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Interaction of *Pseudomonas aeruginosa* with human respiratory mucosa *in vitro*

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ABSTRACT: ***Pseudomonas aeruginosa*** **commonly infects the airways of patients with cystic fibrosis and bronchiectasis. It produces several toxins that slow ciliary beat, stimulate mucus production and damage epithelium. It adheres to epithelial cells, damaged mucosa (in animal models), and mucus. However, little is known of the interaction of *P. aeruginosa* with intact human respiratory mucosa.**

We have studied the interactions of a nonmucoid clinical isolate of *P. aeruginosa* with adenoid tissue in a novel organ culture model with an air-mucosal interphase *P. aeruginosa* ($5.9\pm0.9\times10^6$ colony-forming units (cfu)) was pipetted onto the organ culture surface, and incubated for 15 min, 1, 2, 4, 8, 12, 16, and 24 h, at 37°C in 5% CO_2 in a humidified atmosphere. Assessment has been made by transmission and scanning electron microscopy.

Transmission electron microscopy (TEM) showed that uninfected organ cultures had normal ultrastructure. TEM of infected organ cultures at 8 h showed significant epithelial damage: 43.9±10% of cells extruding from the epithelial surface, 17.7±3% of cells with loss of cilia, 32.9±10.2% of cells with mitochondrial damage, and 11.6±3% of cells with cytoplasmic blebbing. *P. aeruginosa* only infrequently adhered to normal epithelium, but adhered to areas of epithelial damage and to basement membrane. Scanning electron microscopy (SEM) of organ cultures up to 2 h found *P. aeruginosa* only infrequently associated with mucus. SEM at 4 h revealed *P. aeruginosa* predominantly associated with mucus and extruded damaged epithelial cells, but also occasionally associated with cilia, and very occasionally with unciliated cells. SEM also revealed loss of epithelial tight junctions in *P. aeruginosa* infected organ cultures, and *P. aeruginosa* were frequently seen in the gaps between epithelial cells. An extracellular matrix, possibly of bacterial origin, was seen bridging the space between bacteria and cell surface.

We conclude that *P. aeruginosa* infection of this organ culture caused tissue damage and that *P. aeruginosa* preferentially adhered to mucus, damaged epithelium and basement membrane.

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Pseudomonas aeruginosa commonly infects the airways of patients with cystic fibrosis (CF) and other forms of bronchiectasis. Once the infection is established, it is often impossible to eradicate even with intensive antibiotic therapy. Much of the morbidity and mortality of CF patients is due to chronic respiratory tract infection caused by *P. aeruginosa* [1].

P. aeruginosa is extremely versatile biochemically, and can grow in environments as diverse as jet fuel and distilled water [2]. However, it seldom, if ever, infects the lower respiratory tract of healthy people. Previous studies have identified numerous virulence factors for *P. aeruginosa* including: exotoxin A [3]; lipopolysaccharide [2]; the phenazine pigments, pyocyanin and 1-hydroxyphenazine [4, 5]; protease enzymes, such as elastase [6]; several haemolysins [7], including rhamnolipid [8]; and exoenzyme S [9]. *P. aeruginosa* has been shown to adhere to a number of mammalian cell types, including buccal epithelial cells [10, 11], the cilia of nasal and tracheal epithelium [12, 13], nasal epithelial cell culture monolayers [14], and damaged epithelial cells and exposed collagen [15, 16]. It is also known to adhere to respiratory mucin [17], and artificial surfaces [18]. However, these studies either utilize cell systems or animal tissue, and little is known of the interaction of *P. aeruginosa* with intact human respiratory mucosa.

Organ culture experiments usually involve tissue being immersed in cell culture fluid, so that bacteria can grow

in the medium as well as growing on the tissue surface. We have studied *P. aeruginosa* infection of a novel organ culture model of intact human respiratory mucosa exposed to humidified air. We have investigated the effects of *P. aeruginosa* infection on the ultrastructure of intact human respiratory mucosa, and have studied the association of *P. aeruginosa* with the mucosal surface by transmission and scanning electron microscopy.

