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Selective Inhibition of the Accumulation of Extracellular Proteases of *Pseudomonas aeruginosa* by Gentamicin and Tobramycin

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Gentamicin and tobramycin inhibited the accumulation of extracellular proteases secreted by *Pseudomonas aeruginosa*. The secretion of protease was inhibited at concentrations of these drugs that were below the level required to inhibit general protein synthesis. Neither magnesium ions nor high ionic strength antagonized the ability of the aminoglycosides to block secretion of the proteases. Under these culture conditions magnesium ions were shown to antagonize the effects of the aminoglycosides on protein synthesis and aminoglycoside-mediated lysozyme lysis of *P. aeruginosa*. These results suggested that the drugs blocked secretion of the proteases by acting at the level of the outer membrane.

One of the earliest steps in the uptake of aminoglycoside antibiotics is the ionic binding to the outer membrane (8). This step is antagonized by divalent ions (3). Hancock et al. (10) demonstrated that the initial binding of gentamicin increased the permeability of the outer membrane of *Pseudomonas aeruginosa*. The effect of the drug on the outer membrane was antagonized by magnesium ions or by the overproduction of an outer membrane protein, H1 (10, 20, 21). The permeabilization of the outer membrane by gentamicin occurred rapidly and under conditions that blocked uptake of the drug into the bacteria (10, 20, 21).

In this study we report another effect of the initial binding of gentamicin and tobramycin to the outer membrane of *P. aeruginosa*. With concentrations of the drugs that had no significant effect on either growth or protein synthesis, the secretion of extracellular protease by *P. aeruginosa* was inhibited. The ability of these drugs to inhibit the accumulation of extracellular proteases in culture supernatant fluids was not significantly antagonized by experimental conditions shown to inhibit some of the earliest effects of the drugs on *P. aeruginosa* (8).

MATERIALS AND METHODS

Bacterial strain and media. The *P. aeruginosa* strain used in this study was PAO1161 (*leu*$^-$ FP2$^+$) obtained from B. Holloway, Department of Genetics, Monash University, Clayton, Victoria, Australia. FP2 is a transmissible plasmid. Peptone broth dialysate (PBD) medium was prepared by dissolving 20 g of Bacto-Peptone (Difco Laboratories, Detroit, Mich.) in 100 ml of distilled water and dialyzing this solution against 900 ml of deionized distilled water overnight at 4°C. Then, 1.4 g of $MgSO_4$, 10 g K_2SO_4, and 20 ml of glycerol were added to the peptone dialysate, the pH was adjusted to 8.0, and the volume was brought to 1 liter. Nutrient broth (NB; Difco) was prepared according to the instructions of the manufacturer. Magnesium ions were added to NB from a stock solution of sterile 1 M $MgSO_4$. All cultures were grown at 37°C with vigorous shaking. The *P. aeruginosa* cells were grown overnight in PBD broth. The cells were harvested and washed twice in either NB or PBD broth and resuspended in a volume equal to the volume of the overnight culture. The optical density at 550 nm of the starting cultures was 0.8.

Antibiotics and reagents. Gentamicin sulfate was purchased from Sigma Chemical Co., St. Louis, Mo. Tobramycin was a generous gift from Eli Lilly Co., Indianapolis, Ind.

