ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1995, p. 34–39 Vol. 39, No. 1
0066-4804/95/$04.00+0


# Macromolecular Mechanisms of Sputum Inhibition of Tobramycin Activity

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Received 14 February 1994/Returned for modification 7 July 1994/Accepted 24 October 1994

**Tobramycin, an aminoglycoside antibiotic, is used in the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis patients. Tobramycin bioactivity, however, is antagonized by sputum. Glycoproteins (mucins) and high-molecular-weight DNA make up 2 to 3% (P. L. Masson and J. F. Heremans, p. 412–475, *In* M. J. Dulfano, ed., *Sputum: Fundamentals and Clinical Pathology*, 1973) and 3 to 10% (W. S. Chernick and G. J. Barbero, Pediatrics 24:739–745, 1959, and R. Picot, I. Das, and L. Reid, Thorax 33:235–242, 1978) of the dry weight of sputum, respectively. Tobramycin binds to both mucins and DNA obtained from sputum (R. Ramphal, M. Lhermitte, M. Filliat, and P. Roussel, J. Antimicrob. Chemother. 22:483–490, 1988). In vitro, recombinant human DNase (rhDNase) hydrolyzes high-molecular-weight DNA of >50 kb within sputum to fragments of 2 to 4 kb. Studying dialyzable tobramycin, we examined drug binding to whole sputum and to "mock sputum," which consisted of porcine gastric mucin and calf thymus DNA. We also studied the effects of rhDNase treatments of sputum, mock sputum, and calf thymus DNA on tobramycin binding. We found that treatments of sputum, mock sputum, and calf thymus DNA with rhDNase did not significantly increase the tobramycin bioactivity within the dialysates; surprisingly, sputum binding of tobramycin was increased by rhDNase. We conclude that rhDNase does not increase the bioactivity of tobramycin in sputum.**

*Pseudomonas aeruginosa* is the most common cause of chronic endobronchial infection in cystic fibrosis (CF) patients; this infection is the major cause of morbidity and mortality in these patients. Tobramycin, an aminoglycoside antibiotic, is used for antipseudomonal chemotherapy in CF patients. In vitro, tobramycin is active against most *P. aeruginosa* organisms in the absence of sputum; however, in the presence of sputum, tobramycin bioactivity is significantly reduced. Previous investigators have attributed the sputum antagonism of aminoglycoside antibiotics to acidic pH (3, 4, 9), high ionic strength (2, 12, 14), the presence of divalent cations (2, 7–10, 12, 19, 24), and antibiotic binding to sputum (12, 15) or to such sputum components as glycoproteins (18, 20) or DNA (17, 18, 23).

One of the major components of CF sputum is DNA. High-molecular-weight chromatin DNA is released from leukocytes that accumulate and lyse within purulent sputum (1). In vitro, recombinant human DNase (rhDNase) hydrolyzes the DNA within sputum, and this is associated with a visible reduction of sputum viscosity (22). rhDNase is commercially available for improving lung function in CF patients. During a phase III placebo-control trial, rhDNase treatment improved lung function over a 6-month period (unpublished data). Coadministration of tobramycin by the intravenous or aerosol route with rhDNase may have a potential benefit for CF patients by decreasing the antagonism of tobramycin in CF sputum.

We sought to determine if rhDNase treatment of sputum, in vitro, would reduce the antagonism of tobramycin bioactivity by reducing the binding of tobramycin to the high-molecular-weight DNA. To determine if the effect of the rhDNase treatment was specific to CF sputum and its major components, we also examined the interaction of tobramycin with "mock sputum" (gastric mucin and calf thymus DNA) and with mock sputum that had been treated with rhDNase.

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## MATERIALS AND METHODS

**Reagents.** Unless stated otherwise, all chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.), and all solutions were prepared in sterile deionized water. Working stock solutions of tobramycin were prepared once and stored at 4°C. Calf thymus DNA (Calbiochem, San Diego, Calif.) and gastric mucin (76 to 90% pure; ICN Pharmaceuticals Inc., Cleveland, Ohio) were prepared fresh for each experiment. rhDNase (Genentech Inc., South San Francisco, Calif.) was prepared fresh for each experiment in sterile 0.9% sodium chloride. A Spectra/Por 2 dialysis membrane with a molecular-weight cutoff of 12,000 to 14,000 and a dry cylinder width of 16 mm (Spectrum Medical Industries, Inc., Houston, Tex.) was cut into 8-cm pieces, boiled for 10 min in 2% (wt/vol) sodium bicarbonate and 1 mM EDTA (pH 8.0), rinsed thoroughly in deionized water, boiled for 10 min in 1 mM EDTA (pH 8.0), and then stored in sterile deionized water at 4°C.