Materials and methods

Inoculation of P. aeruginosa

A clinical isolate of a nonmucoid and piliated strain of *P. aeruginosa* (P455) was stored in liquid nitrogen and retrieved on number 2 agar plates (Oxoid, Basingstoke, UK). Passage was limited to three times prior to experiments. We have previously shown that P455 produces alkaline protease, elastase, phenazine pigments, lipase, deoxyribonuclease (DNase) and rhamnolipid [4, 19]. One colony was touched and dispersed in 3 ml of brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) and cultured for 6 h on a roller stage at 37°C. The culture was centrifuged and bacteria washed 3 times, before being finally resuspended in phosphate-buffered saline (PBS) (Oxoid, Basingstoke, UK).

Organ cultures

Human adenoid tissue was resected from children who had adenoid hypertrophy and transported to the laboratory in minimal essential medium (MEM); (Gibco, Paisley, UK), which contained antibiotics (50 $\mu g \cdot ml^{-1}$ streptomycin, 50 $IU \cdot ml^{-1}$ penicillin, and 50 $\mu g \cdot ml^{-1}$ gentamicin). Dissection was performed carefully to yield smaller pieces of adenoid of approximately 3×3 mm^2 with a thickness of 1–3 mm. Adenoid pieces with at least two completely cilia-free edges were selected. Tissue was immersed in MEM with antibiotics for a minimum of 4 h and a maximum of 6 h to eradicate commensal bacteria. The time varied because of the time needed to transport the tissue to the laboratory and the time required for dissection. The adenoid pieces were then immersed in 30 ml of MEM without antibiotics for one hour.

A 3 cm petri dish (Sterilin, Stone, UK) was placed in the centre of a 5 cm Petri dish (Sterilin, Stone, UK) aseptically (fig. 1). Four millilitres of MEM without antibiotics were added to the 5 cm Petri dish carefully, so that the inside of the 3 cm Petri dish remained dry. A strip of filter paper measuring 5 cm × 5 mm (Whatman 1, Maidstone, UK) was soaked in sterile MEM and then laid aseptically onto and across the diameters of the two Petri dishes. The two ends of the filter paper strip were positioned using a pair of sterile fine forceps, so that they were immersed in MEM. One piece of the adenoid tissue was placed with its ciliated surface upwards onto the filter paper strip at the centre of the smaller inner Petri dish. Approximately 0.25 ml of 1% semi-molten agar (Oxoid No. 1, Basingstoke, UK) at 40°C was carefully pipetted around the edge of the adenoid tissue in order to seal its cut edges. This solidified as it cooled and created an approximately 3 mm edge of agar.

Ten microlitres of a suspension of washed bacteria or PBS alone (for uninfected control) was dropped directly onto the centre of the organ culture. Uninfected and infected pairs of organ cultures were incubated at 37°C in 5% CO_2 in a well humidified atmosphere for 15 min (scanning electron microscopy, (SEM) n=2); 1 h (SEM n=2); 2 h (SEM n=1); 4 h (SEM n=2); 8 h (SEM n=2) and (transmission electron microscopy, (TEM) n=6); 12 h (TEM n=3); 16 h (TEM n=3); and 24 h (TEM n=4). Thereafter, each of the four edges of the organ culture were touched gently with a sterile plastic disposable loop in order to assess the purity of bacterial growth or sterility of control organ cultures. The organ culture with its adherent edge of agar and a small strip of filter paper was then removed from the Petri dishes and fixed for electron microscopic assessment.

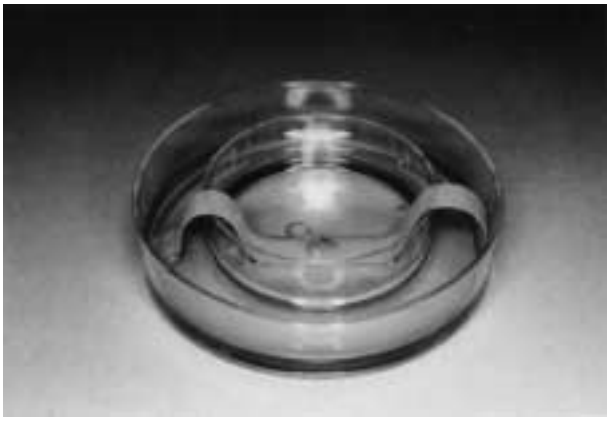

Fig. 1. – A photograph of the organ culture model showing the arrangement of the two Petri dishes and the adenoid tissue with an agar edge. The adenoid tissue was placed on a strip of filter paper, the ends of which were immersed in 4 ml of minimal essential medium.

