Eur Respir J 2003; 21: 932–938
DOI: 10.1183/09031936.03.00097302
Printed in UK – all rights reserved

European Respiratory Journal
ISSN 0903-1936

Pseudomonas aeruginosa adherence to human basement membrane collagen *in vitro*

K.W. Tsang*, D.K. Shum#, S. Chan*, P. Ng*, J. Mak*, R. Leung*, I.H. Shum*, G.C. Ooi¶, G.L. Tipoe+, W.K. Lam*

Pseudomonas aeruginosa *adherence to human basement membrane collagen* in vitro. *K.W. Tsang, D.K. Shum, S. Chan, P. Ng, J. Mak, R. Leung, I.H. Shum, G.C. Ooi, G.L. Tipoe, W.K. Lam.* ©*ERS Journals Ltd 2003.*

ABSTRACT: The mechanisms for *Pseudomonas aeruginosa* colonisation in the airways of patients with bronchiectasis and cystic fibrosis are poorly understood. *P. aeruginosa* could evade mucociliary clearance by adhering to the basement membrane at areas denuded of intact respiratory epithelium.

The authors have developed an *in vitro* model to study *P. aeruginosa* adherence to human basement membrane type-IV collagen by using scanning electron microscopy. *P. aeruginosa* adherence density was determined as the number of *P. aeruginosa* per 20 microscope fields (2,000×) to log inocular size after incubation at 37°C for 45 min.

The presence of phytohaemagglutinin (PHA)-E, which binds specifically to D-galactose-β1–4-D-*N*-acetylglucosamine, significantly reduced *P. aeruginosa* adherence density compared with control. The presence of heparin and calcium also significantly reduced *P. aeruginosa* adherence density. *P. aeruginosa* adherence was not affected by the presence of proline, trans-hydroxyproline, glycine, galactose, *N*-acetylneuraminic acid, *N*-acetylglucosamine or *Arachis hypogea*.

***Pseudomonas aeruginosa* adherence probably acts *via* recognition of the D-galactose-β1–4-D-*N*-acetylglucosamine sequence on type-IV collagen and this process could be inhibited by heparin and calcium. As persistent *Pseudomonas aeruginosa* colonisation is detrimental to patients with cystic fibrosis and bronchiectasis and there is currently no effective treatment for its eradication, these results could lead to novel therapy for persistent *Pseudomonas aeruginosa* infection.**

Eur Respir J 2003; 21: 932–938.

University Depts of *Medicine, #Biochemistry, ¶Diagnostic Radiology and +Anatomy, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong SAR, China.

Correspondence: K.W. Tsang, Division of Respiratory and Critical Care Medicine, University Dept of Medicine, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong SAR, China.
Fax: 852 28725828
E-mail: kwttsang@hkucc.hku.hk

Keywords: Bacterial adherence
basement membrane
collagen
Pseudomonas aeruginosa

Received: October 23 2002
Accepted after revision: January 23 2003

This study was supported by a research grant from the Hong Kong Research Grants Council, Hong Kong SAR, China.

Bronchiectasis, defined as pathological permanent dilatation of the bronchial tree, is a common respiratory disease among East Asians. There is no effective therapy for bronchiectasis and many severely affected patients are chronically infected with *Pseudomonas aeruginosa*, which accounts for significant morbidity and mortality [1]. At present, the only treatment for *P. aeruginosa* lung infection is administration of antibiotics, which is ineffective in eradicating *P. aeruginosa*. A better understanding of the mechanism of *P. aeruginosa* persistence in the lungs of these patients holds the key to the development of potential new and novel therapies for this resistant infection.

It is widely believed that bacterial adherence to the target mucosal surface has an important role in the pathogenesis of disease, since adherence establishes anchorage for further interactions with the host [2]. Bacteria may achieve this process by expressing surface adhesins, which bind to epithelial surface receptors in a specific fashion. *P. aeruginosa* adheres to a number of mammalian cell types including buccal epithelium [3], respiratory epithelium [4], respiratory mucin [5] and exposed collagen [6]. An *in vivo* study recently showed that *P. aeruginosa* adhered to exposed bronchial connective tissue and intraluminal secretions rather than intact respiratory mucosa in patients with cystic fibrosis (CF) [7]. Collagen-binding proteins have been identified for *Streptococcus pneumoniae* and Staphylococci, which mediate their adherence to mammalian extracellular matrix material [8]. By using transmission electron microscopy, the present group has recently shown that *P. aeruginosa* has a high affinity for human basement membrane collagen fibrils *in vitro* [9]. Adherence to basement membrane is, therefore, an important issue that has not been studied previously. Therefore, the authors have recently established a model to study bacterial adherence to basement membrane collagen and applied this to evaluate the effects of various chemicals on the adherence of *P. aeruginosa* to collagen *in vitro* [10].