**Sputum.** Sputum was obtained from children and adults with CF who were not receiving tobramycin or rhDNase for at least 1 week prior to the collection of the sample. The sputum samples were kept frozen at −20°C until used. The DNA concentration was determined by the fluorometric method of Kissane and Robins (11).

**Organisms.** Six *P. aeruginosa* strains (four mucoid and two nonmucoid) isolated from six CF patients were used in this study. The MIC of tobramycin for each strain was determined by the Sensititer system (Radiometer America Inc., Westlake, Ohio). The MIC of tobramycin for four of the strains was 0.25 μg/ml; the MICs for the other two strains were 1.0 and 2.0 μg/ml. These strains were kept frozen in skim milk at −70°C. Fresh subcultures were used for each experiment.

**Culture medium.** A divalent cation-deficient broth (MB) was used both as the growth medium for the *P. aeruginosa* strains and as the dialysis medium. The MB consisted of 1× M9 salts (6), pH 7.4 (1× M9 salts are 42 mM $Na_2HPO_4$, 22 mM $KH_2PO_4$, and 19 mM $NH_4Cl$); 50 mM monosodium glutamate; 1% glucose (wt/vol); 1% glycerol (vol/vol); 1× minimal essential medium vitamin solution (Gibco BRL, Grand Island, N.Y.); and 1× minimal essential medium amino acids solution (Gibco BRL). The MB was filter sterilized (pore size, 0.22 μm; Millipore, Bedford, Mass.) and stored at 4°C.

**Sputum DNA hydrolysis by rhDNase.** Sputum (1 g) was incubated with 10 μg of rhDNase for 1, 5, 15, or 30 min at 37°C. Additional $Ca^{2+}$ and $Mg^{2+}$ were not included in the rhDNase treatment: sputum contains an adequate concentration of these ions to allow rhDNase activity (22). The DNA was isolated from the sputum by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation (22) and then subjected to 0.7% agarose gel electrophoresis (21).

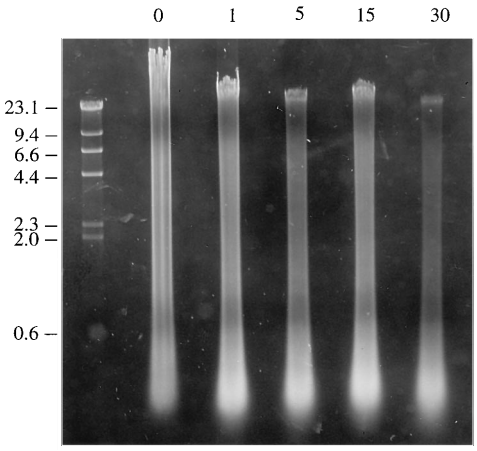


FIG. 1. Effects of rhDNase activity on DNA within CF sputum. Agarose gel electrophoresis of DNA isolated from CF sputum after the sputum was incubated with 10 μg of rhDNase per g for 0, 1, 5, 15, or 30 min at 37°C was performed. Size markers (in kilobases) are given at the left.

**rhDNase effect on tobramycin binding.** Sputum (1 g) was pretreated either with 10 μg of rhDNase or with rhDNase diluent (no enzyme) for 15 min at 37°C. The sputum was placed into dialysis tubing (nominal cutoff, 12,000 Da); tobramycin was added to the sputum to yield a final concentration of 0, 0.4, 4.0, or 40 μg/ml in the MB at equilibrium; and the ends of the tubing were secured with string. The dialysis bag was placed in 10 ml of MB, and the mixture was dialyzed for 2 h at 4°C with gentle shaking (100 rpm). After dialysis, the bag containing the sputum was removed, and the dialysate was filter sterilized (0.22-μm-pore-size Millex-GV disposable filter units; Millipore). A *P. aeruginosa* killing curve was then performed with the dialysate to determine the antipseudomonal activity. Filter sterilizing the dialysates did not affect the killing curves: we conducted killing curves in filtered and nonfiltered dialysates and obtained the same results (data not shown).