TEM assessment

The adenoid tissue was fixed in 0.05 M sodium cacodylate-buffered 2.5% glutaraldehyde (pH 7.2) and postfixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2), followed by serial dehydration in alcohol and embedding in Araldite. A single section (70–90 nm thick) was taken through the centre of each organ culture for TEM assessment using a Hitachi H-7000 (Katsuta-Shi, Ibaraki-Ken, Japan) and typically contained 150–250 cells. The mean basement membrane length of a section was 2,473 µm (range 2,044–2,841 µm). For TEM, the specimens were coded so that the observer was unaware of their treatment. Each cell was scored for four parameters [20]: 1) loss of cilia from ciliated cells, 0 (fully ciliated), 1, 2, 3 (only one or two visible cilia); 2) extrusion of the cells from the epithelial surface, 0 (absent), 1, 2, 3 ,4 (cell completely

extruded from epithelial surface but still in contact with other epithelial cells); 3) cytoplasmic blebbing, 0 (absent), 1 (minor) or 2 (major); and 4) mitochondrial damage, manifested as swelling and disruption of mitochondrial cristae, 0 (absent) or 1 (present). As the relative significance of the TEM parameters was unclear, the score attributed to each was adjusted, so that a final maximum possible damage score of 100 was obtained for each of the four parameters. The total damage score for the tissue was obtained by summation of the adjusted scores of each of the four parameters (maximum possible 400). The percentages of cells with extrusion from the epithelial surface was calculated by the difference between 100 and the percentage of cells with no extrusion. Likewise, the percentages of cells with loss of cilia, cytoplasmic blebbing and mitochondrial damage were calculated [20].

SEM assessment

The methodology used was described previously [21]. Briefly, adenoid tissue was fixed in 2.5% glutaraldehyde for a minimum of 24 h before routine processing through gentle buffer washes, then 1% osmium tetroxide for 1 h, followed by dehydration through graded ethanols to acetone and critical point drying in CO_2. Tissue was mounted on aluminium stubs, sputter-coated with gold, and examined in a Hitachi S-4000 scanning electron microscope (Katsuta-Shi, Ibaraki-Ken, Japan) by an examiner blind to the experimental protocol.

Statistical analysis

Wilcoxon signed ranked test was employed to analyse the data [22]. A p-value less than 0.05 was taken as a statistical significant difference between two groups of data.

Results

Bacteria

The mean inoculum of *P. aeruginosa* for the infected organ cultures was $5.9\pm0.9 \times 10^6$ colony forming units (cfu). *P. aeruginosa* infected organ cultures produced pure growth of *P. aeruginosa* from all four edges after incubation and the controls were sterile.

Gross appearance of the organ cultures

After incubation, by naked eye examination the uninfected organ cultures appeared unchanged. However, *P. aeruginosa* infected organ cultures developed a greenish blue colour that was detectable from 12 h.

TEM assessment (table 1)

The uninfected control organ culture had an essentially normal ultrastructure in all the experiments. After 8 h infection with *P. aeruginosa*, there was a significant ($p<0.05$) increase in the percentage of cells displaying extrusion from the epithelial surface, loss of cilia, mitochondrial damage and cytoplasmic blebbing (fig. 2). The mean combined mucosal damage score of the infected organ cultures was significantly ($p<0.05$) increased when compared with the uninfected controls. However, damage to the epithelium was patchy, and some areas examined had relatively normal ultrastructure. This is reflected in the combined mucosal damage score, which has a possible maximum value of 400.

