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Fibronectin and α5β1 integrin mediate binding of *Pseudomonas aeruginosa* to repairing airway epithelium

P. Roger*, E. Puchelle*, O. Bajolet-Laudinat+, J-M. Tournier*, C. Debordeaux*, M-C. Plotkowski#, J.H.M. Cohen‡, D. Sheppard§, S. de Bentzmann*

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ABSTRACT: Initial infection of the airway by *Pseudomonas aeruginosa* may occur through a variety of bacterial strategies including binding to epithelial receptors present at the surface of the respiratory epithelium.

In order to characterize the adherence sites for *P. aeruginosa* in damaged and repairing bronchial tissue, an *ex vivo* model of airway epithelial injury and repair was developed using primary cell cultures of nasal cells from 14 subjects with polyposis.

***P. aeruginosa* strongly adhered to flattened dedifferentiated (FD) bronchial and nasal cytokeratin 13-positive epithelial cells in the process of migration for repair. In *in vitro* experiments, competitive binding inhibition assays demonstrated that α5β1 integrins and cellular fibronectin, in particular the RGD sequence, are receptors involved in *P. aeruginosa* adherence to FD nasal epithelial cells. Fluorescent cell sorting analysis and immunofluorescence techniques revealed that the α5β1 integrins are overexpressed and apically exposed in FD nasal epithelial cells. One 50 kDa outer membrane protein was identified in piliated and nonpiliated strains of *P. aeruginosa* that was involved in binding to cellular fibronectin and α5β1 epithelial integrins.**

These results demonstrate that *Pseudomonas aeruginosa* adherence is related to the dedifferentiation of airway epithelium during the repair process which unmasks and upregulates the α5β1 integrin expression and induces active synthesis of cellular fibronectin. These epithelial receptors are then used by a *Pseudomonas aeruginosa* 50 kDa outer membrane protein as sites of bacterial adherence.

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*INSERM U514, CHR Maison Blanche, Reims, France. +Laboratoire de Bactériologie-Virologie-Hygiène, Hôpital Robert Debré, Reims, France. #Dept of Microbiology and Immunology, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil. ‡Laboratoire d'Immunologie, Hôpital Robert Debré, Reims, France. §University of California, Dept of Medicine, Lung Biology Center, the Cardiovascular Research Institute, San Francisco, USA.

Correspondence: S. de Bentzmann
INSERM U514 IFR53 CHR Maison Blanche
45 rue Cognacq Jay
51092 Reims Cédex
France
Fax: 33 326065861

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Pseudomonas aeruginosa is an opportunistic pathogen responsible for severe respiratory infections in patients with bronchial inflammatory diseases, particularly with cystic fibrosis (CF) and in patients with compromised host defences [1, 2]. The airway initial infection by *P. aeruginosa* may occur through a variety of bacterial strategies, including binding to mucins and epithelial receptors present at the surface of the respiratory epithelium [3–5]. Injury of the airway epithelium can occur during exposure to a variety of inhaled and infectious agents such as *Haemophilus influenzae* [6] and *P. aeruginosa* [7, 8] and during airway inflammation. Following injury, airway epithelial cells neighbouring the injured and denuded basement membrane areas undergo repair [9, 10]. The repair process includes spreading, migration and proliferation of the epithelial cells neighbouring the injured area. During the repair process, migrating airway epithelial cells actively synthesize cellular fibronectin [11] required for cell migration. Integrins, transmembrane proteins that mediate cell–substratum and cell–cell adhesion, are a key part of this process. There is an α5β1 transient integrin-induction at the leading edge of cells during early phase epithelial repair; this integrin being a fibronectin receptor [11, 12].

It has previously been reported *in vitro* that CF and non-CF respiratory epithelial cells engaged in the repair process [13, 14] are dedifferentiated and exhibit a high affinity for *P. aeruginosa*, and that gangliotetraosylceramide, containing the sequence Gal*N*Ac β1–4 Gal, (asialo GM1) receptors are partly responsible for this bacterial-cell interaction. Integrins and fibronectin have been described as host receptors for a variety of pathogens, including *Pneumocystis carinii* [15] and *Mycobacterium leprae* [16], in the attachment to lung and nasal epithelial cells. It has been reported that *P. aeruginosa* binds to cellular fibronectin and moreover, that cellular fibronectin promotes bacterial binding to non-CF dedifferentiated airway epithelial cells [17], suggesting that cellular fibronectin and upregulated cellular fibronectin receptors, α5-β1 integrins, may account for increased *P. aeruginosa* adherence on non-CF repairing airway epithelia.

The main objectives of this study were to identify and characterize the cellular receptors involved in *P. aeruginosa* adherence to damaged and repairing airway epithelium in acute initial infectious episodes and more precisely to investigate the role of α5β1 integrins and of cellular fibronectin in *P. aeruginosa* adherence to non-CF airway epithelium undergoing repair. The *P. aeruginosa* adhesins

involved in the interaction with cellular fibronectin and α5β1 integrins were also identified.

Materials and methods

Bacteria

P. aeruginosa (PO10 serotype), a non-mucoid piliated strain, is a clinical isolate obtained from airway secretions of a non-CF patient. PAK (a well-characterized reference laboratory strain), PAK*p*- (a pilus-lacking isogenic strain), and PAKN1 (a *rpoN* mutant) strains were kindly provided by W. Paranchych and S. Lory (Department of Microbiology, University of Alberta, Canada and University of Seattle, WA, USA, respectively). The different strains were cultured overnight at 37°C in trypticase soy broth (TSB, Sanofi Diagnostics Pasteur, Marnes la Coquette, France) under mild agitation.

