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Activity of Gentamicin, Tobramycin, Polymyxin B, and Colistimethate in Mouse Protection Tests with *Pseudomonas aeruginosa*

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Mouse protection tests were carried out with four antibiotics and six strains of *Pseudomonas aeruginosa*. All strains were susceptible to all four antibiotics by an in vitro test. A heavier bacterial inoculum increased the mean effective dose of gentamicin and tobramycin, but not polymyxin B. Second and third doses of gentamicin in the mouse protection test made little change in the mean effective dose. In the mouse protection tests, tobramycin was the most active antibiotic if the results were analyzed in terms of the therapeutic index or ratio of toxicity to efficacy. Colistimethate was poorly inactive in vivo. Polymyxin B was most active on an absolute basis but also was the most toxic. One strain of *Pseudomonas* was classified as resistant to gentamicin in vivo although it was susceptible in vitro. Strains of *Pseudomonas* that were uniformly susceptible to antibiotics in vitro were not uniformly susceptible in the mouse protection test to low doses of antibiotic.

Although there are numerous publications on susceptibility of *Pseudomonas aeruginosa* to antibiotics in vitro, there are few on chemotherapy of experimental infections (5). Polymyxin B and colistimethate, the two forms of polymyxin available for parenteral use in humans, have rarely been directly compared in mouse protection tests on *P. aeruginosa*. The aminoglycosides have seldom been compared with the polymyxins on *P. aeruginosa* in vivo.

The purpose of this study was to determine the activity of gentamicin, tobramycin, polymyxin B, and colistimethate on six strains of *P. aeruginosa* in the mouse protection tests.

MATERIALS AND METHODS

The methods were generally those previously described (3). Important changes will be noted.

Antibiotics. Tobramycin sulfate was supplied by Eli Lilly and Co. Gentamicin, polymyxin B, and sodium colistimethate were purchased from the University Hospital Pharmacy. Antibiotic dilutions were freshly prepared, and unused portions were discarded.

Bacteria. A group of 13 strains of *P. aeruginosa* were obtained from the Clinical Microbiology Laboratory of the University Hospital. All strains were isolated from blood culture between July 1973 to June 1974. Nine strains were discarded from the study. Five strains were resistant in vitro to gentamicin, and four strains had low virulence for mice.

Two strains were added to the study that were not recent clinical isolates. Strain 41501 was characterized in a publication (11) and entered in the American Type Culture Collection (14). Strain PA-103, a gift of P. V. Liu, produces exotoxin A and has been maintained in his laboratory for over ten years (10).

The Clinical Microbiology Laboratory of the University Hospital performed agar diffusion antibiotic susceptibility tests by the standard methods (12, 17).

For mouse protection tests, the following method of culture was adopted because it gave reproducibly virulent cultures. Typical colonies from agar plates were inoculated into 5 ml of brain-heart infusion broth (Baltimore Biological Laboratories, Baltimore, Md.), and the broth suspension was incubated 18 h at 37 C. A 0.5-ml sample was inoculated into 5 ml of brain-heart infusion broth, and the suspension was incubated for an additional 24 h at 37 C. Viable cell counts were about 3×10^9 ml.

Immunotype. This was determined with typing sera supplied by H. B. Devlin (7).

Virluence. Virulence of broth cultures was established by injecting groups of mice intraperitoneally (i.p.) with 0.2 ml of serial 10-fold dilutions of broth cultures. Results were analyzed by Reed-Muench method (13) and expressed as mean infectious dose per milliliter (ID_{50}/ml).

Mouse protection tests. Mice were infected i.p. with 0.2 ml of bacterial suspension and for most studies were given single graded doses of antibiotics 1 h later. Animals were observed for 48 h. Data were analyzed by Spearman-Karber technique with the aid of a Wang computer (6). The virulence of the inoculum was determined with each test by inoculating groups of mice i.p. with serial dilutions of the culture. Experiments with inocula that were too high or too low were discarded.

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RESULTS

Antibiotic susceptibility, immunotype, and virulence of the six strains of *P. aeruginosa* in the study are shown in Table 1. All strains were classified as susceptible by agar diffusion tests to gentamicin, tobramycin, polymyxin B, and colistin. The virulence of strain PA-103 for mice was considerably greater than that of the other five strains.

To test for toxins in the original inoculum, bacterial suspensions of all six strains were made free of living bacteria by heat (3 min in a boiling water bath) or by micropore filtration with a 0.22-μm filter. There were no deaths among groups of mice injected i.p. with 0.2 ml of the treated suspensions.

To determine the influence of the size of the bacterial inoculum on the mean effective dose (ED_{50}) of antibiotics in the mouse protection test, groups of mice were infected i.p. with three different inocula of *Pseudomonas* PA-103 and were treated 1 h later with single graded doses of antibiotic given subcutaneously (s.c.; Table 2). A 10-fold increase in the bacterial inoculum produced a fourfold rise in the ED_{50} for gentamicin or tobramycin. There was no change in ED_{50} for polymyxin B.

