BACTERIAL PATHOGENICITY

Effects of characterised *Pseudomonas aeruginosa* exopolysaccharides on adherence to human tracheal cells

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This study evaluated, *in vitro*, the role of different *Pseudomonas aeruginosa* exopolysaccharides (EPS) in mediating adherence to human respiratory epithelial cells. Two mucoid and non-mucoid isogenic pairs of *P. aeruginosa* strains isolated from patients with cystic fibrosis (CF) and bronchiectasis were used. Adherence was tested with human tracheal epithelial cell lines from CF and normal fetuses. The CF cells bound significantly more bacteria than the normal cells. The strain from the bronchiectasis patient was significantly more adherent than that from the CF patient and this difference was consistently most marked with the non-mucoid variant and with normal epithelial cells. The differing behaviour of mucoid CF and non-mucoid bronchiectasis strains reflected the chemical composition of their EPS: mainly alginate in the former and neutral polysaccharides in the latter. Additive inhibition experiments with chemically characterised EPS indicated that neutral polysaccharides associated with alginate may act as ligands for the adherence of *P. aeruginosa* to CF epithelial cells.

Introduction

Pseudomonas aeruginosa is a common human respiratory pathogen and is particularly important as a cause of chronic respiratory infections in cystic fibrosis (CF) and bronchiectasis. Two mechanisms contribute to the complex pathogenesis of these respiratory infections. First, bacterial adhesion to the respiratory epithelial cells initiates infection [1]. Secondly, inhibition of phagocytosis by macrophages and polymorphonuclear leucocytes favours progression [2]. During both infections, the *P. aeruginosa* strains become mucoid by secreting an exopolysaccharide (EPS) containing alginate [3]. The molecular basis of this association is not known. *P. aeruginosa* EPS is a complex mucoid exopolysaccharide composed not only of alginate but also of other polysaccharides, and a knowledge of its chemical composition is essential to understanding its role in adherence. Several studies have shown that alginate is produced not only by mucoid *P. aeruginosa* but also, in smaller amounts, by non-mucoid isolates, including non-mucoid revertants of mucoid strains [4–6].

Alginate is among the numerous exoproducts and virulence factors produced by *P. aeruginosa*. It has been reported as playing a role in the adhesion of the bacterium to epithelial cell surfaces. In particular, it seems to be the adhesin for tracheal cells [7] and for tracheobronchial mucins [8]. However, some discrepancies are apparent: alginate purified from some *P. aeruginosa* strains binds to buccal and tracheal epithelial cells, whereas alginate from other strains does not do so, maybe reflecting structural diversity [9]. In a recent study, Ramphal *et al.* [10] showed several classes of adhesins on the surfaces of mucoid and non-mucoid *P. aeruginosa*, not only alginate and pilin, but also other adhesins requiring an alternative sigma factor. Other authors [11] have suggested the presence of such a 'dependent' adhesin in the adherence of *P. aeruginosa* cells to human lung pneumocytes. A recent review [12] emphasised that alginate does not represent a major ligand to the respiratory epithelium.

A previous study extracted and characterised different EPSs from mucoid and non-mucoid *P. aeruginosa* strains isolated from CF and bronchiectasis [5]. These differed in the alginate and neutral sugar content, and in the degree of O-acetylation of the alginate. A recent study showed [13] that the alginate and neutral

polysaccharides content of *P. aeruginosa* EPS may influence susceptibility to phagocytosis and to the macrophage oxidative burst. Less is known about the relationship between the chemical composition of the EPS and adherence. In view of the specific cellular receptors in CF, the role of EPS may not be very significant. Nevertheless, the injured respiratory epithelium binds mucoid *P. aeruginosa* more readily than intact respiratory epithelium [14, 15] and Woods *et al.* [16] showed that buccal epithelial cells from CF patients bound greater numbers of *P. aeruginosa* cells than those from healthy individuals. Recently, Saiman [17] demonstrated a difference between the binding of *P. aeruginosa* to cell lines and primary polyp nasal cells from CF and non-CF origins.

This study determined the ability of two mucoid and non-mucoid isogenic pairs of *P. aeruginosa* strains to adhere *in vitro* to monolayers of human tracheal epithelial cells and compared their adherence to CF and normal cell lines. It also investigated the potential role of chemically different exopolysaccharides extracted from *P. aeruginosa* in mediating this adherence.

