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Inability of Purified *Pseudomonas aeruginosa* Exopolysaccharide to Bind Selected Antibiotics

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It has been proposed that the exopolysaccharide (alginate) of mucoid *Pseudomonas aeruginosa* strains which infect cystic fibrosis patients might bind and hence protect this pathogen from antibiotics. To test this hypothesis, we employed equilibrium dialysis to measure the binding between several antibiotics and purified *Pseudomonas* alginate. Binding was calculated from the residual concentrations of antibiotics in free solution by a biological assay. The detectable binding of antibiotics to alginate was consistent with expectations; the positively charged antibiotics steptomycin and tobramycin, bound to the polyanion (0.047 and 0.024 μmol/mg of alginate, respectively), whereas the neutral species, clindamycin and penicillin, bound negligibly or not at all (0.0011 and 0 μmol/mg of alginate, respectively). When these experiments were performed in the presence of physiological concentrations of saline, none of the antibiotics bound to the polysaccharide. Since the binding observed was abrogated by salt concentrations typical of the tracheobronchial secretions of cystic fibrosis patients, the data suggest that tight binding of antibiotics to the exopolysaccharide of a mucoid *P. aeruginosa* strain does not provide increased antibiotic resistance.

Pseudomonas aeruginosa is commonly found in patients with cystic fibrosis and other chronic lung diseases, particularly in diseases associated with bronchiectasis (8, 9, 19, 25). Patients are apparently colonized first with nonmucoid strains of *P. aeruginosa*, but as the disease progresses to its chronic stage, mucoid variants emerge (7). The mucoid capsular material of these *P. aeruginosa* strains is a heteropolymer of mannuronic and guluronic acid moieties (alginate) (14, 15). It has been proposed that this polyanionic exopolysaccharide binds certain antibiotics (possibly in a manner analogous to that of a cation exchange column), thereby preventing them from reaching the cell surface and thus conferring antibiotic resistance to the mucoid strains (5, 6, 10). Such a barrier function is supported by the demonstration of a retardation of diffusion of netilmicin through agar growth media seeded with crude *P. aeruginosa* exopolysaccharide (20). Studies in which whole bacteria have been employed have generated data both supporting and refuting the hypothesis that mucoid pseudomonad strains are more resistant than nonmucoid strains to various antibiotics (1, 11, 17). Refuting the hypothesis described above are data indicating a heterogeneity of the response of mucoid pseudomonad strains to antibiotics and a correlation of increased antibiotic susceptibility, not to the mucoid nature of the organism, but to the presence of a membrane protein(s) (12).

However, all of the studies in which whole bacterial cells have been used to assess the protective nature of the alginate are difficult to interpret. For antibiotic susceptibility to be tested, the cells must first be suspended in saline before they are brought into contact with the antibiotic. This process of cell suspension, in our hands, sheared off the very polysaccharide coating that might serve as the putative protective barrier (C. Tannenbaum, personal observation). Thus, with the putative barrier removed, antibiotics would have free access to their targets, thereby precluding the ability to truly assess the actual contribution of the exopolysaccharide to resistance. To circumvent this problem, we employed equilibrium dialysis to measure the affinity of several antibiotics for alginate purified from a mucoid strain of *P. aeruginosa* isolated from a cystic fibrosis patient. Such affinity studies would demonstrate the potential of the exopolysaccharide to protect bacteria from antibiotics, independent of possible changes in membrane composition and β-lactamase levels that confuse interpretation when whole cells of *P. aeruginosa* are used.

MATERIALS AND METHODS

A clinical isolate of mucoid *P. aeruginosa* was obtained from the sputum of a 21-year-old patient with cystic fibrosis (St. Christopher's Hospital for Children, Philadelphia, Pa.). Colorless (i.e., non-lactose-fermenting), oxidase-positive mucoid colonies from MacConkey agar plates were identified as *P. aeruginosa* by the two-tube selective-growth procedure (N/F System; Flow Laboratories, Roslyn, N.Y.). Nonmucoid *P. aeruginosa* colonies were derived by serial passages of the primary mucoid isolates on the medium of Sokol et al. (21), a formulation enhancing the discernment of various morphotypes of *P. aeruginosa* (13). Using the standard tube dilution technique for determination of MICs of antibiotics, we found the nonmucoid derivative of *P. aeruginosa* to be more susceptible than the parent mucoid strain to all four of the antibiotics tested. (For tobramycin, streptomycin, clindamycin, and penicillin, the MICs for the nonmucoid and mucoid *P. aeruginosa* strains were, respectively [in micrograms per milliliter], 0.030 and 0.244; 3.9 and 62.5; 1,000 and >4,000; and 125 and >4,000.) Mucoid material was obtained in sufficient quantity for binding studies by growing lyophilized mucoid isolates in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) to 12 h post-stationary phase (optical density at 675 nm, 1.70). Cells were removed from the medium by centrifugation at 25,000 × *g* for 40 min, and the supernatant was precipitated with 3 volumes of isopropanol. Exopolysaccharide was purified by performing three rounds of 95% ethanol precipitation, lyophilization, and resuspension in doubly distilled water (pH 7.4). To maximize binding capacity and minimize viscosity of the

