Chemical composition of the extracellular slime glycolipoprotein of *Pseudomonas aeruginosa* and its relation to gentamicin resistance

G. ARSENIS and G. DIMITRACOPOULOS*

Department of Microbiology, National University of Athens, and *Department of Microbiology, School of Medicine, University of Patras, Greece

Summary. The slime glycolipoproteins (GLPs) extracted from *Pseudomonas aeruginosa* strain C2 and its laboratory-induced gentamicin-resistant variant were analysed for gross chemical composition. The GLP of the wild-type strain contained significantly greater amounts of neutral sugars, uronic acid and thiobarbituric-reactive material ($p < 0.001$) than the GLP of the gentamicin-resistant variant. Also significantly higher ($p < 0.01$) was the amino-sugar content of the GLP from the wild-type strain. Paper chromatographic analyses of the hydrolysates of the GLPs revealed that two neutral sugars, rhamnose and mannose, were absent from the GLP of the resistant variant. The GLP of strain C2 contained significantly less protein than the GLP of the gentamicin-resistant variant.

Introduction

The more general use of the aminoglycoside antibiotic gentamicin against *Pseudomonas aeruginosa* has led to an observed increase in resistance and several lines of research point towards the envelope of *P. aeruginosa* having a significant role in resistance by an exclusion mechanism (Sykes and Morris, 1975). Many of the clinical gentamicin-resistant isolates of *P. aeruginosa* do not contain known forms of gentamicin-modifying enzymes and show a reduced permeability to gentamicin (Bryan and Van Den Elzen, 1977). It has also been reported that the amount of surface lipids in laboratory-induced resistant variants of *P. aeruginosa* is greater than that of the parent sensitive strains (Pechey and James, 1973).

The production of extracellular slime is considered to be characteristic of *P. aeruginosa* (Haynes, 1951) and has been studied as a potential virulence factor (Sensakovic and Bartell, 1974; Dimitracopoulos and Bartell, 1980). It has even been proposed that the slime is responsible for the high degree of resistance of *P. aeruginosa* to antibiotics (Brown and Richards, 1964).

In a previous paper, evidence was presented for the existence of two additional protein bands at isoelectric points of about pH 5.62 and pH 5.3 in the glycolipoprotein (GLP) fraction of the extracellular slime layer of a laboratory-induced gentamicin-resistant variant of *P. aeruginosa* (Lambris *et al.*,

1980). In this paper we report quantitative and qualitative differences in the chemical composition of GLP between the wild type and a laboratory-induced gentamicin-resistant variant of *P. aeruginosa*.

Materials and methods

Bacterial strains

Pseudomonas aeruginosa strain C2 was isolated from a clinical specimen and the minimal inhibitory concentration (MIC) of gentamicin was 0.4 mg/L. A laboratory resistant variant grown in gentamicin 250 mg/L was produced from the sensitive wild strain by repeated passage in Trypticase Soy Broth (BBL Microbiology Systems, Cockeysville, MD, USA) containing increasing concentrations of gentamicin (Dimitracopoulos *et al.*, 1979). The resistant variant differed from the wild strain in lytic phage pattern, phage adsorption and agglutination with the seven Fisher's antisera. Reversion to the original properties of the wild strain was demonstrated after ten serial subcultures in antibiotic-free broth (Dimitracopoulos *et al.*, 1979).

Extraction and purification of slime GLP

The GLP fraction was obtained from the extracellular slime layer of *P. aeruginosa* strain C2 and its resistant variant (Bartell *et al.*, 1970; Sensakovic and Bartell, 1974). Slime was extracted in 0.15 M NaCl from bacterial cultures grown for 18 h on sheets of cellophane overlying Trypticase Soy Agar (BBL). The extract was precipitated with ethanol, clarified by centrifugation at 16 300 g for 30

