Relationship Between Gentamicin Susceptibility Criteria and Therapeutic Serum Levels for *Pseudomonas aeruginosa* in Mouse Infection Model

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In this study estimations of in vivo and in vitro gentamicin susceptibility for a series of strains of *Pseudomonas aeruginosa* were compared. The series included an extremely susceptible strain, typically susceptible strains by current susceptibility criteria, and strains with enzymatic and permeability-mediated resistance. In vivo testing was done by using a mice protection test involving six 1-h doses of gentamicin and an inoculum of 50 50% lethal doses of *P. aeruginosa*. Both normal mice and cyclophosphamide-treated mice were used. It was found that peak serum levels and serum levels of gentamicin obtained just prior to the sixth dose (fifth dose trough levels) required for protection were much higher than minimal inhibitory concentrations (MICs) or minimal bactericidal concentrations (MBCs) obtained in high-cation medium. However, first dose trough levels were similar to MICs or MBCs. Only an extremely susceptible strain, 280, could be treated at antibiotic dosages and serum levels which are considered likely to be safe in humans. A distinct inoculum effect was found in the mice tests, with a 10-fold increase in inoculum producing a 4-fold increase in the amount of gentamicin required, but no inoculum effect was found for MICs. These results suggest that current susceptibility criteria in use for gentamicin and *P. aeruginosa* overestimate gentamicin susceptibility, particularly when low-cation growth medium is used for susceptibility testing and when treating disseminated infection.

Gentamicin, either alone or in combination with carbenicillin, is currently considered to be one of the most useful antibiotics for treatment of infection by *Pseudomonas aeruginosa*. Despite many years of clinical experience, there is still considerable doubt about the efficacy of gentamicin in the treatment of *Pseudomonas* sepsis. Poor survival rates reported in several studies of *Pseudomonas* bacteremia suggest that gentamicin is not highly effective in disseminated infections (8, 14, 16), yet most strains of *P. aeruginosa* are considered susceptible by current laboratory in vitro susceptibility testing results.

In the present study we examined the relationship of gentamicin susceptibility to in vivo efficacy of gentamicin in mouse models of disseminated infection by using a series of isogenic *P. aeruginosa* strains with different R-factors and a nonisogenic series varying in gentamicin susceptibility and virulence. Currently if strains have minimal inhibitory concentrations (MICs) of ≤ 6 they are regarded as susceptible (17). We have included infection of cyclophosphamide-treated mice as a model of infection of hosts

with compromised host defenses. Because the apparent susceptibility of *P. aeruginosa* to aminoglycosides is very highly influenced by media conditions (9, 10, 20, 26), we examined several media in an attempt to determine which one gives an evaluation of susceptibility that is most predictive of in vivo results.

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MATERIALS AND METHODS

Bacteria. Six well characterized strains of *P. aeruginosa* were obtained from the culture collection of L. E. Bryan. Strain PA103 was provided by S. D. Davis (Medical College of Wisconsin, Milwaukee, Wis.). Strain characteristics are shown in Table 1. Three of the strains are derivatives of strain 280 that carry R-factors producing a set of strains varying mainly in gentamicin susceptibility.

Mice. Locally bred male ICR mice weighing 25 to 31 g were used.

Antibiotics. Gentamicin sulfate injectable (40 mg base/ml) from Schering Corp. was used.

MIC. MICs on solid media were determined with a

TABLE 1. *Properties of P. aeruginosa strains used in mouse protection tests*

Strain	Characteristics used for identification on recovery from animals ^a	Other characteristics	Source or reference
280	Stable brown pigment, requires methionine, extremely susceptible to all aminoglycosides		(22)
280(RUA7)	Stable brown pigment, rifampin resistance (Rif ^r), R-factor-specified resistance to Cb, Sm, Tc	2''-O-adenylylation of gentamicin	(22)
280(R130)	Stable brown pigment, rifampin resistance (Rif ^r), R-factor-specified resistance to Sm, Su, Hg	3-N-acetylation of gentamicin	(22)
280(R151)	Stable brown pigment, rifampin resistance (Rif ^r), R-factor-specified resistance to Cb, Cm, Su	2''-O-adenylylation of gentamicin	(22)
PA-103	Pyocine type 10	Exceptional virulence for mice	S. Davis, Medical College of Wisconsin
10804	Pyocine type 1h		Burn isolate (6)
1136	Pyocine type 16, resistant to Gm, Tm, Km, Sm, sisomicin, amikacin, neomycin	Generalized impairment of permeability to aminoglycosides	Clinical isolate (4)

^a Abbreviations: Cb, carbenicillin; Tc, tetracycline; Sm, streptomycin; Su, sulfonamide; Hg, mercury; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Tm, tobramycin.

