonproducing strains following a 4-day treatment with isepamicin or amikacin

Drug	Mean $\log_{10}$ CFU/g of vegetation ± SD (no. of sterile vegetations/no. of total vegetations)		
	KPn491-1Rif	Tc491	Tc191
None[a]	7.9 ± 1.9 (0/8)	5.9 ± 1.9 (0/8)	6.2 ± 1.1 (0/8)
Isepamicin	3.2 ± 1.5 (5/8)	3.6 ± 1.8 (4/8)	4.4 ± 2.55 (2/8)
Amikacin	2.8 ± 0.9 (5/8)	3.6 ± 2.4 (4/8)	3.7 ± 2.44 (3/8)
Comparison (*P*) between groups	0.0001	0.055	0.065

[a] $P = 0.056$ when the three strains were compared.

free strain and its stronger virulence in mice has not been investigated further. Table 2 also gives the bacterial densities in the vegetations of the animals treated with amikacin or isepamicin, indicating that both drugs were highly effective against the non-enzyme-producing strain ($P = 0.0001$) but much less so against both AAC(6′)-I producers, with the *P* values 0.055 for Tc491 and 0.065 for Tc191 being at the limit of significance.

When the reductions in bacterial densities induced with the three strains in treated versus control animals were compared, there were highly significant differences, with a *P* of 0.0097 for isepamicin and a *P* of 0.0069 for amikacin (Table 3). Also, the differences observed individually between either the low-level or the high-level AAC(6′)-I producer and KPn491-1Rif were statistically significant, with *P* values of <0.05. However, there was no such difference between the two enzyme-producing strains, with a *P* value of >0.05 (Table 3). Comparison of the efficacies of isepamicin and amikacin against any of the strains tested revealed no significant difference, with *P* values of ≥ 0.52 for all strains (Table 3).

The amikacin and isepamicin concentrations in sera, measured 1 hour after the final i.m. injection, were 30.3 ± 3.9 and 28.5 ± 5.05 μg/liter, respectively, in agreement with previous observations (13). Trough levels of the two compounds were below the level of detection. At the end of therapy, colonies differing in size or appearance were found among the bacteria persisting in the vegetations. The antibiotic susceptibilities of these colonies were tested as described above for the uniformly resistant variants observed in vitro. Surprisingly, however, no such resistant variants were found in vivo.

DISCUSSION

Adequate and efficient antibiotic therapy rests largely on the knowledge of in vitro bacterial drug susceptibility. Extrapolation to in vivo efficacy takes into account pharmakokinetic and pharmacodynamic parameters but remains empirical and may require some interpretation of the in vitro susceptibility data. Here we used experimental endocarditis in rabbits, recognized as a model of severe bacterial infection (9), with isogenic strains of *K. pneumoniae* and assayed the efficacies of amikacin and isepamicin monotherapies against low- and high-level AAC(6′)-Ib producers in order to test whether apparent in vitro susceptibilities of resistance-gene-bearing bacteria allowed a reliable prediction of their behavior under antibiotic treatment in vivo. We chose once-a-day administration of both drugs in order to study their intrinsic efficacies under suboptimal conditions, a mode of administration in rabbits validated previously (9).

AAC(6′)-Ib production is the most common cause for amikacin and isepamicin resistance in members of the family *Enterobacteriaceae*, and in *K. pneumoniae* it is frequently associated with the production of an AAC(3)-II and ESBL (2, 22). The AAC(6′)-Ib-, AAC(3)-II-, and ESBL-producing isolates used in this study were therefore representative of problem-causing *Klebsiella* spp. in the clinical setting.

In vitro, strains KPn491, KPn1319, and KPn191 and their respective isogenic transconjugants, showed decreasing aminoglycoside susceptibilities which were paralleled by decreasing bactericidal activities of amikacin and isepamicin, the two drugs tested against the transconjugants (Fig. 2). This decrease in susceptibility was most likely the result of increased AAC(6′)-Ib production, as suggested, although not formally proven, by the intensities of the enzyme-containing bands shown in Fig. 1. The reasons for the difference in enzyme production have not been explored, but variations in the copy numbers of the conjugative plasmids might be a factor, because the susceptibilities of the three transconjugants to gentamicin, whose modification was mediated by a distinct but cotransferred gene, *aac(3)-II*, were decreased in the same order as those to the compounds modified by AAC(6′)-Ib.

The in vitro activities of amikacin and isepamicin were noticeably different. The MICs of isepamicin for the isolate producing a low level of AAC(6′)-Ib and the corresponding transconjugant were 8 to 16 times lower than those of amikacin, while they were 4 times lower for the strains producing a high level of AAC(6′)-Ib (Table 1), in agreement with previous observations (23). Also, the relative bactericidal activity of isepamicin in vitro appeared somewhat greater than that of amikacin. In fact, isepamicin killed the low-level-AAC(6′)-Ib producer at least as rapidly as and to the same extent (Fig. 1D) that amikacin killed the fully susceptible strain (Fig. 1A), while the activity of the latter against the enzyme producer appeared clearly impeded (Fig. 1B). In contrast, both compounds were equally inefficient in killing uniformly aminoglycoside-resistant variants (data not shown).