Competition enzyme-linked immunoassay. A two-step competition enzyme-linked immunoassay was used to quantitate the amount of *Pseudomonas* elastase in culture supernatants. The methods used for this assay were described by Engvall (5). Microtiter plates were coated overnight with 0.2 ml per well of 1,000 ng of elastase per ml in 0.05 M carbonate buffer (pH 9.6). In separate tubes, serial twofold dilutions of purified elastase ranging from 200 to 3.12 ng/ml were prepared in phosphate-buffered saline (NaCl, 8 g; KH_2PO_4, 0.2 g; Na_2HPO_4, 1.15 g) supplemented with 0.05% Tween 20 and 1% bovine serum albumin (Fraction V; Sigma). Samples of culture supernatant fluids were diluted in phosphate-buffered saline supplemented with Tween 20 and bovine serum albumin. Equal volumes of either purified elastase or the culture supernatant fluid samples were mixed with a 1:1,000 dilution of rabbit anti-elastase antibody. These antigen antibody mixtures were placed on ice for 1 h. A 0.2-ml sample of the antigen-antibody mixture was added to the precoated microtiter wells. After 2 h the wells were washed three times with phosphate-buffered saline to remove any rabbit antibody that did not bind to the immobilized elastase. A 0.2-ml sample of a 1/500 dilution of goat anti-rabbit immunoglobulin G alkaline phosphatase-conjugated antibody (Sigma) was added to each well. The plate was then incubated for an additional hour at room temperature. The plate was washed three times with phosphate-buffered saline to remove any unbound conjugated antibody. Alkaline phosphatase substrate (1 mg of *p*-nitrophenylphosphate per ml in 10% diethanolamine) was added to each well in a volume of 0.2 ml. After 30 min of incubation at room temperature, the reaction was stopped by adding 0.05 ml of 3 M NaOH. The absorbance of each well was measured at 405 nm by using a microELISA reader (Dynatech Laboratories, Inc., Alexandria, Va.). Each dilution of the samples was run in triplicate. Anti-elastase antibody was prepared by injecting New Zealand white rabbits with purified elastase (Nagase, Ltd., Osaka, Japan) as described previously (26).

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Assay for total proteolytic activity. Proteolytic activity was determined by using the azocasein assay as described previously (14). Activity was expressed as the change in A_{390} units per milliliter after 60 min of incubation at 37°C. The activity was reported as the mean of six determinations per sample with the standard deviation.

Measurement of protein synthesis. The washed cells were resuspended in 5 μCi of [^{3}H-]leucine (4,5-^{3}H; specific activity, 2 Ci/mmol; New England Nuclear Corp., Boston, Ma.). Three 50-μl samples of the cells were removed and spotted on Whatman 3MM filter paper, and the acid-insoluble counts were determined after washing these filters with 10% trichloroacetic acid. The results were expressed as the mean counts per minute of these three samples with the standard deviation.

Statistical analysis. The rates of incorporation of [^{3}H-]leucine into acid-insoluble material was determined by linear regression analysis with a 95% confidence limit. This value was determined by multiplying the standard error of the regression coefficient by the t value at $P = 0.05$ with $n - 2$ degrees of freedom. Difference between the rates was determined by Student's t test.

RESULTS

Effects of gentamicin and tobramycin on the accumulation of extracellular protease in culture supernatants. The effect of subinhibitory concentrations of tobramycin or gentamicin on the accumulation of extracellular protease was tested on washed *P. aeruginosa* cells resuspended in fresh PBD broth. The concentration of drug used ranged from 0.05 to 1.0 μg/ml. In PBD broth the MIC of both drugs was 4 to 8 μg/ml, and the MBC was 125 to 250 μg/ml. Gentamicin had no significant effect on the rate of growth or the rate of incorporation of [^{3}H-]leucine into protein (Fig. 1A and B). However, after 180 min of incubation, the amount of total active protease and elastase antigen released into the PBD medium containing 0.5 μg of gentamicin per ml was 23 to 24% of that released by cells grown in antibiotic-free PBD medium (Table 1). The addition of 0.05 or 0.1 μg/ml to cells growing in PBD medium had no significant effect on the production of extracellular proteases ($P = 0.05$).

Concentrations of 0.5 or 0.1 μg of tobramycin per ml inhibited both the rate of growth and the protein synthesis of *P. aeruginosa* (Fig. 1C and D). The viability of the cells after 150 min of incubation with 0.5 or 0.01 μg of tobramycin per ml was not significantly different from that of the control cells ($P = 0.05$). The amount of [^{3}H-]leucine incorporated into protein after 150 min of incubation with either 0.05 or 0.01 μg of tobramycin per ml was 85% that of the untreated cells (Table 1). The secretion of total protease was 6% or less of that secreted by the control cells, whereas the secretion of elastase was 12% that of the control cells (Table 1).