Steers replicator (23) by using 10^5 bacteria per spot. MICs in broth were determined by using an inoculum of 10^5 bacteria in 1 ml of broth in 16 by 125-mm tubes. End points were determined visually after 24 h of incubation at 37°C. Inocula were prepared by growing strains in the same medium used for MIC determinations or in the corresponding broth in the case of solid media. Calcium and magnesium levels of commercial media were determined at the University of Alberta Hospital by an atomic absorption method. Mueller-Hinton medium was adjusted to physiological levels of calcium and magnesium (see Table 2). Minimal medium used was a low-phosphate, tris(hydroxymethyl)aminomethane-buffered medium previously described (4).

Virulence testing. Virulence was established by injecting mice intraperitoneally (i.p.) with 0.2 ml of serial 10-fold dilutions of bacterial suspensions. Inocula were prepared by growing cultures in BBL brain heart infusion (BHI) broth at 37°C with shaking to an optical density at 600 nm of 0.5 unit, collecting cells by centrifugation, and resuspending them in BHI. Deaths were counted after 48 h as preliminary experiments demonstrated no further deaths after this time. Virulence was expressed as 50% lethal dose (LD₅₀) determined by the method of Reed and Muench (19). Each virulence determination used 20 to 25 mice, with 5 mice for each dilution of bacteria. Virulence determinations were replicated three or more times, and the results were pooled for determination of the LD₅₀. Reproducibility between repetitions was within a factor of $10^{0.1}$.

Cyclophosphamide-treated mice. Cyclophosphamide treatment of mice was used to reduce their resistance to infection with the various *P. aeruginosa* strains. The method was that described by Pierson et al. (18), using a single i.p. dose of 300 mg of cyclophosphamide per kg. Total white blood cell counts were 15 to 25% of normal at 4 days after treatment and re-

mained in this range for a further 48 h before elevating. Neutrophil counts were less than 50% of initial values for this same time period. Virulence and protection tests on cyclophosphamide-treated animals were started 4 days after cyclophosphamide treatment, and experiments were terminated in a further 48 h. No deaths were seen in 20 control animals injected with 300 mg of cyclophosphamide per kg.

Mouse protection tests. Mice were injected i.p. with 50 LD₅₀ of the strain in question and treated by subcutaneous injections of serial twofold dilutions of gentamicin, covering the range of 2.5 to 80 mg/kg, beginning 1 h after infection. Deaths were counted at 48 h and the results were analyzed by the Spearman-Kärber method (7). Each 50% protective dose (PD₅₀) determination used 30 mice with 5 mice at each dose level. PD₅₀ determinations were repeated three or more times, and the data pooled for the PD₅₀ were reported. Reproducibility between repetitions was within a factor of $10^{0.2}$. The virulence of the inoculum was checked with each test by inoculating groups of mice with 50 LD₅₀ and 0.5 LD₅₀. Experiments were discarded in which inocula of 50 LD₅₀ were not uniformly lethal or inocula of 0.5 LD₅₀ caused deaths. Bacteria were recovered from the heart tissue of animals dying from infection and were used to confirm the identity of the infecting strain (Table 1).

Serum gentamicin levels. Serum levels of gentamicin were determined by a radioenzymatic assay based on that described by Haas and Davies (12) modified by the use of the gentamicin 3-N-acetyl transferase from *Escherichia coli* JR89. Serum levels were measured at 5 or 10 min after gentamicin injection for peak serum levels and at 5- to 10-min intervals thereafter for up to 1 h to determine the rate of gentamicin elimination. Blood collected from three mice by tail bleeding was pooled for each measurement, and three to five measurements were averaged for each value reported. Measurements were repro-

ducible within $\pm 10\%$. Peak or trough serum levels after gentamicin injection were plotted against dosage, and this plot was used to estimate serum levels at the PD_{50} .

Bactericidal levels in broth and serum. Antibiotic dilutions were prepared in 0.1-ml volumes of serum, serum-broth (1:1, vol/vol), or broth and inoculated with 10^4 of the bacteria under test. After 24 h of incubation at 37°C , $10\ \mu\text{l}$ of each dilution was spread on Trypticase soy agar (BBL) and was incubated for 24 h at 37°C . Concentrations from which less than three bacterial colonies appeared were considered bactericidal.