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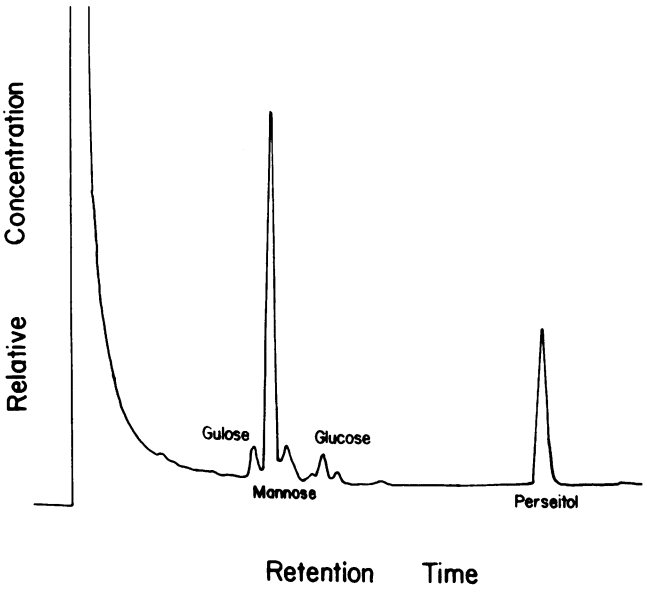

FIG. 1. Gas-liquid chromatography of reduced, hydrolyzed, and derivatized *P. aeruginosa* exopolysaccharide. A total of 3.2 nmol of a reduced, hydrolyzed, and Tri-Sil "Z"-derivatized alginate sample was chromatographed together with 1.6 nmol of the internal standard, perseitol. Two peaks eluted at the relative retention times characteristic of the two (α and β) mannose peaks; another peak eluted at a relative retention time that is the arithmetic mean of the retention time expected for the two gulose peaks. These data indicate that, as expected, mannose and gulose are components of the reduced *P. aeruginosa* alginate. Two peaks with relative retention times characteristic of glucose (0.534 and 0.5635, respectively) also eluted from the gas chromatograph when the exopolysaccharide was analyzed. Glucose is apparently produced when 1,6-anhydrogulose (one of the two products formed when guluronic acid is chemically reduced with carbodiimide and sodium borohydride) interacts with the derivatizing reagent Tri-Sil "Z". Formation of the glucose derivative from guluronic acid suggests that the ratio of mannuronic acid to guluronic acid of this alginate is approximately 8:1 and that the polyuronide is free of detectable levels of extraneous, contaminating polysaccharides.

mucoid exopolysaccharide, we chelated the divalent cations associated with the polyuronide and removed them by first resuspending 25 mg (dry weight) of the polymer in 10 ml of water containing 50 mM NaCl and 25 mM EDTA (pH 7.4) and then dialyzing it for 24 h against five changes of glass-distilled water (pH 7.4). The final product was shown spectrophotometrically (absorbance measured at 280 and 260 nm) and colorimetrically (2, 16) to have less than 2% protein or nucleic acid contamination. Gas-liquid chromatography was performed on a model 3200 instrument (Varian Associates, Inc., Palo Alto, Calif.) equipped with a flame ionization detector to determine the composition of the alginate. Purified mucoid material was chromatographed after reduction, hydrolysis (2 M HCl in MeOH, 12 h, 100°C), and formation of the trimethylsilyl-derivatized neutral sugars (23, 24). The purified mucoid material was found to be a heteropolymer of mannuronic and guluronic acid moieties present in a ratio of approximately 8:1 (see Fig. 1).

Analysis of the isolated alginate by gas-liquid chromatography also indicated that 8.7% of the precipitated, reduced, and hydrolyzed exopolysaccharide was composed of glucose (Fig. 1). Since alginate is a polymer of mannuronic and guluronic acid moieties, reduction and hydrolysis of the polymer would be expected to yield mannose and gulose but not glucose monomers. However, glucose is produced when 1,6-anhydrogulose (one of the two products formed when guluronic acid is chemically reduced with carbodiimide and sodium borohydride [22]) interacts with the derivatizing reagent Tri-Sil "Z" (Pierce Chemical Co., Rockford, Ill.). This is supported by our inability to observe glucose before reduction. The results of gas-liquid chromatography indicate, therefore, that lipopolysaccharide or other contaminants were below the level of detection.

To determine the ability of purified mucoid material to bind antibiotics, we performed equilibrium dialysis experiments. In these experiments, the pH of all fluids was adjusted to 7.4 with 0.1 M HCl or 0.1 M NaOH and, within detectable limits, did not change during the period of binding. Dialysis bags (exclusion limit, 12,000 in molecular weight) containing either 2 ml of water (as a control) or 5 mg of purified alginate in a final volume of 2 ml were placed in tubes containing 9 ml of a known concentration of antibiotic. All four antibiotics subjected to equilibrium dialysis with alginate were used in concentrations favoring the detection of even minimal amounts of binding; in all experiments, the antibiotic concentration was such that there was a large molar excess of ionized uronic acid monomers in relation to antibiotic molecules. Antibiotic concentrations of 200 μg of streptomycin, 10 μg of tobramycin, 7.5 μg of clindamycin, and 7.5 μg of penicillin per ml resulted in potential binding site/antibiotic molecule ratios of 9:1, 146:1, 185:1, and 146:1, respectively.