Materials and methods

Inoculation of Pseudomonas aeruginosa

A clinical isolate of a nonmucoid and piliated strain of *P. aeruginosa* (PACS001) was stored in brain/heart infusion that contained 20% glycerol in liquid nitrogen. *P. aeruginosa* was retrieved on brain/heart infusion agar (Oxoid, Basingstoke, UK) plates and incubated overnight at 37°C. Passage was limited to three times prior to experiments. Following overnight incubation, a colony of *P. aeruginosa* was agitated in 4 mL of brain/heart infusion in a 6 mL clear plastic tube mounted on a roller stage for 24 h at 37°C. The resultant bacterial suspension was then centrifuged for 10 min at 2,000×*g*. The supernate was discarded and replaced with

4 mL of phosphate-buffered saline (PBS; Oxoid). This was repeated three times to wash the bacteria, which were finally resuspended in PBS. The final *P. aeruginosa* suspension was used for incubation with the Eppendorf lids (see description below).

Collagen coating

Sterile human type-IV collagen (Sigma, St. Louis, MO, USA) solution (2 $mg \cdot mL^{-1}$ in 1% acetic acid) was prepared immediately before each experiment. According to the manufacturer, the collagen had three major bands after sodium dodecylsulphate-polyacrylamide gel electrophoresis under reducing conditions consistent with basement membrane collagen [11]. Lids of plastic Eppendorf (microcentrifuge) tubes (Sorenson, Salt Lake City, UT, USA) were carefully trimmed and removed from the body of the tubes and sterilised by autoclaving. Collagen solution (50 μL) was added to the inside of an inverted Eppendorf lid and allowed to air-dry in an incubator maintained at 37°C for 24 h. Collagen-coated lids were washed by immersing in sterile PBS three times and air-dried for 30 min in an unhumidified incubator at 37°C. This protocol provided consistent and uniform coating of type-IV collagen onto the Eppendorf lids (fig. 1).

Incubation of Pseudomonas aeruginosa *with collagen-coated lids*

P. aeruginosa suspension (50 μL in PBS), which contained either none or various concentrations of test agents, was carefully added onto the collagen-coated lids by gentle pipetting. Viable count of the inoculating *P. aeruginosa* suspension was also performed to determine the bacterial concentration and purity. The lids were then incubated in the *P. aeruginosa* suspension for 45 min at 37°C in an unhumidified atmosphere. The authors had previously determined that 45 min was optimal for maximal adherence without any significant alteration in *P. aeruginosa* viable count. After incubation, the *P. aeruginosa* suspension was carefully decanted from the collagen-coated lids. The lids were rinsed in sterile PBS solution (5 mL) three times to remove nonadherent bacteria. Following that, the lids were fixed in 4% glutaraldehyde and stored at 4°C until processing for electron microscopy.

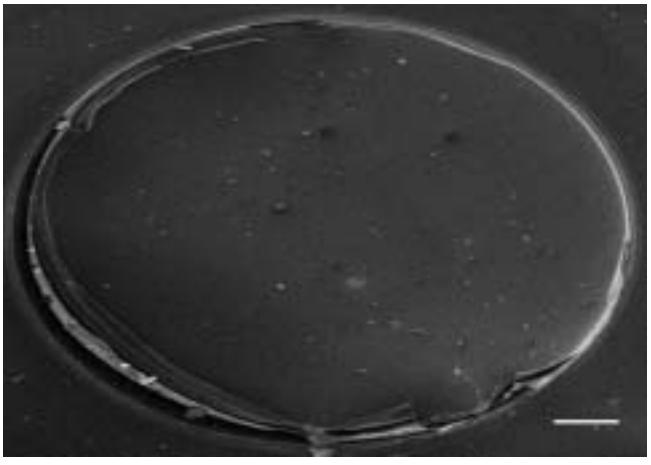

Fig. 1. – A scanning electron micrograph showing the test surface coated with a smooth layer of human type-IV collagen, the edges of which became slightly wrinkled after processing. Scale bar=500 μm.

Scanning electron microscopy processing

Collagen-coated lids incubated with *P. aeruginosa* were fixed in 4% glutaraldehyde for 24 h before rinsing in sodium cacodylate buffer, and postfixed in 1% osmium tetroxide for 1 h. Standard dehydration in graded ethanol then followed (three times in 50%, three times in 70%, three times in 90%, and three times in 100% for 5 min each) to 100% acetone. Specimens were then critically dried in carbon dioxide and mounted on aluminium stubs before being sputter-coated with gold. These specimens were randomly coded and stored in individual desiccated tubes prior to scanning electron microscopy examination by an observer who was unaware of the treatment.

Scanning electron microscope assessment of Pseudomonas aeruginosa *adherence to collagen-coated Eppendorf lids*

Each lid was placed on the stage of a scanning electron microscope (SEM) and viewed at low magnification (200×) to confirm uniform collagen coating, as shown in figure 1; otherwise the specimen would be rejected. For each specimen, 20 random SEM fields were examined at 2,000× magnification at the centre of the lid. The number of bacilli was counted manually for each of the SEM fields. The total number of *P. aeruginosa* bacilli was then calculated as *P. aeruginosa* density on collagen surface, which was a reflection of *P. aeruginosa* affinity towards collagen under the specific experimental condition. Adherence density was calculated as the total number of *P. aeruginosa* bacilli detected in 20 SEM fields divided by the logarithm of inocular size of *P. aeruginosa*, determined by viable counting as colony-forming units.