min, and dialysed overnight against water. The dialysate was centrifuged at 105 000 *g* for 3 h and the supernatant fluid containing the GLP fraction was lyophilised. Column chromatography was performed with both Sephadex G-100 and G-200 (Pharmacia Fine Chemicals, Fullerton, CA, USA) and elution was affected with 5mM Tris buffer pH 7.2. Anion exchange chromatography was performed on diethylaminoethyl (DEAE) cellulose (Cellex D, BioRad Laboratories, Richmond, CA, USA) in Tris-HCl buffer (0.05 M Tris, pH 7.2) and elution was accomplished with a continuous gradient of KCl from 0.3 to 1.0 M. All eluates were monitored for protein and carbohydrate. Cellulose acetate electrophoresis was performed on Sepraphore II strips (Gelman Instrument Co., Ann Arbor, MC, USA) in sodium barbital-HCl buffer, pH 6.8 and barbital-sodium barbital buffer, pH 8.6, at 5 mA/strip for 60 min followed by staining with Ponceau S for protein and periodic acid Schiff's reagent for carbohydrate. Immunodiffusion was performed in Special Noble Agar (Difco) 1.5% in barbital-sodium barbital buffer (veronal buffer), pH 8.6, overnight at room temperature. The antiserum used was prepared in rabbits by injecting purified GLP as previously described (Bartell *et al.*, 1970). Immuno-electrophoresis was performed on glass slides overlaid with Special Noble Agar 2.0% in veronal buffer, pH 8.6, at 5 mA/slide for 60 min at 4°C. Ultracentrifugation of GLP was performed at a concentration of 10 mg/ml in 0.2 M NaCl at 20°C. The sedimentation pattern was visualised by Schlieren optics.

Chemical analysis

The chemical composition of the GLPs was determined by standard methods and average values are presented based on six determinations of each component. Total neutral sugars were determined by the anthrone reaction (Spiro, 1966), total amino-sugars by the method of Belcher *et al.* (1954), protein by the method of Lowry *et*

al., (1951), uronic acid by the method of Bitter and Muir (1962), thiobarbituric-reactive material by the method of Osborn (1963) and Ellwood (1970), and phosphorus by the method of Chen *et al.* (1956). For the identification of amino sugars, the GLP fractions were hydrolysed in 6 N HCl at 100°C for 24 h. The hydrolysates were developed in butanol-pyridine-distilled water (6:4:3) on descending paper chromatograms and the separated amino-sugars were visualised with acetylacetone and *p*-dimethylamino-benzaldehyde. For the identification of neutral sugars, the GLPs were hydrolysed in 6 N HCl at 100°C for 24 h. The hydrolysates were developed in a mixture of butanol-ethanol-distilled water (4:1:2) containing 1% v/v of ammonia solution 25% on descending paper chromatograms and the separated neutral sugars were visualised with HCl-*p*-anisidine.

Statistical analysis

The significance of differences in the chemical composition of GLP between the wild and the laboratory-induced gentamicin-resistant variant was determined by the two-tailed *t* test.

Results

The glycolipoproteins extracted from the slime layer of *P. aeruginosa* C2 and its gentamicin-resistant variant were analysed for gross chemical composition and the results, which are shown in table I, represent mean values of six determinations of each component on one lot of each GLP. Compared with that of the gentamicin-resistant variant, the GLP of the wild-type strain C2 contained significantly greater amounts of neutral sugars, uronic acid and thiobarbituric-reactive

Table I. Chemical composition of extracellular slime GLPs derived from *P. aeruginosa* C2 and its gentamicin-resistant variant grown in gentamicin 250 mg/L

Component	Percentage (mean $\pm$ SEM)* of dry weight of slime GLP in			
	<i>P. aeruginosa</i> C2	gentamicin-resistant variant	<i>t</i> Value	<i>p</i>
Total neutral sugars	22.33 $\pm$ 0.38	15.50 $\pm$ 0.56	+10.06	<0.001
Total amino sugars	11.48 $\pm$ 0.75	8.30 $\pm$ 0.44	+3.62	<0.01
Uronic acid	6.50 $\pm$ 0.22	4.00 $\pm$ 0.20	+8.20	<0.001
Thiobarbituric-reactive material	0.93 $\pm$ 0.02	0.68 $\pm$ 0.01	+9.26	<0.001
Protein	20.80 $\pm$ 0.24	22.40 $\pm$ 0.21	-4.91	<0.001
Phosphorus	0.67 $\pm$ 0.01	0.70 $\pm$ 0.02	-0.97	>0.1

*Results of six determinations of each component on one batch of each GLP.

