Different Aminoglycoside-Resistant Phenotypes in a Rabbit Staphylococcus aureus Endocarditis Infection Model

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The impact of different types of enzymatic resistance on the in vivo antibacterial activity of aminoglycosides (amikacin, gentamicin, and netilmicin) was studied in the rabbit endocarditis model with four strains of Staphylococcus aureus. Animals were treated in a manner simulating the administration of a single daily human dose. Amikacin had no effect on the three kanamycin-resistant strains despite apparent susceptibility in the disk diffusion test. Gentamicin appears to be the preferable aminoglycoside for treatment of staphylococcal infections.

More than 40% of the Staphylococcus aureus strains isolated in hospitals are resistant to methicillin and show associated resistance to other antibiotics, especially aminoglycosides (8, 9, 11). The treatment of methicillin-resistant S. aureus infections is based principally on glycopeptides (mainly vancomycin). When combination therapy is required, aminoglycosides are used because of their rapid and intense killing power (16, 17, 21). Enzymatic inactivation is the major mechanism of S. aureus resistance to aminoglycosides (15, 19). Three types of enzymes, APH(3’), ANT(4’), and APH(2’)-AAC(6’)) are involved, respectively, in resistance to kanamycin (K’), kanamycin and tobramycin (K’), and kanamycin, tobramycin, and gentamycin (KTG)1. Other aminoglycosides are also modified by these enzymes; namely, netilmicin is modified by APH(2’)-AAC(6’)) and amikacin is modified by all three enzymes, whereas their bacteriostatic activity against S. aureus is preserved (1, 2; R. Bismuth, J. R. Pirault, H. Drugeon, and P. Courvalin, 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 705, 1989).

The purpose of this in vivo study in a rabbit S. aureus endocarditis infection model was to assess the impact of these enzymes on the antibacterial activity of amikacin and gentamicin, alone or in combination with a cell wall-active antibiotic (such as vancomycin), and to determine whether a disk diffusion test can indicate which suitable aminoglycoside with which to combat a staphylococcal infection.

Four S. aureus strains were studied; they were isolated from different clinical samples kindly supplied by R. Bismuth. Two of them were methicillin resistant (MecA+), and the other two were methicillin susceptible (MecA−). A previous hybridization study (R. Bismuth, unpublished data) showed that the plasmid content of these strains coded for enzymes modifying aminoglycosides. One carried no enzyme and displayed a susceptible wild-type phenotype (S-SA, methicillin resistant). The other three produced an enzyme: APH(3’)-producing K-SA (methicillin susceptible), ANT(4’)-producing KT-SA (methicillin resistant), and AAC(6’)-APH(2’)-producing KTG-SA (methicillin susceptible). Disk diffusion tests were performed in accordance with the standard agar plate method recommended by a committee of the French Society for Microbiology (5). Mueller-Hinton media, in which calcium (50 mg/liter) and magnesium (25 mg/liter) had been adjusted, were seeded with a 106-CFU/ml suspension and read after a 24-h incubation at 37°C. The MIC was determined by microdilution in liquid medium (6) after a 24-h incubation at 37°C. Strains were rated as susceptible, intermediate, or resistant with respect to zone diameters and critical concentrations (5). The same method was used to determine the MIC of vancomycin for each strain.

Aortic endocarditis (14) was induced in female New Zealand rabbits by insertion of a polyethylene catheter into the left ventricle, followed 24 h later by intravenous inoculation of 106 CFU of each S. aureus strain. Twenty-four hours after inoculation, control animals were sacrificed and treatments for a 48-h period were begun. Five treatments were studied: gentamicin (Shering-Plough Laboratories, Levallois-Perret, France), amikacin (Bristol-Myers Squibb Laboratories, Paris-La Défense, France), vancomycin (Lilly Laboratories, St-Cloud, France), and the combinations gentamicin-vancomycin and amikacin-vancomycin. Netilmicin (Shering-Plough Laboratories, Paris, France) was only modified by the bifunctional enzyme and was thus tested only on the KTG-SA strain in order to validate the results observed with gentamicin on this strain. Aminoglycosides were administered to rabbits in a single daily dose, in agreement with current recommendations concerning the best dosage regimen for these drugs (7, 10, 12, 18), simulating the kinetics of a human dosage of amikacin of 15 mg/kg/day, of gentamicin of 3 mg/kg/day, or of netilmicin of 6 mg/kg/day, by using a computerized system of automatic syringes managed by software to adapt the infusion rate. Vancomycin was administered at a constant intravenous infusion rate in order to simulate a total human dose of about 30 mg/kg, allowing the achievement of a steady-state concentration of around 25 mg/liter of serum. After sacrifice, thoracotomy, and car...
endocarditis vegetations were removed, pulverized, stored in ice, and ultimately ground in a 0.5-ml volume of a saline solution. The homogenate obtained was then spread on Tryptase-soja agar plates in pure form or after a 1:100 or 1:1,000 dilution by using a Spiral seeder (Interscience). After a 24-h incubation at 37°C, colonies were counted and the results were expressed in log_{10} CFU per gram of vegetation. The sensitivity limit of this method was one colony for 50 µl seeded.