Effects of lectins, cations, sugars and other reagents on Pseudomonas aeruginosa *adherence*

A number of reagents, purchased from Sigma, unless otherwise stated, which were previously shown to affect *P. aeruginosa* adherence or were biochemically appropriate in the form of collagen constituents, were mixed with the *P. aeruginosa* suspension to evaluate their effects on *P. aeruginosa* adherence. Ca^{2+} was presented as calcium chloride ($CaCl_2 \cdot H_2O$) solution (Merck, Berlin, Germany). The concentrations of each of these reagents used in the *P. aeruginosa* suspension are shown in tables 1–4.

Statistical analysis

Data are expressed as mean±SEM, unless otherwise stated. Wilcoxon-signed rank tests were employed to compare paired data from the same experiments. A p-value of <0.05 was taken as a statistically significant difference between two groups of data.

Results

General observation

There was a consistent pattern on the SEM examination of *P. aeruginosa* adherence to collagen surface. The vast majority of SEM fields examined showed singular identical bacilli adherent to the collagen, usually with the long axis of the bacilli in direct contact with the latter (fig. 2).

In <1% of the SEM fields examined, the *P. aeruginosa* bacilli appeared in a cluster, like a bunch of grapes. There was little evidence of detachment of originally adherent bacilli,

Table 1. – The effects of lectins on the adherence densities of *Pseudomonas aeruginosa* adherence to human collagen type-IV

Lectins	Concentration $mg \cdot mL^{-1}$	Total *P. aeruginosa* bacilli in 20 SEM fields	Log *P. aeruginosa* inoculum cfu	*P. aeruginosa* adherence density	p-value
PHA-E#	0	284.0±104.6	8.8±0.2	31.7±11.2	
	0.01	219.2±96.8	8.9±0.1	24.2±10.5	0.07
	0.1	95.4±25.9	8.9±0.1	10.7±2.8	0.03*
	1	164.4±59.0	8.8±0.1	18.4±6.4	0.03*
Arachis hypogea#	0	396.0±61.2	8.8±0.1	44.8±6.4	
	0.01	412.8±120.8	8.9±0.1	46.1±13.0	0.92
	0.1	311.5±50.6	8.9±0.1	34.9±5.4	0.17
	1	281.5±50.0	8.9±0.1	31.5±5.3	0.07

Data are expressed as mean±SEM, unless otherwise stated. SEM: scanning electron microscope; cfu: colony-forming units; PHA-E: phytohaemagglutinin. #: n=6. *: $p<0.05$ when compared with absence of reagent using Wilcoxon-signed rank test.

Table 2. – The effects of charge on the adherence densities of *Pseudomonas aeruginosa* adherence to collagen type-IV

Ionic species	Concentration	Total *P. aeruginosa* bacilli in 20 SEM fields	Log *P. aeruginosa* inoculum cfu	*P. aeruginosa* adherence density	p-value
Ca^{2+} mM#	0	177.3±47.9	8.7±0.1	20.4±5.7	
	0.1	141.8±26.9	8.7±0.1	16.3±3.2	0.17
	1	81.0±18.3	8.8±0.1	9.3±2.1	0.03*
	5	82.8±25.5	8.8±0.1	9.4±3.0	0.03*
	10	115.3±14.3	8.8±0.1	13.2±1.7	0.03*
Heparin $IU \cdot mL^{-1}$¶	0	248.8±80.8	8.5±0.2	28.6±8.9	
	10	114.7±28.0	8.5±0.2	13.4±3.2	0.02*
	100	117.8±18.1	8.4±0.2	13.8±2.0	0.01*
	1000	137.8±25.4	8.5±0.2	16.1±2.9	0.12

Data are presented as mean±SEM, unless otherwise stated. SEM: scanning electron microscope; cfu: colony-forming unit. #: n=6; ¶: n=8. *: $p<0.05$ when compared with absence of reagent using Wilcoxon-signed rank test.

Table 3. – The effects of collagen component amino acids on the adherence of *Pseudomonas aeruginosa* adherence to collagen type-IV

Amino acid	Concentration $mg \cdot mL^{-1}$	Total *P. aeruginosa* bacilli in 20 SEM fields	Log *P. aeruginosa* inoculum cfu	*P. aeruginosa* adherence density	p-value
Proline#	0	269.9±63.3	8.8±0.1	30.8±7.2	
	0.1	256.1±35.8	8.7±0.1	29.3±4.2	0.92
	1	288.7±54.0	8.8±0.1	32.6±6.0	0.75
	10	263.6±30.8	8.8±0.1	29.8±3.3	0.92
Trans-hydroxyproline¶	0	212.2±41.6	9.0±0.1	23.7±4.8	
	0.1	197.1±39.7	8.9±0.1	22.1±4.5	0.31
	1	238.9±48.9	9.0±0.1	26.7±5.6	0.74
	10	242.8±45.9	9.0±0.1	27.1±5.1	0.50
Glycine#	0	153.9±41.0	8.7±0.2	17.5±4.7	
	0.1	172.0±39.7	8.8±0.2	19.5±4.7	0.75
	1	171.7±46.0	8.8±0.2	19.5±5.5	0.60
	10	138.8±37.9	8.7±0.2	15.6±4.4	0.75

Data are presented as mean±SEM, unless otherwise stated. SEM: scanning electron microscope; cfu: colony-forming unit. #: n=6; ¶: n=7.

as there were no bacterial "footprints" or other tell-tale distortion of the collagen surface. Bacterial polar pili were also found to be attached to the collagen surface (fig. 3).

