First-Exposure Adaptive Resistance to Aminoglycoside Antibiotics In Vivo with Meaning for Optimal Clinical Use

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Received 6 June 1990/Accepted 12 October 1990

The first exposure of gram-negative bacilli to an aminoglycoside antibiotic in vitro induces a biphasic bactericidal response and adaptive drug resistance (G. L. Daikos, G. G. Jackson, V. T. Lolans, and D. M. Livermore, J. Infect. Dis. 162:414–420, 1990; G. G. Jackson, G. L. Daikos, and V. T. Lolans, J. Infect. Dis. 162:408–413, 1990). The therapeutic implications were examined in netilmicin treatment of a Pseudomonas aeruginosa infection of normal and neutropenic mice. For 2 h after the first dose, the bactericidal rates were rapid, 0.75, 1.0, and 1.5 log10 CFU/h with doses of 10, 30, and 60 mg/kg, respectively. Each twofold increase in dosage reduced the number of surviving bacteria fivefold. Between 2 and 6 h, the second-phase bactericidal rate was slow, i.e., 0.3 log10 CFU/h, regardless of the dose. In a multiple-dose regimen, the same amount of netilmicin given in one dose was 70% and 90% more effective than two or three doses, respectively. Doses calculated to keep the drug level in plasma above the minimal inhibitory concentration were less effective than regimens giving first exposure to a high drug concentration. Adaptive resistance occurred when doses were given more than 2 h after the start of treatment. Temporary survival of bacteremic neutropenic mice was 60 to 70% greater with a second dose at 2 h than after a longer interval. In a thigh infection of neutropenic mice treated every 2 h, doses 4, 6, and 8 h after the first one showed no bactericidal effect. A drug-free interval of 8 h (20 times the drug half-life) renewed bacterial susceptibility to drug action. The results in vivo confirm the biphasic bactericidal action and induction of adaptive resistance that characterize first exposure of gram-negative bacilli to aminoglycoside antibiotics. The phenomena have meaning for the optimum clinical use of aminoglycosides.

The rate of the bactericidal action of an aminoglycoside antibiotic on Pseudomonas aeruginosa and other aerobic gram-negative bacilli has been observed to be biphasic in vitro (23). An initial phase of rapid bacterial killing is induced by passive ionic binding of the drug to bacterial lipopolysaccharide (16, 23, 24, 30). The killing rate is directly related to the initial drug concentration. A second phase of slower bacterial killing is associated with decreased energy-dependent uptake of the aminoglycoside, and the rate is independent of the initial or persistent drug level (6, 14, 23, 28). Bacteria surviving the first exposure develop adaptive resistance, which is mediated by impermeability to all aminoglycosides (7, 22b). Adaptive resistance is unstable and is reversed during growth in drug-free media.

If the same interactions between aminoglycosides and bacteria apply in the treatment of bacterial infections, the data are relevant to selection of the most effective dosing regimen for therapeutic use of these antibiotics. The investigations reported here were designed to determine whether the biphasic bactericidal action of aminoglycoside antibiotics observed in vitro also applies in the treatment of an experimental infection and whether the first-exposure effect includes the development of adaptive resistance.

MATERIALS AND METHODS

Experimental infection. Infection was produced in the thighs of normal or neutropenic female ICR mice weighing 23 to 28 g (Sprague Dawley Laboratory, Madison, Wis.) by injection of 0.1 ml of broth containing approximately 107 CFU of bacteria in the log phase of growth per ml. Experiments were performed with three strains of P. aeruginosa: the laboratory strain, ATCC 27853, and two clinical isolates, PA-D1 and PA-D13. The MIC and MBC of netilmicin were 0.5 and 2.0 μg/ml, respectively, for all three strains. The number of viable organisms was measured at specified times by skinning the thigh and excising the soft tissues from the bone of animals killed by anesthesia. The muscles were homogenized with 1 ml of sterile 0.85% saline with a hand-operated ground-glass tissue homogenizer. The homogenate was kept at 4°C while quantitative cultures were done. Serial dilutions of the homogenate were cultured on Mueller-Hinton agar (MHA) plates, and the CFU were counted after overnight incubation at 37°C.

