Pharm. Pharmacol. Commun. 1998, 4: 489–491
Received June 25, 1998
Accepted July 20, 1998

In-vitro Effects of Sub-inhibitory Concentrations of Gentamicin on *Pseudomonas aeruginosa* Alginate Production

QORBAN BEHZADIYAN-NEJAD, EFFAT SOURI* AND PARVIZ OWLIA

*Department of Microbiology, Faculty of Medical Sciences, University of Tarbiat Modarres, *Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

Abstract

The mucoid strains of *Pseudomonas aeruginosa* produce hyperviscous substances consisting mainly of alginate, which have several roles in pathogenesis. The in-vitro effects of sub-inhibitory concentrations of gentamicin on the production of alginate by mucoid *P. aeruginosa* were investigated.

The production of alginate by mucoid *P. aeruginosa* cells cultured on agar media in the presence of sub-inhibitory concentrations of gentamicin was quantified by high-performance liquid chromatography. Alginate production was significantly ($P < 0.01$) reduced by 0·5 and 0·25 minimum inhibitory concentrations of gentamicin.

The results suggest that intractable chronic respiratory tract infections involving *P. aeruginosa* might be prevented by sub-inhibitory concentrations of gentamicin.

Pseudomonas aeruginosa is an important opportunistic pathogen in chronic respiratory tract infections such as exacerbation of cystic fibrosis, chronic bronchitis and diffuse panbronchiolitis (Govan & Deretic 1996). The bacteria may trigger infection after adhering to the mucous membranes. As microcolonies develop, they become surrounded by a glycocalyx composed of various exopolysaccharides (Lam et al 1980). Mucoid strains of *P. aeruginosa* produce hyperviscous substances consisting mainly of β1-4-linked D-mannuronic acid and L-guluronic acid (Linker & Jones 1966; Evans & Linker 1973; Jarman 1979). Non-mucoid strains elaborate a polymer made up of sugars such as glucose and fructose (Costerton et al 1978). The alginate provides a protective barrier against antimicrobial agents and the immune system (Russell & Gacesa 1988; Anwar et al 1989a, b). Little is known about the effects of sub-inhibitory concentrations of antimicrobial agents on the glycocalyx (Molinari et al 1993). Some influence might be anticipated, since there are reports showing the effects sub-inhibitory concentrations of various agents on exoenzymes, which are important virulence factors (Woods et al 1986) including exotoxin A, exoenzyme S, elastase, phospholipase C and total protease. At sub-inhibitory concentrations, tobramycin and gentamicin reduce the levels of proteases in-vitro (Warren et al 1985). Erythromycin, clarithromycin and azithromycin inhibit the elaboration of elastase and proteases at concentrations below the minimum inhibitory concentration (MIC) in-vitro (Kirst & Sides 1989; Molinari et al 1993). It therefore seemed reasonable to suppose that some of these agents might influence alginate production by *P. aeruginosa* cells at sub-MIC levels. We therefore investigated the effects of 0·5 and 0·25 MIC gentamicin on the quantitative production of alginate in a mucoid strain of *P. aeruginosa*.

Correspondence: P. Owlia, Department of Microbiology, Faculty of Medical Sciences, University of Tarbiat Modarres, PO Box 14155-4838, Tehran, Iran.

Materials and Methods

Bacteria

Mucoid *P. aeruginosa* 8821M (Leitao et al 1992) was kindly donated by Dr Isabel Sa-Corria, Instituto Superior Tecnico, Lisboa, Portugal. The standard strains of *Pseudomonas aeruginosa* 27853 and *Escherichia coli* 25922 were used for determination of MIC (Anhalt & Washington 1985). The strains were maintained in 10% skimmed milk (Difco Laboratories, Detroit, MI) at −80°C (Ichimiya et al 1994) and were subcultured on Muller-Hinton agar (Difco Laboratories, Detroit, MI) (Ichimiya et al 1994).

MIC determination

Standard solutions of gentamicin (1·25–320 μgmL^{-1} (Sigma Chemical Co., Ltd, St Louis,

MO) were prepared by dissolving gentamicin in sterile distilled water and diluting in Muller–Hinton agar. An agar dilution technique using Muller–Hinton agar and an inoculum of 10^4 colony-forming units (CFU) per spot was used for determination of MIC. The lowest concentration of antimicrobial agent which inhibits visible growth after 16 h incubation at 37°C was defined as the MIC (Anhalt & Washington 1985).

Standard solution of alginate

A standard solution of alginate was prepared by dissolving alginic acid (Sigma Chemical Co., Ltd, St Louis, MO) in distilled water (0·5 mg mL^{-1}) and was used as positive control. Distilled water without alginic acid was used as negative control (Toyoda et al 1985).

