ELSEVIER

Journal of Cystic Fibrosis 7 (2008) 44–53

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Feasibility of nasal epithelial brushing for the study of airway epithelial functions in CF infants☆

Katharina Mosler [a], Christelle Coraux [a], Konstantina Fragaki [a], Jean-Marie Zahm [a], Odile Bajolet [a,b], Katia Bessaci-Kabouya [c], Edith Puchelle [a], Michel Abély [a,c], Pierre Mauran [a,d,*]

[a] *INSERM, U514, Reims, F-51092, France*

[b] *CHU Reims, Hôpital Maison Blanche, Unité d'Hygiène, Reims, F-51092, France*

[c] *CHU Reims, American Memorial Hospital, Service de Pédiatrie A, Centre de Ressources et de Compétences pour la Mucoviscidose, Reims, F-51092, France*

[d] *CHU Reims, American Memorial Hospital, Service de Pédiatrie A, Explorations Fonctionnelles Pédiatriques, Reims, F-51092, France*

Received 2 October 2006; received in revised form 11 March 2007; accepted 20 April 2007

Available online 5 June 2007

Abstract

Background: For a better understanding of the early stages of cystic fibrosis (CF), it is of major interest to study respiratory epithelial cells obtained as early as possible. Although bronchoalveolar lavage has been proposed for this purpose, nasal brushing, which is a much less invasive technique, has seldom been used in CF infants. The aim of the present study was to examine in a few infants the feasibility of a nasal brushing technique for studies of airway epithelial functions in very young CF infants.

Methods: In 5 CF (median age 12, range 1–18 months) and 10 control infants (median age 5, range 1–17 months), a nasal brushing was performed by means of a soft sterile cytology brush, after premedication with oral paracetamol (15 mg/kg body weight) and rectal midazolam (0.2 mg/kg body weight). Samples were used for microbiological, cytological and functional studies.

Results: The procedure was well tolerated. Number of cells collected was similar in CF and non-CF patients (CF: median 230×10^3, range 42×10^3–900×10^3; non-CF: median 340×10^3, range 140×10^3–900×10^3). Median number of viable cells was 67% (range 31–84%). Freshly obtained samples were successfully used for studies of ciliary beating frequency and cAMP-dependent chloride efflux. In 7 out of 17 cell cultures, confluence was obtained (CF: 2 out of 7; non-CF: 5 out of 10). The feasibility of studying protein release and mRNA expression of IL-8, IL-6 and TNF-α, under basal conditions and after stimulation by *Pseudomonas aeruginosa*, was demonstrated.

Conclusions: By means of a simple nasal brushing technique easily performed and well tolerated, it is feasible, in infants, to harvest respiratory cells in sufficient amounts to study the airway epithelium using a broad range of techniques including cell culture.

Keywords: Cystic fibrosis; Cell physiology; Cell culture

1. Introduction

Cystic fibrosis (CF), the most common lethal genetic disease in the Caucasian population, is characterized by airway inflammation and infection leading to progressive destruction of lungs. Nevertheless, the events that occur very early during the progression of the disease at the airway level in infants are not known.

☆ Data from present manuscript have been presented at the 29th European Cystic Fibrosis Conference: Copenhagen, Denmark, 2006. [1].

* Corresponding author. Unité d'explorations Fonctionnelles Pédiatriques, Service de Pédiatrie A, American Memorial Hospital, CHU de Reims, 49 rue Cognacq-Jay, 51092 Reims, France.

E-mail address: pmauran@chu-reims.fr (P. Mauran).

1569-1993/$ - see front matter © 2007 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jcf.2007.04.005

One of the most important abnormalities in CF is an abnormal processing of the mutated CFTR protein through the endoplasmic reticulum that causes abnormal location or even absence of the protein at the apical plasma membrane of airway epithelial cells [2]. This abnormality results in a defective cAMP-regulated chloride transport associated with a marked dehydration of the airway surface fluid, decreased mucus transport and airway obstruction. In CF adult patients with the most common mutation (homozygous ΔF508), it has been shown that the clinical manifestations may vary considerably and that mild phenotype could be partly related to a residual CFTR which could explain a normal cAMP-dependent chloride conductance [3,4]. At cellular level, we have also reported that the CFTR expression and localization could be related to the differentiation state of the airway epithelium [5]. In CF infants, such studies are still lacking.

