Epithelial respiratory cells from cystic fibrosis patients do not possess specific *Pseudomonas aeruginosa*-adhesive properties

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Summary. Nasal polyp cells in primary culture from cystic fibrosis (CF) and non-CF patients were compared for the ability to bind *Pseudomonas aeruginosa* cells and for the presence of sulphated glycoconjugates at the epithelial cell surface. Quantitation of bacterial adhesion, by scanning electronmicroscopy, showed no significant difference between the cells cultured from CF and non-CF patients. Micro-organisms associated with ciliated cells were mainly aggregated, in contrast with those from non-ciliated cells. Sulphated glycoconjugates were identified on cells cultured from both CF and non-CF patients, regardless of whether or not these cells had attached bacteria. A matrix-like material that surrounded the aggregated bacteria was more prominent on cells cultured from CF patients than on those from non-CF patients. The interaction of aggregated *P. aeruginosa* cells with polyp cells cultured from both CF and non-CF patients appeared to occur by means of this matrix material. Our findings suggest that chronic colonisation of the airways of CF patients cannot be explained by an increased affinity between the *P. aeruginosa* cells and the respiratory cell surface receptors in the CF patient. Nevertheless, the in-vitro observation that the matrix surrounding the bacteria reacted with a monoclonal antibody against respiratory mucins allows us to speculate that increased mucin secretion by cells from CF patients might, *in vivo*, play a decisive role in the interaction between *P. aeruginosa* and the respiratory epithelium.

Introduction

Chronic colonisation and infection of the respiratory tract with *Pseudomonas aeruginosa* occurs commonly in patients with cystic fibrosis (CF). Several factors may contribute to the susceptibility of these patients to infection by the organism. In normal individuals, entrapping of inhaled micro-organisms by respiratory mucus and subsequent elimination by ciliary beating protects the respiratory tract against infection. However, when the ciliary clearance is decreased, bacteria may multiply and infect the epithelial cells. In CF, severe reduction of the mucociliary transport has been described.¹ Moreover, *P. aeruginosa* cells adhere avidly to human respiratory mucins.^{2,3} This property, as well as the presence of chemotactic factors in the mucins from CF patients,⁴ may play a decisive role in the infection of these patients. This hypothesis was supported by immunohistopathological studies on the airways of CF patients, which showed that the *P. aeruginosa* cells remained sequestered within an exudate, rather than attached to undamaged epithelium.⁵

To explain the specificity of *P. aeruginosa* as a respiratory pathogen in CF patients, the presence of some component of respiratory mucins unique to CF patients must be assumed. However, recent in-vitro studies showed that most mucin-like glycopeptides from the sputum of CF patients allowed less adhesion of *P. aeruginosa* cells than did glycopeptides from the respiratory secretions of bronchitic patients.⁶

The specific interaction between cell surface glycoconjugate receptors and microbial adhesins is an important step in the pathogenesis of infectious diseases. Although the adhesion of *P. aeruginosa* to normal respiratory cells from different animal species, including man, has been studied exhaustively,^{2,7–12} the adhesion to respiratory cells from CF patients has not been investigated, to our knowledge.

Epithelial cells from CF patients differ from those of non-CF patients in permeability to chloride ions^{13,14} and in releasing high-mol.-wt glycoconjugates which are more highly sulphated than are those from the cells of non-CF patients.¹⁵ This high sulphate content of the cell-surface glycoconjugates may affect the interaction of the bacteria with the airways.^{15,16} Alternatively, CF results in defective glycosylation of the transmembrane conductance regulator protein (CFTR),¹⁷ and defective glycosylation of cell surface

glyconjugates may also occur, imparting specific adhesive properties for *P. aeruginosa* epithelial respiratory cells.

The present study evaluated whether the adhesion of *P. aeruginosa* cells to cultured respiratory epithelial cells from CF patients differed from that to cells from non-CF patients. Because increased sulphation of cell-surface glycoconjugates might augment the adhesion of *P. aeruginosa* to CF cells, we also investigated the occurrence of sulphated carbohydrates on the surface of respiratory epithelial cells cultured from CF and non-CF patients.

