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Determinants of the Efficacy of Tobramycin Therapy against Isogenic Nonmucoid and Mucoid Derivatives of *Pseudomonas aeruginosa* PAO1 Growing in Peritoneal Chambers in Mice

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Mice which were supporting the growth of *Pseudomonas aeruginosa* in chambers implanted in their peritoneums were given two intramuscular injections of tobramycin (15 mg/kg of body weight each) at an interval of 8 h. Three hours later, chambers were removed and their contents were assessed for viable counts. Controls revealed that tobramycin levels in the chambers were 3.8 μg/ml 15 min after injection of 15 mg of tobramycin per kg and remained above the in vitro MICs (0.5 to 1 μg/ml) for the tested strains for 8 h. It was demonstrated that tobramycin therapy was less effective with higher inocula and with longer delay before administration. Thus, in vivo, the concentration of bacteria in the chambers at the time of the first tobramycin injection had a profound effect on the bactericidal efficacy of tobramycin therapy. No such concentration dependence was observed in mock in vitro therapy experiments. A phage-selected mucoid derivative of *P. aeruginosa* PAO1 showed only a marginal increase in in vitro aminoglycoside susceptibility and no major alteration in in vivo susceptibility compared with its isogenic nonmucoid parent strain.

Pseudomonas aeruginosa is an opportunistic gram-negative pathogen which causes infections that are difficult to treat by conventional antibiotic therapy (2, 9). Indeed, in some clinical situations it has been concluded that therapeutic intervention is rarely successful (4, 7). This is largely due to the high intrinsic resistance of *P. aeruginosa* to antibiotics, a property that is in part caused by the low permeability of the *P. aeruginosa* outer membrane (9). Nevertheless, this cannot be the only factor, since levels of antibiotics that might be predicted, on the basis of in vitro MIC tests, to eliminate *P. aeruginosa* infections are often unsuccessful in patient therapy.

This is particularly evident in cystic fibrosis patients with characteristic chronic *P. aeruginosa* lung infections. In this case, antibiotic therapy, while transiently successful with appropriate dosing (19), does not prevent reinfection by the same strain. Furthermore, after several episodes of reinfection, antibiotics become less effective even in the apparent absence of bacterial resistance development. In the case of aminoglycoside therapy of such lung infections, aminoglycoside tolerance in vivo has been suggested to be due to poor penetration, sequestration of aminoglycosides by polyanions, adaptation (i.e., transient, nonmutational antibiotic resistance), or inhibition of aminoglycoside uptake by the bacterial exopolysaccharide (alginate) which is produced by mucoid isolates from the lungs of cystic fibrosis patients (2, 19).

To shed some light on these problems, we have turned to an animal model which allows growth of *P. aeruginosa* on available in vivo nutrients (11, 12). In this model, 10^2 to 10^3 bacteria are enclosed in a cylindrical plastic chamber sealed at each end with membrane filters (Millipore Corp., Bedford, Mass.). These chambers are then inserted into the peritoneal cavities of laboratory mice. The bacteria then undergo a classical bacterial growth curve to a peak of 10^8 to 10^9 bacteria per ml within 16 h, after which numbers of viable bacteria remain stable for some months. Removal of the chambers and extraction of their contents allow full recovery of the in vivo-grown organisms. Since mouse cells are excluded from the chambers, the influence on bacterial tobramycin susceptibility of growth on in vivo nutrients could be studied in the absence of phagocytic cells. We report here that the time of initiation of therapy and the bacterial concentration in the chamber strongly influenced the outcome of therapy.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. Our laboratory wild-type *P. aeruginosa* PAO1 H103, a serotype 5 isolate, has been described previously (17). Phage 7 was obtained from T. L. Pitt (Public Health Laboratory, London, England) and propagated on strain H103. This phage had the unusual property of being able to form plaques on all 17 IATS serotype type strains of *P. aeruginosa* (R. E. W. Hancock, unpublished observations). Twenty-eight other phages in our laboratory collection, with a variety of cell receptors, were described previously (17). Phage susceptibility was determined as described previously (21). Approximately 50% of the phage 7-resistant mutants of strain H103 demonstrated classical mucoid morphology after growth on rich-medium agar plates for 24 to 36 h and were indistinguishable from several mucoid *P. aeruginosa* isolates from patients with cystic fibrosis. One of these, strain H328, was selected for this study, since it demonstrated phage typing patterns identical to those of the parent strain, H103, when tested against our laboratory phage collection.