It has been suggested that in CF patients, inflammation may precede infection [6,7]. Whether the CFTR defect is directly linked to an enhanced inflammatory process beginning before the first infection [8], or the airway inflammation is only a consequence of infection due to the impaired bacteria clearance [9,10] is still debated. In bronchoalveolar lavages (BAL) of CF infants with negative bacterial and viral cultures, Khan et al. [11] found that the number of neutrophils and the interleukin 8 (IL-8) levels were increased as compared with healthy controls. In contrast, in similar studies, Armstrong et al. [12,13] could not confirm these results. However, while BAL is the standard method to obtain material for studies of the respiratory epithelium, it is an invasive technique which cannot be easily performed in asymptomatic infants. In addition, the lavages are not only the reflection of the only epithelium because they contain also inflammatory cells and their products. Thus, studies are scarce, and the situation in the CF infant lung before the first infection remains an open question.

Taking into account the recent initiation of systematic neonatal CF screening, our objective was to find a simple and repeatable minimally invasive technique to analyze respiratory epithelial cells as early as possible in order to obtain information on the state of inflammation, infection, cAMP-dependent chloride conductance and histological characteristics of the airway epithelial sample. Interestingly, it has been recently reported that the use of non-bronchoscopic brushing to study the paediatric airways for clinical and research purposes was safe and easy to perform [14,15]. However, this technique was applied to healthy and asthmatic children prior to a surgical treatment that is under general anaesthesia and after tracheal intubation. Therefore, this technique does not appear appropriate to study asymptomatic babies. It is generally admitted that nasal epithelium characteristics and functions well reflect those of the lower airway epithelium [16,17,18] although some contradictory results about inflammation levels in CF and control patients have been reported. Noah et al. reported that nasal lavage fluid and BAL IL-8 levels are not significantly correlated [7]. However, Pitrez et al. have found that nasal IL-8 values well reflect lower airway levels [19], and Bergoin et al. that IL-8 concentrations measured in nasal lavages are significantly higher in CF patients than in healthy controls, similarly to what is found when using BAL [20]. Although several previous studies have shown that nasal brushing is safe and useful in adults and in children [21–28] this technique has been rarely used in CF [21,29] and only one study included CF infants [30].

In the present report, we describe a nasal brushing technique that can be easily performed in very young CF and non-CF infants and gives the ability to analyze airway cells and their functional properties, from native cell sheets and primary cell cultures. We believe this technique might be useful for further studies including a larger number of infants, in order to compare the airway epithelial functions between CF and non-CF infants.

2. Materials and methods

2.1. Patients

The present study was approved by the Regional Ethics Committee (Comité de Protection des Personnes de Champagne-Ardenne) and written informed consent was obtained from the parents of the infants prior to sampling. CF infants were recruited from the outpatient clinics devoted to CF care (Centre de Ressources et de Compétences pour la Mucoviscidose) of the American Memorial Hospital, Reims, France. Non-CF infants were recruited in the same hospital, among patients needing mild sedation for diagnostic investigation or minor surgery. At the time of nasal brushing, all non-CF patients were free of clinical signs of respiratory infection, and CF patients had no exacerbation and no or only mild respiratory symptoms (Table 1).

Nasal brushings were performed in 5 CF (median age 12 months, range 1–18 months) and 10 non-CF control infants (median age 5 months, range 1–17 months). Four out of the 5 CF patients were diagnosed by neonatal screening; in the other one, neonatal screening was falsely negative and diagnosis was done because he had chronic diarrhea. Four CF infants had ΔF508/508 homozygous genotype, the other one was compound heterozygous ΔF508/G542X. In one CF infant, nasal brushing was repeated 3 times, at ages 8, 13 and 17 months. The respiratory status (symptoms, bacterial colonization and medication) of all the infants are summarized in Table 1.