**Killing curves.** The *P. aeruginosa* strains from the frozen stock were plated onto Luria agar plates and incubated overnight at 37°C in air. One colony from these plates was grown in MB at 37°C to an optical density at 600 nm of 0.3 to 0.5. The bacteria were inoculated into the sterile dialysates to obtain an initial density of $10^6$ CFU/ml (10 μl of inoculum per 10 ml of dialysate). These cultures were incubated with shaking at 37°C for 4 h. Bacterial densities were determined at 0, 1, 2, and 4 h. Samples (100 μl) were diluted serially in phosphate-buffered saline, plated on Luria agar plates in duplicate, and then incubated overnight in air at 37°C.

**Kinetics of tobramycin efflux.** Tobramycin was added to rhDNase-treated (10 μg/ml) and untreated samples of sputum, gastric mucin with calf thymus DNA, calf thymus DNA, and gastric mucin to obtain a final concentration of 40 μg/ml in the sample and MB. Since rhDNase requires $Ca^{2+}$ and $Mg^{2+}$ for activity, $CaCl_2$ and $MgCl_2$ were added to obtain final concentrations of 5 mM each in the rhDNase treatments of gastric mucin with calf thymus DNA and calf thymus DNA. Dialysis as described above was performed for 4 h at either 4°C or 37°C. A control, consisting of tobramycin alone in a dialysis bag, was included in each experiment. Samples (200 μl each) of the dialysates were taken at 0.5, 2, 5, 15, 30, 60, 120, and 240 min; the concentration of tobramycin in the samples was determined by fluorescence polarization immunoassay with the TDX apparatus (Abbott Laboratories, Dallas, Tex.). The standard curve was prepared in MB. The published within-run precision and day-to-day precision have a coefficient of variation of 5% or less at tobramycin concentrations of 1.0, 4.0, and 8.0 mg/liter. The sensitivity, according to the manufacturer's specification, is 0.18 mg/liter.

**Statistical methods.** Linear regression was performed with tobramycin efflux as the outcome measure and DNA concentration or gastric mucin concentration as the independent variable. The F ratio test was performed to test the slope's significance. Paired *t* tests were used to test the significance of the bacterial kill with and without DNase in the presence of tobramycin and tobramycin efflux with or without DNase. Paired tests were also performed at the 0- and 4-h time points for bacterial growth or kill. Statistical Package for Social Sciences software (SPSS Inc., Chicago, Ill.) was used in all analyses.

## RESULTS

**rhDNase activity on DNA within CF sputum.** Hydrolysis of sputum DNA with 10 μg of rhDNase per g of sputum for various incubation times is shown in Fig. 1. This sputum sample contained 7.4 mg of DNA per g. By 5 min, the majority of the high-molecular-weight DNA had been hydrolyzed, and by 30 min, the DNA had been hydrolyzed to fragments of <0.6 kb. We chose to treat the sputum with 10 μg of rhDNase per g of sputum for 15 min at 37°C.

**Effect of rhDNase treatment on tobramycin bioactivity.** To determine if rhDNase hydrolysis of the DNA within sputum would decrease the binding of tobramycin, we measured the biological activity of tobramycin in rhDNase-treated sputum and untreated sputum by performing killing curves with the dialysate. rhDNase treatment of the sputum did not significantly change the amount of bioactive tobramycin in the dialysates ($P > 0.1$): similar numbers of bacteria were killed with