Neutropenia was produced in mice by intraperitoneal injection of 150 and 100 mg of cyclophosphamide per kg 3 and 1 days, respectively, before the experiment. On the days of the experiments, 16 uninfected mice were exsanguinated to confirm the neutropenia. A fatal bacteremic infection was initiated in groups of neutropenic mice by intraperitoneal injection of 0.1 ml of broth containing approximately 106 CFU of bacteria. The survival record of the animals and the treatment schedule included observations after 2, 4, 6, 8, 12, and 24 h.

Antibiotic treatment. Netilmicin was made up fresh from a powder standard in a stock solution of 15 mg/ml. For use in treatment, dilutions were prepared from this stock solution in which the prescribed dose was present in 0.1 ml. Antibiotic treatment was given by intraperitoneal injection of the dose at specified times. The level in plasma was determined by using an agar-well diffusion microbiologic assay. Blood was pooled from three to five uninfected mice exsanguinated 15, 60, and 120 min after the administration of drug. As therapy, the first dose was given after initiation of the

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infection in the mouse thigh. Retreatment of different groups of mice was varied between only the initial dose and a dose every 2 h for five doses. The total amount of drug given per mouse varied between 10 and 130 mg/kg. The statistical significance of the variance among groups was determined by the t test, and the differences in survival were calculated by chi square.

RESULTS

Levels of netilmicin in serum during the 2 h following a dose of 15, 30, or 60 mg/kg are shown in Fig. 1. The level 15 min after the 60-mg/kg dose was 50 μg/ml. In the first 2 h, the drug level declined to 3 μg/ml, indicating a half-life (t1/2) for mice of ≈25 min. Doses of 30 and 15 mg/kg produced proportionately lower levels in serum. All doses produced levels in plasma above the MIC for the test strain for at least 2 h.

The bactericidal effect of three regimens that delivered the same total dose of netilmicin with one, two, or six doses was used to evaluate the relative effects of a high peak level and a continuous inhibitory concentration in groups of mice infected with one of two different strains of P. aeruginosa. An initial dose of 60, 30, or 10 mg/kg was given immediately after infection and was followed by no, one, or five doses of the respective amount at intervals of 3 h or hourly from 0 to 5 h. Results of the treatment regimens after 2 and 6 h are shown in Fig. 2. In untreated mice, the number of CFU in the thigh muscles 6 h after infection with 3 × 10⁶ organisms of strain PA-D13 increased five times over the input number; an inoculum of 6 × 10⁶ organisms of strain ATCC 27853 increased 1.6 times. The resultant level of the untreated infection was 7.2 and 7.0 log₁₀ CFU per mouse thigh, respectively, for the two strains.

The bactericidal effect of netilmicin in any treatment regimen was biphasic. During the first 2 h, the largest dose, 60 mg/kg, with the highest first-exposure drug level, killed the inoculum at a rate of 1.5 log₁₀ CFU/h. With a dose one-half as large (30 mg/kg), the bactericidal rate was one-third lower (1.0 log₁₀ CFU/h) and the number of surviving CFU was five times greater. One-third the dose, 20 mg/kg, given in two doses at 0 and 1 h had a bactericidal rate one-half as fast, 0.75 log₁₀ CFU/h, and more than 25 times the number of surviving CFU. The results were comparable with the two strains.

Between 2 and 6 h of treatment, the bactericidal rates were reduced and similar for the different dosage regimens, as shown by the parallel slope of the lines in Fig. 2. In animals infected with strain PA-D13, the second-phase bactericidal rate was 0.3 log₁₀ CFU/h regardless of the dosage regimen. For strain ATCC 27853, the second-phase bactericidal rates were 0.1, 0.16, and 0.31 log₁₀ CFU/h with regimens of one, two, and six doses. The net bactericidal effect after 6 h among mice given the same cumulative dose of netilmicin on different schedules was significantly different. The single dose with a high initial level reduced surviving CFU 6 and 12 times more than the same amount of drug given in two or six doses, respectively. Two cycles of treatment with an intermediate-size dose reduced surviving CFU more effectively than six doses that were calculated to keep the drug level above the MIC. Thus, the height of the level and not the pressure of a constant MIC was the most bactericidal regimen. The results with the two strains were comparable, and the differences were statistically significant.