Table II. Paper chromatographic analysis of the neutral sugars of slime GLPs derived from *P. aeruginosa* C2 and its variant grown in gentamicin 250 mg/L

Source of GLP	Presence (+) or absence (–) of			
	glucose	rhamnose	mannose	galactose
<i>P. aeruginosa</i> C2	+	+	+	+
Gentamicin-resistant variant	+	–	–	+

material ($p < 0.001$). The amino-sugar content of GLP derived from the wild-type strain C2 was also significantly higher than that of GLP derived from the gentamicin-resistant variant ($p < 0.01$). In contrast, the GLP of the wild-type strain C2 contained significantly less protein than the GLP of the gentamicin-resistant variant (mean values 20.80 and 22.40, respectively). No significant difference was found between the phosphorus contents (mean values 0.67 and 0.70) of the two strains.

The differences observed in the neutral and amino-sugar content of the GLPs led us to investigate these components further. Paper chromatographic analyses revealed four neutral sugars in GLP hydrolysate of strain C2 and two in a similar preparation of its gentamicin-resistant variant (table II). The four neutral sugars of strain C2 were glucose, rhamnose, mannose and galactose, and those of its resistant variant were glucose and galactose. The paper chromatographic analyses revealed the presence of two amino-sugars in each GLP; these were identified as glucosamine and galactosamine.

Discussion

The data presented indicate that the laboratory-induced resistance of *P. aeruginosa* to gentamicin is accompanied by quantitative and qualitative changes in the chemical composition of slime GLP.

In a previous report (Lambris *et al.*, 1980) isoelectric focusing of the slime GLP of strain C2 showed four protein bands within the pH range 5.46–5.6; two further bands at about pH 5.62 and pH 5.3 appeared in the slime GLP of the variant grown in the presence of gentamicin 250 mg/L. The results of the present study are consistent with these findings, the GLP of wild-type strain C2 containing significantly less protein than the GLP of the gentamicin-resistant variant ($p < 0.001$). Taken together, these results suggest that laboratory-induced resistance to gentamicin effects both quan-

titative and qualitative changes in the protein component of slime GLP.

Moreover our observations indicate that laboratory-induced resistance to gentamicin caused quantitative alterations in other components of slime GLP, namely neutral sugars, uronic acid, thiobarbituric-reactive material and amino-sugars. The amount of these components derived from the resistant variant was significantly lower than that from the wild-type strain. Paper chromatographic analyses revealed that the GLP of the resistant variant lacked two neutral sugars, but no qualitative differences were found in the amino-sugar content of the two strains.

Slime GLP by its inactivation of phages, is seen to possess receptor-like properties (Bartell *et al.*, 1971; Reese *et al.*, 1974). The significant reduction in the amount of neutral and amino-sugars of the GLP from our resistant variant may explain the inability of the cells to adsorb the phages that were active against the parent strain, and the absence of rhamnose and mannose may indicate the functional importance of these neutral sugars in phage-adsorption.

Besides possessing receptor-like properties, the slime GLP of *P. aeruginosa* seems to contribute to the pathogenesis of infection with the organism (Sensakovic and Bartell, 1974; Lynn *et al.*, 1977; Dimitracopoulos and Bartell, 1980), has antiphagocytic activity, exerts a mitogenic effect on human peripheral blood and cord blood lymphocytes and activates human complement via the alternative pathway (Papamichail *et al.*, 1980; Lambris *et al.*, 1982). Lysogenisation of *P. aeruginosa* can result in considerable changes in the chemical composition and antigenicity of the slime GLP and, perhaps more significantly, in the pathogenicity of the cell (Dimitracopoulos and Bartell, 1979). The extensive alterations in the surface of the *P. aeruginosa* cell described in this communication may also have implications in the biological activities of slime GLP, but such a relationship remains to be clarified.

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