Growth conditions. Bacteria were grown in vitro in Proteose Peptone no. 2 (PP2) (Difco Laboratories, Detroit, Mich.) (12) or in vivo in polypropylene chambers capped by Millipore filters (0.2-μm pore size) and inserted into the peritoneal cavities of mice (11, 12) exactly as described previously.

Standard in vivo tobramycin therapy. By using the chamber model, strains H103 and H328 were grown in mice. The chamber inoculum was standardized by total counts to $2.0 \times$

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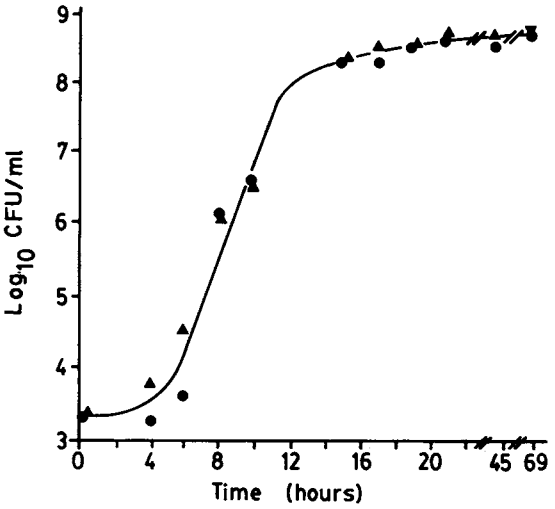

FIG. 1. Growth in vivo in mouse peritoneal chambers of *P. aeruginosa* H103 (●) and its mucoid derivative, H328 (▲). Each point represents the average viable count of 4 to 12 chambers implanted into the peritoneal cavities of mice at time zero and then removed at the indicated times; contents were diluted and plated for viable counts. Individual values varied not more than fourfold from the given geometric means. Statistical comparison of the data obtained for strains H328 and H103 by using Fisher's exact test showed that only the threefold difference in geometric means at 4 h of growth was significantly different ($P < 0.05$). We considered this difference to be due to differential clumping at this time, since no significant differences ($P > 0.2$) were seen at other times.

10^4 cells per ml, and four chambers were implanted in each mouse. Inoculum bacteria were diluted in saline but not washed to allow for potential maintenance of mucoid exopolysaccharide association with strain H328. At least one of these chambers was inoculated with the nonmucoid strain H103 and the remaining (color-coded) chambers contained H328, so that both mucoid and nonmucoid bacteria received similar tobramycin treatments. A standard therapeutic regimen involving two intramuscular injections at 8-h intervals was adopted; however, the time of initiation of this regimen was varied. Mice were injected with tobramycin (15 mg/kg of mouse body weight) at 4, 6, 8, and 10 h postimplantation (Fig. 1), and a second injection was given 8 h after the first. Control mice were injected with sterile saline. The mice were sacrificed 3 h after the second injection, and the chambers were removed. The sample from each chamber was individually diluted with sterile saline and plated on PP2 agar plates for a viable count. Bacteria from chambers inoculated with strain H328 retained the mucoid phenotype.

For mock in vitro tobramycin treatments, an overnight culture of strain H103 was diluted in PP2 broth to the given initial concentration of cells, and then 1 ml was placed in a sterile test tube on a tube roller and rotated for 1 h at 37°C. At this time, 3 μg of tobramycin per ml (the approximate concentration of tobramycin in mouse peritoneal chambers after intramuscular injection) was added to one set of tubes. Then the tubes were rotated for a further 3 h at 37°C, and the contents were diluted and plated for viable counts.