In a similar experiment, but with extension of the treatment period to 12 h, three groups of mice were treated with a single dose of 30 mg/kg of netilmicin per kg given as one, two, or three doses at 0, 0, and 6 or at 0, 6, and 12 h after initiation of infection. The numbers of surviving bacteria with each regimen were compared after 6 h (one dose on each regimen) and 24 h when the amount of drug received was the same (Table 1). The kinetics of the bactericidal action were the same: early killing at a faster rate, 1.4 to 0.8 log₁₀ CFU/h, was directly related to the size of the first dose. After 6 h, a dose of 30 mg/kg was 80% (five times fewer CFU) and 87% (eight times fewer CFU) more effective in reducing the number of surviving bacteria than smaller first doses of 15 mg (one-half) and 10 mg (one-third), respectively.

In the second phase, from 6 to 24 h, the rates of decline in viable bacteria were slower and comparable for each of the treatment schedules. The net CFU in the latter period included the postantibiotic effect and ability of normal mice to contain the treated local infection. After 24 h, when all animals had received the same cumulative dose of netilmicin in 12 h, first exposure to a high drug level from a single dose reduced surviving CFU by 50 to 80% and 63 to 90% more than regimens that delivered the drug in two or three divided doses, respectively. The differences are statistically significant.

The 6-h dosing interval used was approximately every 15th half-life of the drug in mice. From the observed levels in serum in Fig. 1, the aggregate times that the drug level in serum was above the MIC for the infecting strain were approximately 3, 4, and 5 h for the one-, two-, and three-dose regimens, respectively. Thus, the time that the level in serum was above the MIC was inversely rather than directly related to the final bactericidal effect, and the concentration of the drug upon first exposure correlated directly with bacterial reduction.

The bactericidal effects of the first and second doses were compared directly in the treatment of a pseudomonal thigh infection with strain ATCC 27853 (Fig. 3). Treatment was initiated with a first dose of either 15, 30, or 60 mg/kg; 2 h later, the bactericidal rates were observed to be 1.0 ± 0.27 (standard deviation), 1.5 ± 0.2, and 1.9 ± 0.3 log₁₀ CFU/h, respectively. Mice initially given the lowest dose, 15 mg/kg, were reassigned to one of three new subgroups and retreated with a second dose of either 15, 30, or 60 mg/kg. Two hours
later, the CFU per thigh were again enumerated. The bactericidal rates during 2 h after the second dose were 0.9, 1.0, and 0.9 \( \log_{10} \) CFU/h for the 15-, 30-, and 60-mg/kg doses, respectively. Thus, the bactericidal rate following the second dose was the same and was not determined by the size of the dose. The inset in Fig. 3 compares the kinetics of similar observations made in vitro with another strain of \( P. \) aeruginosa (23).

To further evaluate the importance of the first-exposure effect noted above and the in vivo expression of adaptive resistance, mice were made neutropenic to remove that aspect of host immunity. On the day of the experiments, the leukocyte count of 16 leukopenic mice was 700 ± 140/mm\(^3\). No more than two granulocytes were found on each blood smear. In eight normal mice, the leukocyte count was 3,400 ± 850/mm\(^3\), and granulocytes predominated in the blood film.

The bactericidal effects of a single dose and different schedules of repetitive dosing with evaluation over an extended treatment period of 12 h were compared in the mouse

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**TABLE 1.** Effect of dosing regimen of netilmicin in reducing the number of surviving bacteria during \( P. \) aeruginosa infection in neutropenic mice

<table>
<thead>
<tr>
<th>Treatment regimen (total given)</th>
<th>Strain PA-D13</th>
<th>Strain ATCC 27853</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mice</td>
<td>( \log_{10} ) CFU/thigh ± SD</td>
</tr>
<tr>
<td>No treatment</td>
<td>10</td>
<td>6.8 ± 0.10</td>
</tr>
<tr>
<td>(a) 10 mg/kg every 6 h (30 mg/kg)</td>
<td>7</td>
<td>4.9 ± 0.30</td>
</tr>
<tr>
<td>(b) 15 mg/kg every 3 h (30 mg/kg)</td>
<td>10</td>
<td>4.7 ± 0.40</td>
</tr>
<tr>
<td>(c) 30 mg/kg at 0 h</td>
<td>10</td>
<td>4.0 ± 0.40</td>
</tr>
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</table>