Assays of aminoglycoside concentrations in serum were performed at the peak (30 min after the start of infusion) and trough (24 h after administration) points. The immunoenzymatic method performed with a COBAS MIRA unit used EMIT reagents (Behring Diagnostics Inc., Cupertino, Calif.) for amikacin (detection threshold, 1 mg/liter; coefficient of variation, 3.9%) and gentamicin (detection threshold, 0.3 mg/liter; coefficient of variation, 2.7 to 6.7%) and TDx/TDXPLx reagent (Abbott) for netilmicin (detection threshold, 0.85 mg/liter; coefficient of variation, 2.7 to 3.1%). Vancomycin assays were performed at the time of sacrifice (steady state) by the same method with EMIT reagents (detection threshold, 2.5 mg/liter; coefficient of variation, 4.1 to 6.9%).

The main judgment criterion was the number of surviving bacteria in vegetations (expressed in log_{10} CFU per gram). The efficacy of the different therapeutic regimens for each strain was first determined by analysis of variance (ANOVA). Pairwise comparison by Scheffe’s test was then performed when ANOVA showed a significant difference (Statview; Abacus Concepts, Berkeley, Calif.). For the group infected with K-TSA, a Fisher exact test allowed comparison of the rates of surviving animals.

The results of the disk diffusion tests and the MICs for the four strains are shown in Table 1. All four strains were susceptible to amikacin and netilmicin. Table 2 shows the results of experimental endocarditis due to the four strains expressed as the mean log_{10} CFU per gram of vegetation ± the standard deviation. In groups treated with amikacin, a significant decrease in the bacterial count in vegetations, compared to control animals, was observed only for S-SA strains sensitive to all antibiotics, i.e., those not producing an enzyme. In groups treated with gentamicin, the decrease compared to the controls was significant for all of the strains except KTG-SA (which produced the bifunctional enzyme). No treatment was effective against this strain. Amikacin and gentamicin were active against S-SA, with the latter producing a significant difference. A comparison of the percentages of surviving animals infected with the K-TSA strain showed that all six of the animals treated with amikacin had died versus only 25% (two of eight) of the gentamicin-treated group ($P = 0.0009$; Fisher exact test).

The results of antibiotic assays for the peak and trough concentrations of aminoglycosides and the steady-state concentration of vancomycin confirm that the objectives set were reached. The peak and trough concentrations in serum, respectively, were as follows: amikacin, 49.5 ± 1.6 and 1.2 ± 0.5 mg/liter; gentamicin, 15.8 ± 1.2 and 1.2 ± 0.2 mg/liter; netilmicin, 45.7 ± 4.3 and 0.3 ± 0.1 mg/liter. The steady state concentration of vancomycin in serum was 22.3 ± 8.7 mg/liter. Under our experimental conditions, the apparent aminoglycoside elimination half-times and concentration peaks were close to those observed in humans (4).

Studies on the resistance of S. aureus to aminoglycosides have defined the enzymatic mechanisms involved and characterized the enzymes in terms of molecular structure, substrate spectrum, and epidemiology (3, 8, 11, 15, 19, 20). The presence of enzymes has been correlated with different resistance phenotypes defined by MICs (13). However, the impact of the enzymatic modification of aminoglycosides on their killing effect both in vitro and in vivo has rarely been investigated, particularly for amikacin and netilmicin. A reduction of the bactericidal activity of these antibiotics in vitro in the presence of an enzyme and a loss of synergy during associations with vancomycin or oxacillin have been reported, including the strains used in this study (2; R. Bismuth, F. Vermee, H. Drugeon, and P. Courvalin, 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1331, 1988). These two studies used the time-kill curve method to evaluate the early bactericidal effect of aminoglycosides on S. aureus. They indicated that the killing effect of amikacin and netilmicin is reduced in the presence of enzymes that modify aminoglycosides but dissociated from the bacteriostatic activity shown in disk diffusion tests. This dissociation led these authors to propose an interpretive reading of the tests (1, 2; Bismuth et al., 29th ICAAC). They suggested that enzyme-induced modifications compromise the susceptibility of the strains to the antibiotics of interest.