Materials and methods

Bacteria

Two mucoid *P. aeruginosa* strains isolated from sputa were used, one from a CF patient (M-CF) and one from a bronchiectatic patient (M-BR). The two corresponding isogenic non-mucoid derivatives (nM-CF and nM-BR) were spontaneous variants and were used for comparison. All these organisms were non-piliated, and have been studied previously [5]. O-Serotyping by the International Antigenic Typing System showed similar results within both pairs: the CF pair were of serotype 10 and the pair from the bronchiectasis patient were polyagglutinating.

Bacteria labelled with [³H] uracil were obtained by inoculating one colony from an overnight 37°C culture on Pseudomonas Isolation Agar (PIA; Difco, France) into 5 ml of Bacto Nutrient Broth (NBY; Difco) containing labelled uracil (Amersham, France) 20 µCi/ml. This culture was incubated at 37°C for 18 h without agitation. The bacteria were then washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 10 mM phosphate buffer, pH 7.4) containing unlabelled uracil 100 mg/L, then resuspended in PBS (5 ml). The viable count was adjusted to 10⁹ cfu/ml and the radioactivity associated with the cells was evaluated in a liquid scintillation counter (Pharmacia LKB1217 Rackbeta).

Determination of chemical content of the EPS

The chemical composition of the EPS from the four organisms was determined by gas-liquid chromatography and ¹H-NMR spectroscopy, as described previously [5]. In addition, EPS from *Laminaria hyperborea*, a

brown seaweed, was used; this was kindly provided by Sobalg SA, Landerneau, France. In some experiments, the neutral and acidic fractions obtained after chromatography of the EPS on DEAE-Trisacryl were tested, after elution with 0.1 M and 0.8 M NaCl, respectively.

Preparation of cell monolayers

Two human tracheal epithelial cell lines transfected with SV40 virus were used. Both were from fetuses, one normal (NT cells) and the other with CF. The CF cells were homozygous for the most common deletion, ΔF508 and were kindly provided by J. Picard, Unité 181, Institut National de la Santé et de la Recherche Médicale, Paris. In adherence assays, the cells were used after nine to 15 passages. Under these conditions, they retained typical epithelial morphology, and the CF cells exhibited the abnormal regulation of chloride conductance characteristic of the disease [18]. Plastic micro-titration plates (Costar, France) containing sterile glass coverslips to prevent adherence of *P. aeruginosa* cells to the plastic were used to prepare the cell monolayers: 400 µl of the epithelial cell suspension were layered on to the 10-mm diameter glass coverslips in 48-well plates and incubated for 5 days in a CO₂ incubator. The morphology, confluence and condition of the monolayers were studied by light microscopy after May-Grünwald and Giemsa staining. The cells were washed twice with DMEM (Gibco, France) (44 mM NaHCO₃ buffer, pH 7.2) immediately before use.

Adherence assays

Radiolabelled bacterial suspensions (50 µl) were applied to the cell layers and incubated for 60 min at 37°C in a atmosphere of air 95%, CO₂ 5%. The cell monolayers on the coverslips were then washed three times with warm (37°C) PBS to remove non-adherent bacteria. The coverslips were removed and placed in individual vials, where cell-associated radioactivity was released by adding 150 µl of 1 N NaOH, followed by neutralisation with 150 µl of 1 N HCl. The radioactivity in 5-ml amounts of scintillation liquid (Amersham) was then counted in a liquid scintillation counter as previously described [19]. After each test of adherence, the coverslips were air-dried and examined by light microscopy with May-Grünwald and Giemsa staining. Bacterial counts were expressed as adherent bacterial cfu/well based on the means and SD of four separate experiments, each performed in quadruplicate.

Inhibition experiments

Studies on the inhibition of adherence by EPS and its fractions were performed with the nM-BR *P. aeruginosa* strain, which was the most adherent of the four organisms. Cell monolayers were preincubated for 30 min at 37°C, in CO₂ 5% with 250 µl EPS or EPS fraction (1 mg/ml) instead of the 250 µl of DMEM medium, then the radiolabelled bacteria were added

and the binding assay was continued in the usual way. Results were expressed as mean percentage inhibition of binding and SD, based on four separate experiments, each performed in quadruplicate.

To assess the role of alginate, mixed (i.e., alginate-containing) EPS was treated with an alginase obtained from the marine bacterium ATCC 433367, which is able to hydrolyse *P. aeruginosa* alginate isolated from CF patients [20]. The EPS was incubated for 18 h at 37°C with the alginase (0.13 µg of enzyme/mg of EPS; specific activity, 80 units/mg of protein) before addition to cell monolayers. The enzyme activity was checked by measuring the increase of optical density at 235 nm, which assesses formation of unsaturated uronic acids. One enzyme unit produced 1 µmol of unsaturated termini per min, with 3-deoxy-D-manno-octulosonic acid (Sigma) as the standard. Results were compared with those observed with alginase the untreated EPS.