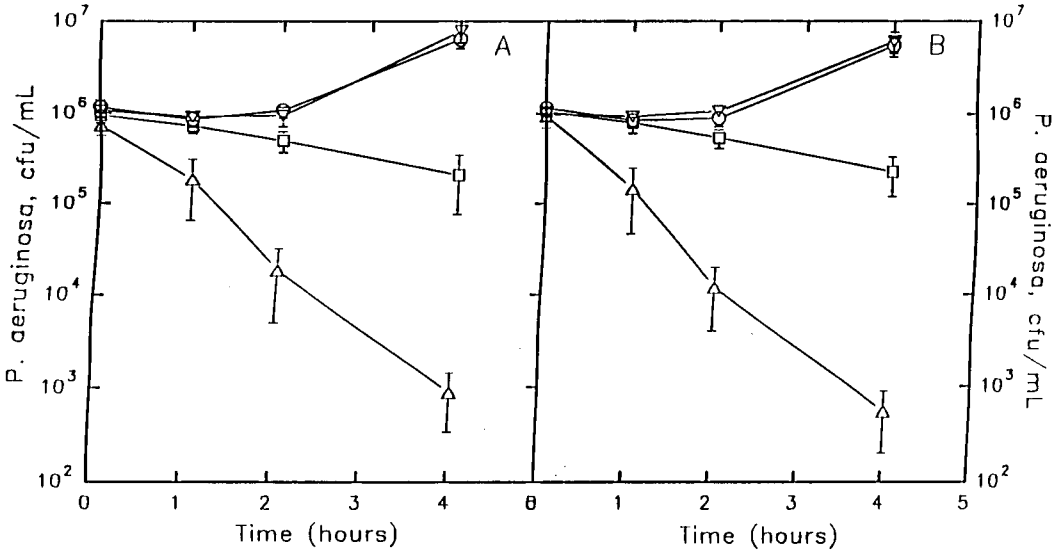


FIG. 2. Antipseudomonal activity in MB after dialysis against sputum from a single CF patient. The sputum was pretreated either with rhDNase (10 μg/g) (A) or with rhDNase diluent (B) at 37°C for 15 min. Tobramycin was added to the sputum to yield a final concentration of 0.4 (▽), 4.0 (□), or 40 μg/ml (△) in sputum and MB. Control growth curves (○) were determined in the absence of tobramycin. Datum points are the means of six experiments (six *P. aeruginosa* strains were tested; the MIC of tobramycin range was 0.25 to 2.0 μg/ml); the vertical bars indicate the standard errors of the means (SEM).

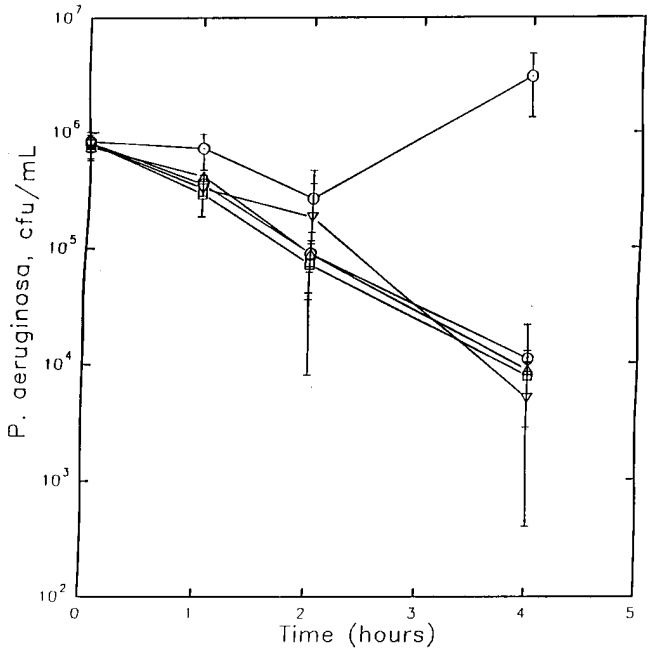


FIG. 3. Antipseudomonal activity in MB after dialysis against CF sputum containing tobramycin. Sputum samples were pretreated with rhDNase at 0 (○), 2 (▽), 10 (□), or 50 μg/g (△) at 37°C for 15 min. Tobramycin was added to the sputum to obtain a final concentration of 4 μg/ml in sputum and MB. Control growth curves (⊙) were determined in the absence of tobramycin. Datum points are the means of three experiments (sputum samples were obtained from three CF patients with DNA concentrations of 0.7, 1.5, and 4.8 mg/g [wet weight]); the vertical bars indicate the SEM. One *P. aeruginosa* strain (2549) was used for all three experiments (the MIC of tobramycin equaled 0.25 μg/ml).

treated and untreated sputum (Fig. 2). At 2 and 4 h, 7 of 8 means had higher bacterial counts with rhDNase. The number of CFU was reduced by only one-half an order of magnitude with 4 μg of tobramycin per ml and by 1,000-fold with 40 μg of tobramycin per ml. These two concentrations of tobramycin are 2 to 160 times greater than the MICs for the six *P. aeruginosa* strains tested. The same results were obtained when these experiments were performed with the same six strains of *P. aeruginosa* and sputum (homogenized with sputolysin) from a different patient (data not shown).

Increasing the rhDNase concentration to 50 μg did not significantly change ($P > 0.05$) the bactericidal effect of tobramycin in the 2-h dialysates (Fig. 3). Bactericidal effects similar to this were seen with 2, 5, and 10 μg of rhDNase per g and with sputum not treated with rhDNase (Fig. 3). The number of CFU with 4 μg of tobramycin per ml over 2 h was reduced only 100-fold. This concentration is 16 times greater than the MIC for the *P. aeruginosa* strain (2549) used in this experiment.