Effects of lectins on Pseudomonas aeruginosa *adherence density*

Table 1 shows that phytohaemagglutinin (PHA)-E had inhibitory effects on *P. aeruginosa* adherence density. PHA-E at concentrations of 0.1 and 1 $mg \cdot mL^{-1}$ significantly reduced *P. aeruginosa* adherence density when compared with absence of PHA-E ($p<0.05$). The presence of *Arachis hypogea* appeared to decrease *P. aeruginosa* adherence density, although there was no statistical significance ($p>0.05$).

Effects of charge on Pseudomonas aeruginosa *adherence density*

Table 2 shows that the presence of Ca^{2+} at concentrations of 1, 5, and 10 mM, but not 0.1 mM, significantly reduced *P. aeruginosa* adherence density when compared with the absence of Ca^{2+} ($p<0.05$). There appeared to be no dose-dependent inhibition of *P. aeruginosa* adherence to collagen in the range of Ca^{2+} tested. Heparin also reduced *P. aeruginosa* adherence density significantly at concentrations of 10 and

Table 4. – The effects of sugars on the adherence densities of *Pseudomonas aeruginosa* adherence to collagen type-IV

Sugar	Concentration mg·mL^{-1}	Total *P. aeruginosa* bacilli in 20 SEM fields	Log *P. aeruginosa* inoculum cfu	*P. aeruginosa* adherence density	p-value
Galactose#	0	161.1±44.9	8.9±0.1	18.2±5.2	
	0.01	204.2±55.4	8.9±0.2	23.4±6.7	0.25
	0.1	227.2±78.4	8.9±0.2	26.1±9.4	0.35
	1	201.6±59.2	8.9±0.2	23.2±7.4	0.35
N-acetylneuraminic acid#	0	182.0±41.5	8.6±0.2	21.1±4.6	
	0.01	134.3±22.0	8.7±0.2	15.4±2.5	0.07
	0.1	173.3±44.6	8.6±0.2	20.3±5.1	0.60
	1	236.2±60.8	8.6±0.2	27.3±6.8	0.92
N-acetylglucosamine#	0	139.3±26.7	8.7±0.1	15.9±3.1	
	0.01	105.7±27.4	8.7±0.1	12.1±3.1	0.25
	0.1	97.5±36.4	8.7±0.1	11.2±4.2	0.25
	1	112.1±37.8	8.7±0.1	12.9±4.4	0.35

Data are presented as mean±SEM, unless otherwise stated. SEM: scanning electron microscope; cfu: colony-forming unit. #: n=6.

100 International Units (IU)·mL^{-1}, but not 1,000 IU·mL^{-1}, when compared with no heparin ($p<0.05$).

Effects of collagen components on Pseudomonas aeruginosa *adherence density*

Table 3 shows that proline, trans-hydroxyproline and glycine at concentrations of 0.1, 1, and 10 mg·mL^{-1} did not have any significant effects on *P. aeruginosa* adherence to collagen when compared with absence of test reagent ($p>0.05$).

Effects of sugars on Pseudomonas aeruginosa *adherence density*

Table 4 shows that galactose, *N*-acetylneuraminic acid, and *N*-acetylglucosamine at concentrations of 0.01, 0.1, and 1 mg·mL^{-1} did not have any significant effects on the adherence of *P. aeruginosa* to collagen when compared with absence of test agent ($p>0.05$).