Quantification of alginate

The modified method of Ichimiya et al (1994) was used for the preparation of bacterial alginate. After 8 h incubation at 37°C in Muller-Hinton broth (Difco Laboratories, Detroit, MI) the mucoid *P. aeruginosa* 8821M strain was adjusted to 10^3 CFU mL^{-1} with 0·06 M phosphate-buffered saline (PBS) pH 7·2, and 0·1 mL of the resultant suspension was inoculated onto Muller-Hinton agar plates, with or without gentamicin. Plates were incubated for 24 h at 37°C. The colonies formed were collected on a cotton swab and suspended in 10 mL PBS. Viable bacterial counts were determined by transferring 100 μL decimal dilutions onto Muller-Hinton agar plates. Six suspensions were prepared to allow the average viable count to be determined. Each suspension was centrifuged at 500 *g* for 5 min to remove bacteria. The supernatant was used for the determination of alginate by high-performance liquid chromatography (HPLC), according to the modified method of Toyoda et al (1985). Two millilitres of a copper-hydrochloride solution (1 mL 2·5% copper sulphate solution, 9 mL distilled water and 40 mL 36% hydrochloric acid) and 1 mL 0·4% (w/v) naphthoresorcinol (Sigma Chemical Co., Ltd, St Louis, MO) were added to 1 mL of sample. The mixture was vortexed and heated in a hot-water bath at 100°C for 40 min, then cooled to room temperature in a ice bath. The solution was shaken with 4 mL butyl acetate (Merck, Frankfurter Strasse 250, Germany). After centrifugation, the aqueous layer was discarded and the organic layer was washed with 3 mL 20% (w/v) aqueous NaCl. The organic layer (100 μL) was diluted with 200 μL acetonitrile (Merck, Frankfurter Strasse 250, Germany) and 25 μL was injected onto the HPLC system.

HPLC

The HPLC system consisted of a Model 510 HPLC pump, a Model 480 UV detector and a Model 476 integrator (Waters, Milford, MA). Determination was performed on a Resolve C_{18} column (3·9×150 mm, Waters, Milford, MA) using acetonitrile–water–butyl acetate (55:42:3) as the mobile phase at a flow rate of 0·6 mL min^{-1} and UV detection at 565 nm.

Statistical analysis

The results were presented as mean ± s.d. and the differences between control and gentamicin samples were analysed using the paired *t*-test. In all cases a P value of $< 0·05$ was considered to be significant.

Results and Discussion

The MIC values of the mucoid strain of *P. aeruginosa* 8821M, *P. aeruginosa* 27853 and *E. coli* 25922 were 8, 2 and 1 μg mL^{-1}, respectively. Alginate produced by *P. aeruginosa* had a retention time identical to that of the standard solution of alginate (Figure 1). Control experiments showed a linear relationship between the alginate peak and concentration of solution (data not shown).

Alginate production (per log CFU) was expressed as a percentage of control. The results showed a

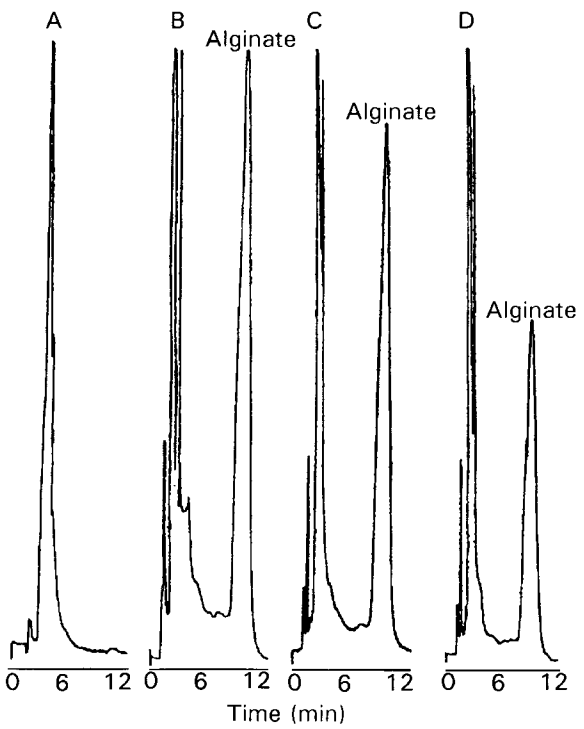

Figure 1. Representative HPLC chromatograms of negative control (A), *P. aeruginosa* 8821M cultured on Muller-Hinton agar without gentamicin (B), with 0·25 minimum inhibitory concentration of gentamicin (C), with 0·5 minimum inhibitory concentration of gentamicin (D).

significant ($P < 0{\cdot}01$) difference between alginate production by control (untreated) cultures ($100 \pm 6{\cdot}8\%$) and that produced by bacteria grown in the presence of 0·5 ($78{\cdot}0 \pm 0{\cdot}6\%$) and 0·25 ($60 \pm 7{\cdot}1\%$) MIC gentamicin.