Materials and methods

Cell culture

Epithelial respiratory cells were cultured from 13 small explants, each *c.* 1 mm² in size, which were dissected from nasal polyps from five CF patients and from four non-CF patients, who were undergoing polypectomy for nasal obstruction. The explants were cultured on type I collagen matrix in hormone-supplemented serum-free defined RPMI 1640 medium (Sigma), as described elsewhere.¹⁸

Bacteria

A non-mucoid strain of *P. aeruginosa* isolated from the expectorated sputum of a CF patient was maintained in Trypticase Soy Broth (TSB; Institut Pasteur) containing glycerol 20% v/v at -20°C. For adhesion assays, bacteria were cultured overnight in TSB at 28°C, harvested by centrifugation and resuspended in RPMI 1640 medium containing 20 mM N-2 hydroxyethylpiperazine-N'2 ethanesulphonic acid (RPMI-HEPES). The optical density of this suspension was adjusted to 0.5 at a wavelength of 640 nm (Beckman M9 Spectrophotometer). This optical density corresponded to *c.* (5–8) × 10⁸ cfu/ml. Immediately before the adhesion assays, the bacteria were dispersed with a syringe fitted with a 25 gauge needle.

Adhesion assays

After incubation for 4–6 days (as above), the supplemented RPMI medium in the culture wells was removed and replaced by 200 µl of the *P. aeruginosa* suspension. The cultures were incubated for 1 h at 37°C, then rinsed four times with gentle manual agitation with 0.5 ml of 0.1 M phosphate-buffered saline, pH 7.2 (PBS), and fixed with glutaraldehyde 2.5% in PBS for 2 h at 4°C, unless otherwise stated.

Quantitation of bacterial adhesion. Fixed epithelial cells were rinsed with PBS, dehydrated through a graded series of washes with ethanol, dried to the critical point, then coated with gold-palladium and examined with a scanning electronmicroscope (SEM) (Philips Model 525). Because the contours of non-ciliated cells were not always discernible, we could not

directly determine the number of adherent bacteria per epithelial cell. Therefore, to quantitate adhesion of *P. aeruginosa* cells, we used an image analyser (Biocom 500) connected to the SEM to draw the outlines of ciliated cells and, for each microscope field, to determine the surface areas, in µm², of both ciliated (CC) and non-ciliated cells (NCC). In each culture examined, adherent bacteria were counted in at least 150 microscope fields at a magnification of × 2700. The working distance (10 mm), angle (20°) and voltage (15 kV) remained constant throughout the study.

Preliminary assays showed that the *P. aeruginosa* cells adhering to CC or NCC cultured from CF or non-CF patients were often present as clumps. The numbers of bacteria in these clumps could not be determined. So, each clump was counted as one bacterium and the distribution of clumps was assessed. This approach underestimated the total adhesion of *P. aeruginosa* cells.

Localisation of sulphated carbohydrates on the surfaces of epithelial cells

Glutaraldehyde-fixed epithelial respiratory cells cultured from three CF and three non-CF nasal polyps were rinsed in PBS and exposed, for 24 h at room temperature, to the High Iron Diamine (HID) solution, prepared as described by Spicer *et al.*¹⁹ Subsequently, the specimens were rinsed in PBS, dehydrated through graded ethanol washes, embedded in an epoxy resin and observed, without uranyl or lead acetate staining, in a transmission electronmicroscope (TEM, Jeol 200 CX). Control cultures were exposed for 24 h to a solution of HID from which FeCl₃ was omitted.

Evaluation of the role of mucins in bacterial aggregation

The role of the mucins secreted by respiratory cells in causing the aggregation of *P. aeruginosa* cells was evaluated by fluorescence microscopy and electron-microscopy. Epithelial respiratory cells with adherent bacteria were fixed with paraformaldehyde 4% in PBS for 2 h at 4°C, rinsed with PBS, and exposed for 1 h to the monoclonal antibody (MAb) 17B1 against the respiratory mucin of rhesus monkeys²⁰ (kindly provided by Dr R. Wu, University of California, Davis, CA, USA). The antibody was used at a concentration of 10 mg/L in PBS containing bovine serum albumin 1% (PBS-BSA). Subsequently, the cultures were rinsed in PBS and exposed to an anti-mouse IgG-biotin complex (Amersham International, Bucks) diluted in PBS-BSA. For fluorescence microscopy, the cells were exposed to a 1 in 25 streptavidin-FITC complex at a 1 in 25 dilution. To favour the visualisation of aggregated bacteria, cultures were then exposed to a rabbit antibody against the somatic *P. aeruginosa* antigen (Institut Pasteur) at a 1 in 10 dilution and, finally, to an anti-rabbit IgG-Texas Red complex (Amersham). The cultures were then observed with a light micro-