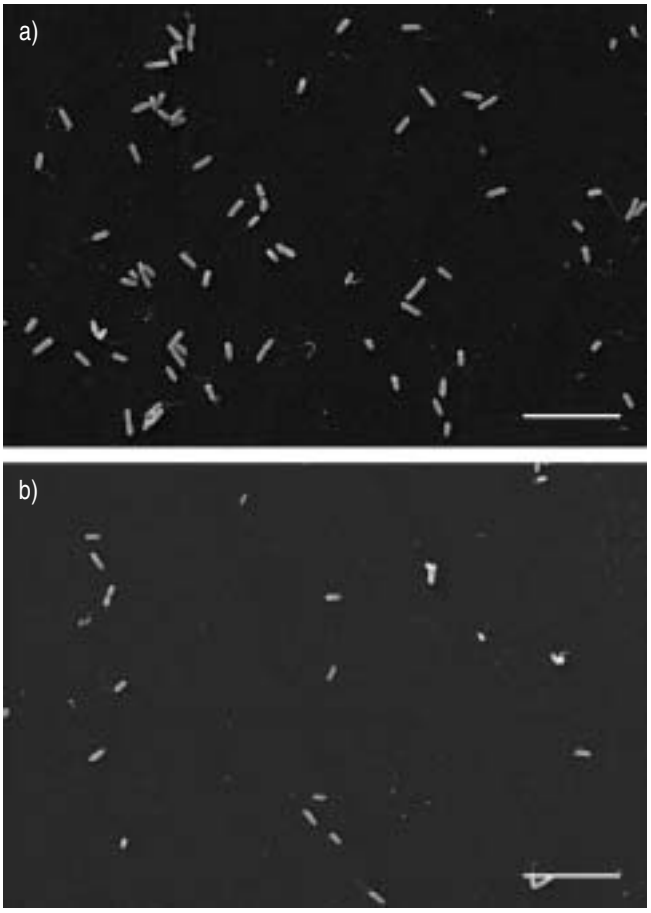

Fig. 2. – Scanning electron micrographs showing the collagen-coated surface with adherent *Pseudomonas aeruginosa* bacilli after 45 min of incubation in phosphate-buffered saline containing a) no phyto-haemagglutinin (PHA)-E and b) 0.1 mg·mL^{-1} of PHA-E, which significantly reduced *P. aeruginosa* adherence. Scale bars=10 μm.

Discussion

The authors have described a new model to directly study bacterial adherence to basement membrane using scanning electron microscopy [10]. By using direct manual counting of surface adherent *P. aeruginosa* bacilli with scanning electron microscopy, they have determined the exact number of adherent bacteria on the collagen surface. This could be a more direct and specific, albeit more laborious, method to determine bacteria adherence than previous indirect assays of bacterial adherence, such as radiolabelling techniques. By recent use of this model, the authors have shown that

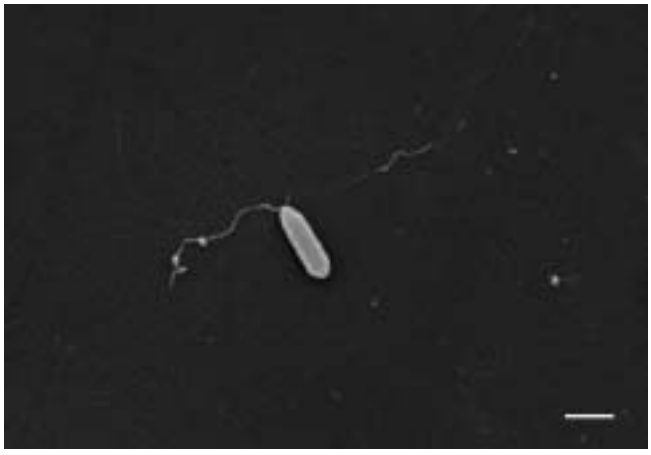

Fig. 3. – A high-power scanning electron micrograph showing the adherence of *Pseudomonas aeruginosa* bacilli to the collagen surface, usually on the bacterial long axis. There were polar pili on the bacillus, which appeared to be attached to the collagen surface. Scale bar=1 μm.

P. aeruginosa adherence to basement membrane collagen is reduced in the presence of low-dose erythromycin, probably partly due to alteration of bacterial morophology [10]. These results showed that the lectin PHA-E, but not *A. hypogea*, significantly inhibited *P. aeruginosa* adherence to collagen. PHA-E appeared to inhibit *P. aeruginosa* adherence at 0.01, 0.1 and 1 mg·mL^{-1}, although only the latter two concentrations inhibited adherence significantly. Ca^{2+} inhibited *P. aeruginosa* adherence at a concentration of >0.1 mM, although there was no obvious dose-dependent effect. Heparin only inhibited *P. aeruginosa* adherence at 10 and 100 IU·mL^{-1} but not at 1,000 IU·mL^{-1}. Major amino acid constituents of collagen, namely proline, trans-hydroxylproline and glycine, did not affect *P. aeruginosa* adherence significantly. Similarly, sugars, including galactose, *N*-acetylneuraminic acid and *N*-acetylglucosamine, did not alter *P. aeruginosa* adherence significantly.

Deoxyribonucleic acid fingerprinting techniques suggest that most CF patients harbour genetically related *P. aeruginosa* strains in their respiratory tract over long periods of time [12]. However, little is known of the mechanism(s) of *P. aeruginosa* persistence in the bronchiectatic airway. The preferential adherence of *P. aeruginosa* to damaged tissue is also largely unexplained, although damaged airway epithelial cells express asialo-G_{M1} oligosaccharide, which could be a *P. aeruginosa* receptor [13]. It is possible that *P. aeruginosa* bacilli evade mucociliary clearance by adhering to basement membrane at mucosal sites denuded of intact ciliated epithelium. *P. aeruginosa* exotoxins, such as pyocyanin, 1-hydroxylphenazine and rhamnolipid, can also expose the basement membrane to *P. aeruginosa* bacilli through slowing of ciliary beating, separation of epithelial tight junctions, and sloughing of damaged respiratory mucosa [7, 9]. As many intraluminal bacteria are adherent to respiratory mucus, many workers believe that this could be a reservoir for persistent airway pathogens, such as *P. aeruginosa* and *Haemophilus influenzae* [7, 14]. However, respiratory mucus is eventually expectorated and cannot subsequently retain these pathogens in the airways. The hypothesis described above could, therefore, better explain the persistent airway colonisation by respiratory pathogens, such as *P. aeruginosa* and nontypable *H. influenzae*. However, the mechanism(s) of *P. aeruginosa* adherence to basement membrane have not been studied systematically.