**Tobramycin efflux from CF sputum.** We examined the kinetics of tobramycin effluxes from rhDNase-treated sputum and untreated sputum at 4°C and 37°C over a 4-h period by fluorescence polarization immunoassay for two reasons: (i) to measure directly the concentration of free tobramycin in the dialysate and (ii) to confirm that the system was at equilibrium at the temperature and duration of dialysis we used for the killing curve experiments (4°C for 2 h) (Fig. 2). The sputum used in this experiment was obtained from the same patient whose sputum was used for the killing curves and contained 4.8 mg of DNA per g (wet weight).

At 4°C, the maximum tobramycin concentrations in the dialysates from both rhDNase-treated and untreated sputum samples were reached in 2 h (Fig. 4A). The peak tobramycin concentration in the rhDNase-treated sample (Fig. 4A, solid symbols) was similar to the concentration of the untreated sample (Fig. 4A, open symbols). These values represent concentrations about 14 to 114 times greater than the MICs of tobramycin for the *P. aeruginosa* strains used for the killing curves. At 37°C, tobramycin concentrations in both dialysates were similar to those at 4°C and were reached in 1 h (Fig. 4B). By 30 min, greater than 90% of the tobramycin added to the dialysis bag without sputum (Fig. 4A and B) had moved into the MB at 4°C and 37°C.

**Tobramycin efflux from mock sputum.** CF sputum is a complex matrix comprising many different types of molecules. To

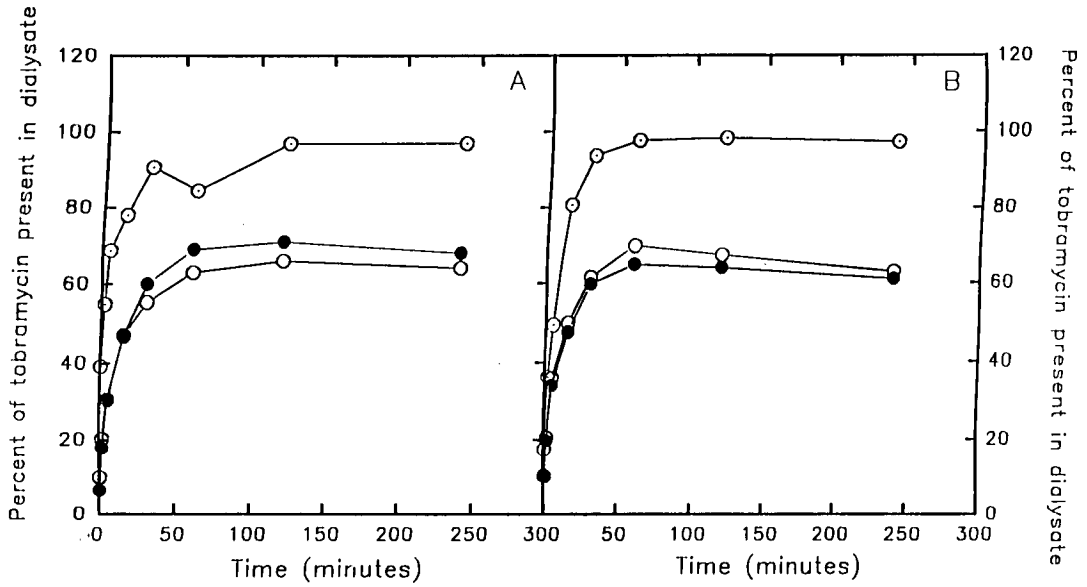


FIG. 4. Kinetics during dialysis at 4°C (A) and 37°C (B) of tobramycin efflux into MB from dialysis bags containing sputum from a single CF patient. The sputum (1 g) was pretreated with either 10 μg of rhDNase (solid circles) or rhDNase diluent (open circles) at 37°C for 15 min. Tobramycin was added to the sputum to obtain a final concentration of 40 μg/ml in the sputum and MB. Tobramycin efflux from the dialysis bag without sputum is indicated (⊙).