Cell cultures

In vitro dedifferentiated nasal epithelial cells were obtained using two primary cell culture conditions with nasal cells obtained from 14 non-CF subjects with polyposis: 1) the explant-outgrowth cell culture model [18], and 2) an original dissociated cell culture model. In the explant-outgrowth model, well-differentiated (WD) nasal epithelial cells could be observed, while at the periphery of the outgrowth, flattened dedifferentiated (FD) nasal epithelial cells were identifiable. In the dissociated cell culture model, surface nasal epithelial cells were obtained after overnight incubation of nasal tissues in 0.05% (w/v) pronase E (Sigma, St Louis, MO, USA) in culture medium at 4°C. Matched FD and WD cell cultures were performed for each patient. WD nasal epithelial cell cultures were obtained after seeding epithelial cells onto a type 1 collagen gel and culturing to confluency. FD nasal epithelial cell cultures were obtained in separated dishes by sparsely seeding epithelial cells in the culture dishes. The number of FD nasal epithelial cells was increased by creating an additional chemical wound. Twelve hours later, cells around the edges of the injured area dedifferentiated and spread to the centre of the damaged epithelial areas. The culture medium in all culture dishes was a serum-free RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA), supplemented with 10 ng·mL^{-1} epidermal growth factor, 1 μg·mL^{-1} insulin, 0.5 μg·mL^{-1} hydrocortisone, 1 μg·mL^{-1} transferrin, 10 ng·mL^{-1} retinoic acid and antibiotics (200 U·mL^{-1} penicillin and 200 μg·mL^{-1} streptomycin).

Ex vivo *model of injury and repair*

In order to be sure that the *P. aeruginosa* binding to nasal dedifferentiated epithelial cells undergoing repair could be observed in experimental conditions close to the *in vivo* situation, an *ex vivo* model of injury and repair was performed on human bronchial tissue from a patient undergoing surgery for bronchial carcinoma on areas that were microscopically normal and distant from the tumour. Pieces of bronchial tissue were damaged using a probe cooled by immersion in liquid nitrogen placed in contact with the bronchial tissue by applying a calibrated pressure of 33 kPa for 10 s. The damaged tissue, with a damaged zone of ~15 mm^2, was then incubated in RPMI 1640 culture medium at 37°C in a 5% CO_2 atmosphere, allowing it to undergo repair for a period of 24 h. Bacteria at 5×10^8 colony-forming units (cfu)·mL^{-1} were added for 1 h at 37°C.

Competitive binding assays

The implication of α5β1 epithelial integrins acting as host receptors for *P. aeruginosa* adherence to FD nasal epithelial cells was evaluated for the *in vitro* explant-outgrowth culture model in the presence of monoclonal antibodies against β1 (P5D2; a gift from E. Warner, University of Minnesota, Minneapolis, MN, USA), α3 and α5 integrin subunits (Gibco BRL) at dilutions 1:5, 1:200 and 1:200, respectively, prior to contact with bacterial inoculum. The same experiments were performed with a monoclonal antibody raised against cellular fibronectin (Sigma) at 1:50 and in the presence of GRGDSP (0.1 and 1 mg·mL^{-1}) and GRGESP (1 mg·mL^{-1}) peptides (Gibco BRL) for 1 h at 37°C, prior to contact with the bacterial inoculum adjusted to 5×10^8 cfu·mL^{-1} for 1 h at 37°C. Simultaneous control experiments were carried out with immunoglobulin (Ig)M (1:20) or IgG (1:100), depending on the nature of the specific immunoglobulins, or with the culture medium only.

Scanning electron microscopy and quantification of bacterial adherence

In vitro cell cultures (explant-outgrowth and dissociated cell culture models) and the bronchial *ex vivo* repair model, were prepared for scanning electron microscopy (SEM) by fixation with 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), dehydration through graded concentrations of ethanol and critical point drying and coating with 15 nm gold-palladium beads. Quantification of bacterial adherence to FD nasal epithelial cells in the explant-outgrowth model was performed using a computer-assisted scanning electron microscope, as previously described [19].

Immunocytochemical characterization of the ex vivo *model of injury and repair*

The *ex vivo* bronchial repairing tissue was embedded in OCT compound (Tissue-tek, Miles Inc., Elkhart, IN, USA) and immediately frozen in liquid nitrogen. Frozen samples were cut (5 μm thick) at -20°C and sections were transferred to gelatin-coated slides. The immunodetection of *P. aeruginosa* was performed by incubating slides for 1 h with monovalent serum raised against *P. aeruginosa* O10 serotype (1:10) (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). After rinsing with PBS 0.1 M (pH 7.2), sections were incubated with biotinylated anti-rabbit secondary antibody (1:50) (Amersham, Little Chalfont, UK) for 1 h, rinsed and then further incubated with streptavidin-fluorescein isothiocyanate (FITC) (1:50) (Amersham). Cell cytokeratin characterization was performed at the same

time, with antibodies against cytokeratins 13 (Sigma) and 14, which are typical basal cell markers, on the same sections using the same procedure. Cell cytokeratins were detected using streptavidin-Texas red (Amersham). Immunodetection of α5 and β1 integrin subunits was investigated in parallel with *P. aeruginosa* immunodetection, with anti-α5 (1:300) and anti-β1 (1:10) antibodies revealed with FITC, the bacteria being revealed with Texas red.

