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Protection of *Pseudomonas aeruginosa* against Ciprofloxacin and β-Lactams by Homologous Alginate

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***Pseudomonas aeruginosa*-derived alginate but no other neutral and negatively charged polysaccharides protected mucoid and nonmucoid strains of that organism against ciprofloxacin, gentamicin, ticarcillin, and ceftazidime. Data indicate that alginate has an intrinsic protective effect which is independent of diffusion, charge, or biofilm phenomena.**

The role of *Pseudomonas aeruginosa* in lung infections of patients with cystic fibrosis (CF) is well documented (1, 9, 12), but the means by which mucoid strains survive aggressive antibiotic therapy are ill defined. It has been suggested that the alginate may, among other things, directly protect the bacteria against antibiotics by facilitating a biofilm or microcolony mode of growth (7, 8) or by retarding antibiotic diffusion (5, 10, 15). More-recent work, however, has tended to discount diffusion as a clinically relevant factor (14).

A non-diffusion-related effect of alginate in antagonizing aminoglycoside but not β-lactam activity was described by Baltimore et al. (2), and diminished aminoglycoside activity due to the positively charged antibiotic molecule binding to the electronegative alginate is well established (10, 13). Observations in our laboratory, however, indicated that both ciprofloxacin, which is uncharged at neutral pH, and β-lactams were also subject to alginate inhibition. Hence, the purposes of this present work were to reappraise the potential of alginate in directly inhibiting the activity of CF-related antibiotics and to investigate the charge-attraction basis of antibiotic inhibition by substitution of other uncharged or negatively charged polysaccharides for alginate.

A mucoid strain of *P. aeruginosa* derived from a patient with CF, its nonmucoid revertant, and *P. aeruginosa* NCTC 6750 were maintained at −70°C as frozen suspensions of exponential-phase cultures at approximately 5×10^8 cells per ml in Iso-Sensitest broth containing 10% (wt/vol) glycerol; viable counts did not significantly change throughout this work. On thawing, suspensions were washed and used for antibiotic exposure experiments in which ciprofloxacin (Bayer), ceftazidime (Glaxo,) gentamicin (Sigma), and ticarcillin (SmithKline Beecham) were employed at final concentrations of 0.1, 10, 10, and 20 μg/ml, respectively.

Alginate was extracted and purified from the mucoid strain by the method of Govan and Fyfe (11). Sodium alginate (Kelco/AIL International Girvan, Ayshire, United Kingdom), dextran (grade A; BDH), sodium carboxymethyl cellulose (SCMC; medium viscosity grade; Sigma), and soluble starch (Oxoid) were used at final concentrations of 1.0% (wt/vol) unless otherwise stated.

Inocula (0.10 ml at 5×10^8 cells per ml) were added to prewarmed solutions containing 4.8 ml of double-strength phosphate-buffered saline (PBS; Dulbecco A; Oxoid), 0.10 ml of antibiotic concentrate in PBS, and 5.0 ml of aqueous 4.0% (wt/vol) polymer (normally 0.5% [wt/vol] in the case of *P. aeruginosa* alginate) and slowly shaken at 37°C. Samples (1.0 ml) were removed immediately after inoculation and rapidly serially diluted in PBS (9 ml), and 0.2-ml aliquots were spread on the surface of quintuplicate Nutrient Agar (Oxoid) plates. Subsequent samples were removed at suitable intervals for 30 min and treated similarly. After enumeration of colonies following incubation for 48 h at 37°C, the percentage of survivors was plotted on a logarithmic scale against exposure time. In experiments involving β-lactams, the inoculum was incubated in Iso-Sensitest broth for 1.0 h at 37°C to ensure logarithmic growth before antibiotic addition, and PBS was replaced with Iso-Sensitest broth in the antibiotic exposure medium.

MICs of ciprofloxacin were determined at doubling dilutions in Iso-Sensitest agar containing 0, 1.0, and 2.0% (wt/vol) seaweed alginate by using surface inocula (30 μl) containing approximately 3×10^4 viable cells of the three strains of *P. aeruginosa* described above together with *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571. Zone diameters produced by disks of tobramycin (10 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), and aztreonam (30 μg) were also determined in Iso-Sensitest agar containing 0, 1.0, and 2.0% (wt/vol) seaweed alginate inoculated throughout with approximately 10^6 viable cells of the same organisms per ml.