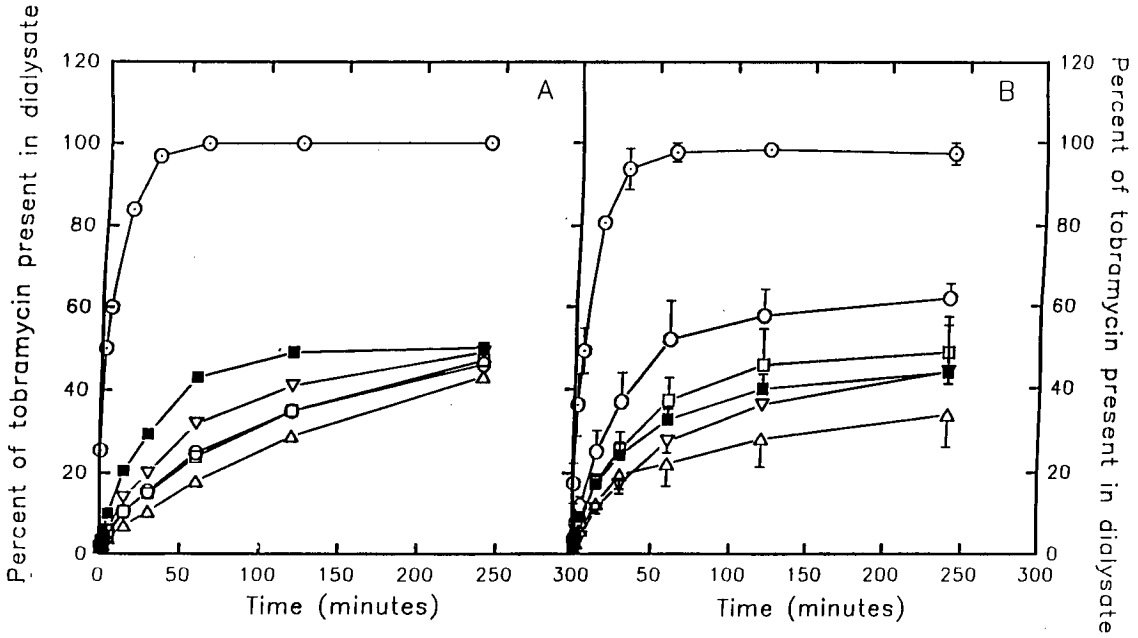


FIG. 5. Kinetics during dialysis at 37°C of tobramycin efflux into MB from dialysis bags containing 12.5% (wt/vol) gastric mucin with and without calf thymus DNA. The gastric mucin samples (1 ml) with and without DNA either were incubated with 10 μg of rhDNase for 15 min at 37°C (A) or did not receive the rhDNase treatment (B) before the addition of the tobramycin and dialysis of the samples: 12.5% gastric mucin (■); 12.5% gastric mucin with calf thymus DNA at 0.5 (○), 2.0 (▽), 4.0 (□), and 8.0 mg/ml (△). Tobramycin was added to the samples to obtain a final concentration of 40 μg/ml in the samples and MB. Tobramycin efflux from the dialysis bag without gastric mucin and calf thymus DNA is indicated (⊙). Datum points are either the results of one experiment (A) or the means of three experiments (B); vertical bars indicate the SEM.

gain insight into the mechanism of tobramycin binding, we examined the efflux of tobramycin from two major components of sputum: glycoproteins (mucins) and DNA.

A 12.5% (wt/vol) solution of gastric mucin, which consists of high-molecular-weight glycoproteins from porcine stomachs, and known amounts of added calf thymus DNA were used as mock sputum. Kinetic studies of tobramycin effluxes from gastric mucin with and without DNA and with and without rhDNase treatment were performed at 37°C over 4 h (Fig. 5A and B). At 4 h, in all samples of gastric mucin (with and without calf thymus DNA), the concentrations of free tobramycin in the MB appeared to reach near steady state (Fig. 5A and B). The mean tobramycin concentration was regressed against the DNA concentration at each separate time point. The slopes were not significantly ($P > 0.05$) different from zero in every case. Also, by a paired $t$ test, the effluxes of tobramycin in untreated samples were significantly higher than those in treated samples ($P < 0.05$). In other words, more binding of tobramycin was seen with rhDNase treatment.

**Tobramycin efflux from calf thymus DNA and gastric mucin.** The effluxes of tobramycin from calf thymus DNA alone and from gastric mucin alone were also examined. More tobramycin was observed in the MB with DNA alone than with DNA with 12.5% gastric mucin (Fig. 5A and B and Fig. 6A and B). This suggests that mucin may play a role in tobramycin binding. Again, the DNA concentration was regressed against the tobramycin concentration in MB. At 4 h, the slope was significantly different from zero with an $r^2$ value of 0.93 in untreated samples. This significance was not seen at 2 h. Regression with treated samples was also not significant. Again, paired $t$ tests demonstrated significantly higher effluxes of tobramycin in untreated samples.