Immunocytochemical distribution of epithelial integrins

After fixation in pure methanol at -20°C for 15 min, cell cultures were incubated for 1 h with antibodies against α5 (1:300) and β1 (1:10) integrin subunits, rinsed with PBS, and incubated with biotinylated anti-mouse secondary antibody (Amersham, Les Ulis, France) (1:50) and with streptavidin-FITC (Amersham, Les Ulis, France). Entire cultures were mounted in a Citifluor anti-fading solution (Agar Scientific, Stansted, UK) and observation of immunolabelling was performed with a MRC 600 Biorad Confocal system mounted on a Zeiss Axioplan microscope (Ivry, Seine, France), on 0.2 µm interval XY sections collected on a vertical axis from the apical to the basal surface through the cell cultures.

Fluorescent cell sorting analysis of epithelial integrins

The level of *in vitro* expression of the α5β1 fibronectin-binding integrin was estimated on WD and FD matched nasal epithelial cells from the same patient separated from the matrix with 15 mM ethylene diamine tetraacetic acid (EDTA) at 37°C for 15 min. Cells were harvested by centrifugation at 100 × *g* and 4°C for 5 min, incubated with anti-α5 (1:200), or anti-β1 (1:20) integrin subunit antibodies for 40 min at 4°C and the labelling was revealed with a fluorescein-conjugated anti-mouse secondary antibody (Tebu, Le Perray en Yvelynes, France). FITC-surface labelling was measured at 585 nm over 20,000 events on a Beckton Dickinson FACScan cytofluorimeter (Becton Dickinson, Plymouth, UK). The fluorescent intensity of FITC was expressed in arbitrary units on a logarithmic scale.

Extraction, sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blot of P. aeruginosa *outer membrane proteins*

P. aeruginosa outer membrane proteins (OMP) were extracted as previously described by CARNOY *et al.* [20]. Crude bacterial cell wall extracts were dialysed overnight against bi-distilled water and lyophilised. *P. aeruginosa* OMP were resuspended in an electrophoresis buffer containing 10 mM Tris, 1 mM EDTA, 1 mM sodium dodecyl sulphate (SDS) and 5% β-mercaptoethanol and separated on 4–15% polyacrylamide gels with 20 µg of protein in each lane for standard electrophoresis. Gels containing *P. aeruginosa* OMP were either silver-stained or electrotransferred to nitrocellulose sheets which, after fixation with 0.2% Ponceau red (Sigma) in 3% trichloroacetic acid solution, were blotted in one of two ways: 1) with cellular fibronectin at 10 $\mu g \cdot mL^{-1}$ in PBS containing 1% BSA for 2 h at room temperature under mild agitation, then after rinsing, with monoclonal anti-fibronectin antibody (1:200) in PBS/1% BSA overnight at 4°C and with biotinylated anti-mouse secondary antibody (1:1,500, Amersham) and streptavidin-horse radish peroxidase (1:1,500); or 2) with epithelial α5β1 integrins from FD dissociated cell cultures at 10 $\mu g \cdot mL^{-1}$ obtained by classical immunoprecipitation techniques [21], followed, after rinsing, with anti-α5β1 monoclonal antibody (P3D10H5; a gift from D. Sheppard, University of California, San Francisco, CA, USA) and peroxidase anti-mouse secondary antibody (1:1,500). Controls were performed by omitting the first ligand and then incubating with the primary antibody, or incubating with the first ligand and then incubating with a non-immune primary antibody. Detection of immunoreactive bands was performed with the electrochemiluminescence technique.

Statistical analysis

The number of adherent bacteria obtained in the bacterial quantification study was expressed as median values and ranges. Each incubation of epithelial cells (controls, incubations with specific antibodies, non-immune IgG or IgM and peptides) was performed on six different explant-outgrowth cell cultures for each patient. Results from the incubation of epithelial cell cultures with monoclonal antibodies or peptides were analysed using a nonparametric Mann–Whitney U-test in comparison with control epithelial cell cultures incubated with non-immune IgG or IgM, or in culture medium alone. Results were considered significant for p-values <0.05.

Results

Remodelling in the bronchial ex vivo *model of injury and repair*

After injury of human bronchial tissue by a cooled probe, the process of repair was followed for 24 h. The pseudostratified organization of the non-injured area was maintained, whereas in the area undergoing repair, the typically round or triangular basal cells were present. Basal cells in the process of dedifferentiation and repair (FD cells) were also identified (fig. 1A). In SEM observation, the transitional area between the non-injured and the injured and repairing areas was easily identifiable by the decrease in the ciliated cell number and by the elongation and the orientation of cells belonging to the repairing area towards the centre of the wound (fig. 1B). All cells belonging to the epithelial basilar cell layer in contact with the basement membrane were cytokeratin 13-positive cells, in the non-injured area and in the injured area (fig. 1C). In the basilar cell layer, cytokeratin 14 labelling was discontinuous in the non-injured area and in the injured area (data not shown). The FD airway epithelial cells (bronchial or nasal) undergoing repair, all express the cytokeratin 13 marker [22], irrespective of the model used.