Statistical analysis

Data are expressed as means and SEM, and statistical significance was assessed with Student's *t* test for unpaired observations.

Results

The surface characteristics of the *P. aeruginosa* cultures and the chemical composition of their EPS in the culture medium used are described in Table 1. Neither of the bronchiectasis organisms, but both CF organisms produced alginate. On the other hand, both the bronchiectasis organisms produced a neutral EPS composed of neutral sugars including rhamnose, mannose, glucose, galactose, N-glucosamine and N-galactosamine.

Bacterial adherence to human tracheal epithelial cell monolayers

As shown in Fig. 1, adherence was time-dependent, with the number of adherent bacteria increasing progressively during the first hour of contact, then reaching a maximum which thereafter remained constant. On this basis, an incubation period of 60 min was selected for further experiments. As shown in Table 2,

Fig. 1. Kinetics of adherence of *P. aeruginosa* to CF cells. Radiolabelled bacterial suspensions (50 µl), adjusted to 10^9 cfu/ml, were incubated with tracheal epithelial cells for different periods. Results represent the means and SD of four experiments at each time interval. Results are for organisms as follows: —■—, nM-BR; —□—, M-BR; —●—, nM-CF; —○—, M-CF.

Table 2. Comparison of adherence of *P. aeruginosa* to CF and normal cell monolayers

<i>P. aeruginosa</i> strain	Mean (SD) adherence to epithelial monolayers*		
	CF-cells	normal cells	p
nM-BR	46 (2)	12 (2)	<0.001
M-BR	38 (1)	7 (1)	<0.001
nM-CF	25 (1)	2 (0.1)	<0.001
M-CF	21 (5)	2 (0.1)	<0.001

*Adherence is expressed as the mean number (SD) of adherent bacteria ($\times 10^5$)/well, as estimated from the radioactivity remaining on the cell monolayer after 60 min of contact, as indicated in *Materials and methods*.

the CF cells bound significantly more bacteria than did normal cells ($p < 0.001$). The bronchiectasis organisms, especially nM-BR (Fig. 1), demonstrated greater rates of adherence to the CF epithelial cells than did the other cultures. The mucoid bronchiectasis strain was significantly less adherent than its non-mucoid revertant ($p < 0.01$).

Table 1. Chemical composition of the EPS of the four mucoid and non-mucoid *P. aeruginosa* strains used in adherence assays

Strain	Relative molar ratio of sugars composing the EPS*						Characteristics of the alginate		
	Rha	Man	Gal	Glc	GalN	GlcN	Alginate (%)*	M/G ratio†	O-acetyl (%)†
M-CF	1	0.3	1	1	1	0.1	70	89/11	37
nM-CF	0.14	0.01	0.04	1	0.18	0.16	33	79/21	46
M-BR	1	0.2	0.03	1	0	0.1	<1	—	—
nM-BR	0.2	0.02	0.04	1	0.2	0.3	<1	—	—

*Estimated by GLC of the methylglycosides from the methanolysis of EPS, as described previously [5]. Rha, rhamnose; Man, mannose; Gal, galactose; Glc, glucose; GalN, N-galactosamine; GlcN, N-glucosamine.

†Ratio of D-mannuronic acid to L-guluronic acid and degree of acetylation, calculated from $^1\text{H-NMR}$ spectra, as described previously [5].

There was a correlation between the predominance of neutral sugars in the EPS (Table 1) and the extent of bacterial adherence: non-mucoid *P. aeruginosa* adhered better than the mucoid strains.

Influence of EPS components on bacterial adherence to human tracheal epithelial cells

Each of five EPS preparations tested decreased the adherence of *P. aeruginosa* nM-BR strain to human tracheal cells compared with control cultures (Table 3). EPS from organisms M-BR-1 and M-BR-2 contained more neutral sugars than alginate and were the strongest inhibitors of adherence, causing a significant reduction in bacterial binding ($p < 0.001$). Less pronounced inhibition of adherence was also seen with the EPS preparations from organisms M-CF and M-BR-3, which predominantly contained alginate. The *Laminaria* EPS, which had a non-acylated alginate with a high ratio of guluronic acid, did not influence adherence significantly.