RESULTS

In vitro susceptibility of strains to gentamicin. Properties of the strains included in this study are shown in Table 1. These strains exhibited a broad range of MICs of gentamicin regardless of which growth medium was used for susceptibility testing (Table 2). MICs for the strain 280 series, when tested with high-cation media, ranged from 0.125 to $8\ \mu\text{g}/\text{ml}$. Strains 280 and 280(RUA7) in particular would be regarded as susceptible strains by current susceptibility criteria (16) as would the nonisogenic strains PA103 and 10804. Strain 1136 would be regarded as clearly resistant by these criteria. The remaining strains fall into the intermediate or indeterminate category. Thus, using a variety of testing media, these strains represent a series covering the spectrum of susceptibility as judged by standard laboratory susceptibility testing criteria currently being used for gentamicin. Minimal bactericidal concentrations (MBCs) were twofold higher than MICs (see Table 4).

Susceptibility testing in low-cation media gave MICs which were significantly lower than in high-cation medium (Table 2).

Virulence tests. Table 3 gives LD_{50} values

for the various strains of *P. aeruginosa* when given i.p. to normal and cyclophosphamide-treated mice. These studies were used to standardize the bacterial inoculum in an animal model of *P. aeruginosa* infection of normal hosts as well as hosts with impaired defense mechanisms.

The strains as shown in Table 3 varied in virulence as judged by LD_{50} . Strain PA103 is exceptionally virulent and has been recommended as a reference strain for mouse protection tests (6). The presence of R-factor R130 and RUA7 did not significantly affect the virulence of strain 280 in mice. These strains thus provide a set of strains of nearly identical virulence but varying in MIC of gentamicin over the range of clinical susceptibility. R-factor R151 enhanced virulence of strain 280, although it did not alter the virulence of another *P. aeruginosa* strain, ML4262.

Cyclophosphamide-treated mice were clearly more susceptible to infection with all strains tested (Table 3). These mice represent a model of infection with *P. aeruginosa* in which host defense has been compromised. Such circumstances are common in human infections with *P.*

TABLE 3. LD_{50} of *P. aeruginosa* strains for normal and cyclophosphamide-treated mice

Strain	LD_{50} (bacteria/inoculum)	
	Normal mice	Cyclophosphamide-treated
280	4.5×10^7	2.0×10^6
280(RUA7)	5.0×10^7	3.1×10^6
280(R130)	4.5×10^7	3.1×10^6
280(R151)	8.0×10^6	5.6×10^5
PA103	1.6×10^6	6.3×10^4
10804	5.6×10^7	1.5×10^6
1136	4.8×10^7	3.7×10^6

TABLE 2. MICs of gentamicin in various media^a

Strains	Low-cation media			High-cation media				BHI
	Minimal ^b		NB (Ca, 9; Mg, 4)	MHA (Ca, 75; Mg, 20)	MHB (Ca, 75; Mg, 20)	OxSa (Ca, 28; Mg, 4)	Minimal (Ca, 28; Mg, 20)	
	(Ca, 0; Mg, 4) ^c	(Ca, 15; Mg, 4)						
280	0.016	0.06	0.016	0.5	0.25	0.25	0.125	0.25
280(RUA7)	0.06	0.25	0.125	2	2	2	1	1
280(R130)	0.125	0.5	0.25	8	4	8	4	4
280(R151)	0.25	1	0.25	8	8	8	4	8
PA103	0.06	0.25	0.125	2	2	1	1	2
10804	0.125	0.5	0.25	4	4	2	2	2
1136	2	4	1	64	64	32	16	32

^a Abbreviations: NB, nutrient broth (BBL); MHA, Mueller-Hinton agar (BBL); MHB, Mueller-Hinton broth (BBL); OxSa, Oxoid isosensitest agar; BHI, brain heart infusion broth (BBL).

^b Minimal medium (4) varied only in calcium and magnesium concentrations (as CaCl_2 and MgCl_2).

^c Numbers in parentheses are concentrations of cations in milligrams per liter.

aeruginosa. In some instances the parallel is very close, since systemic *P. aeruginosa* infections develop in humans treated with cyclophosphamide (8).

Protection tests. The principal objective of this study was to determine if in vitro gentamicin susceptibility criteria that are in wide use in diagnostic laboratories effectively predict the therapeutic response of model animal *P. aeruginosa* infections to treatment with gentamicin.