In vitro tobramycin susceptibility measurements. An agar dilution technique as described in the National Committee for Clinical Laboratory Standards handbook was used to determine the MIC of tobramycin (a gift from Eli Lilly Canada Inc., Toronto, Canada) for strains H103 and H328. The tobramycin was used at serial twofold dilutions ranging from 8.0 to 0.0625 μg/ml in Mueller-Hinton agar (Difco Laboratories). Ten microliters of diluted overnight cultures or cultures from the mouse peritoneal chambers (10^5 CFU/ml) was spotted on the antibiotic plates, which were incubated at 37°C overnight. Tests were done in duplicate, and the MIC was recorded as the lowest test concentration that inhibited growth.

Tobramycin levels in serum and chambers implanted in mice. As recommended by T. Parr (Eli Lilly and Co., Indianapolis, Ind.), agar plates were prepared by the addition of 10 ml of agar medium no. 5 (6.0 g of peptone, 3.0 g of yeast extract, 1.5 g of beef extract, and 15.0 g of agar per liter). This was followed by the addition of another 5 ml of agar inoculated with *Bacillus subtilis* ATCC 6633. Seven 6-mm-diameter wells were cut symmetrically on each plate. Standard curves were prepared, in triplicate, by the addition of 0.05 ml of serial dilutions of tobramycin in either serum or saline. Test samples of serum and saline from chambers were taken at times ranging from 0.25 to 8 h after injection of tobramycin. These samples were diluted and dispensed into the wells in duplicate. Plates were incubated at 30°C for 16 to 18 h. Zones of inhibition were measured to the nearest 0.1 mm. Results of the tobramycin levels in serum were supported by data obtained by high-pressure liquid chromatography. However, chamber samples could not be analyzed in this way because of the lack of a suitable standard curve.

RESULTS

Isolation of mucoid variants. To allow direct assessment of the role of mucoid exopolysaccharide in tobramycin susceptibility of *P. aeruginosa*, spontaneous phage 7-resistant, mucoid mutants of strain H103 were isolated at a frequency of 10^{-6} by the strategy of Martin (14). One of these, strain H328, was apparently an isogenic mucoid exopolysaccharide-producing derivative of strain H103. This mucoid phenotype was stable on repeated subculture on PP2 agar plates. Strain H328 (MIC of tobramycin = 0.5 μg/ml) demonstrated a marginal (twofold) increase in tobramycin susceptibility compared with strain H103 (MIC = 1.0 μg/ml), as measured by the agar dilution method after prior growth in vitro or in vivo in peritoneal chambers.

Tobramycin treatments in vivo. Chambers containing 2×10^3 bacteria from strain H103 or its mucoid derivative, H328, were implanted into the peritoneal cavities of mice. At various time points, chambers were removed and their contents were plated for viable counts (Fig. 1). After a lag period of 4 h, bacteria grew logarithmically for approximately 10 h with a doubling time of approximately 50 min (cf. a doubling time in vitro in PP2 broth of 45 min). Thereafter, growth plateaued at a concentration of 2×10^8 to 5×10^8 cells per ml, and viable counts remained at this level for at least 53 h. The growth curves for strain H103 and its mucoid derivative, H328, were quite similar (Fig. 1). Colonies grown from cells taken from chambers at all sampling times retained their mucoid (for H328) and nonmucoid (for H103) appearances.

Since we wished to determine the influence of in vivo growth on tobramycin susceptibility, it was necessary to establish a therapeutic regimen which delivered sufficient tobramycin to the chambers. Initial experiments suggested that 15 mg of tobramycin per kg injected intramuscularly resulted in adequate killing under the appropriate conditions. Therefore, at various times after injection, the levels of tobramycin in the blood and in saline-containing chambers implanted in the peritoneum were assessed (Table 1). Con-

TABLE 1. Levels of tobramycin achieved in the serum and in chambers implanted in the peritoneums of mice

Time (h) postinjection[a]	Mean tobramycin level (μg/ml)[b] in:	
	Serum	Peritoneal chamber
0	0	0
0.25	>12.8	3.8 ± 2.3
0.5	>12.8	2.9 ± 1.6
1.0	6.5 ± 2.5	3.0 ± 0.5
2.0	3.3 ± 1.8	3.4 ± 0.8
4.0	0.4 ± 0.3	2.5 ± 0.1
8.0	<0.05	1.2 ± 0.5