In all TEM sections of the infected organ cultures at 8 h, bacteria were seen closely associated with the mucosal surface. *P. aeruginosa* was found predominantly adherent to damaged epithelial cells and mucus. In all the

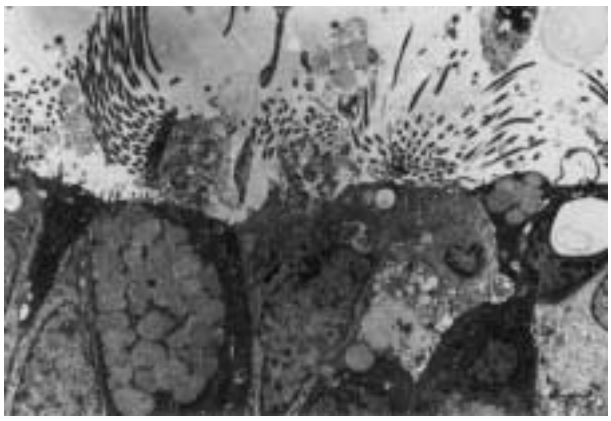

Fig. 2. – Transmission electron micrograph of an adenoid organ culture infected with *Pseudomonas aeruginosa* for 8 h, showing damage to the epithelium. There is loss of cilia (cell to right of centre), cell extrusion (part of cell to left of centre), and mitochondrial damage (all cells). (scale bar=2.0 μm).

Table 1. – Transmission electron microscopy assessment of adenoid organ cultures at 8 h

Organ culture	Cells with loss of cilia	Cells extruding from the epithelial surface	Cells with cytoplasmic blebbing	Cells with mitochondrial damage	Combined mucosal damage score
	%	%	%	%	
Uninfected	7.8±1.1	13.1±1.2	1.7±0.4	1.8±0.5	9.5±1.0
Infected with PA	17.3±3.0*	43.9±10*	11.6±3.0*	32.9±10.2*	32.9±18*

All data are mean of six separate experiments±SEM. For explanation of the morphometric scoring system please see text. *: $p<0.05$ *versus* uninfected organ culture. PA: *Pseudomonas aeruginosa* (P455 strain).

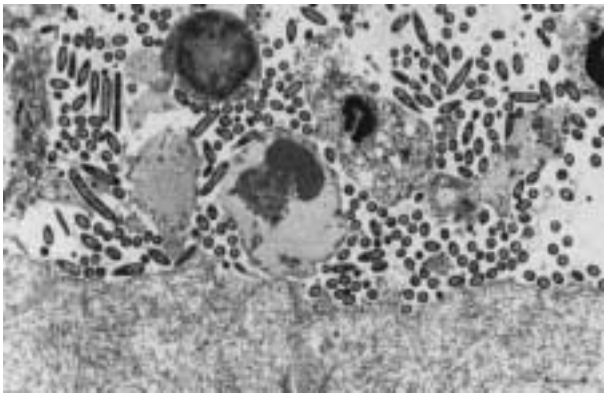

Fig. 3. – Transmission electron micrograph of an adenoid organ culture infected with *Pseudomonas aeruginosa* for 8 h, showing adherence of bacteria to basement membrane. (scale bar=2.22 μm).

infected organ cultures, large colonies of bacteria were found infiltrating the epithelium and adhering to the basement membrane (fig. 3).

In the organ cultures infected by *P. aeruginosa* for 12, 16 and 24 h, complete disintegration of the epithelial structure and subepithelial collagen layer was seen. Bacteria were seen infiltrating the disintegrated mucosa. No intracellular bacteria were detected.

SEM assessment

In organ cultures incubated for 15 min, 1 and 2 h, *P. aeruginosa* was only found infrequently in small colonies which were associated with mucus, and with occasional extruded cells. After 4 h, the bacterial numbers had increased substantially and mucosal damage had occurred. Most of the surface mucus, cell debris and extruded cells, had associated *P. aeruginosa* (fig. 4). When *P. aeruginosa* was seen associated with mucus, there were often a large number of bacteria in the microcolony. *P. aeruginosa* were found only occasionally associated with cilia and unciliated cells. When *P. aeruginosa* were seen associated with ciliated epithelial cells, they were usually associated with the tips of cilia. Separation of

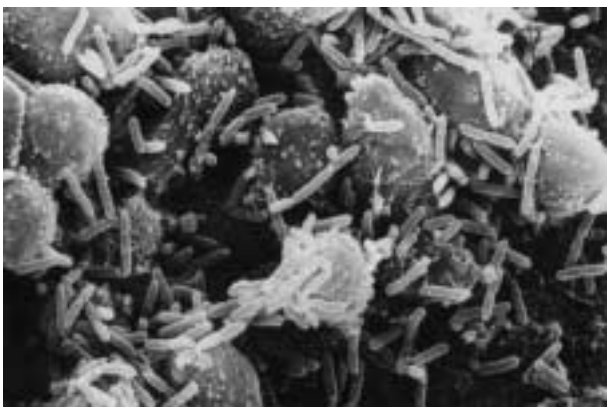

Fig. 4. – Scanning electron micrographs of an adenoid organ culture infected with *Pseudomonas aeruginosa* for 4 h, showing bacteria adhering to extruded and damaged epithelial cells (scale bar=1.79 μm).