After 4 h of dialysis, tobramycin was still moving into the MB from all concentrations of gastric mucin (Fig. 7). The amount of free tobramycin in the dialysates was dependent on the concentration of gastric mucin. The percentages of gastric mucin were regressed against the tobramycin concentrations in MB at 2 and 4 h. The slopes were significant, with $P$ values of 0.02 and 0.01, respectively, and the $r^2$ values were 0.966 and 0.983, respectively. When tobramycin was again added to the dialysis bag without gastric mucin, greater than 90% of the tobramycin appeared in the dialysate by 30 min (Fig. 7).

## DISCUSSION

In vitro, rhDNase (at a concentration of 1 μg/g of sputum) hydrolyzes high-molecular-weight sputum DNA: this is associated with a reduction in sputum viscosity by one-half within 15 min (22). We have shown that the concentration of rhDNase and the duration of rhDNase treatment we chose to use (10 μg/g for 15 min) were sufficient to effectively hydrolyze DNA within the sputum samples used in this study (Fig. 1).

Tobramycin binds to components of sputum; hence its bioactivity in sputum is reduced (12, 15, 18). Since DNA makes up 3 to 10% (5, 16) of sputum (dry weight), we sought to determine if treating sputum with rhDNase would reduce the amount of tobramycin binding. When *P. aeruginosa* killing curves were performed for sputum dialysates, we found that the amount of bioactive tobramycin was not increased when the sputum (unmanipulated or homogenized) was treated with rhDNase. We also determined that increasing the rhDNase concentration fivefold did not reduce the antagonism of tobramycin bioactivity.

Since biological assays for antibiotic activity (e.g., killing curves) measure the free antibiotic indirectly with some inherent error, we also measured the concentrations of free tobramycin in the dialysates directly by fluorescence polarization immunoassay. Our studies of tobramycin efflux from whole

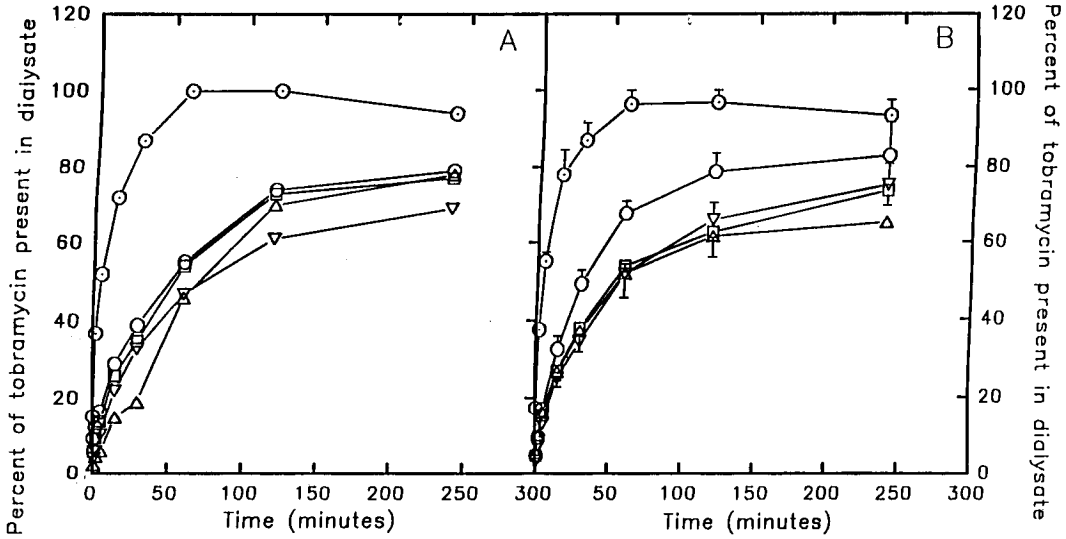


FIG. 6. Kinetics during dialysis at 37°C of tobramycin efflux into MB from dialysis bags containing calf thymus DNA. Calf thymus DNA samples (1 ml) either were incubated with 10 μg of rhDNase at 37°C for 15 min (A) or did not receive the rhDNase treatment (B) before the addition of the tobramycin and dialysis. The DNA concentrations were 0.5 (○), 2.0 (▽) 4.0 (□), and 8.0 mg/ml (△). Tobramycin was added to the DNA to obtain a final concentration of 40 μg/ml in the DNA and MB. Tobramycin efflux from the dialysis bag without calf thymus DNA is indicated (⊙). Data points are either the results of one experiment (A) or the means of three experiments (B); vertical bars indicate the SEM.