P. aeruginosa *adherence sites in repairing bronchial and nasal epithelium undergoing repair*

In the *ex vivo* repair model, *P. aeruginosa* strongly adhered to desquamated bronchial epithelal cells, to the denuded basement membrane, as well as to cytokeratin 13-positive epithelial cells undergoing repair, either round basal cells or FD cytokeratin 13-positive bronchial epithelial cells (fig. 2A and B). In the non-injured area, *P. aeruginosa* did not adhere to the pseudostratified bronchial epithelium. Some bacteria were bound to cytokeratin 13-negative cells anchored to the basilar cytokeratin 13-positive epithelial cell layer. The SEM images showed the association of adherent *P. aeruginosa* and FD bronchial epithelial cells with fillipodia in the injured and repairing area (fig. 2C and D). Furthermore, *P. aeruginosa* was colocalized with β1 (fig. 2E and F) or α5 (fig. 2G and H) integrins in bronchial epithelial cells undergoing repair. In *in vitro* cell cultures, *P. aeruginosa* showed a high affinity for FD nasal epithelial cells, whereas *P. aeruginosa* did not exhibit any affinity for WD nasal and bronchial epithelial cells.

Fibronectin and α5β1 integrins are involved in P. aeruginosa *adherence to repairing epithelial cells*

To investigate the role of epithelial integrins as receptors for *P. aeruginosa* adherence to FD nasal epithelial cells, the explant-outgrowth cell cultures were incubated with an anti-β1 integrin subunit monoclonal antibody and a significant reduction in *P. aeruginosa* adherence to FD nasal epithelial cells (15.1±3.5 bacteria·μm^{-2}, $p<0.05$) was observed, compared to FD cells in control explant-outgrowth cell cultures (39±10.1 bacteria·μm^{-2}).

Incubation of the explant-outgrowth cell cultures with anti-α5 integrin subunit antibody significantly reduced *P. aeruginosa* adherence to FD nasal epithelial cells (34.2± 8.9 to 8.9±6.2 bacteria·μm^{-2}, $p<0.05$), whereas incubation with antibody raised against α3 integrin subunit had no significant effect on *P. aeruginosa* adherence to FD nasal epithelial cells (33.6±2 to 33.8±2 bacteria·μm^{-2}). Pretreatment with non-immune mouse IgG did not modify *P. aeruginosa* adherence to FD nasal epithelial cells (data not shown).

The role of cellular fibronectin in *P. aeruginosa* adherence to FD nasal epithelial cells was investigated by blocking cellular fibronectin with a monoclonal anti-cellular fibronectin antibody. Incubation of explant-outgrowth cell cultures with the monoclonal anti-cellular fibronectin antibody significantly reduced *P. aeruginosa* adherence to FD nasal epithelial cells (31±4.1 to 19.5±1.9 bacteria·μm^{-2}, $p<0.05$), whereas incubation with non-immune IgM did not affect *P. aeruginosa* adherence, compared to control explant-outgrowth cell cultures incubated with culture medium alone (data not shown). A significant decrease in *P. aeruginosa* adherence was observed with the GRGDSP peptide (33.2±9 to 16.1±3.9 and 13.2±2.2 bacteria·μm^{-2}, $p<0.05$ for 0.1 and 1 mg·mL^{-1} respectively), whereas the GRGESP peptide did not affect *P. aeruginosa* adherence to FD nasal epithelial cells (33.2±9 to 34.1±7.3 bacteria·μm^{-2}).