To determine the influence of second and third doses of antibiotic on the ED_{50}, groups of mice were infected i.p. with 5 ID_{50} of *Pseudomonas* PA-103. Graded doses of gentamicin were given 1 h later, 1 and 5 h later, or 1, 5, and 22 h later. Animals were observed for 48 h. The ED_{50} and 95% confidence intervals for one dose of gentamicin was 0.5 mg/kg (0.2 to 0.9), for two doses was 0.5 mg/kg (0.2 to 0.9), and for three doses was 0.2 mg/kg (0.1 to 0.5). The results are expressed as mg/kg per dose.

The activity of gentamicin, tobramycin, polymyxin B, and colistimethate was determined in mouse protection tests on six strains of *P. aeruginosa* (Table 3). Mouse protection tests with all six strains were carried out with relatively light bacterial inocula. Antibiotics were given in single s.c. doses 1 h after i.p. infection. Colistimethate was relatively ineffective. It had essentially no detectable activity against three of the strains and poor activity against two other strains.

Polymyxin B was the most effective on an absolute basis. However, it was also the most toxic. Acute mean lethal doses (LD_{50}) for 18- to 22-g mice were previously established as 430 mg/kg for tobramycin, 279 mg/kg for gentamicin, and 306 mg/kg for sodium colistimethate (3). For this study, the acute LD_{50} of polymyxin B s.c. in mice was 65 mg/kg by Spearman-Karber technique (6). When the results of the mouse protection test were expressed as therapeutic index or ratio of the LD_{50} to the acute ED_{50} of the antibiotic, tobramycin was the most effective drug. The lowest therapeutic index for tobramycin was 13. In contrast, the therapeutic index was less than 10 for three strains with gentamicin, four strains with polymyxin B, and five strains with colistimethate.

TABLE 1. *Antibiotic susceptibility, immunotype, and virulence of six strains of P. aeruginosa*

Strain	Agar diffusion inhibition				Immunotype	Virulence (ID_{50}/ml)
	Gentamicin	Tobramycin	Polymyxin	Colistin		
383	16	19	17	15	1	40
420	18	21	18	14	2	70
764	19	23	19	16	4	130
18	15	18	17	15	2	100
41501	20	23	18	16	1	200
PA-103	18	20	15	13	2	6,000

[a] All zones were determined on 10-μg disks, except polymyxin B disks contained 300 μ.

TABLE 2. *Influence of bacterial inoculum on susceptibility of Pseudomonas PA-103 to antibiotics in vivo*

Bacterial inoculum (ID_{50})	ED_{50} (mg/kg)		
	Gentamicin	Tobramycin	Polymyxin B
14	1.6 (0.8-3.0)	1.2 (0.7-1.9)	1.6 (1.0-2.6)
140	5.8 (3.6-9.2)	5.4 (3.2-8.0)	1.5 (1.2-1.9)
1400	23 (13-40)	21 (11-42)	Not done

DISCUSSION

This study demonstrated that six strains of *P. aeruginosa* that were uniformly susceptible to antibiotics by in vitro tests were not uniformly susceptible to antibiotics in mouse protection tests.

If the results are considered in terms of therapeutic index or ratio of toxicity to efficacy, tobramycin was the most effective antibiotic.

Colistimethate was markedly ineffective. It had significant activity against only one strain of *Pseudomonas*. There is apparently no data in the literature proving that colistimethate is effective in vivo against *P. aeruginosa* (5). The paper that contains much of the basic data on colistimethate had results of in vivo trials on *E. coli* and *Klebsiella* infections in mice (15). Sous et al. reported the ED_{50} for one strain of *Pseudomonas* as 53 mg/kg and LD_{50} s.c. for colistimethate of 200 mg/kg, giving a therapeutic index of 4 (16). Hepding reported an average

TABLE 3. *Bacterial inoculum and results of mouse protection tests with four antibiotics on six strains of P. aeruginosa*

Strain	Inoculum (ID_{50})	Gentamicin		Tobramycin		Polymyxin B		Colistimethate	
		ED_{50}[a] (mg/kg)	T.I.[b]	ED_{50}[a] (mg/kg)	T.I.	ED_{50} (mg/kg)	T.I.	ED_{50}[a] (mg/kg)	T.I.
383	10	62 (42-92)	4.5	30 (19-47)	15	7.6 (5.2-10)	8.5	>120	<2.5
420	16	47 (24-92)	6	32 (18-57)	13	13 (7.1-23)	5	>120	<2.4
764	6	43 (27-67)	6.5	19 (12-31)	23	7.8 (5.1-12)	8.3	65 (42-97)	4.8
18	3	18 (10-32)	16	18 (8.9-37)	24	8.2 (4.6-15)	7.9	>120	<2.5
41501	6	3.5 (2.2-5.5)	80	1.4 (0.2-2.2)	310	2.0 (1.1-3.6)	33	60.2 (37-97)	5.1
PA-103	14	1.6 (0.8-3)	174	1.2 (0.7-1.9)	360	1.6 (1.0-2.6)	41	12.7 (6.6-24.4)	24

[a] 95% confidence intervals.
[b] Therapeutic index or LD_{50}/ED_{50}.

therapeutic index of 4.6 in the mouse protection test with six strains of *Pseudomonas* compared with an average therapeutic index of 9.3 for polymyxin on eight strains and an average 17.5 for gentamicin on eight strains (9). In a recent study on local therapy of experimental *Pseudomonas* keratitis in guinea pigs, colistimethate was only one-twelfth as effective as tobramycin (S. D. Davis and J. W. Chandler, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 14th, San Francisco, Calif., Abstr. 45, 1974). The data presented here and those in the literature show that colistimethate has little activity in experimental infections on *P. aeruginosa*.