Fig. 1. Scanning electronmicrographs of respiratory epithelial cells from a CF patient in culture showing: **a**, the presence of strands of secretion with adherent bacteria (arrows) and, **b**, an amorphous material (arrow) associated with the bacteria. Bars = 4 μm .

scope equipped with epifluorescent illumination (Axioptot, Zeiss). For electronmicroscopy, the cells were treated, as above, as far as exposure to the anti-mouse IgG-biotin complex. Subsequently, they were treated with a streptavidin-colloidal gold (5 nm) complex (Bio Cell), fixed with osmium tetroxide 1% in 0.1 M cacodylate buffer, pH 7.4, containing ruthenium red 0.05% w/v, dehydrated with graded ethanols prepared with cacodylate-ruthenium red buffer and embedded in epoxy resin. Ultrathin (90 nm) and thick (1.0 μm) sections were examined in the TEM operating at 80 or 200 kV.

Statistical analyses

The Wilcoxon matched-pairs test was used to compare the mean numbers of adherent bacteria/ μm^2 on CC and NCC, and the percentage of aggregated bacteria adherent to CC and NCC in each culture examined. The comparisons between the cultures from CF and non-CF patients for the mean number of adherent *P. aeruginosa* cells, the percentage of CC with aggregated micro-organisms, and the percentage of aggregated bacteria adherent to CC and NCC were done by the Mann-Whitney test.

Results

Characteristics of the epithelial cell cultures

Explants dissected from nasal polyps from CF and non-CF patients yielded cellular outgrowth in which rapid ciliary activity was evident by optical microscopy. Both CC and NCC were observed, by SEM, in the cellular outgrowth but the CC were more frequent near the excised polyp. At the periphery of the cultures, migrating cells, with pseudopodial projections extending towards the collagen matrix and unfolded apical cell membranes, were identified (not shown). These migrating cells have a specific adhesive

property for *P. aeruginosa*²¹ and were excluded from the quantitative study.

In five of the 13 cultures from CF patients, we observed strands of secretion that linked one CC to another. Adherent bacteria were identified in these strands (fig. 1a). In the other cultures from CF patients, or in those from non-CF patients, these strands of secretion were rarely observed. Moreover, in most of the cultures from CF patients we detected an amorphous material lying over the epithelial cells. The bacteria were closely associated with this material (fig. 1b).

Adhesion of *P. aeruginosa* to epithelial respiratory cells

P. aeruginosa cells were seen, by SEM, to adhere to both CC and NCC cultured from CF and non-CF patients, but a marked difference was noted between the patterns of adhesion to these two cell types. Bacteria associated with CC were mainly present as aggregates (fig. 2), whereas bacteria associated with NCC usually were not aggregated. This difference was

Fig. 2. Scanning electronmicrograph of respiratory epithelial cells from a CF patient, showing aggregated *P. aeruginosa* cells associated with the cilia (arrows). Bar = 6 μm .

Fig. 3. Percentages of aggregated *P. aeruginosa* cells associated with ciliate (CC) and non-ciliate cells (NCC) in cell cultures from CF and non-CF patients. The Wilcoxon matched-pairs test showed a significant difference between the percentage of bacteria associated with the CC and NCC ($p < 0.01$).

statistically significant for cultures from both CF and non-CF patients ($p < 0.001$, fig. 3). No difference was noted between cultures from CF and non-CF patients with respect to the percentages of aggregated *P. aeruginosa* cells associated with CC or NCC (fig. 3).

The adhesion of *P. aeruginosa* cells to CC surfaces was significantly higher than to NCC surfaces, regardless of whether the cells were from CF or non-CF patients ($p < 0.01$, fig. 4). However, no significant difference was observed between cell cultures from CF and non-CF patients with regard to either the number of bacteria adhering to CC or NCC surfaces (fig. 4), or to the percentage of CC with attached bacteria (fig. 5).

Fig. 4. Adhesion of *P. aeruginosa* cells to ciliate (CC) and non-ciliate (NCC) respiratory cells in culture from CF and non-CF patients.

Fig. 5. Percentages of ciliate cells from CF and non-CF patients showing attached *P. aeruginosa* cells.

Transmission electronmicroscopy

During observation by TEM of the cells cultured from CF and non-CF patients, *P. aeruginosa* cells were never seen to be interspersed between the cilia but were observed at the extremities of the cilia, usually as aggregates of bacteria surrounded by a matrix-like material. Examination of thick sections of cells from CF patients showed a continuity between the cilia and this matrix-like material (fig. 6). The interaction of aggregated bacteria with NCC also appeared to occur by means of this surrounding matrix (fig. 7c).