[a] Intramuscular injection of 15 mg of tobramycin per kg of body weight.
[b] Means ± standard deviations of three independent experiments.

centrations in serum were very high at early times after injection and declined with a half-life of approximately 40 min. At 4 h postinjection, concentrations in serum had already declined to a level below the in vitro MICs for H103 and its derivatives. Similar behavior has been observed previously for aminoglycosides (16, 18). In contrast, in the peritoneal chambers, maximal tobramycin concentrations of only 3.8 μg/ml were obtained after 15 min, but concentrations declined much more slowly (half-life of approximately 5 h) and remained above the MICs for *P. aeruginosa* for 8 h after the tobramycin injection.

To determine the susceptibility of in vivo-grown bacteria to tobramycin, therapy was initiated at 4, 6, 8, and 10 h after implantation of chambers. The therapeutic regimen comprised two intramuscular injections of tobramycin (15 mg/kg each), with an 8-h interval between them. Three hours after the second injection, chambers were removed and viable counts on the chamber contents were done. There was substantial killing of both strains when therapy was initiated at 4 or 6 h after chamber implantation (Table 2). However, therapy was less successful when initiated at 8 or 10 h after chamber implantation. Similar therapeutic efficiencies were observed for the mucoid strain H328 and its nonmucoid parent, H103 (Table 2), and the mean viable counts for therapy initiated at a given time after chamber implantation were not significantly different when strains H103 and H328 were compared ($P > 0.2$ by Fisher's exact test). No bacterial tobramycin resistance development was observed during the lifetime of these experiments, since the MICs of tobramycin for bacteria from the chambers were identical to those reported above (i.e., 0.5 μg/ml for strain H328 and 1.0 μg/ml for strain H103).

TABLE 2. Influence of intramuscular tobramycin therapy on the viability of strain H103 and its mucoid derivative, H328, in mouse peritoneal chambers

Strain	Time (h) of initiation of therapy after chamber implantation	Viable count/ml 11 h after initiation of therapy[a] in mice injected with:		Therapeutic efficiency index[b]
		Saline	Tobramycin	
H103	4	1.5×10^8	2.5×10^0	7.8
	6	1.5×10^8	6.0×10^2	5.5
	8	2.6×10^8	1.5×10^6	2.2
	10	4.7×10^8	2.0×10^7	1.4
H328	4	3.2×10^8	4.5×10^0	7.8
	6	2.9×10^8	3.1×10^1	6.9
	8	2.8×10^8	1.0×10^6	2.5
	10	3.3×10^8	2.5×10^6	2.1

[a] Values are the geometric means of three independent experiments, each of which involved four to six chambers in three mice. Individual datum values varied about 3- to 10-fold from the geometric mean.
[b] Logarithm (base 10) of the ratio of bacterial counts in chambers from saline-treated mice to counts in chambers from tobramycin-treated mice.

TABLE 3. Effect of chamber inoculum size on the outcome of intramuscular tobramycin therapy against strain H103

Time (h) of initiation of therapy after chamber implantation	Inoculum size (cells/ml)	Viable count/ml 11 h after initiation of therapy[a] in mice injected with:		Therapeutic efficiency index[b]
		Saline	Tobramycin	
4	10^4	2.4×10^8	2.5×10^0	8.0
	10^5	2.9×10^8	1.7×10^3	5.2
	10^6	3.3×10^8	2.2×10^4	4.2
	10^7	2.6×10^8	2.4×10^5	3.0
6	10^4	2.4×10^8	6.0×10^2	5.6
	10^5	2.3×10^8	8.8×10^3	4.4
	10^6	2.2×10^8	2.9×10^5	2.9
	10^7	1.8×10^8	4.1×10^6	1.6

[a] Values are the geometric means of three independent experiments, each of which involved four to six chambers in three mice. The range of individual values was 3- to 10-fold from the geometric mean.
[b] Logarithm (base 10) of the ratio of bacterial counts in chambers from saline-treated mice to counts in chambers from tobramycin-treated mice.