2.2. Nasal brushing technique in infants

Nasal brushing was performed on an in-patient basis. The infants were given a premedication with oral paracetamol (15 mg/kg of body weight) and rectal midazolam (0.2 mg/kg of body weight) 20 min before nasal brushing. Nasal brushing was performed by means of a soft sterile brush (Scrinet Diam.5.5, Laboratoire C.C.D., Paris, France). After nasal lavage with physiological saline in order to remove mucus, a

Table 1
Nasal brushing in CF and control infants: clinical characteristics, bacterial status and respiratory symptoms

Patient	Group	Genotype	Sex	Age	Medication	Bacterial status	Respiratory symptoms
1	CF	ΔF508/ΔF508	m	18	None	*S. aureus* chronic colonization	None
2	CF	ΔF508/ΔF508	f	1	Omeprazole	None	None
3	CF	ΔF508/ΔF508	f	12	None	*S. aureus* chronic colonization	None
4	CF	ΔF508/ΔF508	m	9	None	None	None
5-1	CF	ΔF508/ΔF508	m	8	None	*S. aureus* chronic colonization	None
5-2				13	None	*S. aureus* chronic colonization	Daily morning cough
5-3				17	Dornase alpha, tobramycine	*P. aeruginosa* first colonization	Daily morning cough
6	Control		f	12	None	None	None
7	Control		m	6	None	None	None
8	Control		m	5	Clonazepam	None	None
9	Control		f	3	None	None	None
10	Control		f	5	None	None	None
11	Control		m	17	None	None	None
12	Control		f	3	None	None	None
13	Control		m	4	Amox-clavul., salbumol, budezonid	None	None
14	Control		f	9	None	None	None
15	Control		m	1	None	None	None

m: male, f: female, *S. aureus*: *Staphylococcus aureus*, *P. aeruginosa*: *Pseudomonas aeruginosa*.

brushing was performed in both nostrils by a gentle circular movement. If mucus attached to the brush was visible, it was discarded by means of sterile tweezers. Brushes were immersed in 2 ml RPMI 1640 medium with Hepes and Glutamax I (Gibco, Grand Island, NY, USA) without antibiotics (further called "transport medium" throughout the text), manually shaken and immediately brought to the laboratory. After the nasal brushing which was performed between 09 and 11 h a.m., patients were carefully monitored for vital and more minor signs, comfort and pain and they were not discharged on the same day before 06 h p.m.

2.3. Samples processing

Within 15 min after nasal brushing, epithelial cells (dissociated epithelial cells and non-dissociated epithelial cells called cell sheets) were detached from the brushes by scraping the bristles with tweezers which were then agitated within the transport medium. Samples obtained from the two nostrils were pooled and then centrifuged 5 min at 250×*g* at room temperature.

Supernatants of these centrifuged transport media were used for microbiological investigations. The cell pellets were resuspended in 2 ml RPMI 1640 medium with Hepes and Glutamax I and directly used for evaluation of cell phenotypes, ciliary beating frequency analysis and cAMP-dependant chloride efflux study. The remaining cells were used for cell culture after cell counting and cell viability study.

2.3.1. Microbiological investigations

Aliquots of the centrifuged transport media supernatants were cultured on horse blood and chocolate blood agar incubated at 37 °C during 48 h. Identification was performed for the following micro-organisms: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Branhamella catarrhalis*, *Corynebacterium* sp., and *Neisseria* sp.