The inhibition of the adherence by EPS was dose-related and only the preparations from organisms M-BR-1 and M-BR-2 remained inhibitory when diluted to 100 mg/L, whereas the other EPS preparations ceased to inhibit adherence at concentrations below 500 mg/L.

To investigate further the role of the various EPS components (alginate and neutral sugars) in affecting the adherence of *P. aeruginosa*, two control procedures were performed. First, the effect of treating the EPS with alginase was examined (Fig. 2). Alginase treatment reduced the inhibition of pseudomonal binding for each of the EPS preparations except that from organism M-BR-1, which was devoid of alginate. Nevertheless, EPS from organism M-BR-1, as well as that from M-BR-2 retained a significant ability to inhibit adherence of *P. aeruginosa*, even after the alginase treatment. For the other EPS preparations, the alginase caused loss of any significant ability to inhibit adhesion of *P. aeruginosa*. Secondly competition experiments tested the ability of neutral and acid

Fig. 2. Inhibition of the adherence to human tracheal epithelial cells of *P. aeruginosa* nM-BR by different EPS preparations and reversal of this inhibition by alginase, ▨. The five EPS preparations were chosen as having different chemical compositions (see Table 3). Each bar represents the mean of quadruplicate experiments, and the error bar indicates the SD. ** $p < 0.001$ and * $p < 0.05$, by *t* test, as compared to the control without EPS.

fractions of the EPS to inhibit adhesion of *P. aeruginosa* (Table 4). The neutral fractions significantly inhibited adherence whereas the acidic fractions did not do so.

Discussion

Adherence of bacteria isolated from CF bronchial secretions logically should be studied with cells in a state that most closely imitates the natural biology of the disease. In most studies, however, the adhesion support is an inert material such as dacron fibres [21] or normal respiratory cells from different animal species [22], or cells injured by acids [7]. Adherence to respiratory cells from CF patients has been little-investigated and, even then, often only with laboratory strains of *P. aeruginosa*. This study used *P. aeruginosa*

Table 3. Inhibition by five different EPS preparations of the adherence of the *P. aeruginosa* strain nM-BR to human tracheal epithelial cells

EPS tested*	Relative molar ratio of sugars composing the EPS [†]						Characteristics of the alginate [†]			Inhibition of adherence [‡] % (SD)
	Rha	Man	Gal	Glc	GalN	GlcN	Alginate (%) [†]	M/G ratio	O-acetyl (%)	
M-BR-1	0.17	0.2	0.03	1	0.02	0.01	ND	—	—	90 (3) [§]
M-BR-2	0.4	0.2	0.04	1	0.2	0.1	33	79/21	46	80 (3) [§]
M-BR-3	1	0.2	0.03	1	0	0.1	83	97/3	2	67 (2) [§]
M-CF	1	0.3	1	1	1	0.1	70	89/11	37	65 (10)
<i>Laminaria</i>	ND	ND	ND	ND	ND	ND	99	45/55	0	20 (5)

ND, not detected.

*Origin of the five EPS tested: the numbers correspond to different EPS types produced by the same *P. aeruginosa* strain on various culture media: *Laminaria* is the EPS produced by *Laminaria hyperborea*. These EPS preparations were chosen as having differences in chemical composition.

[†]The chemical characteristics were determined by GLC and RMN spectroscopy, see Table 1.

[‡]Data are means and SD based on four separate experiments performed in quadruplicate. Inhibitors were used at 1 mg/ml. [§] $p < 0.001$ and

^{||} $p < 0.05$ compared with a control without EPS.

Table 4. Inhibition of the adherence of the nM-BR *P. aeruginosa* strain to human tracheal epithelial cells by purified fractions of EPS

EPS	Purified fraction*	Percentage (SD) inhibition of adherence†
M-CF	Neutral	61 (4)‡
	Acidic	30 (3)
M-BR-3	Neutral	59 (4)‡
	Acidic	20 (1)

*The neutral fraction (neutral sugars) and acidic fraction (alginate) were obtained by chromatography on DEAE-Trisacry with elution by 0.1 M and 0.8 M NaCl, respectively.

†Data are means and SD based on at least four separate experiments performed in quadruplicate. All inhibitors were used at 1 mg/ml.