The adhesion of *P. aeruginosa* to respiratory mucosa is complex and multiple *P. aeruginosa* adhesins and epithelial receptors appear to be involved. *P. aeruginosa* pili are highly strain-specific proteinaceous appendages, which are adhesins mediating adherence to human tracheal mucosa [15]. Pili present on the surface of *P. aeruginosa* recognise the D-*N*-acetylgalactosamine-β1–4-D-galactose (GalNAcβ1–4Gal) disaccharide of asialo-G_{M1} and -G_{M2} receptors [16]. Mucoid strains of *P. aeruginosa* produce an exopolysaccharide that forms a loose capsule of organised linear strands of polysaccharide radiating outwards from the cell surface. This has been shown to mediate attachment to human respiratory epithelium [9, 17]. The authors have also observed a direct apposition of *P. aeruginosa* polar pili to the collagen surface in many SEM fields, although in many instances the *P. aeruginosa* bacilli were also directly attached to the collagen surface themselves.

P. aeruginosa and other common respiratory pathogens, such as nontypable *H. influenzae* and *S. pneumoniae,* bind to glycoconjugates on glycolipids and mucins. Specifically, the GalNAcβ1–4Gal disaccharide found in glycosphingolipid of epithelial cell surfaces of human lung explants is a candidate receptor [18]. Cell surface sialic acid has been identified as a vital component of epithelial receptors for *P. aeruginosa* adhesin(s) [19]. Several other respiratory pathogens, such as *Mycobacterium pneumoniae* utilise sialic acid-containing glycoconjugates as receptors [20]. Surface-bound neuraminidase could play a part in the initial recognition system, in addition to its removal of sialic acid residues to allow increased binding affinity between adhesin and asialo-terminal residues of cell surface receptors [21]. Available data also show that sialic acids and *N*-acetylglucosamine are components of mucin receptor(s), and both type 1 D-galactose-β1-3-D-*N*-acetylglucosamine (Galβ1–3GlcNAc) and type 2 D-galactose-β1–4-D-*N*-acetylglucosamine (Galβ1–4GlcNAc) disaccharide units are involved in the binding to *P. aeruginosa* [22]. Recently, *P. aeruginosa* has also been shown to possess high-affinity binding sites for sialyl-Lewis X conjugate, an *N*-acetylneuraminic acid α2–3-D-galactose-β1–4(D-fucoseα1-3)-D-*N*-acetylglucosamine oligosaccharide sequence that is commonly found in the mucins of CF patients [23]. This suggests that in addition to the recognition of neutral carbohydrate determinants, there are *P. aeruginosa* adhesins specific to acidic glycoconjugates produced as a response to local inflammation of the airway mucosa.

Basement membranes are predominantly comprised of type-IV collagen, laminin, fibronectin, and heparan sulphate proteoglycans. They underlie epithelial and endothelial cells and surround peripheral nerve and muscle cells [24]. Type-IV collagen is the most abundant nonfibril-forming collagen within the lung and provides the scaffolding for other basement membrane components to attach to. *P. aeruginosa* adheres to type-I collagen matrix [25], fibronectin [26], and laminin *via* a nonpilus-mediated mechanism [27]. *P. aeruginosa* adherence to type-I and -II collagen is inhibited by D-galactose, D-mannose and *N*-acetylneuraminic acid [28], and this suggests that saccharides could play a role in *P. aeruginosa* adherence to type-I and -II collagen. However, the adherence of *P. aeruginosa* to type-IV collagen, the most abundant framework of basement membrane, has not been studied previously.

The lectins PHA-E and *A. hypogea* were used to antagonise the adherence of *P. aeruginosa* to type-IV collagen in the present model. PHA-E specifically binds Galβ1–4GlcNAc linked to the Manα1–6 arm of complex-type *N*-glycans [11], and is likely to compete with the *P. aeruginosa* adhesin that recognises receptors bearing this disaccharide unit [22]. As the 7S domain of type-IV collagen bears loci for asparagine-linked glycans of the bi- and triantennary type with terminal β1–4-D-galactose-D-*N*-acetylgalactosamine (Galβ1–4GalNAc) [29] and the current results showed that PHA-E reduced *P. aeruginosa* adherence to collagen, *P. aeruginosa* adherence to type-IV collagen could involve the Galβ1–4GalNAc sequence. *A. hypogea* agglutinin binds specifically to β1–3-D-galactose-D-*N*-acetylgalactosamine (Galβ1–3GalNAc), which is the terminal sequence of gangliotetrosylceramide. The binding of *P. aeruginosa* to the latter suggests that this glycolipid might be an epithelial receptor for *P. aeruginosa* [18]. However, the lack of effects of *A. hypogea* on *P. aeruginosa* adherence in this study suggests that the terminal Galβ1–3GalNAc sequence is not involved in *P. aeruginosa* adherence to type-IV collagen. The lack of effect on adherence by the sugars, including galactose, *N*-acetylneuraminic acid, and *N*-acetylglucosamine (table 4), suggests that the three-dimensional structure of the determinant disaccharide or clusters of these are more important for adhesin recognition than individual sugars, and is consistent with the findings from the lectin studies.