Colistimethate apparently is active in a single s.c. dose in mouse protection tests on other organisms. Sous reported the ED_{50} for one strain of *E. coli* as 3.6 mg/kg (16). Schwartz et al. obtained an ED_{50} of 10.7 mg/kg on one strain of *E. coli* and 1.2 mg/kg on one strain of *Klebsiella* (15).

When compared on an absolute basis, polymyxin B was the most effective antibiotic. It may be the antibiotic of choice for topical therapy, in which case systemic toxicity would not be a concern. It should probably be preferred to colistimethate in systemic therapy of infections by *P. aeruginosa* resistant to less toxic drugs.

Waitz et al. compared the activity of gentamicin and tobramycin in mouse protection tests with six strains of *P. aeruginosa* (18). Five strains were recent clinical isolates. The mean ED_{50} for gentamicin was 7.8 mg/kg with a range of 0.5 to 19. For tobramycin the mean ED_{50} was 3.8 mg/kg with a range of 0.4 to 6.1. Heifetz et al. performed mouse protection tests with gentamicin on eight strains of *P. aeruginosa* (8). The average ED_{50} was 11.5 mg/kg with a range of 7.2 to 16.1. The mean ED_{50} in the present study for gentamicin was 25 mg/kg with a range of 1.6 to 62. One factor that may account in part for the higher ED_{50} observed in this study is that four strains were recently isolated from blood culture.

An increase in the size of the bacterial inoculum led to an increase in the ED_{50} for gentamicin and tobramycin. Acred et al. reported similar findings with *P. aeruginosa* and carbenicillin (1). It is evident that the size of the bacterial inoculum must be carefully controlled if consistent results are to be obtained in the mouse protection tests. There is apparently no concensus as to the best bacterial inoculum for the mouse protection test. Acred et al. did not define the inoculum in terms of virulence (1). In several studies, the inoculum was set at 10^7 organisms without regard to virulence (18-20). In another report, the inoculum was defined as a certain dilution of a broth culture without regard to virulence (16).

A tremendous amount of work has gone into standardizing in vitro antibiotic susceptibility tests, but apparently little has been done to standardize in vivo antibiotic susceptibility tests. The suggestions which follow are offered in an effort to improve the comparability of data obtained by various laboratories with the mouse protection test. Several strains of *P. aeruginosa* should be included in every study. Intuitively, recent clinical isolates that have had few passages in the laboratory ought to have the greatest clinical relevance. Some well-

standardized strains with carefully documented, stable susceptibility in vivo might also be included. Strain PA-103 may be a useful reference strain. The virulence of the bacterial inocula should be determined with every experiment. Male white mice of 18–22 g have been used for many years as experimental animals and have the advantage of being relatively cheap. The i.p. route is a convenient one for infections. The optimum timing in number in doses of antibiotic should be established by additional investigation. One definition of optimal timing and dosage is that the optimum schedule should result in the lowest ED_{50} expressed as mg/kg per day. The paper by Acred et al. has an example of this (1). The ED_{50} of carbenicillin in a single dose was 410 mg/kg and in four doses given every 2 h was 184 mg/kg of the total dosage. Experiments should be planned to cover the full dose response curve from 100% survivors to 100% deaths if possible. Results should be analyzed by Spearman-Karber or a comparable method to give ED_{50} with 95% confidence intervals. Determination of ED_{50} without confidence intervals is less useful. New drugs should be compared with standard drugs.

By using tentative criteria previously put forward (2), strains 41501 and PA-103 can be classified as susceptible in vivo to gentamicin and tobramycin since the infections were cured by blood levels achievable in patients. At the other extreme, it seems reasonable to classify strain 383 as resistant in vivo to gentamicin because of the narrow margin of safety. This is another example of a strain of *Pseudomonas* that is susceptible by in vitro test and apparently resistant by in vivo tests (3, 4).

The four recent clinical isolates were considerably more resistant to antibiotics in the mouse protection test than were the two strains that had been maintained in the laboratory for some time. This observation suggests that resistance in the mouse protection test in strains of *P. aeruginosa* may be a characteristic that can be lost by repeated passage upon laboratory media.

Whether results obtained by mouse protection test on *P. aeruginosa* have any validity for human infections remains to be determined. That must be determined by trials in humans. However, the results of mouse protection tests should have as much relevance for human infections, on theoretical grounds at least, as any given in vitro test.

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