\(^a\) Significance: a versus c, \( P = 0.01 \) at 6 and 24 h for PA-D13, \( P = 0.05 \) at 6 and 24 h for ATCC 27853; b versus c, \( P = 0.01 \) at 6 h and 0.05 at 24 h for strain PA-D13, not significant at either time for ATCC 27853.
FIG. 3. In vivo bactericidal rate in a *P. aeruginosa* infection of the mouse thigh following different doses of netilmicin given as the first and second dose. The results are plotted as the hourly log number of bacteria killed against the log of the dose of drug given (15, 30, or 60 mg/kg). The lines show the mean and the brackets show the standard deviation for groups of five mice for each point. The coefficient of correlation between the kill rate and the size of the first dose is \( r = 0.98 \). The correlation for the second dose is \( r = -0.12 \). (Inset) Similar plot of the kill rate during the first hour and 2 to 6 h after exposure of a culture to netilmicin in vitro (23). The abscissa in the inset is stated as drug concentration per log CFU. In the in vivo study, all of the mice in comparable groups received the same size bacterial inoculum, and therefore the unit for the two is the same.

Thigh infection of neutropenic mice (Fig. 4). Infection with *P. aeruginosa* was initiated 2 h before treatment was begun. All except the control group of mice were initially treated with 30 mg of netilmicin per kg. Different groups of mice were then given either no additional treatment, one additional dose of 20 mg/kg after 2, 4, 6, or 8 h, or repeated doses of 20 mg/kg every 2 h for five doses. The total amounts of netilmicin given on the two-dose regimen were 1.67 times and on the multiple-dose regimen 4.33 times as much drug as in a single dose. The bactericidal effect of each dose was assessed after 2 h by comparing the surviving CFU in five treated animals and their respective controls.

The first dose reduced CFU by 0.6 log\(_{10}\) CFU per mouse thigh. Without further treatment, bacterial growth proceeded at a rate of 0.15 log\(_{10}\) CFU/h, which was comparable to growth in the untreated control but starting with a lower inoculum. A return to the initial level of infection was reached 5 to 6 h after the single-dose treatment. In one subset of mice, a second dose given 2 h after the first one (before the development of adaptive resistance in vitro [7]) further reduced the number of CFU by 0.5 log\(_{10}\) CFU over the next 4 h (the second-phase bactericidal rate was 0.2 log\(_{10}\) CFU/h for 2 h after the dose). Sequential third, fourth, and fifth doses at 4, 6, and 8 h had no detectable antibacterial effect. Six hours after first drug exposure, an increase in the level of infection began which occurred in spite of treatment calculated to give a constant level in plasma above the MIC. The bacterial growth rate was parallel to that in mice given only the initial dose or no drug, each starting with a lower number of viable organisms. Among animals stratified to receive a second dose after intervals of 4 or 6 h, neither dose had a bactericidal effect, and in fact each was followed by a slight increase in the number of bacteria.

The unresponsiveness to doses given between 4 and 8 h as either interval or serial doses was reversed after an 8-h interval of no intervening treatment (when unstable adaptive resistance was observed to be reversed in vitro [7]). A second dose at that time reduced the high level of infection that had been achieved by 0.4 log\(_{10}\) CFU during 2 h following the dose. This was two-thirds the dose and two-thirds the
rate observed after the first dose. In absolute number of bacteria killed, the percents reduction were the same for the first and second dose given after 8 h. A simultaneous 8-h dose given as the fifth serial dose to mice treated every 2 h had no bactericidal effect and caused no slowing of bacterial growth. Twelve hours after the initiation of infection, the single initial dose had appreciably reduced the level of infection from that observed in the untreated control mice. Only regimens in which doses were given at 0 and 2 h or 0 and 8 h were followed by a further reduction in viable bacteria and were the only regimens that restrained the infection at or below the initial level. Adaptive resistance to the bactericidal effect of treatment was present for all doses given between 4 and 8 h after the first drug exposure.