HID staining of CF and non-CF epithelial respiratory cells

Sulphated glycoconjugates were identified by the HID method on respiratory cells from CF and non-CF patients regardless of whether these cells had attached bacteria or not, as shown in fig. 7. No general difference was noticed between the electron density of the surfaces of apical cells cultured from CF and non-

Fig. 6. Transmission electronmicrograph from a thick section of cells cultured from a CF patient. Aggregated *P. aeruginosa* cells are surrounded by a matrix-like material (arrowheads) which seemed to promote the interaction between the bacteria and the ciliate cells (arrows). Bar = 2.5 μm .

Fig. 7. Transmission electronmicrographs of cells cultured from CF (a, c) and non-CF (b, d) patients labelled with the HID solution. Sulphated glycoconjugates are revealed by the high electron density at the epithelial surfaces (arrows): note a, HID-labelled matrix material is between the cilia (arrows); c, the bacteria are surrounded by a matrix (arrows) which is more abundant and more heavily stained than is the matrix around bacteria associated with cells from a non-CF patient d, and the uppermost cell (double arrows) is diffusely stained. Bars = 0.5 μ m.

CF patients, but in one of the three cultures from CF patients examined, diffuse HID staining of all the uppermost epithelial cells was noticed (fig. 7c). In contrast, although a slight electron density was seen in the matrix-like material surrounding aggregated bacteria around the cells cultured from non-CF patients, this material was more abundant and stained more heavily in cultures from CF patients (figs. 7a and 8b).

Immunocytochemical localisation of mucin

Fluorescence assays showed that most of the epithelial respiratory cells were labelled by the MAbs against rhesus monkey mucin (not shown). A fibrillar fluorescent material was seen surrounding the aggregated *P. aeruginosa* cells and, occasionally, linking bacteria to the ciliate cells.

Electronmicroscopy revealed that both CC and

Fig. 8. Transmission electronmicrographs of respiratory ciliate (a) and non-ciliate cells (b, c) labelled with the anti-mucin MAb. Insets in b and c (1 and 2) show higher magnifications of the microvilli (double arrows) from a secretory (b) and a non-secretory cell (c) labelled with the 5-nm colloidal gold granules. Labelling is not present along all the surface of the cells (arrowheads). In d, the labelling of the matrix-like material surrounding aggregated *P. aeruginosa* cells by the anti-mucin MAb is shown (arrows). Bars = 0.5 μm generally, but 0.1 μm for insets.

NCC were labelled by the anti-mucin MAb, but that labelling was restricted to the uppermost surfaces of cilia and microvilli (fig. 8a-c). Mucin was also identified in the matrix-like material which surrounded the bacteria (fig. 8d).

Discussion

In otherwise healthy persons, tracheobronchial infection by aerobic gram-negative bacilli is usually preceded by failure of the normal respiratory defence

mechanisms.²² The available evidence suggests that the respiratory tracts of CF patients may not be damaged prior to infection.²³ The factors predisposing CF patients to bacterial colonisation remain to be determined. However, the predominant mechanism allowing successful bacterial colonisation of the tracheobronchial tree is adhesion to epithelial cell receptors. Adhesion may prevent micro-organisms from being washed from the epithelial surface and may permit more effective delivery of bacterial products to the cell membrane.²⁴ In this study, we evaluated whether specific adhesive properties of the

epithelial respiratory cells from CF patients for *P. aeruginosa* might be one of the factors triggering the initial colonisation of the respiratory airways by this micro-organism. We selected a non-mucoid strain because, in CF, bacterial colonisation first occurs with this type of organism.

No significant difference was observed between the adhesion of the *P. aeruginosa* strain to epithelial cells from CF and non-CF patients. Nor could we establish a relationship between increased sulphation of apical cell surfaces from CF patients and adhesion of *P. aeruginosa*: sulphated glycoconjugates were identified on cells regardless of whether or not these had attached bacteria. In cultures from CF and non-CF patients, bacteria were seen to associate with CC mainly as aggregates. This association appeared to occur via a matrix-like material surrounding the bacterial cells. This observation raises the question of whether or not *P. aeruginosa* can adhere to uninjured respiratory cells. The adhesion of clinical isolates of non-mucoid *P. aeruginosa* to actively-beating CC has been reported previously,⁷ but long incubation periods were required to allow detection of the adherent micro-organisms. *P. aeruginosa* produces various exotoxins and it is conceivable that, during these long incubation periods, the bacterial products may have accumulated in the culture medium, cleaving host cell glycocalyx components and exposing masked receptors. The proteases of *P. aeruginosa* have been reported to induce the release of glycoconjugates from the apical surfaces of dog tracheal cells in culture.²⁵ In our study, the cilia continued to beat actively throughout the assays and the incubation period of the respiratory cells with micro-organisms was not so long as to allow accumulation of extracellular bacterial products. The fact that bacteria were not seen adhering to the cilia leads us to believe that non-mucoid *P. aeruginosa* adhere poorly, if at all, to uninjured respiratory cells.