Amikacin and isepamicin were tested in a rabbit endocarditis model against a set of in vitro-constructed isogenic strains in order to minimize possible strain-dependent factors that might

TABLE 3. Reduction of bacterial densities, in cardiac vegetations, of AAC(6′)-Ib-producing and -nonproducing strains following a 4-day treatment with isepamicin or amikacin

Drug	Mean reduction of $\log_{10}$ CFU/g of vegetation ± SD[a]			Comparison of results with indicated strains (*P*)			
	KPn491-1Rif	Tc491	Tc191	All strains	KPn491-1Rif		Tc491
					Tc491	Tc191	Tc191
Isepamicin	4.8 ± 1.5	2.4 ± 1.6	2.2 ± 1.9	0.0097	<0.05	<0.05	>0.05
Amikacin	5.1 ± 0.9	2.7 ± 1.7	2.9 ± 1.9	0.0069	<0.05	<0.05	>0.05

[a] Comparisons of results after treatment with isepamicin with those after treatment with amikacin yielded *P* values of 0.52 for KPn491-1Rif, 0.69 for Tc491, and 0.55 for Tc191.

influence the therapeutic response. Only the transconjugants producing low and high levels of AAC(6′)-Ib were used, with the fully susceptible recipient being a control. Unexpectedly, the two enzyme-producing strains induced lower bacterial densities in the cardiac vegetations of the untreated animals than the fully susceptible strain, but the course of the untreated disease was similar in each of the three groups (Table 2).

In contrast to what was observed in vitro, there was no noticeable difference in efficacy between amikacin and isepamicin in vivo. Furthermore, the low-level-enzyme-producing strain was no more susceptible than the high-level-enzyme-producing strain. As expected, both aminoglycosides were highly active against the susceptible control strain ($P = 0.0001$). For this strain, the peak concentration/MIC ratios were 114 for isepamicin and 60 for amikacin and the efficacy of isepamicin was equivalent to that of amikacin, suggesting a lower level of intrinsic activity of isepamicin in cardiac vegetations. However, neither treatment proved to be efficacious against Tc491 ($P = 0.055$) at peak concentration/MIC ratios of 57 for isepamicin and 7.5 for amikacin or against Tc191 ($P = 0.065$) at ratios of 3.6 for isepamicin and ca. 1 for amikacin (Table 1 and data not shown). These conclusions were reinforced when the reductions in the bacterial densities were considered and when the susceptible and the enzyme-producing strains were compared pairwise (Table 3).

The fact that there was no difference in the responses of the two AAC(6′)-Ib-producing strains to either treatment ($P > 0.05$), demonstrating that the apparent in vitro susceptibility of Tc491 was not maintained in vivo, might be explained by the possibility that aminoglycoside concentrations reached in cardiac vegetations were appreciably lower than the concentrations in sera, as suggested by previous studies with gentamicin (6), or that bacterial drug uptake was reduced in the vegetations or both. In this situation, the concept of competition between the rates of bacterial drug accumulation and drug modification (3) would apply, according to which the enzyme even at low concentration should be able to modify efficiently the small amount of drug taken up into the bacterial cell and thereby cause resistance.

As far as the selection of mutants is concerned, uniformly aminoglycoside-resistant variants were isolated in vitro from all the AAC(6′)-I-producing strains. The importance of the aminoglycoside concentration/MIC ratio for preventing the emergence of non-enzyme-dependent resistance in vitro has been pointed out for *K. pneumoniae*, with a ratio of greater than 8 being required for netilmicin (1). The present study does not validate this result for isepamicin, since we isolated such mutants of Kpn491 and Tc491 in vitro at an isepamicin concentration/MIC ratio of 64. By contrast, we found no uniformly resistant variants among the bacteria persisting in the cardiac vegetations, not even variants of the high-level-AAC(6′)-Ib producer Tc191, for which only the low peak concentration/MIC ratios for isepamicin and amikacin mentioned above were obtained. Consequently, the discrepancy cannot be explained by differences in these ratios but possibly by differences in the durations of contact between the drugs and the bacteria. In vitro, we observed an increase in the number of small colonies with a time of contact of up to 24 h, whereas in vivo, this time was much shorter because of the short half-lives of the aminoglycosides. An alternative possibility, however remote, is that uniformly resistant variants are less virulent and that there is a link between the loss of virulence and the development of non-enzyme-dependent aminoglycoside resistance. In support of this possibility is our observation that after intraperitoneal injection of 10^8 CFU of uniformly aminoglycoside-resistant variants isolated in vitro, all mice survived. Whatever the reason for the emergence of such variants in vitro may be, it does not seem to be of predictive value for their selection under therapy in vivo.

Severe infections caused by gram-negative bacilli are frequently treated with aminoglycosides in combination with β-lactam antibiotics. The present model study was limited to aminoglycoside monotherapy in order to evaluate one parameter, i.e., the importance of the level of expression of a common resistance gene, *aac(6′)-Ib*, in apparently susceptible strains of *K. pneumoniae*. For these strains, in vitro susceptibility data did not allow the prediction of the in vivo efficacies of amikacin and isepamicin. Furthermore, and in spite of what might have been expected from the clearly distinct MICs and kill-curve data, the in vitro advantage of isepamicin over amikacin was not reflected in the endocarditis model. The question remains open whether low-level enzyme production in apparently aminoglycoside-susceptible strains also affects the response to the synergistic action of aminoglycoside–β-lactam combinations. The results of a previous study (13) provide circumstantial evidence that this might be so, since there was no synergistic effect in vivo of amikacin or isepamicin and ceftriaxone against an AAC(6′)-Ib-, ESBL-producing strain of *K. pneumoniae*, while there was such an effect of the combination of the β-lactam and gentamicin, a compound not modified in that particular strain.

Considering all the data, we feel that care should be taken in clinical practice when the presence of an *aac(6)-I* gene, expressed at low level, is suspected. A reduced inhibition zone size around the amikacin disk in the standard diffusion assay should raise suspicion, which may be confirmed when 6′-*N*- and 2′-*N*-ethylnetilmicin disks are used and substantially smaller zone sizes around the latter are observed (16, 21).

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