The effect of the timing of doses was further investigated with a two-dose regimen given as treatment for a fatal bacteremic pseudomonal infection in neutropenic mice. Mice in four subgroups were all given an initial dose of 60 mg/kg. A second dose of the same size was given either 2, 4, 6, or 8 h later. The numbers of animals in each group surviving the overwhelming bacteremia for 12 h, that is, 4 to 12 h after the last dose, are given in Table 2. Two sequential doses within the prerefractory period (0 and 2 h) prolonged the lives of all of the mice in the group and did so for the longest time after the last dose. When the second dose was given 4, 6, or 8 h after the first one, survival after 12 h was reduced 60 to 70%. The later doses had insignificant benefit over the single dose alone, and this result is compatible with the development of adaptive resistance. Without further treatment, none of the neutropenic mice survived the infection for 24 h.

**DISCUSSION**

Treatment of an infection with *P. aeruginosa* in the thigh muscle of mice with netilmicin reproduced with striking similarity the observations in vitro showing biphasic bactericidal kinetics of aminoglycoside antibiotics and the development of unstable adaptive resistance as a consequence of the first drug exposure (7, 16, 23). The inductive role of ionic

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**TABLE 2.** Effect of in vivo induction of first-exposure downregulation of netilmicin uptake on survival with two-dose therapy (60 mg/kg) during *P. aeruginosa* infection in neutropenic mice.

<table>
<thead>
<tr>
<th>Interval to 2nd dose (h)</th>
<th>Total drug (mg/kg)</th>
<th>No. of 10 mice surviving at 12 h (% survival)</th>
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<tbody>
<tr>
<td>2</td>
<td>120</td>
<td>10 (100)</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>4 (40)</td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>4 (40)</td>
</tr>
<tr>
<td>8</td>
<td>120</td>
<td>3 (30)</td>
</tr>
<tr>
<td>0</td>
<td>60</td>
<td>2 (20)</td>
</tr>
</tbody>
</table>

* Chi square (Yates) = 8.8, df 3; *P* < 0.05.
binding initiating the early phase of rapid bactericidal action is passive and entirely dependent on the drug concentration. The accelerated unidirectional bacterial transport of the bound drug was shown by the direct relationship between dose and the high rate of bactericidal action immediately after the first dose. The second phase of antibacterial action, in which drug uptake is rate limited, independent of the dose, and has a slow fixed bactericidal rate, was also observed. Together they represent the unique first-exposure effect of aminoglycosides on gram-negative bacteria (16, 23).

Adaptive resistance appeared before 4 h after the first dose. It was revealed by the failure of doses between 4 and 8 h after the first one to produce any appreciable bactericidal action while the number of bacteria was increasing. That the resistance is adaptive and not the result of selection of resistant mutants is shown by the requirement for an adaptive period for development of drug refractoriness and reversal to susceptibility after an interval without drug exposure. Further evidence from other studies has shown adaptive resistance to be inducible by either one-fourth or four times the MIC with the same kinetics and that the effect induced by a single member of the class produced uniform adaptive cross-over impermeability resistance to other aminoglycosides (7, 22b). None of these effects suggest that the observations are the result of drug-selected resistant mutants. The mechanism of adaptive resistance was shown to be mediated by failure to transport drug into the bacterial cell (7). This ability of aminoglycosides to alter the bacterial processes necessary for their own uptake has been observed in various ways (1, 13, 14, 22a, 24).

Netilmicin and P. aeruginosa were chosen for investigation, but other gram-negative bacilli and aminoglycosides interact in a similar fashion. However, the evolution of the phase, completeness of the refractory state, and its reversal may vary among different species and requires additional investigation. Biphasic bactericidal kinetics and unstable adaptive resistance were demonstrated by Kapuscic and coworkers treating experimental pneumonia in nonneutropenic guinea pigs with tobramycin (18). Similar results may be observed in the results of other investigators using different experimental models of infection (4, 9–12, 18, 19, 21, 31).