The influence of chamber inoculum on therapeutic effectiveness was examined by using strain H103 (Table 3). Progressive increases in the chamber inoculum resulted in progressive decreases in the therapeutic effectiveness when therapy was initiated at 4 or 6 h after chamber implantation. To demonstrate that the concentration of tobramycin in the chambers during therapy was sufficient to cause killing, we treated strain H103 in vitro with 3 μg of tobramycin per ml (a concentration maintained within the in vivo chambers for more than 2 to 4 h after a single intramuscular injection). While the untreated controls grew 11- to 23-fold within 3 h, the tobramycin-treated bacteria were totally killed at this time, irrespective of the inoculum (Table 4). Similar data were obtained for strain H103 grown on PP2 broth (Table 4) or Mueller-Hinton broth (data not shown).

DISCUSSION

These studies indicate that the in vivo therapeutic efficacy of tobramycin against *P. aeruginosa* is strongly dependent on the bacterial numbers at the time of initiation of tobramycin therapy. This is consistent with previous studies in mouse protection models (5, 6, 18). However, in these other studies, therapeutic efficacy was measured in terms of pre-

TABLE 4. Efficacy of tobramycin treatment of strain H103 in vitro

Initial concn (cells/ml)	Viable count 3 h after initiation of treatment[a] in:		Therapeutic efficiency index[b]
	Control tubes	Tobramycin tubes	
10^4	2.3×10^5	0	>5.4
10^5	1.3×10^6	0	>6.1
10^6	1.4×10^7	0	>7.1
10^7	1.1×10^8	0	>8.0

[a] Mock therapy was done as described in Materials and Methods. Results are the averages of two independent experiments in PP2 broth. Similar data were obtained for Mueller-Hinton broth.
[b] Logarithm (base 10) of the ratio of bacterial counts in control tubes to counts in tubes with 3 μg of tobramycin per ml.

vention of death from sepsis which followed intraperitoneal inoculation of *P. aeruginosa*. These studies were limited by the necessity to infect with 10- to 1,000-fold the lethal dose killing 50% of mice of the particular *P. aeruginosa* strain in order to achieve the desirable endpoint (i.e., death) in control mice (5, 6, 18). Since *P. aeruginosa* strains usually have 50% lethal doses of 10^6 to 10^8 bacteria per mouse, only limited conclusions could be made about the role of bacterial inoculum and tobramycin therapy. In our model of in vivo growth, the peritoneal chamber model (11, 12), we were able to operate over a much wider range of bacterial concentrations and could measure therapeutic efficacy as a function of numbers of bacteria surviving therapy. On the basis of in vitro mock therapy experiments in two different media (Table 4), it was clear that sufficient tobramycin was delivered into the chambers (Table 1) to potentially cause complete bacterial killing. Nevertheless, the ability of tobramycin to kill *P. aeruginosa* in vivo was limited by the time of initiation of therapy relative to the time of chamber implantation in the peritoneum (Table 2) and by the bacterial inoculum in the chambers (Table 3). These two factors may indeed be the same, since the bacteria started growing rapidly within 4 to 6 h after chamber implantation (Fig. 1), and thus the number of bacteria in the chambers at initiation of therapy in the experiments reported in Table 2 would depend on the time after chamber implantation.