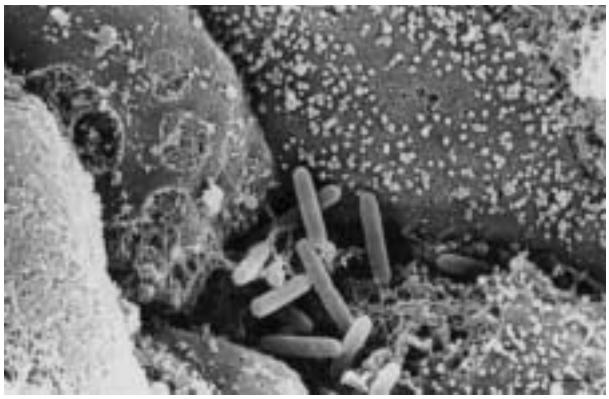

Fig. 5. – Scanning electron micrograph of an adenoid organ culture infected with *Pseudomonas aeruginosa* for 4 h showing bacteria in a gap between adjacent epithelial cells. There was an extracellular matrix material bridging the space between bacteria, and between bacteria and cell surfaces. The same appearance was seen between bacteria and other mucosal structures, such as mucus and cilia. (scale bar=0.89 μm).

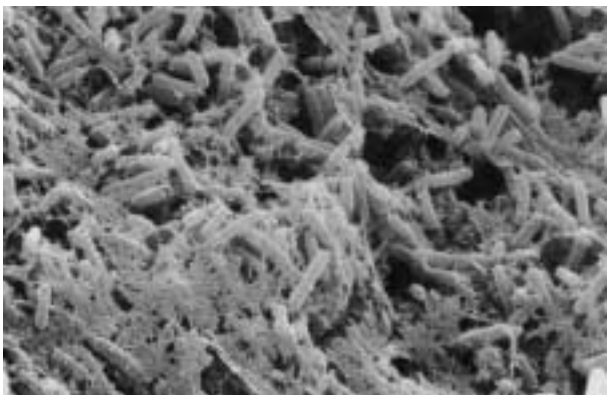

Fig. 6. – Scanning electron micrograph of an adenoid organ culture infected with *Pseudomonas aeruginosa* for 8 h, showing a biofilm of bacteria with a lace-like extracellular matrix material obscuring most of the organ culture surface. (scale bar=1.33 μm).

epithelial cell junctions was evident in many parts of the organ culture surface. Frequently, but not always, colonies of *P. aeruginosa* were seen in the gaps formed between epithelial cells, confirming the impression gained from TEM that *P. aeruginosa* penetrates the epithelium between cells to reach the collagen layer (fig. 5). After 8 h, most of the surface of the organ cultures was covered with bacteria that formed continuous sheets covering the surface of a large proportion of the organ culture (fig. 6). This concealed the structures that the bacteria were associated with and made SEM assessment difficult. Bacteria in these large colonies were often seen dividing.

In bacterial microcolonies, particularly those with large numbers of bacteria, an extracellular matrix material was seen closely associated with bacteria. This material was not seen in uninfected controls, but was seen associated with *P. aeruginosa* grown in BHI broth after 6 h, suggesting that it had bacterial origin, although the amount of the material was less than that seen in organ cultures. When *P. aeruginosa* was seen associated with the mucosal surface including mucus, cilia, unciliated cells and extruded cells, the matrix material was often

seen "bridging" the gap between the bacteria themselves (fig. 6), and between bacteria and the mucosal component with which the bacterium was associated (fig. 5).