sputum showed that there was a decrease in the amount of free tobramycin when the sputum was treated with rhDNase. Aminoglycoside bioactivity is known to be affected by the environmental milieu, which includes such variables as pH (3, 4, 9), ionic strength (2, 12, 14), and divalent cations (2, 7–10, 12, 19, 24). Our data suggest that the reduced bioactivity of tobramycin in the killing curve experiment (Fig. 2A) was probably not due to antagonism by cations or the pH within the dialysate; this result most likely was due to the fact that tobramycin was also binding to mucins (18, 20), which make up 2 to 3% of the dry weight of sputum (13).

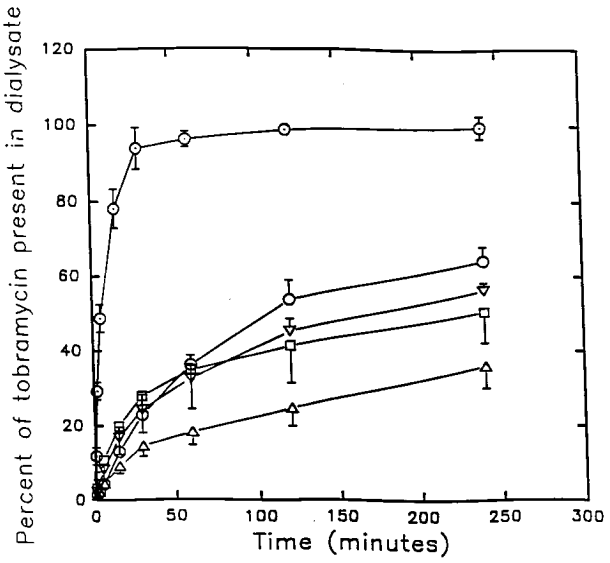


FIG. 7. Kinetics during dialysis at 37°C of tobramycin efflux into MB from dialysis bags containing gastric mucin at the following percentages (wt/vol): 5 (○), 7.5 (▽), 12.5 (□), and 20% (△). Tobramycin was added to the gastric mucin to obtain a final concentration of 40 μg/ml in the gastric mucin and MB. Tobramycin efflux from the dialysis bag without gastric mucin is indicated (⊙). Data points are the means of three experiments; vertical bars indicate the SEM.

Bryant and Hammond (4) found that the reduction of aminoglycoside activity by purulent material was the same at 4°C and 37°C. We found that tobramycin binding to sputum was not temperature dependent and that the tobramycin concentration within the dialysate had reached a plateau in 2 h, confirming that our system was in equilibrium when we performed the *P. aeruginosa* killing curves.

We used mock sputum (12.5% gastric mucin with calf thymus DNA at 0, 2, 4, and 8 mg/ml) to determine if higher concentrations of DNA would increase the amount of tobramycin binding and to try to separate the effects of mucins and DNA on tobramycin binding. Our data suggest that tobramycin binding was not dependent on the concentration of DNA with gastric mucin. rhDNase treatment of the gastric mucin with added DNA decreased the amount of free tobramycin, suggesting that mucin played a larger role in binding tobramycin than did the DNA and that hydrolyzed DNA products may bind tobramycin better than high-molecular-weight DNA. Results from the study of DNA alone support this hypothesis. We found that tobramycin binding was dependent on the concentration of gastric mucin, which is in agreement with the results of Ramphal et al. (18). However, Ramphal et al. (18) also showed that the amount of free tobramycin increased by about 30% when a DNA-rich fraction of sputum was treated with 3.3 μg of DNase (bovine) per ml for 6 h. We found an increase of tobramycin binding in our mock sputum and no significant change in the antibacterial effect in sputum treated with rhDNase.

Our data demonstrated that although the rhDNase had hydrolyzed the high-molecular-weight DNA within sputum, the tobramycin bioactivity remained low. Possible explanations are that (i) the tobramycin continued to be bound to the hydrolyzed DNA and/or (ii) the tobramycin that may have been released after rhDNase treatment may be bound to the mucin component of sputum. This study does not demonstrate any in vitro synergism between rhDNase and tobramycin.

## ACKNOWLEDGMENTS

This work was supported by NIH grant HL50253 and in part by the Cystic Fibrosis Foundation.

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