Effect of gentamicin and tobramycin on the outer membrane. It was possible that the aminoglycosides blocked the accumulation of extracellular proteases by altering the permeability of the outer membrane of *P. aeruginosa*, rather than selectively affecting the synthesis of the proteases. Hancock et al. (10) demonstrated that gentamicin promoted lysozyme lysis of *P. aeruginosa*. The drug increased the permeability of the outer membrane, and this allowed lysozyme access to the cell wall. The addition of lysozyme and gentamicin to cells resuspended in PBD had no effect on the rate of growth or protein synthesis (Fig. 2A and B). There appeared to be no aminoglycoside-promoted lysozyme lysis of these cells.

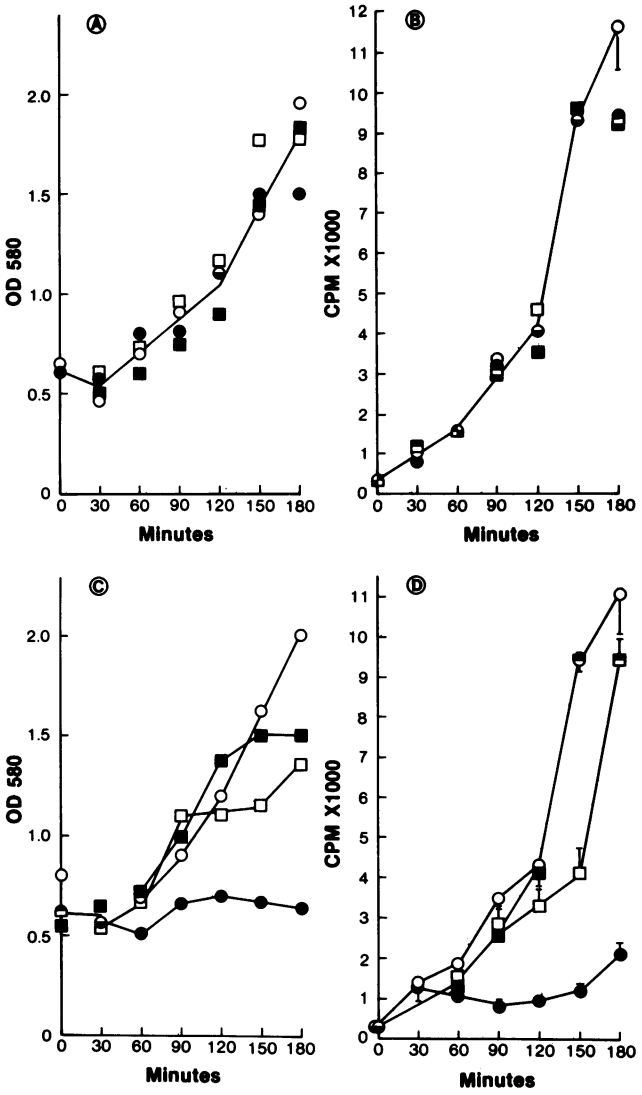

FIG. 1. Effect of sublethal concentrations of aminoglycosides on the rate of growth and protein synthesis. *P. aeruginosa* PAO1161 was grown overnight (18 h) in PBD. The cells were harvested washed twice with fresh PBD broth and then split into eight equal samples. The cells were then resuspended in PBD broth supplemented with gentamicin (A and B) or tobramycin (C and D) at concentrations of 0 μg/ml (○), 0.05 μg/ml (■), 0.1 μg/ml (□), and 0.5 μg/ml (●). The optical density (OD) of the culture was measured every 30 min. Three 50-μl samples were removed every 30 min, and the amount of [^{3}H-]leucine incorporated into acid-insoluble material was determined. The standard deviation of the mean was plotted as a bar from the symbol. Symbols without error bars had a standard deviation less than the range of values covered by the symbol.