Figure 1 shows the influence of *P. aeruginosa* alginate concentration on the survival of nonmucoid cells exposed to 0.1 μg of ciprofloxacin per ml. The protection afforded is clearly concentration dependent, with approximately 1, 2, 30, and 60% survivors after 15 min of exposure in the presence of 0, 0.125, 0.25, and 0.50% (wt/vol) alginate, respectively. Qualitatively similar but less-marked effects were observed when autologous alginate was added to washed cells of the mucoid strain (data not shown). The influences of various polymers on survival of the nonmucoid strain exposed to ciprofloxacin and gentamicin are shown in Fig. 2 and 3, respectively; again, qualitatively similar results were obtained with other strains. In each case, only alginate was protective, and that from *P. aeruginosa* was much more protective than that from seaweed. Controls (data not shown) demonstrated that none of the polymers influenced the growth or survival of any of the strains used, and the order of mixing of polymer, antibiotic, and inoculum had no influence on results. The protective effects of *P. aeruginosa* alginate on growing cultures of the nonmucoid strain ex-

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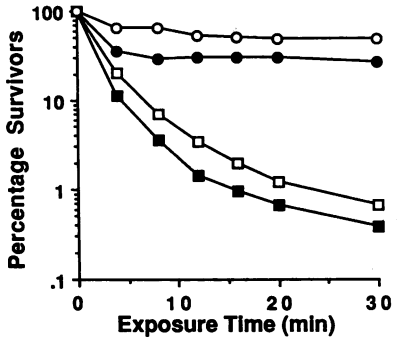

FIG. 1. Effect of *P. aeruginosa* alginate on ciprofloxacin activity against nonmucoid *P. aeruginosa* at alginate concentrations (weight/volume) of 0.0% (■), 0.125% (□), 0.25% (●), and 0.5% (○).

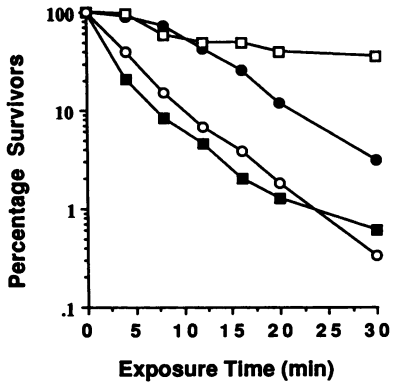

FIG. 3. Effects of PBS (■), 2% (wt/vol) seaweed alginate (●), 2.0% (wt/vol) SCMC (○), and 0.25% (wt/vol) *P. aeruginosa* alginate (□) on gentamicin activity against nonmucoid *P. aeruginosa*.

posed to ticarcillin and ceftazidime are shown in Fig. 4A and B, respectively.

The morphology of growing cells exposed to β-lactams was monitored microscopically, and the proportions showing lysis, spheroplast formation, or other antibiotic-induced changes with time corresponded well with the survival data illustrated in Fig. 4.

Incorporation of alginate in Iso-Sensitest agar at concentrations which were observed by viable-count techniques to protect against ciprofloxacin permitted no increase in the MIC of ciprofloxacin for any of the five test organisms, nor were decreases in inhibition zone diameters for sensitivity disks of ciprofloxacin, ceftazidime, or aztreonam observed. In general, ciprofloxacin and the β-lactams exhibited zone diameters in 2.0% (wt/vol) alginate which were 95 to 100% of those of the controls without alginate. The aminoglycoside tobramycin, however, produced zone diameters which were between 61 and 73% of the control value.

Hitherto, most in vitro studies implicating bacterial alginate in antibiotic resistance have illustrated protection only against aminoglycosides (2, 10, 13), but the data in this paper clearly show that alginate protects *P. aeruginosa* against antimicrobial agents of three chemically distinct types, viz., quinolone, aminoglycoside, and β-lactam (Fig. 1, 3, and 4, respectively). While there have been isolated reports of enhanced β-lactam resistance in mucoid cultures of *P. aeruginosa* (4), the present data are to our knowledge the first which demonstrate protection by alginate against ciprofloxacin. The mechanisms involved are unclear, but Fig. 2 demonstrates that the protection is not generally afforded by other carbohydrate polymers. The SCMC used possessed a negative charge density of 0.7 (compared with 1.0 for alginate), yet it was inactive. This, together with the absence of any net charge on ciprofloxacin (isoelectric point, 7.4), indicated that factors other than charge were responsible for the antagonism.