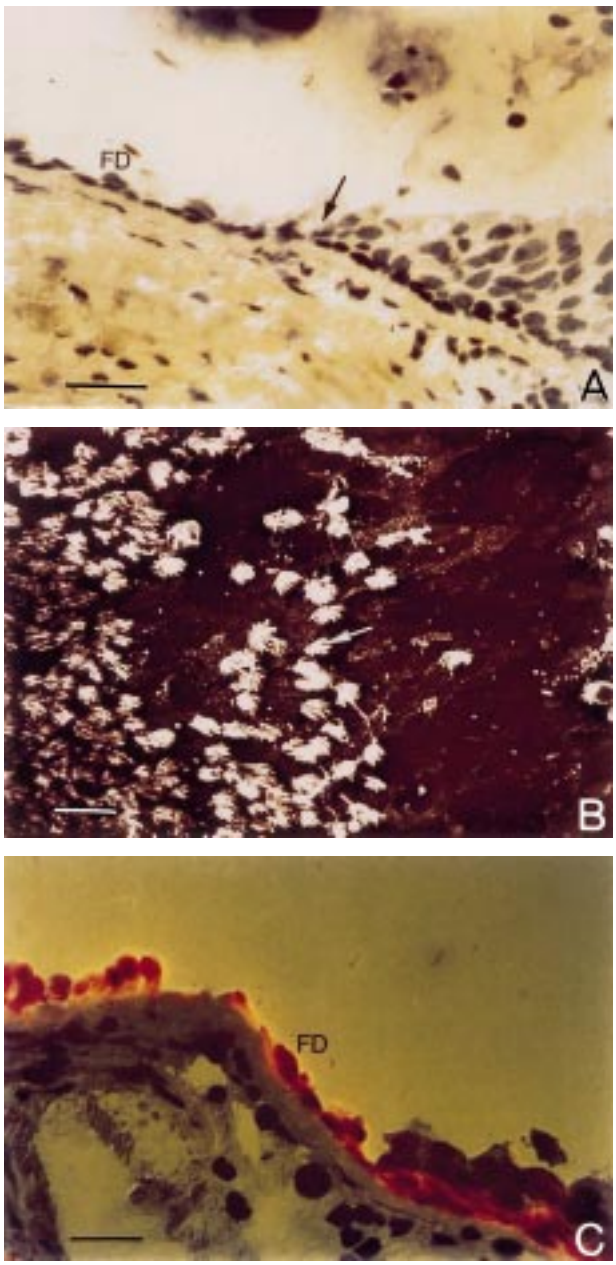

Fig. 1. – Histological characterization of the repair of the surface respiratory epithelium induced by a cryoprobe injury in a bronchial *ex vivo* repair model (A). Pseudostratified bronchial epithelium can be observed near the edge of injury (arrow), whereas flattened dedifferentiated (FD) epithelial cells are observable in the injured area undergoing repair. On the scanning electron microscope view (B), the transitional area (arrow) between the non-injured, the injured and repairing areas was indentifiable by the decrease in ciliated cell numbers, as well as by the orientation of cells towards the centre of the injured area. Texas red labelled-cytokeratin 13 was identified in typical round cytokeratin 13-positive basal cells still attached to the basement membrane, as well as flattened cytokeratin 13-positive basal cells in the process of migration (FD) (C). (Internal scale bars A=250 μm, B=10 μm and C=150 μm.)

Expression and distribution of epithelial integrins in dedifferentiated respiratory epithelial cells

Immunodetection of the β1 and α5 integrin subunits, which was performed on entire explant-outgrowth cultures in 4% paraformaldehyde fixative conditions (without cell

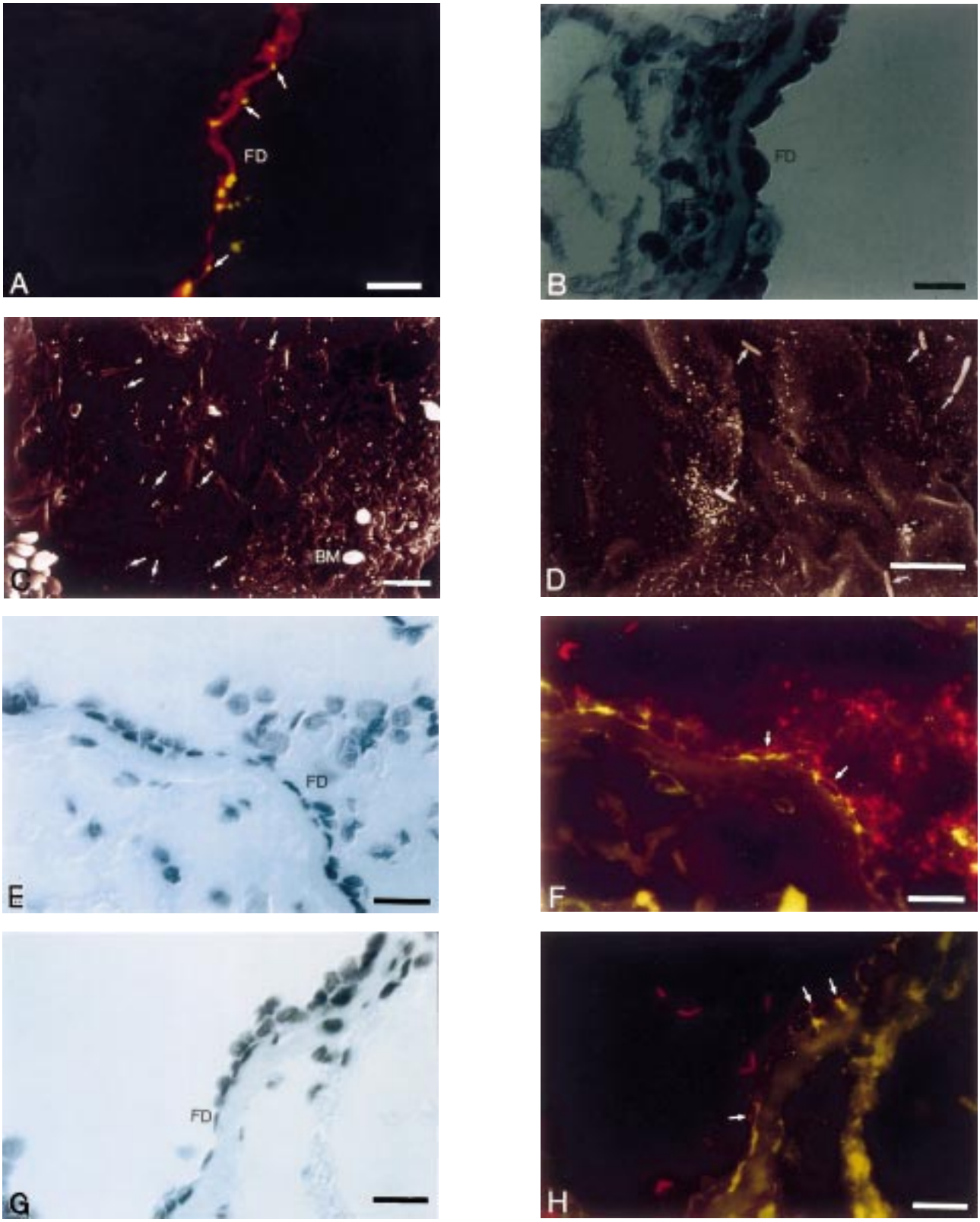

Fig. 2. – Immunohistological localization of *Pseudomonas aeruginosa* in the *ex vivo* repair model. Colocalization of *P. aeruginosa* (fluorescein isothiocyanate) and flattened dedifferentiated (FD) cytokeratin 13-positive basal cells (Texas red). Bacteria (arrows) can be seen attached to the apical side of the FD cytokeratin 13-positive basal cells (A), with the corresponding Nomarski view (B). In the scanning electron microscope views (C and D) of the injured and repairing areas, bacteria (arrows) are bound to FD bronchial cells which migrate over the denuded basement membrane (BM). *P. aeruginosa* (arrows) is colocalized with β1 (E and F) and α5 integrins in bronchial epithelial cells (FD) undergoing repair (G and H). (Internal scale bars A, B, E–H =150 μm, C=10 μm and D=5 μm.)