2.3.2. Evaluation of cellular phenotypes

A 200 μl of nasal brushing suspension was cytocentrifuged 10 min at 800 rpm (Cytospin 2, Shandon, Astmoor, GB) on slides that were stored at −20 °C until use. Identification of neutrophil and secretory epithelial cells was performed by May–Gruenwald–Giemsa and Alcian blue–periodic acid–Schiff stainings, respectively. Identification of epithelial cells was realized by peroxydase immunodetection using Pan cytokeratin antibodies. Briefly, slides were treated with 0.3% hydrogen peroxyde for 15 min to quench endogenous peroxidase activity and then saturated for 15 min with 1% BSA in PBS 0.1 M, pH=7.2 (Gibco). Slides were then incubated for 1 h with a 1/50 diluted monoclonal Pan cytokeratin-antibody (Anti Pan cytokeratin clones C-11, PCK-26, CY-90, KS-1A3, M20 and A53-B/A2, Sigma, St. Louis, MO, USA). After several washes with PBS, the slides were successively incubated with biotin-coupled anti-mouse antibody (Dako, Glostrup, Denmark) then with a horseradish peroxidase-conjugated streptavidin (Dako). The peroxidase activity was revealed using the LSAB kit (Dako). The slides were briefly counterstained with Mayer's haematoxylin, mounted and observed under a Zeiss Axiophot microscope. Staining was performed on one CF and on 3 non-CF samples.

2.3.3. Ciliary beat frequency analysis

A 20 μl cell suspension aliquot was placed on the heated stage (37 °C) of an inverted microscope (Nikon TMS, Champigny sur Marne, France) between a glass slide and a coverslip (separated by a 1-mm-thick silicon ring). Video-recordings of epithelial cell sheets from the nasal brushing were performed at ×40 magnification by using a CCD camera (JAI PULNIX TM-760, San Jose, CA, USA) and a

DVD recorder. The recorded images were then displayed on a video monitor where ciliated cells were selected for ciliary beat analysis. The variations in light intensity induced by the ciliary beat were detected by a photodetector placed on the screen of the monitor. The signal was then digitised by a computer and the resulting data converted by a Fast Fourier Transform method into a frequency spectrum from which a mean ciliary beat frequency (CBF) was calculated [31]. Measurements were performed on at least 12 different ciliated cells per sample.

2.3.4. cAMP-dependent chloride efflux

Cell sheets were incubated with the 6-methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ) probe (Sigma) in a hypotonic chloride buffer solution (130 mM NaCl, 2.4 mM K_2HPO_4, 10 mM D-glucose, 1 mM $CaSO_4$, 1 mM $MgSO_4$ and 10 mM Hepes diluted at 1/2 in water) for 30 min at 37 °C. Thereafter, cells were washed twice in the hypotonic chloride buffer to remove residual extracellular SPQ. The hypotonic chloride buffer was then replaced by an isotonic buffer in which the NaCl was replaced by 130 mM of $NaNO_3$ and the cell suspension was placed in a 35 mm culture dish on the heated stage of an inverted microscope (TE 300, Nikon). A cell sheet was held by means of a micro-capillary (Vacutips, Eppendorf, Le Pecq, France) connected to a vacuum syringe and driven by a micromanipulator (Transferman NK2, Eppendorf). Amiloride (10^{-5} M), and 3 min later, forskolin (25 μM), were added to the nitrate buffer. Throughout the experimental process, fluorescence images of the SPQ probe within the cell sheet (obtained from excitation light at 365 nm and emission light at 395 nm) were recorded every 30 s for 3 min using a Micromax CCD camera and Metafluor software (Universal Imaging, US). Chloride efflux by non-CF cells and CF cells were calculated by measuring the variations in SPQ fluorescence over the first minute incubation period with amiloride or forskolin ($\Delta F/\Delta t$), as described by Bonnet and Zahm [32]. Fluorescence variations were measured within the whole cell sheet. We performed one assay on three different cell sheets per sample.

2.3.5. Airway epithelial cell culture

Epithelial cell sheets were dissociated with trypsine Versene solution (composed of PBS containing 0.33 g/l EDTA, 1.42 g/l NaCl and 1.67% (V/V) 2.5%-trypsine; Gibco) for 1.5 min at room temperature. Epithelial cells were counted using a Malassez Cell and the viability of the cells was assessed by the Trypan blue dye exclusion assay.

Epithelial cells were seeded at a concentration of 85,000–230,000 viable cells/well on 24-well-plates in 0.5 ml of culture medium. To improve cell adhesion, culture plates were coated with 2% collagen I extracted from rat tail tendons as described previously [27] and 20% fetal calf serum (Gibco) was added to the culture medium.