Baltimore et al. (2) demonstrated that alginate might protect *E. coli* and *S. aureus* against aminoglycosides (but not carbenicillin or piperacillin) by a non-diffusion-based mechanism. Our observations, made by using the same MIC determinations and susceptibility disk methods as those authors, entirely support their findings. However, the fact that a marked degree of ciprofloxacin and β-lactam inhibition was apparent in our study (Fig. 1 and 4, respectively) clearly indicates that the construction of survivor plots for young cells exposed to antibiotic is a more-sensitive method of detecting antibiotic inhibition than those mentioned above.

Failure to eradicate chronic mucoid *P. aeruginosa* in pulmonary infections in patients with CF is a phenomenon which is not restricted to a single group of antibiotics. It is well established that susceptibility to many antibiotics is growth rate dependent (6), and it is probable that the rate of growth of *P. aeruginosa* in the airways of patients with CF would be low as a result of depletion of iron or other

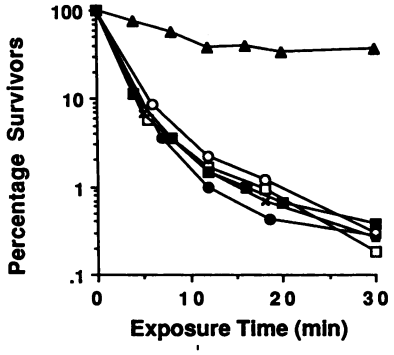

FIG. 2. Effects of PBS (■), 2.0% (wt/vol) seaweed alginate (●), 0.25% (wt/vol) *P. aeruginosa* alginate (▲), 2.0% (wt/vol) SCMC (○), 2.0% (wt/vol) starch (□), and 2.0% (wt/vol) dextran (×) on ciprofloxacin activity against nonmucoid *P. aeruginosa*.

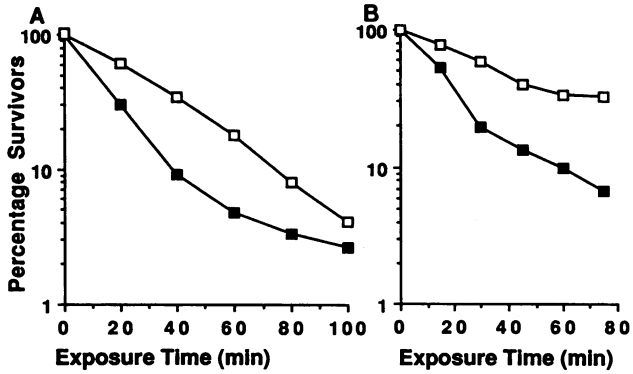

FIG. 4. Effects of PBS (■) and 0.25% (wt/vol) *P. aeruginosa* alginate (□) on ticarcillin (A) and ceftazidime (B) activities against nonmucoid *P. aeruginosa*.

nutrients. The selection of PBS (rather than a conventional culture medium) in which to suspend the inoculum during exposure to ciprofloxacin and gentamicin in this study had the dual advantage of minimizing problems of interpretation of results due to possible interaction with medium components and imparting a more-relevant low growth rate on the exposed cells. The cells of the inoculum, however, were in mid-log phase in a rich medium prior to freezing, and it is probable that endogenous nutrients would have permitted at least some further growth.

Any explanation of the mechanism of protection must account for the effect occurring with three chemically unrelated antibiotics; thus, a non-charge-related binding is an obvious possibility. Alternatively, the alginate might bind an essential nutrient (3) and so impart an even slower metabolic rate and thus a diminished effect. While the mechanisms of biofilm protection are likely to be factors contributing to *P. aeruginosa* survival in vivo, they cannot account for the results reported here because the cells were dispersed in uniform suspension and were all in the same physiological state of active growth prior to their use as inocula. Thus, this intrinsic alginate protection is additional to the protective effect which is naturally associated with the biofilm mode of growth, and if these results were to be reproduced in vivo, the magnitude of the bacteria-alginate antagonism would have obvious implications for antibiotic efficacy. The findings in this study highlight the need to identify those features of both alginate and affected antibiotic molecules which mediate the protective effect.

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