permeabilization), showed a specific and apical expression of these integrins in FD nasal epithelial cells, whereas poor or no labelling was detected in WD nasal epithelial cells (data not shown). In methanol-fixative conditions (which allow cell permeabilization), confocal microscopic observations clearly showed that β1 integrins were only localized at the basal side of WD nasal epithelial cells (fig. 3C and D), whereas these integrins were distributed both apically (fig. 3A) and in contact with the collagen substrate in FD nasal epithelial cells (fig. 3B). Using the *in vitro* dissociated cell culture model, the expression of α5 and β1 integrin subunits was investigated by flow cytometric analysis in FD and WD nasal epithelial cells. The α5 integrin subunits were expressed at higher levels in FD compared to WD nasal epithelial cells (fig. 3E and F). A higher expression of β1 integrin subunits was observed in FD nasal epithelial cells compared to WD nasal epithelial cells (fig. 3G and H).

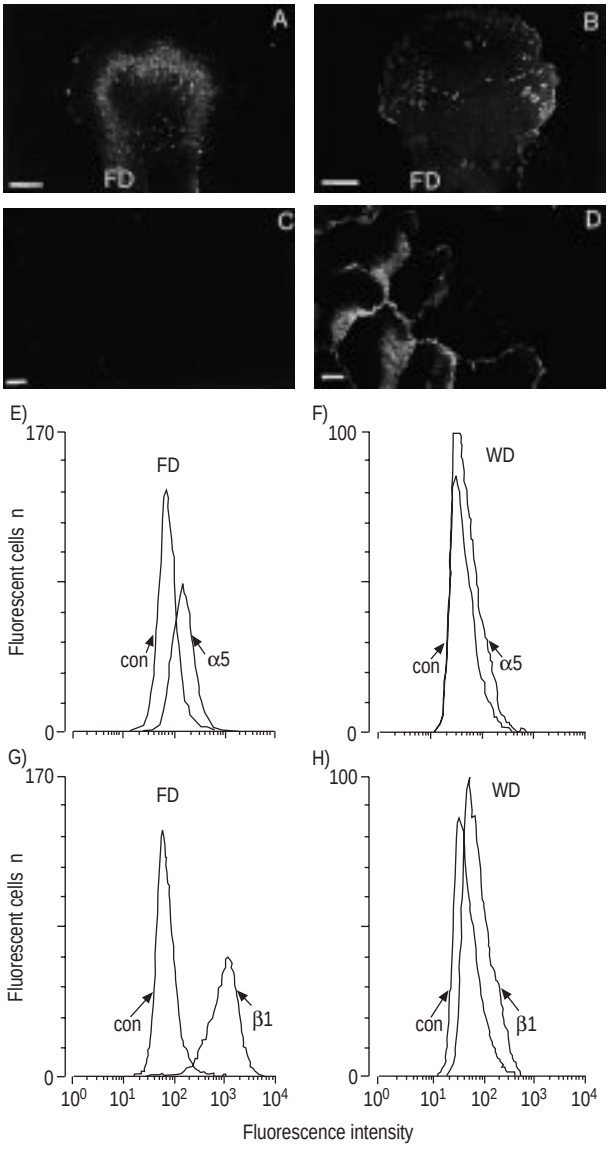

Fig. 3. – Immunolocalization of β1 integrin subunits in nasal epithelial cells in the explant-outgrowth culture model with confocal imaging was performed in permeabilized fixative conditions. The β1 integrin subunit was expressed at both apical (A) and basal (B) surfaces, under the form of focal contacts in flattened dedifferentiated (FD) cells at the periphery of the outgrowth. No apical (C) labelling was detected on the apical surface of well-differentiated (WD) cells in the outgrowth near the explant, whereas β1 integrin subunits were concentrated when in contact with the collagen matrix (D) on the basal side of WD epithelial cells. Flow cytometric analysis of integrins expressed by nasal polyp epithelial cells obtained in the *in vitro* dissociated cell culture model. FD nasal epithelial cells expressed more α5 and β1 integrins (E and G) than their matched WD nasal epithelial cells (F and H). Each labelling was compared to its own control (con).

Outer membrane proteins of P. aeruginosa *involved in binding to host fibronectin and α5β1 integrin*

The 13–113 kDa protein cell wall extract profile obtained with the different *P. aeruginosa* strains by electrophoresis is shown in figure 4A. A Western blot analysis of total immobilized OMP with cellular fibronectin showed that the fibronectin-binding OMP (fig. 4B) appeared as a single band with an apparent molecular weight of 50 kDa, observed in piliated strains PAK and PO10, as well as in non-piliated strains PAK*p*- and the PAK *rpoN* mutant.

The Western blot of total OMP from the different *P. aeruginosa* strains incubated with immunoprecipitated α5-β1 epithelial integrins from FD nasal epithelial cells also showed a unique band with an apparent molecular weight of 50 kDa. This band was also reactive with α5β1 integrins (fig. 4C) in piliated and non-piliated *P. aeruginosa* strains. Similar results were obtained when total OMP was blotted with β1 integrin subunits (data not shown). Controls, which were performed either without the primary ligand fibronectin or α5β1 integrin, or with the primary ligand fibronectin or α5β1 integrin and revealed with a non-immune antibody, did not show any immunoreactive band (fig. 4D).