### Table 1. Results of in vitro tests for each of the four strains studied

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diam (mm), MIC (mg/liter), susceptibility*</th>
<th>Vancomycin MIC (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-SA</td>
<td>26, 1, S 26, 0.5, S 27, 0.5, S 32, 0.125, S 1</td>
<td></td>
</tr>
<tr>
<td>K-SA</td>
<td>24, 1, S 25, 0.25, S 26, 0.25, S 30, 0.064, S 0.5</td>
<td></td>
</tr>
<tr>
<td>KT-SA</td>
<td>23, 8, S 6, 16, R 22, 0.5, S 26, 0.125, S 0.5</td>
<td></td>
</tr>
<tr>
<td>KTG-SA</td>
<td>21, 8, S 6, 64, R 6, 64, R 25, 0.5, S 1</td>
<td></td>
</tr>
</tbody>
</table>

* S, susceptible; R, resistant.

### Table 2. Results obtained in vivo after 48-h treatment of the four strains studied

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Mean log CFU/g of vegetation ± SD (no. of rabbits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-SA</td>
<td>K-SA</td>
</tr>
<tr>
<td>Control</td>
<td>9.2 ± 0.5 (7)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2.4 ± 0.4 (5)*</td>
</tr>
<tr>
<td>Amikacin</td>
<td>5.5 ± 0.4 (5)*</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>ND</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>3.3 ± 1.5 (5)*</td>
</tr>
<tr>
<td>Vancomycin + amikacin</td>
<td>3.0 ± 0.9 (7)*</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>3.1 ± 0.8 (6)*</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4.7 ± 1.9 (7)*</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>9.1 ± 0.6 (3)*</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>8.4 ± 0.9 (3)*</td>
</tr>
</tbody>
</table>

* $P < 0.0001$ versus control and amikacin (Sheffe’s test after ANOVA).
* $P < 0.0001$ versus control and gentamicin.
* $P = 0.0009$ versus control and $P = 0.0004$ versus amikacin.
* $P = 0.03$ versus control.
* No difference between vancomycin alone and each combination.
* No difference between gentamicin alone and each combination.
* $P < 0.05$ versus control.
* ND, not done.
* NI, not interpretable (all of the animals in this group died).
therapeutic effect during clinical applications, even though studies indicate that bacteria are just as sensitive to the antibiotic. Our in vivo results obtained with amikacin and netilmicin, which confirm that the loss of bactericidal activity in vitro corresponds to that in vivo, tend to validate this line of reasoning. The loss of kanamycin activity observed in disk diffusion tests was predictive of a loss of amikacin efficacy in vivo, despite the apparent susceptibility of bacteria to this aminoglycoside. The absence of gentamicin activity in disk diffusion tests was predictive of resistance to gentamicin, amikacin, and netilmicin.

In the present study, the human pharmacokinetics of aminoglycosides was simulated in the rabbit (18). Thus, the results obtained are more predictive of the efficacy of treatments used in severe septicemic S. aureus infection. Based on the results of this study, a clinical indication of amikacin in the context of an acute infection with S. aureus cannot be maintained, despite the apparent sensitivity shown in disk diffusion tests. Conversely, gentamicin can provide a real therapeutic benefit against strains with a K' or KT' phenotype. Moreover, the greater in vivo efficacy of gentamicin against the strain with a sensitive phenotype seems to confirm that this aminoglycoside is preferable for the treatment of staphylococcal infections. To our knowledge, this is the first in vivo evidence of the validity of the interpretation proposed by Bismuth et al. (1; Bismuth et al., 29th ICAAC). However, the resistance of strains with a KTG' phenotype to gentamicin precludes the use of an aminoglycoside against infections with these strains.

REFERENCES