Pharmacokinetics of gentamicin were examined so that the therapeutic approach taken in the mouse model would be similar to that taken in humans. The elimination half-life of gentamicin was determined in uninfected mice to be 18 to 23 min. Peak serum levels occurred within 10 min of gentamicin injection. From these results we concluded that hourly injections of gentamicin would produce a range of serum levels comparable to those obtained during treatment of human infections. Preliminary trials demonstrated that a schedule of six 1-h doses of gentamicin was as effective at preventing deaths as eight 1-h doses and more effective than a smaller number of doses. With repeated injections some accumulation of gentamicin did occur by the time of the sixth dose. The value of the serum level taken 10 min after the sixth dose was up to 50% higher than the value for the same time period for the first dose. Trough levels of gentamicin determined just prior to subsequent gentamicin injection underwent a gradual increase for doses of 5 mg/kg and above. Trough levels for various PD₅₀ doses are given in Table 4.

Table 4 presents data comparing MICs and MBCs determined by using high-cation broth

with PD₅₀ values for normal and cyclophosphamide-treated mice. Also shown are gentamicin levels after the first gentamicin dose and trough levels after the fifth dose. Values obtained for the initial peak or trough levels after the fifth dose significantly exceeded MIC values in almost all cases where protection could be achieved. MBCs were also significantly lower than peak serum levels. Initial dose trough levels were frequently similar to MICs or MBCs. However, it should be noted that, in the development of standard susceptibility criteria for bacteria, correlations are made between peak or median blood levels and MICs. The use of correlations between MBCs and trough levels is not widely done and is not the basis of current susceptibility criteria (2).

In cyclophosphamide-treated mice the PD₅₀ values were lower in all cases where successful therapy was possible. This illustrates the role of inoculum on the level of gentamicin required for animal protection. If the inoculum was raised for these mice by 10-fold, the PD₅₀ was significantly increased (Table 5). This is consistent with the inoculum effect described by Davis (5). In contrast, an increase in inoculum from 10⁵ to 10⁶ bacteria per ml did not alter MICs or MBCs determined in Mueller-Hinton broth (MHB).

The inclusion of three strains of nearly identical virulence [280, 280RUA7, 280(R130)] allowed us to examine the effect of variation in gentamicin susceptibility without the complication of variation in virulence. As expected, PD₅₀ values increased with decreased susceptibility. However, for strain 280(RUA7) with a MIC of 2 µg/ml and a MBC of 4 µg/ml, very high doses

TABLE 4. PD₅₀ values for normal and cyclophosphamide-treated mice challenged with 50 LD₅₀ of various strains of *P. aeruginosa*

Strains	MIC ^a (μg/ml)	MBC ^a (μg/ml)	Normal mice				Cyclophosphamide-treated mice			
			PD ₅₀ ^b (mg/kg)	Gentamicin serum level (μg/ml)			PD ₅₀ (mg/kg)	Gentamicin serum level (μg/ml)		
				Peak ^c	Trough ^d			Peak ^c	Trough ^d	
					1st	5th			1st	5th
280	0.25	0.5	9(7.9–10.2)	9	1.0	4.0	7(6.3–7.6)	7	1.0	2.5
280(RUA7)	2	4	37(27.9–50.6)	30	6	23	24(16.2–26.9)	24	5	8
280(130)	8	16	>80				>80			
280(R151)	8	16	33(26.9–46.0)	27	6	21	28(22.9–34.7)	23	4	8
PA103	2	4	23(16.6–32.2)	20	4	8	12(11.3–13.3)	11	1.25	4.5
10804	4	8	56(48.0–66.8)	44	7	28	37(25.6–53.7)	33	6	20
1136	64	128	>80			>80				

^a MICs and MBCs were done in MHB with Mg²⁺ and Ca²⁺ adjusted to 20 and 75 mg/liter, respectively. MBCs represent 99.9% killing.

^b PD₅₀ were calculated by the Spearman-Kärber method. Numbers in parentheses are 95% confidence intervals by Spearman-Kärber calculation.

^c Peak concentration at PD₅₀ dosage after the first gentamicin dose; subsequent levels were equally high or higher.

^d Trough levels after the first (1st) or fifth (5th) gentamicin dose.

TABLE 5. Effect of size of bacterial challenge on gentamicin PD_{50} for cyclophosphamide-treated mice^a

Strain	PD_{50} at infection level of:	
	50 × LD_{50}	500 × LD_{50}
280	7	37
PA103	12	43
280(RUA7)	24	>80
280(R151)	28	>80

^a Protection tests were done as described in text. PD_{50} were calculated by the Spearman-Kärber method (7).

were necessary to achieve 50% protection, and no protection was achieved with strain 280(R130).

In all of these studies only strain 280 could be treated with doses of gentamicin which would be considered nontoxic on a weight basis in humans (11). In addition, peak serum levels required to protect 50% of normal or cyclophosphamide-treated mice from strain 280 would be in the high therapeutic range reported for human infections. The remainder of dosages resulted in very high serum levels and significant accumulation in trough levels.