We have shown previously that *P. aeruginosa* cells were aggregated by RPMI-HEPES medium which had previously been in contact with epithelial respiratory cells in culture for 1 h and we speculated that bacterial aggregation was induced by secretory products from the epithelial cells.²¹ Our hypothesis was supported by the observation that aggregation of *P. aeruginosa* cells was still observed when protein synthesis was blocked by pre-treatment of the micro-organisms with gentamicin 10 mg/L for 1 h. In the present study we observed that a MAb raised against respiratory mucins bound to the matrix-like material which surrounded the aggregated micro-organisms. These results suggest that the reactivity of the mucin may be responsible for bacterial aggregation. The affinity of *P. aeruginosa* for mucins from different origins,^{2,4,26} as well as the aggregation of the organism by salivary mucin,^{27,28} is well documented. Nevertheless, we cannot exclude the possibility that other secretory products from respiratory cells may have participated in the aggregation of *P. aeruginosa* cells. Our observation that the proportion of bacteria present

in the form of aggregates was as high in cultures from non-CF patients as in those from CF patients agrees with recent studies²⁶ in which *P. aeruginosa* was found not to bind to CF respiratory mucin any more than to non-CF intestinal mucin.

Epithelial respiratory cells in primary culture have been reported to package mucins in small vesicles or secretory granules. These are transported to the cell surface, where they fuse with the plasma membrane and discharge the mucin by exocytosis.^{29,30} During exocytosis, part of the mucin is released into the culture medium while part remains tightly associated with the plasma membrane, forming a cell surface mucin layer, as revealed by lectin HPA (*Helix pomatia* agglutinin) reactivity.^{29,31} In our study, mucins could not be detected along all the surfaces of respiratory cells, even secretory ones, but were seen associated with a diffuse material which seemed to occur at the tips of cell microvilli. Mucins were also detected along the cilia, in contrast with the results of Wasano *et al.*³¹ who observed that HPA bound to the surface of hamster secretory cells but not to that of ciliate cells. Our culture model differs from others²⁹⁻³¹ in that (a) excised explants from nasal polyps could represent a source of secretory products, and (b) the percentage of secretory cells, identified by the presence of secretory granules in the outgrowth area, was low. Consequently, the amount of mucin secreted is thought to have been low. Previous work has been shown that hamster tracheal cells in culture for 3 days secrete minimal amounts of mucin and present only a few HPA-reactive sites on their apical surfaces.²⁹ Thus, the low secretory activity of our outgrowth cells may account for the poor mucin labelling of NCC surfaces. In our culture model, mucin, which probably originated from the secretory activity of both outgrowth and excised explant cells, was shown to participate in the aggregation of *P. aeruginosa* cells and to bind to the surface of respiratory cells, including ciliate cells.

In some cell cultures from CF patients, SEM revealed strands of secretion with adherent bacteria as well as an amorphous material lying over epithelial cells. These strands could be the secretory products from respiratory cells. TEM showed that in cultures from CF patients the bacteria were surrounded by a much more abundant mucin-containing matrix than that observed in cultures from non-CF patients. Accordingly, we suggest that respiratory cells from CF patients in culture have greater activity than cells from non-CF patients. This is in agreement with recent results from Merten *et al.*³² who showed that cultured tracheal glandular cells from CF patients had constitutive hypersecretory activity and were hyporesponsive to pharmacological agonists (forskolin and calcium ionophore). In our study, the hypersecretion of mucin by respiratory cells from CF patients did not account for a greater adhesion of *P. aeruginosa*. However, *in vitro*, the secretory products from epithelial cells were diluted in culture medium. *In vivo*, the paucity of water in CF respiratory secretions may alter

their visco-elastic properties and result in the failure of mucociliary clearance.¹⁶ Therefore, it seems reasonable to speculate that, *in vivo*, both the marked mucin secretory activity of respiratory cells from CF patients and the abnormally high viscosity of the mucus may decisively favour the persistence of mucus-associated micro-organisms.

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