This strong effect of inoculum size on tobramycin effectiveness in vivo was not obvious in in vitro experiments measuring tobramycin killing (Table 4). Thus, in the absence of development of resistant mutants, it is clear that there must be important differences between the in vivo and in vitro environments which explain these differences in killing. Several possibilities based on past studies are suggested. For example, although it is generally assumed that Mueller-Hinton agar mimics the ionic conditions of serum, it is possible that peritoneal fluid has a higher ionic strength as a result of other components not present in serum, as suggested for lung fluids (2, 10). However, since tobramycin was successful against lower bacterial inocula, this cannot per se explain the effect of inoculum size. A second possibility is phenotypic adaptation in vivo (also termed microbial persistence [2]) resulting in resistance in vivo which is reversed upon growth in vitro. Aminoglycoside efficacy is strongly determined by the efficiency of aminoglycoside uptake (8). Thus, any in vivo phenotypic adaptation resulting in decreased uptake rates would influence efficacy. Such an adaptation could be due, e.g., to alteration in cellular lipopolysaccharide which is involved in self-promoted uptake of aminoglycosides across the outer membrane of *P. aeruginosa* (8, 9). Lipopolysaccharide alterations after growth in vivo in the chamber model were detected in an earlier study (12), while other studies have demonstrated a correlation between lipopolysaccharide phenotype and aminoglycoside susceptibility in clinical impermeability-type resistance of *P. aeruginosa* (3). Alternatively, since *P. aeruginosa* is an obligate aerobe and we hypothesize that oxygen supply in the peritoneum is somewhat limited (despite the rapid rate of growth in vivo) (Fig. 1), electron transport, upon which aminoglycoside transport depends (8), might be reduced. Several other possibilities for phenotypic adaptations based on the types of antagonists and mutants (8) which affect aminoglycoside uptake could be suggested. However, it must be stressed that the inoculum effects observed in this study can be explained only if such phenotypic adaptations affected only a proportion of the inoculum, such that the possibility of escape from tobramycin killing increased with inoculum size.

In our chamber model, phagocytic cells were excluded. Thus, we were able to utilize chamber inocula that were 5 orders of magnitude lower than the 50% lethal doses of *P. aeruginosa* H103 (i.e., 10^8 bacteria per mouse) (E. Mouat and R. E. W. Hancock, unpublished data). In addition, it must be pointed out that certain studies (10, 15) have indicated that bacteria pretreated with aminoglycosides are more susceptible to phagocytic killing. Thus, while the peritoneal chamber model offers interesting insights into the influence of inoculum size on tobramycin susceptibility in vivo, it must be stressed that the total effectiveness of host defense mechanisms and tobramycin are underestimated in this model.

One of the objectives of this study was to determine whether the alginate mucoid exopolysaccharide could influence susceptibility of *P. aeruginosa* to aminoglycoside treatment. This issue is quite controversial, with various authors suggesting that mucoid derivatives are either more or less susceptible to aminoglycosides than, or as susceptible as, their nonmucoid revertants (1, 13). However, these studies have suffered from a lack of data supporting the isogenic nature of the mucoid and nonmucoid derivatives. To overcome such objections, we used the inverse strategy, phage selection of mucoid variants from a genetically well-characterized parent, *P. aeruginosa* PAO1 H103. Since the derivative H328 was a spontaneous mutant with stable mucoid character and no apparent additional surface alterations, we considered it useful for comparisons of aminoglycoside susceptibility. Our data suggested marginal increases in in vitro aminoglycoside susceptibility of the mucoid mutants compared with the nonmucoid parent, strain H103. However, it could be argued that in vitro susceptibility might not accurately reflect in vivo susceptibility. Since the mouse peritoneal chamber implant model can support the growth of both mucoid and nonmucoid organisms (11), we decided to use this system to compare in vivo susceptibility of these bacteria to tobramycin. Mucoid organisms apparently remained mucoid in this system, judging from their colony morphology after chamber contents were plated. In addition, scanning electron microscopy of the interiors of chambers containing a mucoid derivative of strain H103 after removal from the mouse peritoneum showed a matrix of glycocalyx-like material which was not present in strain H103-containing chambers (N. Kelly, N. Martin, and R. E. W. Hancock, manuscript in preparation). Nevertheless, both strain H103 and its mucoid derivative, H328, displayed similar patterns of growth and similar susceptibility to tobramycin when grown in vivo. These data are consistent with in vitro studies showing that the anionic alginate mucoid exopolysaccharide of *P. aeruginosa* binds aminoglycosides weakly at best (20). In contrast, aminoglycoside binding to the cell surface lipopolysaccharide, an event that is apparently a prerequisite for uptake across the outer membrane of *P. aeruginosa*, is a high-affinity binding event (9).

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