Discussion

In the present study, it has been shown that the α5β1 host integrin, which is apically exposed and overexpressed in dedifferentiated respiratory epithelial cells engaged in repair after injury, as well as the host cellular fibronectin, represent receptors for *P. aeruginosa* adherence. Moreover, it has been shown that *P. aeruginosa* presents an OMP with an apparent molecular weight of 50 kDa, capable of interacting with either α5β1 integrin or fibronectin.

P. aeruginosa adherence to human respiratory epithelium is thought to be important in the initiation of respiratory infections in CF patients and also in non-CF immunocompromised patients [4]. However, several authors have found poor or no adherence to uninjured airway epithelium [7, 23] and major binding in damaged epithelia [7, 24, 25] or repairing epithelia, either tracheal or corneal [14, 26]. The development of an *ex vivo* model of repair performed on human bronchial tissue [9], allowed us to demonstrate that the increased *P. aeruginosa* binding, which occurred in repairing human bronchial epithelium, was not an *in vitro* artefact. These results may also explain why non-CF patients develop pneumonia with non-mucoid *P. aeruginosa* strains with tracheal intubation and mechanical ventilation [1]. These *P. aeruginosa*-targeted FD nasal or bronchial cells have epithelial characteristics and all express cytokeratin 13 as previously reported [22].

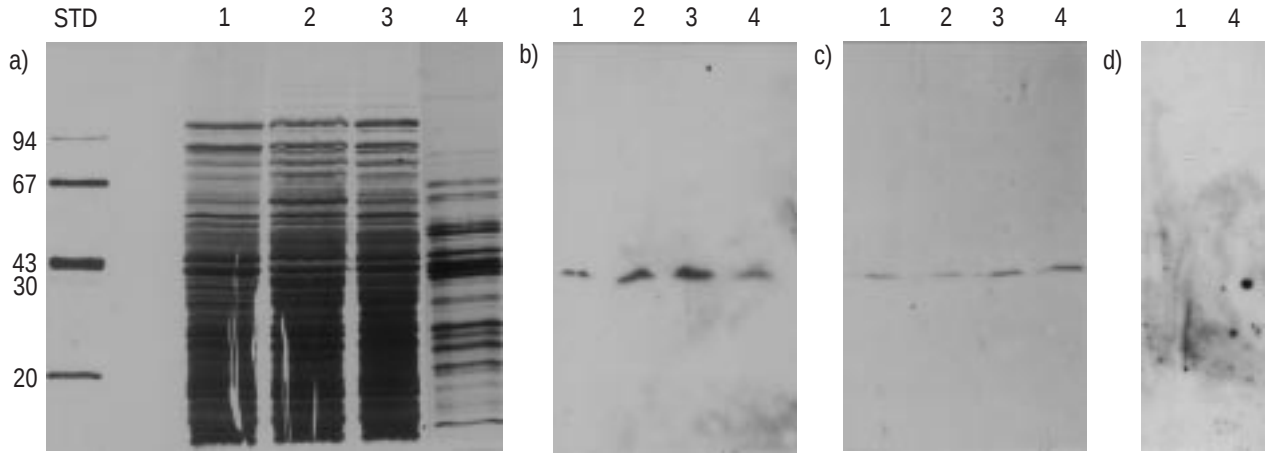

Fig. 4. – Immobilized *Pseudomonas aeruginosa* outer membrane proteins (OMP) were extracted from the PAK (lane 1), the clinical isolate PO10 (lane 4), the pilus-lacking isogenic PAK*p*- (lane 2) and the *rpoN* mutant PAKN1 (lane 3) strains. *P. aeruginosa* OMP were separated by electrophoresis (a). Among the extracted OMP of *P. aeruginosa*, one single band with an apparent molecular weight of 50 kDa, interacts with cellular fibronectin (10 $\mu g \cdot mL^{-1}$), revealed using a monoclonal antibody (mAb) against cellular fibronectin (b). This band also interacts with $\alpha5\beta1$ epithelial integrins (10 $\mu g \cdot mL^{-1}$) revealed with a mAb against $\alpha5\beta1$ integrins (c). Controls performed either with or without the primary ligand and revealed with a non-immune antibody were not immunoreactive (d). STD: molecular weight standards (kDa).

During the repair process, the loss of polarity and the dedifferentiation of airway epithelial cells is associated, *in vitro*, with the loss of microvilli and the emission of fillipodia and lamellipodia towards the centre of the injured area [10], as well as with the upregulation of fibronectin and its $\alpha5\beta1$ integrin receptor [11]. As reported *in vitro* [11] and *in vivo* during repair after mechanical injury in the xenograft model of human bronchial epithelium [12], a higher expression of the fibronectin-binding integrin in FD nasal cells which *P. aeruginosa* binds was identified, opposite to WD cells which *P. aeruginosa* does not bind. In the present study, an apical distribution of this $\alpha5\beta1$ integrin was first reported at the surface of FD nasal cells. PILEWSKI *et al.* [12] showed that this $\alpha5\beta1$ integrin expression is transient and can only be observed in the early phase of repair *in vivo*. This also suggests that the $\alpha5\beta1$ integrin may be involved in *P. aeruginosa* adherence in the initial phase of airway infection rather than in long-term colonization.