After 24 h of equilibrium dialysis at 5°C, the residual concentrations of antibiotic activity remaining in solution outside the control- and exopolysaccharide-containing dialysis bags were determined in a biological assay with *Staphylococcus aureus* ATCC 29213, an organism with well-documented susceptibilities to a large panel of antibiotics. *S. aureus* ATCC 29213 was inoculated into broth cultures containing serial 1:2 dilutions of outer chamber fluids exposed and not exposed to alginate. If equivalent dilutions of these paired solutions were equally inhibitory after 16 h of incubation, no binding of antibiotic to exopolysaccharide was indicated. If, however, serial dilutions of outer-chamber fluid from experimental tubes lost inhibitory activity in fewer dilutions than did the fluid from control tubes, binding was indicated. The starting concentration of antibiotic, the inhibitory concentration of antibiotic for *S. aureus* ATCC 29213 under conditions of the assay, and the comparative number of serial dilutions possible before loss of inhibitory potency of control and experimental outer chamber fluids were used to calculate the micrograms of antibiotic bound to alginate per milliliter (also taking into account the depletion of antibiotic from the outer chamber by diffusion into the 2-ml dialysis bags). As the dialysis bags were originally suspended in 9 ml of antibiotic solution, the total micrograms of antibiotic bound is calculated as: (micrograms of antibiotic bound per milliliter) × 9.

RESULTS AND DISCUSSION

To ensure the efficacy and sensitivity of the equilibrium dialysis binding assay used in these studies, we performed preliminary equilibrium dialysis experiments, taking advantage of the well-established affinity of streptomycin for DNA (4, 18). When 6 mg of calf thymus DNA was placed in dialysis bags, approximately 0.054 μmol of streptomycin was depleted from the solution by binding to each milligram of nucleic acid. Upon replacement of the DNA with purified *P. aeruginosa* exopolysaccharide, the several antibiotics assayed were determined to vary in their extent of binding to

TABLE 1. Binding of antibiotics to polyanionic macromolecules[a]

Antibiotic	Macromolecule	Antibiotic bound (μmol/mg of macromolecule		n	Avg amt of antibiotic bound (μmol/mg of macromolecule under physiological ion conc[b]
		Mean	Range		
Streptomycin	DNA	0.054	0.032–0.075	2	BD
Streptomycin	Alginate	0.047	0.016–0.080	7	BD
Tobramycin	Alginate	0.024	0.015–0.036	6	BD
Clindamycin	Alginate	0.0011	0.00032–0.0019	5	BD
Penicillin	Alginate	0	0	6	BD

[a] The binding tests were carried out as described in the text.
[b] BD, Below detectability limits of assay.

the alginate (Table 1). Streptomycin bound to the exopolysaccharide in an amount 2-fold greater than that of tobramycin and 43-fold greater than that of clindamycin. Importantly, the fact that the greatest amount of binding was found for streptomycin, the antibiotic that had the least-favorable ratio of potential binding sites to antibiotic molecules (see Materials and Methods for ratios of binding sites to antibiotic molecules used), indicates that the assay system employed was adequate for these studies. No difference in the binding capacity of EDTA-treated or nontreated exopolysaccharide was detected. The fact that the positively charged species, streptomycin and tobramycin, bound to the alginate, whereas the neutral species, clindamycin and penicillin, bound negligibly or not at all, suggests that the binding of the cationic antibiotics to the polyanionic exopolysaccharide was of an ionic nature. Ionic binding of the cationic species to the alginate was further suggested by the ability of a solution containing 0.14 M NaCl or ion concentrations mimicking those characteristic of the tracheobronchial fluids of cystic fibrosis patients (0.101 M NaCl, 0.028 M K^+, and 0.0037 M Ca^{2+}) to completely inhibit the association even of streptomycin to the exopolysaccharide. Since organisms infecting the tracheobronchial mucosa of patients with cystic fibrosis would be exposed to ion concentrations of this order (3, 25), we concluded that the bacterial exopolysaccharide itself would not bind enough antibiotics to provide any significant protection for the organisms in vivo. This conclusion is further supported by the fact that a 50% reduction in concentration of 9 ml of a 200-μg/ml solution of streptomycin in distilled water required the binding sites of 5 mg of alginate, an amount equivalent to that of exopolysaccharide harvested under optimal conditions from 3×10^{10} *P. aeruginosa* cells. Obviously, the effectiveness of such a binding mechanism even in fluids of very low ionic strength appears to be rather small. Therefore, the data presented here strongly suggest that mucoid pseudomonads are not more resistant to cationic antibiotics because of the binding of such drugs to alginate.

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