It was possible that the relatively high concentration of magnesium (14.7 mM) in the PBD medium blocked the ability of the aminoglycosides to increase the permeability of these cells. Alternatively, growth of *P. aeruginosa* in PBD could result in the overproduction of an outer membrane protein like H1 (10, 20, 21), which has been shown to block disruption of the outer membrane by this drug. It was unlikely that the cells would be overproducing H1 protein in PBD medium since this outer membrane protein appears to be made under conditions of limited magnesium (10). When the cells were washed and resuspended in nutrient broth

(Fig. 2C and D), gentamicin inhibited both growth and protein synthesis. The cells incubated with both gentamicin and lysozyme showed a decline in the optical density of the culture medium with time. After 120 min, these cells were completely lysed, and there were no signs of clumping in this flask. The addition of lysozyme alone had no effect on either the rate of growth or protein synthesis. Since gentamicin completely blocked protein synthesis of the cells resuspended in NB, few if any changes could occur in the outer membrane composition. These cells were susceptible to aminoglycoside-promoted lysozyme lysis, suggesting that no physiological change associated with growth in PBD was responsible for the failure of the aminoglycosides to increase the permeability of the outer membrane. Either the high ionic strength (114.8 mM K_2SO_4) or the high concentration of magnesium (14.7 mM) in PBD medium could inhibit the aminoglycoside promoted lysozyme lysis of these cells.

Effect of magnesium ions on the inhibition of protease accumulation. To demonstrate that the inhibition of the accumulation of extracellular protease by the aminoglycosides was not antagonized by magnesium ions, *P. aeruginosa* cells grown in PBD broth were washed and resuspended in nutrient broth, a medium low in both ionic strength and magnesium ion content (10). As shown in Fig. 3, 1.0 μg of tobramycin per ml of NB inhibited protein synthesis after a lag of 120 min. Cells resuspended in NB without tobramycin continued to synthesize protein for ca. 210 min. Supplementing the NB with 15 mM $MgSO_4$ or 15 mM $MgSO_4$ and 1.0 μg of tobramycin per ml had no significant effect on the rate of protein synthesis as compared with the cells resuspended in NB ($P = 0.05$). After 240 min the culture supernatant fluids were collected, and the protease activity was determined. Tobramycin antagonized the production of extracellular protease in both the low-magnesium nutrient broth (20% of the control) and the high-magnesium nutrient broth (26% of the control) (Fig. 3, insert). This demonstrated that tobramycin antagonized the production of extracellular proteases under culture conditions that had been demonstrated to block the disruption of the outer membrane of *P. aeruginosa* by these drugs.

TABLE 1. Effects of gentamicin and tobramycin on the accumulation of extracellular protease by *P. aeruginosa* cells growing in PBD medium[a]

Antibiotic (μg/ml)	Effects as measured by:		
	[^{3}H-]leucine (cpm)[b]	Azounits/ml[c]	Elastase (ng/ml)[d]
Gentamicin			
0	11,562 ± 1043	4.9 ± 0.2	171 ± 4
0.5	9,255 ± 91	1.2 ± 0.1	39 ± 5
0.1	9,132 ± 183	4.1 ± 0.1	188 ± 9
0.05	9,191 ± 183	4.1 ± 0.1	171 ± 4
Tobramycin			
0	11,096 ± 1049	5.3 ± 0.6	188 ± 9
0.5	2,102 ± 348	0.1 ± 0.2	21 ± 21
0.1	9,468 ± 35	0.3 ± 0.4	14 ± 7
0.05	9,451 ± 483	0.2 ± 0.1	23 ± 20

[a] Samples were collected after 150 min of incubation at 37°C.
[b] The incorporation of [^{3}H-]leucine into acid-insoluble material is expressed as counts per minute.
[c] Azounits, the change in A_{390} units per milliliter of culture supernatants after 1 h of incubation at 37°C of azocasein substrate.
[d] Concentration in culture supernatants, as determined by competition enzyme-linked immunoassay.