‡*p* < 0.05 compared with a control without EPS.

isolates from patients and monolayers of human cells and found a significant difference between their adherence to epithelial cells from CF and non-CF sources. The CF tracheal cells bound more bacteria than did the normal cells. Three previous studies have also found greater pseudomonal adherence to CF cells than to normal cells [17, 23, 24], whereas one group [25] found no significant difference *in vitro*, but speculated that *in vivo* increased mucin secretion by cells from CF patients might favour binding of *P. aeruginosa*. Epithelial cells from CF patients differ from those of non-CF patients in permeability to chloride ions, in releasing more highly sulphated glycoconjugates, and in harbouring lower amounts of glycosylated glycoconjugates on the cell surface.

Several previous studies found that mucoid *P. aeruginosa* strains adhered better than non-mucoid variants to all cells [21, 22, 26], but others found no difference [1, 27, 28] or that mucoid forms bound less strongly [10, 16]. These differences could be due to differences in the bacteria and cells used. With human isolates and human respiratory cell monolayers, this study found the non-mucoid variants significantly more adhesive.

Previous investigators have suggested that alginate mediates adherence of *P. aeruginosa* to various cells [7, 26, 29] but this has not been confirmed by showing that adherence could be inhibited by addition of free alginate. Moreover, some studies found that some alginates did not bind to buccal epithelial cells, probably reflecting structural diversity [9]. Moreover, to our knowledge, no previous group has really studied the relationship between the clinical composition of *P. aeruginosa* EPS and its role in adherence. The production of alginate, which is only one component of EPS, is highly regulated and is influenced by environmental and nutritional factors [30]. When the carbon source is changed, the yield of alginate may be altered dramatically [5]. Variability among *P. aeruginosa* alginates in terms of amount, chemical composition and idiotypic response had been underlined by Irvin *et al.* [31] and by Pedersen *et al.* [32]. The two types of EPS found in the present study

– alginate-containing and non-alginate containing – are consistent with the study by McArthur *et al.* [33] who showed heterogeneity of EPS based on the presence or absence of neutral and amino-sugars (hexosamines) in addition to mannuronic and guluronic acids. These authors noted the presence of the same sugars as the present study found in the EPS with the greatest inhibitory effect, but did not demonstrate whether these components were substituents of the alginate or if there was more than one polymer present. The present results with the purified fractions indicate the presence of another of another polysaccharide, distinct from the alginate, and this is in agreement with another study [34]. We hypothesise that differences in alginate binding could be explained by the variable chemistry of this material. An alternative hypothesis, that binding was affected by lipopolysaccharides present as contaminants of the EPS, can be discarded, as the O-serotypes of the present strains were constant within the mucoid/non-mucoid pairs.

The predisposition of CF and bronchiectasis patients to respiratory infections with *P. aeruginosa* raises the possibility of specific interactions of the bacteria with the respiratory epithelium. Mucoid and non-mucoid variants have generally equal numbers of pili [35]. In the present study, all organisms were non-piliate (not shown). As for the role of mucoid EPS, alginate seemed to be an adhesin to injured mouse tracheal cells [7] and it was inferred that alginate functions as a bridge between the bacteria and the tracheal surface. These observations appear to be consistent with the results of the present study. Another study [36] on the tracheobronchial mucin receptor for *P. aeruginosa* underlined differences in the role of amino sugars, indicating that they influenced differences in binding between non-mucoid and mucoid strains, and among mucoid strains. Several other recent studies [10, 11, 37] underline the possibility of pseudomonal adhesins other than pilin and alginate.

Alginase treatment decreased the ability of free EPS to inhibit the adherence of *P. aeruginosa*, but did not completely restore adherence, suggesting that alginate was one – but not the sole – EPS component involved in adhesion. Moreover, the neutral EPS fraction appeared to cause greater inhibition of pseudomonal binding than the acidic (alginate) fraction. Thus, the alginate may have only a marginal effect on the adherence of *P. aeruginosa* to respiratory epithelial cells and perhaps, rather, acts to form a matrix in which bacteria become embedded once adherence has taken place. This model has been proposed for the adherence of *P. fluorescens* to surfaces in freshwater [38], where a mucoid mutant also achieved lower levels of attachment than a non-mucoid wild type.

In conclusion, this study shows that the neutral

polysaccharides, which together with alginate compose the *P. aeruginosa* EPS, are involved in adherence. The variability of adherence of mucoid *P. aeruginosa* may be explained in part by the presence, in variable quantities, of alginate and neutral polysaccharides. Further studies are required to assess the behaviour of these components: the alginate may act as a non-specific bridge between cells and bacteria and the neutral polysaccharides could be specific adhesins in primary colonisation by non-mucoid *P. aeruginosa* in cystic fibrosis.

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