Heparin is a glycosaminoglycan similar to heparan sulphate in disaccharide repeats of D-glucuronic acid-D-*N*-acetylglucosamine but different in extensive domains where disaccharide repeats are substituted with N- and O-sulphates. Heparin probably acts *via* competition with heparan sulphate moieties of proteoglycans present in the tissue, inhibiting adherence of urinary pathogens to bladder mucosa [30]. The present results show that heparin significantly inhibited

P. aeruginosa adherence between 10–100 $IU \cdot mL^{-1}$. It is possible that heparan sulphate was present as part of the proteoglycans in the large molecular aggregate component of the type-IV collagen preparation and therefore played a part in *P. aeruginosa* adherence. This interesting phenomenon should be further evaluated, as this low concentration of heparin should be achievable in the airways by nebulisation of a low dosage of heparin without systemic anticoagulative effect. A higher level of heparin, namely 1,000 $IU \cdot mL^{-1}$, was also associated with a lower *P. aeruginosa* adherence density compared with control, although this difference was not statistically significant (table 2). This lack of dose-dependent response is puzzling and cannot be explained by the current patchy understanding of the antiadherence effects of heparin. Further studies are clearly warranted as these could provide insights on *P. aeruginosa* adherence mechanisms and clues for designing experimental novel therapy for *P. aeruginosa* infection using heparin.

MARCUS *et al*. [31] showed that supraphysiological concentrations of Ca^{2+} (15mM) enhance *P. aeruginosa* adherence to hamster tracheal epithelium, suggesting the involvement of metal ions in adhesin-oligosaccharide binding. In contrast, the present authors found that physiological concentrations of Ca^{2+} inhibited *P. aeruginosa* adherence (table 2). In view of the possibility of heparan sulphate proteoglycans associated with type-IV collagen, Ca^{2+} binding to heparan sulphate moieties could have altered the available heparan sulphate for *P. aeruginosa* adherence. The major constituent amino acid components of collagen, namely proline, trans-hydroxyproline and glycine, had no effects on *P. aeruginosa* adherence to type-IV collagen (table 3). This suggests that these amino acids are not directly involved in the adherence process between *P. aeruginosa* and type-IV collagen. This is consistent with the above findings that the adherence process is more likely to involve Galβ1–4GlcNAc but not amino acids. It is also highly possible that these component amino acids are locked in the collagen skeleton and not directly exposed for the adherence process.

The results from this study show that *Pseudomonas aeruginosa* adherence to type-IV collagen probably acts *via* specific mechanism(s) involving adhesin recognition of the D-galactose-β1–4-D-*N*-acetylglucosamine sequence. In addition, heparin and Ca^{2+} also appear to be inhibitory for *Pseudomonas aeruginosa* adherence to proteoglycan components associated with basement membrane type-IV collagen. As persistent *Pseudomonas aeruginosa* colonisation is detrimental to patients with cystic fibrosis and bronchiectasis and there is currently no effective treatment for its eradication, these results could lead to a novel approach to treatment of persistent *Pseudomonas aeruginosa* infection. Further research should be pursued using this model on *Pseudomonas aeruginosa* adherence to other basement membrane components.