The unique drug-reactive phenomena, including the development of adaptive resistance, give importance to the observations in developing optimum therapeutic regimens for clinical use of aminoglycoside antibiotics. It has been suggested in other reports that the area under the time-concentration curve (AUC) is a reliable pharmacokinetic determinant for aminoglycoside efficacy (22, 35). Although this correlation can be verified by the present work, its rationalization lies in the effect of the height of the initial drug level and its reflection in the AUC; and the correlation with AUC applies only to the first and not serial doses if the interval is short. The proportional amount of time that the drug level in plasma was above the MIC for the infecting strain was unimportant. This irrationality in comparison with beta-lactam antibiotics, for which maintenance of an inhibitory drug level is the major pharmacokinetic determinant of efficacy, can be explained by the differences in site and mechanisms of action; the self-promoted internalization of bound aminoglycoside, the concentration-independent second phase of aminoglycoside action, and adaptive resistance are all involved.

Some clinical experience supports the importance of the first dose and the height of the peak plasma drug levels achieved (25–27, 29). In the treatment of experimental infection in mice, a second dose during the prerefractory period also increased the antibacterial effect of the first dose. In vitro and in neutropenic mice, evidence of increasing drug refractoriness is apparent within 3 to 4 h after the first dose. Both in vitro and in vivo experience indicates that after this time, the continued presence of drug in the medium increases and prolongs adaptive resistance. The common use of low-dose, short-interval administration of aminoglycosides and the aim of maintaining the level above the MIC could both augment adaptive resistance and minimize the bactericidal potential (2, 5, 7, 17). Both effects work to the detriment of patients.

Reversal from adaptive resistance to full susceptibility, which was clearly demonstrable in vitro, was also observed in vivo. However, it is more difficult to show in vivo because of the intervening importance of host status. In some experiments, independent recovery of normal animals or failure to prevent death of neutropenic mice with bacteremia occurred before the time and bacterial growth required for observation of reemergence of susceptibility were fulfilled. Whether drug-free periods of adequate length to permit return of drug susceptibility can be recommended clinically must depend on the immunologic competence of the host and the antibacterial effectiveness of the high first dose and cyclic levels. Once-daily dosing approaches the principle and appears to be at least as effective as multiple daily dosing (20, 33, 34).

Toxicity has always been a major concern in the use of aminoglycoside antibiotics. Implications of the first-exposure effect and development of adaptive resistance suggest the uselessness of doses given during the unrecognized refractory period. These doses could double or quadruple the amount of drug, increasing toxicity without a parallel benefit from increased antibacterial activity. Increasingly, experience and opinion indicate that severe oto- and nephrotoxicities are primarily caused by duration of treatment with a persistent drug level and not by brief periods of high drug levels (3, 8, 31, 32, 36). Transient levels of amikacin and tobramycin higher than 300 and 40 μg/ml, respectively, in patients caused no evidence of neuromuscular blockade, nephrotoxicity, or ototoxicity (15, 31). The margin with netilmicin is greater. Ensuring an adequate initial dose to obtain the early bactericidal effect is critical, and avoiding repeated doses in the period required for complete renal clearance of the drug before the next dose would be wise. Acceptance of such a regimen could accomplish the twin goals of avoiding severe toxicity and minimizing adaptive resistance, leading to a more favorable toxicity-effficacy ratio. Furthermore, the practice would permit dosing on the basis of known pharmacokinetics and renal function, with elimination of the expense of serial monitoring of the drug level. Thus, not only the toxicity-effficacy but also the cost-benefit ratio could be improved.

The experimental data presented give a rationale for high-dosage, long-interval administration of aminoglycoside antibiotics. Understanding the unique bactericidal kinetics related to the first-exposure effect of aminoglycoside antibiotics, including adaptive resistance, should improve opportunities to use them more effectively and safely in the treatment of severe infections caused by the common aerobic gram-negative bacilli.

ACKNOWLEDGMENTS

The technical assistance of Sue Lyza and Ria Chiu in the conduct of the animal experiments is gratefully acknowledged.
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