The culture medium used was adapted from a culture medium described by Lechner et al. [33], and consisted of 75% DMEM and 25% F12 medium (Gibco), supplemented with 0.87 μM bovine insulin, 65 nM human transferrin, 1.6 nM recombinant human EGF, 1.38 μM hydrocortisone, 30 nM retinyl acetate, 9.7 nM 3,3′,5-triiodo-L-thyronine, 2.7 μM epinephrine, 35 μg/ml bovine pituitary extract, 5 μM ethanolamine, 5 μM *o*-phosphorylethanolamine, 30 nM sodium selenite, 1 nM manganese chloride tetrahydrate, 0.5 μM sodium metasilicate nonahydrate, 1 nM ammonium molybdate tetrahydrate, 5 nM ammonium vanadate, 1 nM nickel sulfate hexahydrate, 0.5 nM stanous chloride dihydrate (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Culture medium was changed after 2 days, and then every 3 days up to confluence. After 2 days of culture, fetal calf serum was discarded from the culture medium.

2.3.6. Analysis of inflammatory mediators in cultured cells

At confluence, the cultured airway epithelial cells were first incubated for 2 h in incubation medium [DMEM/F-12+L-Glutamine+Hepes (Gibco) with 10% fetal calf serum]. After 2 h, cells were rinsed twice with PBS (D-PBS+$CaCl_2$+$MgCl_2$, Gibco) and then incubated for 24 h with either lipopolysaccharide (LPS) of *P. aeruginosa* 10 μg/ml (Calbiochem, San Diego, CA, USA) in incubation medium, or without LPS in the incubation medium (control samples). Cell supernatants were centrifuged at 10,000×*g* for 5 min and the pellets were discarded. Cell supernatants and culture plates with adherent cells were stored at −80 °C until further examination. The IL-8, IL-6, and tumor TNF-α protein release in cell supernatants and mRNAs expressions in cells were measured by ELISA and RT-PCR techniques, respectively.

2.3.7. ELISA assays

IL-8, IL-6 and TNF-α concentrations in cell culture supernatants were determined with commercially available enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN, USA). The levels of sensitivity of the assays were 3.50 pg/ml, 0.70 pg/ml and 0.12 pg/ml, respectively. In order to standardize the protein release results, IL-8, IL-6 and TNF-α protein concentrations in culture supernatants were expressed as pg of cytokines/μg of total RNA extracted from each cell culture. Extraction of total RNA and evaluation of their concentration were performed as described in the next paragraph.

2.3.8. RT-PCR analyses

Total RNA extraction was performed with the High Pure RNA isolation kit (Roche, Mannheim, Germany). RNA concentration was evaluated in each sample with the RiboGreen RNA quantitation kit (Interchim, Montluçon, France). RT-PCR was performed with 10 ng of total RNA by using the Thermostable r*Tth* Reverse Transcriptase RNA PCR kit (Applied Biosystem, Foster City, CA) and four pairs of nucleotides (Eurogentec, Seraing, Belgium). Forward and reverse primers for human IL-8, IL-6, TNF-α and 28 S were designed as follow: IL-8 primers [forward 5′-GCCAAGGAGTGCTAAAGAACTTAG-3′, reverse 5′-GAATTCTCAGCCCTCTTCAAAAAC-3′], IL-6 primers [forward