Discussion

In this study, we have used an organ culture with an air-mucosal interphase to study the interaction of a nonmucoid strain of *P. aeruginosa* with human respiratory mucosa. We chose a nonmucoid strain partly because we have investigated this strain (P455) extensively in our laboratory [4, 23], and partly because the initial colonization of the respiratory mucosa is usually by nonmucoid strains, which become mucoid with chronic infection [24]. An organ culture with an air-mucosal interphase has a number of advantages. Organ cultures immersed in cell culture medium are constantly exposed to reinfection from bacteria growing in the medium and their products, which may be toxic. This artificially biases the infection in favour of the bacteria. The air-mucosal interphase is more physiological and the mucociliary system might be expected to function as *in vivo*.

Adherence of bacteria to host cells is known to be affected by the microenvironment in which the cells interact with each other [25]. Recently, *P. aeruginosa* adherence to hamster tracheal cell cultures has been reported to be pH sensitive and Ca^{2+}-dependent [26, 27]. Therefore, the presence of cell culture medium in studies of the interaction of *P. aeruginosa* with respiratory mucosa may influence bacterial adherence. Adenoid tissue was used in our experiments because it is relatively easily available human ciliated epithelium, whereas lower respiratory tract tracheal or bronchial epithelium is much more difficult to obtain and more difficult to dissect during preparation of the organ culture.

Previous studies have mainly concentrated on the adherence of *P. aeruginosa* to epithelial cells in the absence of an intact mucociliary system, and little is known of the effects of *P. aeruginosa* infection on the ultrastructure of the respiratory mucosa. Loss of cilia, extrusion of cells from the epithelial surface, cytoplasmic blebbing and mitochondrial damage were found after 8 h in this study, when respiratory mucosa was infected by *P. aeruginosa*. SEM detected *P. aeruginosa* only infrequently on the mucosal surface in the first 2 h after inoculation despite quite a large initial inoculum. This suggests that although *P. aeruginosa* showed an affinity for mucus later in the experiments, washed bacteria from broth culture do not immediately adhere to mucus with high affinity. However, a substantial increase in bacterial density occurred by 4 h, which was associated with mucosal damage. This latency for the detection of *P. aeruginosa* on the mucosal surface has also been reported previously in hamster and mouse tracheal organ cultures [15, 28–30].

In this study, we have shown that *P. aeruginosa* preferentially adhered to mucus and extruded and damaged epithelial cells, in preference to cilia and unciliated cells. Adherence to damaged epithelial cells has previously been shown in hamster trachea organ culture, in which adherence only occurred after influenza A virus infection or treatment with acid that injured the epithelium [15, 31]. These studies have suggested that both mucoid and nonmucoid strains of *P. aeruginosa* only adhered to damaged but not normal cells [32]. Recently BALTIMORE *et al.* [33] only found adherence of *P. aeruginosa* to intraluminal secretions, damaged epithelium and exposed connective tissue, in a histological study of the airways of patients with cystic fibrosis infected by *P. aeruginosa*. Our results agree with these observations. *P. aeruginosa* also adhered less frequently to ultrastructurally normal cilia, which has been observed previously with human respiratory epithelial cells [12–14]. Although the cilia were ultrastructurally normal, we do not know about their function at the time of bacterial adherence. *P. aeruginosa* produces a number of factors which slow or stop ciliary beating [4, 8], which may precede bacterial adherence to cilia.

In studies using organ cultures of whole trachea or tracheal rings from hamsters, mucoid strains were found to adhere as aggregates, primarily to ciliated cells, with the bacterial extracellular matrix itself binding directly to the cilia [27–30]. Epithelium of different species may have different receptors for *P. aeruginosa* adhesins, and the experimental conditions may influence the results obtained. Nonmucoid *P. aeruginosa* adhered primarily to unciliated cells in a study using hamster tracheal cell cultures [26]; to mucus and damaged cells, and very uncommonly to ciliated epithelium in sulphur dioxide injured canine trachea in organ culture [16]; and to both ciliated and unciliated cells in canine tracheal cell cultures [34].

P. aeruginosa has a high affinity for human tracheobronchial mucin [17, 35–38], and our study shows that it also has a high affinity for mucus in organ culture. *P. aeruginosa* also produces a number of toxins which stimulate mucus production (19, 39, 40). In the 8 h organ cultures, *P. aeruginosa* formed continuous sheets over the organ culture surface. It has been suggested that persistence of *P. aeruginosa* in the lower respiratory tract may be helped by the formation of such biofilms. Biofilms may protect the bacteria against host defences [41], such as opsonophagocytic killing by neutrophils [42].