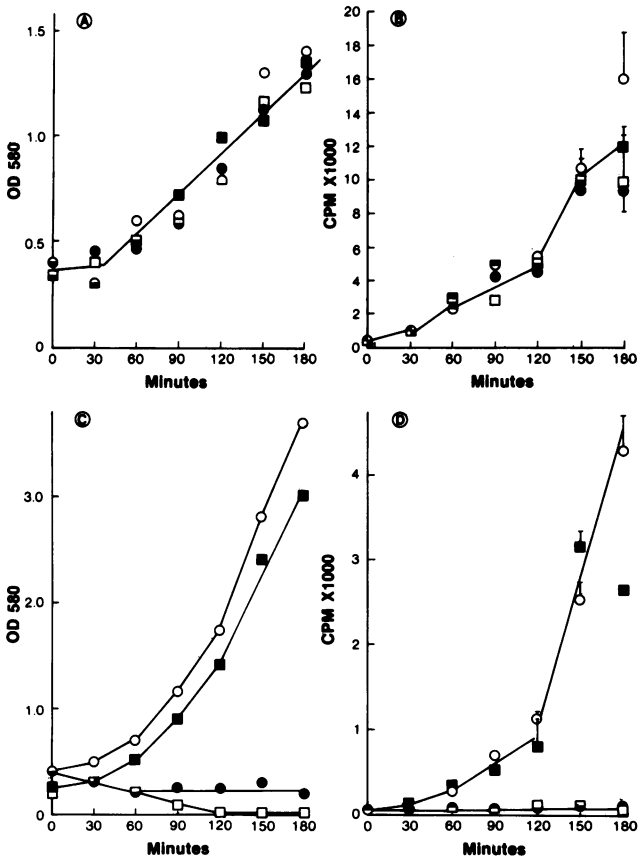

FIG. 2. Effect of medium composition on aminoglycoside-mediated lysozyme lysis. Overnight bacterial cells were prepared as described in the legend to Fig. 1. Half the cells were resuspended in PBD broth (A and B), and half were resuspended in NB (C and D). The medium was used without supplementation (○) or supplemented with 0.5 μg of gentamicin per ml (●), 0.5 μg of gentamicin per ml and 20 μg of lysozyme per ml (□), or 20 μg of lysozyme per ml (■). The optical density (OD) and the amount of [^{3}H-]leucine incorporated into acid-insoluble material were determined as described in the legend to Fig. 1.

DISCUSSION

P. aeruginosa is an opportunistic pathogen which causes fatal infections in immunocompromised hosts (6, 13, 19, 23) and in individuals with cystic fibrosis (12). This microorganism secretes a number of extracellular enzymes that contribute to its pathogenesis, including at least two proteases, elastase and alkaline protease. Of these two proteases, purified elastase has been shown to contribute to tissue damage caused during *Pseudomonas* pneumonias both in in vivo (4, 13, 22, 25, 29) and in vitro studies (2, 7, 16, 18).

Pseudomonas extracellular enzymes must be exported from the cytoplasm to the extracellular environment. There appear to be at least two different mechanisms used to secrete these proteins. Elastase is synthesized as a inactive precursor which accumulates in the periplasmic space before activation and release into the culture medium (15). Exoport of elastase requires that the protein cross both the cytoplasmic and the outer membrane. However, exotoxin A does not accumulate as a precursor in any cell compartment (17). Lory et al. (17) suggested that exotoxin A was exported through both membranes at membrane fusion sites called