5′-GCCAGAGCTGTGCAGATGAGTA-3′, reverse 5′-GCTACATTTGCCGAAGAGCCCT-3′], TNF-α primers [forward 5′-CAGCCTCTTCTCCTTCCTGA-3′, reverse 5′-TGAGGTACAGGCCCTCTGAT-3′] and 28 S primers [forward 5′-GTTCACCCACTAATAGGGAACGTGA-3′, reverse 5′-GGATTCTGACTTAGAGGCGTTCAGT-3′]. Reverse transcription was performed at 70 °C for 15 min. For the IL-8 PCR, an initial denaturation at 95 °C for 2 min was followed by 21 amplification cycles (denaturation at 94 °C for 15 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 10 s) and a final 2-min elongation at 72 °C. For the IL-6 PCR, an initial denaturation at 95 °C for 2 min was followed by 28 amplification cycles (denaturation at 94 °C for 20 s, annealing at 64 °C for 30 s, and elongation at 72 °C for 30 s) and a final 2-min elongation at 72 °C. For the TNF-α PCR, the conditions were as follows: initial denaturation (94 °C, 2 min), 27 amplification cycles (denaturation 94 °C, 30 s, annealing 59 °C, 30 s, and elongation 72 °C, 30 s) and final elongation (72 °C, 7 min). For the 28 S PCR, the conditions were as follows: initial denaturation (95 °C, 2 min), 11 amplification cycles (denaturation 94 °C, 15 s, annealing 66 °C, 20 s, and elongation 72 °C, 10 s), final elongation (72 °C, 2 min). RT-PCR products were separated by acrylamide gel electrophoresis stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) and quantified by fluorimetric scanning (LAS-1000; Fuji, Stamford, CT, USA). The predicted sizes of the RT-PCR products of IL-8, IL-6, TNF-α and 28 S were 222 bp, 223 bp, 302 bp and 212 bp, respectively. The values obtained for IL-8, IL-6 and TNF-α amplifications were normalized to the values obtained for 28 S amplification.

3. Results

3.1. Nasal brushing

3.1.1. Tolerance of procedure

The nasal brushing procedure took about 30 s; the brushing itself took 2 s for each nostril. While they were hold tightly by a nurse, most of the infants cried, but stopped immediately when they were released, so it is unlikely that crying was due to pain during brushing. One of the infants had minor self-limited nasal bleeding after the brushing; otherwise, no adverse events occurred, either in CF or non-CF infants. All patients' vital signs were stable through the 8 h observation period. No patient required oxygen therapy. There was no coughing episode. It is to ensure maximum safety that infants observation after sedation and nasal brushing was maintained up to 8 h; however, within 30 min or less, all infants were doing perfectly well.

3.2. Microbiological assessment

Out of the 7 samples of the centrifuged transport media supernatants obtained in CF infants, one was sterile, 2 were polymicrobial, 4 were monomicrobial (Table 2). Following bacteria were isolated: *S. pneumoniae* (1 sample), *S. aureus* (1 sample), *Corynebacterium* sp. (2 samples), coagulase negative *staphylococci* (3 samples), alpha hemolytic *streptococci* (1 sample) and *B. catarrhalis* (1 sample). All 10 samples obtained in non-CF infants were polymicrobial with 2 or more bacteria. Following bacteria were isolated: *S. aureus* (2 samples), *S. pneumoniae* (1 sample), *H. influenzae* (1 sample), alpha hemolytic *streptococci* (3 samples), coagulase-negative *staphylococci* (5 samples), *Neisseria* sp. including *B. catarrhalis* (6 samples), *Corynebacterium* sp. (5 samples).

Table 2
Nasal brushing in CF and control infants: data from microbiological studies, ciliary beat frequency analysis, cell counts and cell culture

Patient	Microbiology	CBF	Cell count		Cell culture	
		Hz	Total ($\times 10^3$)	Viable (%)	n ($\times 10^3$)/cm^3	Confluence
1	*S. areus*	11.8	300	33	26	No
2	None	8.5	42	98	24	No
3	αH *Streptcocci*, CNS., *Corynebacterium* sp.	11.4	173	87	44	Contaminate
4	*B. catarrhalis*, *Corynebacterium* sp.	9.8	62	86	15	No
5-1	CN*S.*	11.3	900	37	20	No
5-2	CN*S.*	9.2	680	43	86	Yes
5-3	*S. pneumoniae*	10.6	230	76	51	Yes
6	αH *Streptcocci* CN*S.*, *B. catarrhalis*	12.5	nd	nd	nd	No
7	*S. areus*, *Corynebacterium* sp.	9.3	140	50	18	Contaminate
8	CN*S.*, *Neisseria* sp., *Corynebacterium* sp.	9.5	nd	nd	nd	nd
9	CN*S.*, *Corynebacterium* sp.	m	900	70	35	Yes
10	*Neisseria* sp., *Corynebacterium* sp.	10.3	320	50	94	Yes
11	αH *Streptcocci*, *Neisseria* sp.	nd	560	43	114	Yes
12	αH *Streptcocci*, *Neisseria* sp.	10.8	480	84	55	No
13	*S. areus*, CNS	9.9	235	31	43	Contaminate
14	*S. pneumoniae*, *B. catarrhalis*	8.8	355	76	79	Yes
15	CN*S.*, *H. influenzae*, *Corynebacterium* sp.	10	287	67	50	Yes