References

1. Ho PL, Lam WK, Ip MSM, Chan KN, Yuen KY, Tsang KW. The effects of *Pseudomonas aeruginosa* infection in clinical parameters in steady state bronchiectasis. *Chest* 1998; 114: 1623–1629.
2. Beachey EH. Bacterial adherence: adhesin receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J Infect Dis* 1981; 143: 325–345.
3. Woods DE, Bass JA, Johanson WG Jr, Straus DC. Role of adherence in the pathogenesis of *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. *Infect Immun* 1980; 30: 694–699.
4. Niederman MS, Rafferty TD, Sasaki CT, Merrill WW, Matthay RA, Reynolds HY. Comparison of bacterial adherence to ciliated and squamous epithelial cells obtained from human respiratory tract. *Am Rev Respir Dis* 1983; 127: 85–90.
5. Vishwananth S, Ramphal R. Adherence of *Pseudomonas aeruginosa* to human tracheobronchial mucin. *Infect Immun* 1984; 45: 197–202.
6. Ramphal R, Pyle M. Adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to acid-injured tracheal epithelium. *Infect Immun* 1983; 41: 345–351.
7. Baltimore RS, Christie CDC, Smith GJW. Immunohistopathological localization of *Pseudomonas aeruginosa* in lungs from patients with cystic fibrosis. *Am Rev Respir Dis* 1989; 140: 1650–1661.
8. Westerlund B, Korhonen TK. Bacterial proteins binding to the mammalian extracellular matrix. *Mol Microbiol* 1993; 9: 687–694.
9. Tsang KW, Rutman A, Tanaka E, *et al. Pseudomonas aeruginosa* infection of human respiratory mucosa *in vitro*. *Eur Respir J* 1994; 7: 1746–1753.
10. Tsang KW, Ng P, Ho PL, *et al.* Effects of erythromycin on *Pseudomonas aeruginosa* adherence to collagen and morphology *in vitro*. *Eur Respir J* 2003; 21: 401–406.
11. Kobata A, Yamashita K. Fractionation of oligosaccharides by serial affinity chromatography with use of immobilized lectin columns. *In*: Fukuda M, Kobata A, eds. Glycobiology. Oxford, Oxford University Press, 1993; pp. 103–125.
12. Kubesch P, Lingner M, Grothues D, Wehsling M, Tummler B. Strategies of *Pseudomonas aeruginosa* to colonize and to persist in the cystic fibrosis lung. *Scand J Gastroenterol* 1988; 23: Suppl. 143, 77–80.
13. de Bentzmann S, Roger P, Dupuit F, *et al.* Asialo GM1 is a receptor for *Pseudomonas aeruginosa* adherence to regenerating respiratory epithelial cells. *Infect Immun* 1996; 64: 1582–1588.
14. Tsang KW, Rutman A, Kanthakumar K, *et al. Haemophilus influenzae* infection of human respiratory mucosa in low concentrations of antibiotics. *Am J Respir Crit Care Med* 1993; 148: 201–207.
15. Doig P, Todd T, Sastry PA, *et al.* Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. *Infect Immun* 1988; 56: 1641–1646.
16. Lee KK, Sheth HB, Wong WY, *et al.* The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. *Mol Microbiol* 1994; 11: 705–713.
17. Ramphal R, Pier GB. Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in adherence to tracheal cells. *Infect Immun* 1985; 47: 1–4.
18. Krivan HC, Roberts DD, Ginsburg V. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAcβ1-4Gal found in some glycolipids. *Proc Natl Acad Sci USA* 1988; 85: 6157–6166.
19. Doig P, Paranchych W, Sastry PA, Irvin RT. Human buccal epithelial receptors of *Pseudomonas aeruginosa*: identification of glycoproteins with pilus binding activity. *Can J Microbiol* 1989; 35: 1141–1145.
20. Sobeslavsky O, Prescott B, Chanock RM. Adsorption of *Mycoplasma pneumoniae* to neuraminic acid receptors of various cells and possible role in virulence. *J Bacteriol* 1968; 96: 695–705.
21. Schulze IT. The biological active proteins of influenza virus: the haemagglutinin. *In*: Kilbourne ED, ed. The Influenza Viruses and Influenza. New York, Academic Press, 1975; pp. 53–82.
22. Ramphal R, Carnoy C, Fievre S, *et al. Pseudomonas aeruginosa* recognizes carbohydrate chains containing type 1 (Galβ1-3GlcNAc) or type 2 (Galβ1-4GlcNAc) disaccharide units. *Infect Immun* 1991; 59: 700–704.
23. Scharfman A, Degroote S, Beau J, Lamblin G, Roussel P, Mazurier J. *Pseudomonas aeruginosa* binds to neoglycoconjugates bearing mucin carbohydrate determinants and predominantly to sialyl-Lewis conjugates. *Glycobiology* 1999; 9: 757–764.

24. Kleinman HK, Schnaper HW. Basement membrane matrices in tissue development. *Am J Respir Cell Mol Biol* 1993; 8: 238–239.
25. Plotkowski MC, Chevillard M, Pierrot D, *et al.* Differential adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells in primary culture. *J Clin Invest* 1991; 87: 2018–2028.
26. Plotkowski MC, Bernardo-Hilho M, Tournier JM, Puchelle E. *Pseudomonas aeruginosa* bind to cellular soluble fibronectin. *Curr Microbiol* 1993; 26: 91–95.
27. Plotkowski MC, Tournier JM, Puchelle E. *Pseudomonas aeruginosa* strains possess specific adhesins for laminin. *Infect Immun* 1996; 64: 600–605.
28. Stepinska M, Trafny EA. Modulation of *Pseudomonas aeruginosa* adherence to collagen type I and type II by carbohydrates. *FEMS Immunol Med Microbiol* 1995; 12: 187–194.
29. Nayak BR, Spiro RG. Localization and structure of the asparagine-linked oligosaccharides of type IV collagen from glomerular basement membrane and lens capsule. *J Biol Chem* 1991; 266: 13978–13987.
30. Ruggieri MR, Hanno PM, Levin RM. The effects of heparin on adherence of 5 species of urinary tract pathogens to urinary bladder mucosa. *Urol Res* 1984; 12: 199–203.
31. Marcus H, Austria A, Baker NR. Adherence of *Pseudomonas aeruginosa* to tracheal epithelium. *Infect Immun* 1989; 57: 1050–1053.