The *P. aeruginosa*–$\alpha5\beta1$ host integrin interaction occurs in the absence of cellular fibronectin. However, inhibition assays using RGD-containing peptides, representing the cellular binding site of the fibronectin molecule, suggest that fibronectin can also promote bacterial binding to the $\alpha5\beta1$ integrin. This three-way interaction between the host integrin, the fibronectin molecule and the pathogen has been previously reported in *Pneumocystis carinii* attachment to αv- and $\alpha5$-containing integrins from lung epithelial cells [15], as well as for *Mycobacterium leprae* attachment to $\beta1$-containing integrins from nasal epithelial cells [16]. The present data also indicate that the binding of *P. aeruginosa* to $\alpha5\beta1$ host integrins is mediated *via* an OMP from the bacterial cell wall. This suggests that *P. aeruginosa* can bind to the fibronectin molecule, while the other arm of the fibronectin dimer binds to the fibronectin-binding $\alpha5\beta1$ integrin, or can directly bind to the $\alpha5$ subunit of the $\alpha5\beta1$ fibronectin-binding host integrins.

Fibronectin has also been shown to serve as a bridge between the pathogen cell wall and the host integrin [15, 16]. The results confirm that *P. aeruginosa* binds to cellular fibronectin, and moreover that cellular fibronectin promotes bacterial binding to FD nasal cells. This demonstrates the prominent role of the extracellular matrix components [3, 17, 27] in promoting *P. aeruginosa* adherence to the respiratory epithelium. In contrast to the present results, WOODS *et al.* [28] reported an increased *P. aeruginosa* adherence to swabbed CF buccal cells in suspension and a correlation between this increased bacterial adherence and the loss of fibronectin at the surface of these cells, suggesting a protective role of plasma fibronectin against *P. aeruginosa* adherence. However, such a discrepancy can be explained by the fact that buccal cells are histologically quite different from respiratory epithelial cells, since they are squamous epithelial cells and that bacterial adherence to ciliated cells from nose and trachea exceeds bacterial adherence to buccal squamous cells, suggesting that bacterial adherence at these different respiratory sites may involve different mechanisms [29].

Another important issue is that the bacterial adherence to repairing bronchial epithelial tissue is not specific to CF, underlying the questionable role of the CF transmembrane conductance regulator (CFTR) mutant protein in increased number of *P. aeruginosa* binding receptors [30]. The specific presence of apical asialo GM1 receptors on repairing airway epithelial cells has previously been shown [13]. In the present study, the simultaneous availability of the $\alpha5\beta1$ integrins acting as *P. aeruginosa* receptors, expressed during repair, by the same repairing airway epithelial cells was demonstrated. Although the unique specificity of *P. aeruginosa* binding to ΔF508 homozygous cultured nasal epithelial cells compared to other genotypes has been reported [31], it has been shown that nasal or bronchial epithelial cells with either ΔF508/W1282 or unknown/unknown genotype express asialo-GM1 receptors and significantly bind more *P. aeruginosa* than CFTR rescued cells [30, 32]. Although most of the bacteria are identified in the airway lumen and bound to mucins [5], the significance of *P. aeruginosa* adherence to FD repairing cells may be of great importance in the context of the CF disease, since recent studies demonstrate that *P.*

aeruginosa binding triggers the pro-inflammatory cytokine production in CF cells [33]. These data underline the key role of *P. aeruginosa* binding to epithelial receptors in maintaining an inflammatory response.

Furthermore, the highly conserved 50 kDa OMP in the different *P. aeruginosa* strains that were used demonstrate that this OMP represents a new adhesin for *P. aeruginosa*, which is different from pilus-adhesin and flagellin-adhesin [34]. A recent study by KONKEL *et al.* [35] reported a significant homology between the amino-acid sequence of a fibronectin-binding protein encoding for the *cadF* gene of *Campylobacter jejuni* and the OprF from *P. aeruginosa*, a 34 kDa aqueous channel of the *P. aeruginosa* outer membrane. However, the *P. aeruginosa* OprF has never been described as a fibronectin-binding site, whereas other *P. aeruginosa* OMP have been recently described for their binding to host extracellular matrix components [27] and to tracheobronchial mucins [21]. Interestingly, it is generally stated in the literature that the *rpoN* mutant, which lacks pili and flagellum, does not bind to cells. However, a study from SIMPSON *et al.* [36] reported that the *rpoN* mutant strain can still attach to the A549 cell line even though it lacks ability to bind to mucins. The *rpoN* mutant used in the present study is capable of entering epithelial cells, at a low level, suggesting that it does bind to epithelial cells [37]. The present results may also suggest that the *rpoN* mutant is able to bind to epithelial receptors ($\alpha5\beta1$ integrins and cellular fibronectin) that are not present on A549 epithelial cell lines [38].

The overexpression and the apical distribution of fibronectin-binding integrins, as well as the increased synthesis of cellular fibronectin during the dedifferentiation and repair of airway epithelium, may be directly involved in the acute initial infection of the respiratory tract in airway inflammatory diseases by *Pseudomonas aeruginosa*. By binding to the $\alpha5\beta1$ integrin and fibronectin present on the cell surface of dedifferentiated and repairing airway epithelium, *Pseudomonas aeruginosa* may be able to colonize the airways of cystic fibrosis and non-cystic fibrosis patients. It should prove interesting to further explore the potential capacity of these host receptors to bind *Pseudomonas aeruginosa* when these dedifferentiated airway epithelial cells mature and undergo differentiation.

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