S. aureus: *Staphylococcus aureus*, αH *Streptococci*: alpha hemolytic *streptococci*, *S. pneumoniae*: *Streptococcus pneumoniae*, CN*S*: coagulase negative *staphylococcus*, *B. catarrhalis*: *Branhamella catarrhalis*, *H. influenzae*: *Haemophilus influenzae*, CBF: ciliary beat frequency, nd: not done, m: missing.

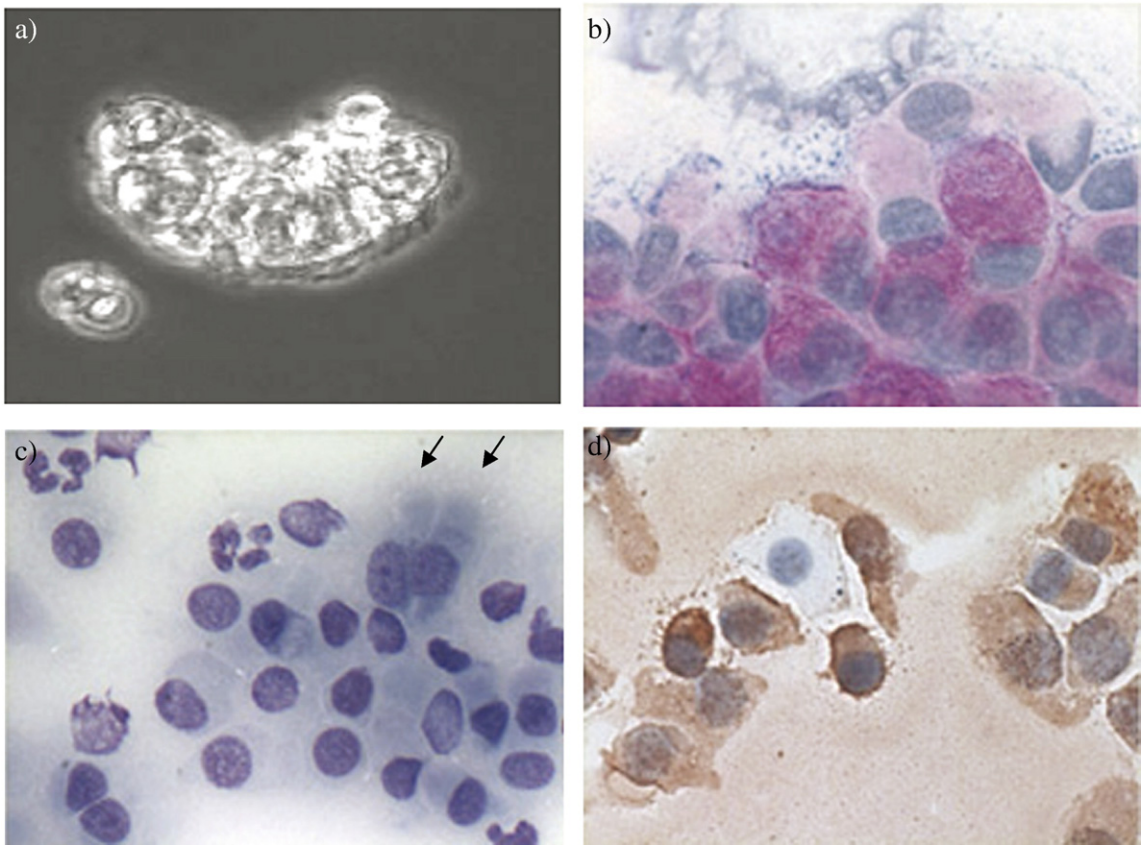

Fig. 1. Cytospin preparations of non-dissociated respiratory epithelial cells freshly collected in infants by means of nasal brushing. a) Microscopic view of nasal epithelial cell sheet (×63, CF infant 4), b) Alcian-blue–PAS staining (×63, CF infant 5-3), c) May–Gruenwald–Giemsa staining (arrows: ciliated cells, ×20, CF infant 5-3), d) Pancytokeratin staining (×40, CF infant 5-2).

3.3. Epithelial cell collection and viability

The number of cells collected by nasal brushing in the two nostrils and counted after centrifugation was similar in CF and non-CF infants (CF: median 230,000, range 42,000–900,000; non-CF: median 340,000, range 140,000–900,000). The median proportion of viable cells detected by Trypan blue dye exclusion was 67% (range 33–98%) in CF patients, and 58% (range 31–84%) in non-CF infants. We obtained more than 400,000 viable cells in 3 samples, 201,000 to 400,000 viable cells in 3 samples, 100,000 to 200,000 viable cells in 5 samples and less than 100,000 viable cells in 4 samples. The raw data are presented in Table 2.

Percentages of viable cells obtained by nasal brushing did not differ according to gender. When *S. aureus* was found in bacterial culture of transport medium supernatant, the percentage of viable cells was <51% (31%, 33%, 50%).

3.4. Evaluation of cellular phenotypes

After nasal brushing, epithelial cells were generally non-dissociated and formed cell sheets (Fig. 1a). In Alcian blue–PAS staining, secretory cells were very difficult to distinguish from others because of mucus which stained as well. However, we observed mixed cells (PAS positive ciliated cells), but no mature goblet cells with membrane bound secretory granules (Fig. 1b). May–Gruenwald–Giemsa staining was performed