In our study *P. aeruginosa* was also found adherent to the basement membrane. Adherence of a nonmucoid strain of *P. aeruginosa* to the tracheal collagen layer was also found in rat trachea injured by brushing [43]. PLOTKOWSKI and co-workers [14, 44, 45] have also reported adherence of nonmucoid *P. aeruginosa* to type I collagen matrix [14], and to submucosal connective tissue obtained from the frog palate [44, 45].

Pili have been identified as an important adhesin for *P. aeruginosa* to buccal cells [11], damaged tracheal epithelial cells [46], and mucin [47], but do not account for all the adhesive properties of *P. aeruginosa* [48], and other adhesins, such as exoenzyme S have been identified [9]. The alginate of mucoid strains may be another *P. aeruginosa* adhesin and nonmucoid strains may also produce alginate [49]. Mucoid strains of *P. aeruginosa* produce an exopolysaccharide that forms a loose capsule of organized linear strands of

polysaccharide radiating outwards from the cell surface. This has been shown to mediate attachment to human respiratory epithelium [50, 51]. In our SEM study, a matrix-like material was closely associated with colonies of *P. aeruginosa* and bridged the space between bacteria and cell surfaces. A similar matrix-like material was seen closely associated with colonies of aggregated bacteria in a recent study performed with primary cultures of human respiratory epithelium obtained from nasal polyps [14]. In this study, the matrix-like material seemed to play a role in the association of aggregated bacteria with cilia, although the adherence of nonaggregated *P. aeruginosa* to unciliated cells occurred without it. A preliminary analysis suggested that because the matrix-like material reacted with antimucin antibody it may have an epithelial origin [14]. However, we have identified similar material around *P. aeruginosa* cultured in broth, and, therefore, suggest that the matrix could have a bacterial origin. In our study, the matrix-like material was frequently seen between bacteria and the point of mucosal contact (including mucus), and it may thus act as an adhesin. The matrix could occur by conversion to the mucoid phenotype during organ culture [52, 53], or be the slime of nonmucoid *P. aeruginosa* [18, 49].

Tight junctions between cells are important in maintaining the integrity of the epithelial surface. They form a barrier to the diffusion of molecules and ions across the epithelial cell layer and their loss may lead to changes in transepithelial electrical resistance, exposure of subepithelial structures to bacteria and their toxins, and leakage of tissue fluid, which contributes to the increased secretions during infection [54]. In our study, both TEM and SEM examination of the tissue infected by *P. aeruginosa* showed epithelial cells separated from their neighbours, and bacteria were sometimes seen invading the epithelium by this route. Cell separation is consistent with loss of epithelial cell tight junctions and may be a precursor to extrusion of cells from the epithelial surface. Current knowledge of the effects of bacterial infection on human respiratory epithelial cell tight junctions is lacking, although it has been shown in animal studies that exposure to cigarette smoke [55, 56], nitrous oxide [57, 58], *P. aeruginosa* elastase [59], neutrophils [60, 61], and reduced Ca^{2+} [62] disrupts epithelial cell tight junctions.

Chronic infection of the respiratory tract by *P. aeruginosa* is difficult to eradicate, even with prolonged use of potent antibiotics. Deoxyribonucleic acid [DNA] fingerprinting techniques suggest that most CF patients harbour genetically related *P. aeruginosa* strains in their respiratory tract over long periods of time [63]. Our study has shown that during infection, *P. aeruginosa* damages the respiratory mucosa and adheres to secretions, damaged epithelial cells and collagen. *P. aeruginosa* adherence to mucus, and its lack of adherence to normal epithelium, may explain why it does not infect the normal airway, which has efficient mucociliary defences. However in bronchiectasis and CF, in which mucus is poorly cleared, *P. aeruginosa* may colonize static secretions and produce toxins which damage the mucosa and further disable remaining host defences. *P. aeruginosa* infection stimulates a florid chronic inflammatory response, which is ineffective in clearing *P. aeruginosa* and damages the lung, encouraging persistence and spread of the infection in the airways [64].

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