Biofilm formation, which correlates with the production of alginate, has several roles in the pathogenesis of *P. aeruginosa* (Russell & Gacesa 1988; Anwar et al 1989a, b; Govan & Deretic 1996). Consequently, any treatment which reduces alginate production, might be effective in counteracting the pathogenesis of *P. aeruginosa*, especially in cystic fibritics and in patients with suppressed immune systems (Ichimiya et al 1994; Govan & Deretic 1996). It has been shown that sub-MIC values of gentamicin reduce the level of proteases in-vitro (Warren et al 1985). Other antimicrobial agents reduce the level of exotoxin A (Molinari et al 1993). However, there is no report regarding the effect of gentamicin on alginate production. In this study, we have demonstrated that sub-inhibitory concentrations of gentamicin, which can be attained in human lungs or other tissues following a normal dosage (Ichimiya et al 1994), reduce alginate production, probably by suppressing biofilm formation. Consequently chronic respiratory tract infections due to mucoid strains of *P. aeruginosa* might be prevented.

Acknowledgement

We thank Professor H. Farsam, Department of Medicinal Chemistry, Tehran University of Medical Sciences, for advising on the HPLC method for determination of alginate.

References

Anhalt, J. P., Washington, H. A. (1985) Antimicrobial susceptibility tests of aerobic and facultatively anaerobic bacteria. In: Washington, J. A. (ed.) Laboratory Procedure in Clinical Microbiology, 2nd edn. Springer-Verlag, New York, pp 295–303

Anwar, H., Biesen, T. V., Dasgupta, M., Lam, K., Costerton, J. W. (1989a) Introduction of biofilm bacteria with antibiotics in a novel in vitro chemostat system. Antimicrob. Agents Chemother. 60: 539–574

Anwar, H., Dasgupta, M., Lam, K., Costerton, J. W. (1989b) Tobramycin resistance of mucoid *Pseudomonas aeruginosa* biofilm grown under iron limitation. J. Antimicrob. Chemother. 24: 647–655

Costerton, J. W., Geesey, G. G., Cheng, K. J. (1978) How bacteria stick. Sci. Am. 238: 86–95

Evans, L., Linker, A. (1973) Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. Bacteriol. 116: 915–924

Govan, J. R. W., Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. 60: 539–574

Ichimiya, T., Yamasaki, T., Nasu, M. (1994) In-vitro effects of antimicrobial agents on *Pseudomonas aeruginosa* biofilm formation. J. Antimicrob. Chemother. 34: 331–341

Jarman, T. R. (1979) Bacterial alginate synthesis. In: Berkeleg, R. C. W., Goodwag, G. W., Ellwood, D. C. (eds) Microbial Polysaccharides and Polysaccharides. Academic Press, New York, pp 35–50

Kirst, H. A., Sides, G. D. (1989) New directions for macrolide antibiotics: structural modifications and in vitro activity. Antimicrob. Agents Chemother. 33: 1413–1418

Lam, J., Chan, R., Lam, K., Costerton, J. W. (1980) Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. 28: 546–556

Leitao, J. H., Fialho, A. M., Sa-Correia, I. (1992) Effects of growth temperature on alginate synthesis and enzymes in *Pseudomonas aeruginosa* variant. J. Gen. Microbiol. 138: 605–610

Linker, A., Jones, R. S. (1966) A new polysaccharide resembling alginic acid isolated from pseudomonas. J. Biol. Chem. 241: 3845–3851

Molinari, G., Guzman, C, A., Pesce, A., Schito, G. C. (1993) Inhibition of *Pseudomonas aeruginosa* virulence factors by subinhibitory concentrations of azithromycin and other macrolide antibiotics. J. Antimicrob. Chemother. 31: 681–688

Russell, N. J., Gacesa, P. (1988) Chemistry and biology of the alginate of mucoid *Pseudomonas aeruginosa* in cystic fibrosis. Mol. Aspects. Med. 10: 1–91

Toyoda, M., Yomota, C., Ito, Y., Harada, M. (1985) High performance liquid chromatographic method for the determination of sodium alginate in foods. J. Food Hyg. Soc. Jpn 26: 189–194

Warren, R. L., Baker, N. R., Johnson, J., Stapleton, M. J. (1985) Selective inhibition of the accumulation of extracellular proteases of *Pseudomonas aeruginosa* by gentamicin and tobramycin. Antimicrob. Agents Chemother. 27: 468–472

Woods, D. E., Schaffer, M. S., Rabin, H. R., Campbell, G. D., Sokol, P. A. (1986) Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. J. Clin. Microbiol. 24: 260–264