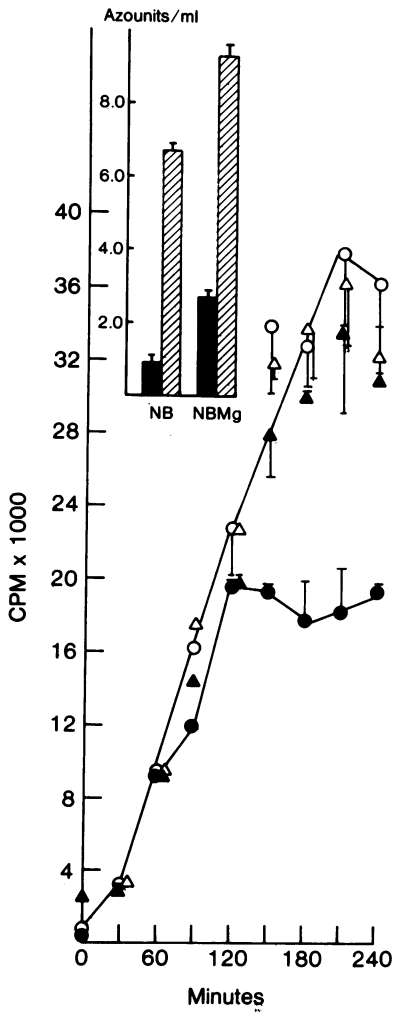

FIG. 3. Effect of magnesium ions on the inhibition of extracellular protease accumulation by tobramycin. Overnight bacterial cells were prepared as described in the legend to Fig. 1. Equal samples of cells were resuspended in either NB or NB supplemented with 15 mM magnesium ions (NBMg). Three 50-μl samples were removed every 30 min, and the amount of [^{3}H-]leucine incorporated into acid-insoluble material was determined. After 240 min of incubation, the cells were removed by centrifugation, and the culture supernatants were passed through a 0.45-μm filter. The total protease activity in the culture supernatant fluid was determined by the azocasein assay. Solid bars represent samples with tobramycin, and cross-hatched bars represent samples without tobramycin. Symbols: ○, NB; ●, NB + 1.0 μg of tobramycin per ml; △, NBMg; ▲, NBMg + 1.0 μg of tobramycin per ml.

Bayer patches. The mechanism by which alkaline protease is exported is not known.

This study showed that the accumulation of extracellular protease (elastase) was markedly inhibited by sublethal concentrations of tobramycin and gentamicin (Table 1). Neither high ionic strength nor high magnesium ion concentrations affected the ability of these antibiotics to block the accumulation of extracellular proteases in culture supernatant (Fig. 3). Both these conditions have been shown to inhibit binding of the drugs to the outer membrane of the cells and to antagonize the lethal effects of these drugs (3, 8). These results suggested that gentamicin and tobramycin inhibited the secretion of extracellular proteases by acting on the outer membrane.

The initial binding of these drugs has been shown to alter outer membrane permeability (10, 11, 20, 21) and to reduce the intercellular levels of cyclic 3′,5′-AMP (1). The effect on membrane permeability was independent of the energy-dependent phase I uptake of the drug (10) and could be completely blocked by the addition of 1 mM magnesium ions. Hancock et al. (10) suggested that passive permeation of the aminoglycoside through the hydrophilic pore of the outer membrane was not the major pathway for uptake of these antibiotics by *P. aeruginosa*; rather, the uptake and killing of aminoglycosides by *P. aeruginosa* required the interaction of the antibiotic with a magnesium-binding protein of the outer membrane. It was this interaction that resulted in the increased permeability of the outer membrane. We demonstrated that the gentamicin blocked secretion of extracellular proteases under conditions that did not increase the permeability of the outer membrane.

Pinkett and Brownstein (24) reported that the synthesis of β-galactosidase by *Escherichia coli* was inhibited by concentrations of streptomycin that failed to inhibit total protein synthesis. In subsequent studies Artman et al. (1) demonstrated that this selective inhibition of β-galactosidase could be reversed by the addition of cyclic AMP to the growth medium. β-galactosidase is a cytoplasmic enzyme, and its expression is dependent upon the levels of cyclic 3′,5′-AMP in the cell. This selective inhibition of β-galactosidase is similar to the observed effects of gentamicin and tobramycin on the accumulation of extracellular *P. aeruginosa* proteases. It is possible that the aminoglycosides affected secretion of extracellular proteases by reducing the levels of cyclic nucleotides of the *P. aeruginosa* cells rather than selectively inhibiting protein synthesis. Unlike β-galactosidase, the production of extracellular proteases by *P. aeruginosa* does not appear to be controlled by catabolic repression (27, 28).

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