Heart blood from moribund mice was collected, diluted in MHB, grown to an absorbance at 600 nm reading of 0.5, and used as an inoculum for determination of MIC. No change in MIC was found for any strain as the result of animal passage.

Minimal bactericidal levels were performed on strains 280 and PA103 in MHB, in MHB and pooled mouse serum (from uninoculated mice) in equal parts, and in mouse serum. MBCs were 0.5 and 4 μ g/ml, respectively, and were the same in each testing condition. Thus gentamicin had identical antibacterial efficacy in broth as in 50 or 100% serum.

DISCUSSION

The normal mouse system used in this study resembles severe disseminated infection with *P. aeruginosa* in humans. The results from the cyclophosphamide-treated mouse model parallel the increase in susceptibility to *P. aeruginosa* seen in patients with complex impairment of host defenses, for example with immunosuppression, various leukemias, burns, and advanced malignancies. We feel that it is in circumstances of disseminated infection that effective antibiotic therapy is most needed. In turn it seems essential that susceptibility testing of *P. aeruginosa* be as dependable as possible for such situations so that not only will the most effective drug(s) be selected for therapy but appropriate dosages will be given. With the exception of strain 280, all of the strains in this study identi-

fied as susceptible by ordinary susceptibility testing criteria required very high dosages of gentamicin to produce 50% protection in normal or cyclophosphamide-treated mice. Even strain 280 required large doses on a weight basis. In summary, none of the strains tested, with the possible exception of strain 280, acted as gentamicin-susceptible strains. All strains tested except strain 280 would be better regarded as resistant.

Strain 280 and its R^+ derivatives of equal virulence show that an increase in MIC is associated with a reduction in susceptibility, as would be expected. All strains illustrate that peak serum levels must exceed MICs by severalfold before 50% protective dose levels are achieved. Similarly, peak serum levels exceeded MBCs by 1.5- to 14-fold. The association between first dose trough levels and MICs was closer. First dose trough levels associated with 50% protection were 0.5 to 3 times MICs and 0.25 to 1.5 times MBCs. Thus, a correlation with trough levels seems to be a more reliable way to predict therapeutic efficacy. Unfortunately this is not the current approach, as MICs are most frequently correlated with peak serum levels. It is equally unfortunate that most of the first dose trough levels required to achieve 50% protection with our strains and models would be considered toxic in humans. Current investigations have shown that trough levels greater than 4 μ g/ml are more likely to be associated with toxicity in humans (11). Fifth dose trough levels would be of even greater concern based on such toxicity criteria, even with strain 280. Thus, whatever the criteria selected, the strains examined in this study with the possible exception of strain 280 cannot be regarded as susceptible to gentamicin if potential toxicity is to be considered, as it must be with gentamicin.

Considerable caution is necessary in forming conclusions on human *P. aeruginosa* infections based on results from animal models. However, despite reservations, animal models clearly take into account many factors that in vitro susceptibility methods do not, and are likely to be a much better test of susceptibility.

From our results we conclude that current criteria for susceptibility based on designation of strains with MICs of gentamicin ≤ 6 μ g/ml as susceptible overestimate the susceptibility of *P. aeruginosa* for disseminated infections. This conclusion holds even with the use of media containing calcium and magnesium at levels similar to those of human serum. Susceptibility testing with a medium containing a low concentration of these cations grossly overestimates susceptibility. These conclusions are supported by the results of many human cases studies (8, 14, 16, 21). Other studies using animals also

support our conclusions in that many strains of *P. aeruginosa* apparently susceptible by in vitro criteria to gentamicin do not respond satisfactorily unless large gentamicin doses are used (1, 5, 13, 15, 24, 25). Our results also demonstrate that MICs are not a satisfactory guide to the peak serum levels needed to insure therapeutic success with *P. aeruginosa* in disseminated infection except that serum levels must exceed MICs by severalfold. In addition, we feel the development of susceptibility criteria based on peak serum levels is unsatisfactory; susceptibility criteria correlating MICs and trough levels have more predictive value. The marked effect of the size of inoculum on the PD₅₀ of gentamicin suggests that intensive treatment of disseminated *P. aeruginosa* infections should be started early when smaller numbers of *P. aeruginosa* are present.

A point of concern is that the present gentamicin susceptibility criteria as applied to *P. aeruginosa* isolates from severe infection may encourage underdosing with gentamicin, particularly if peak serum levels are used to guide therapy. This concern is based on current gentamicin dosages in frequent use and drug levels usually obtained. We feel this is a major reason to reexamine susceptibility criteria for *P. aeruginosa* to gentamicin.

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