in 3 CF and 4 non-CF samples (Fig. 1c). A neutrophil count was performed. The median PMN count was <1% (range <1–4%) in CF samples, and 1.25% (range <1–5%) in non-CF samples. Pan cytokeratin staining showed that a high percentage (>95%) of collected cells were epithelial cells, in CF as well as in non-CF patients. The other cells had the appearance of squamous cells (Fig. 1d).

3.5. Ciliary beating frequency

CBF was measured in epithelial sheets from 15 out of 17 nasal brushing samples. In one non-CF sample, no cilia were observed at the surface of the epithelial sheets. CBF was within a normal range and was not significantly different in CF infants (10.4±1.2 Hz, $n=7$) compared with non-CF infants (10.1±1.1 Hz, $n=8$). The raw data are presented in Table 2.

3.6. cAMP-dependent chloride efflux

We were able to perform a study on chloride efflux on three different cell sheets in 6 out of 10 samples (Fig. 2).

Among the 6 samples where the full number of experiments was performed, 3 were from CF infants (ΔF508 homozygous) and the remaining 3 from non-CF infants. After addition of forskolin, the median chloride efflux in CF samples was 1.1 arbitrary units (AU) (range 0.7–1.6). In non-CF samples, the median chloride efflux was 3.8 (range: 2.4–4.1). The raw data are presented in Fig. 2. The repetition of measures at a 9 months time interval in a same CF infant gave similar results.

3.7. Cell culture

In 7 out of 17 cell cultures (2 CF, 5 non-CF), confluence was obtained in the center of the wells with sometimes areas not confluent in the periphery of the culture (Fig. 3 and Table 2). In the 10 other cultures, cell confluence was not obtained, four times because of the limited number of cells that were seeded (<50,000 viable cells/cm^2), and three times due to a bacterial (1 case) or fungal (2 cases) contamination. When excluding contaminated cultures, we obtained confluence in 6 out of 7 cultures with a seeding concentration of 50,000–140,000 viable cells/cm^2 (85,000–230,000 viable cells/well on 24-well-plates). Confluence (Fig. 3) was reached after 10 days (range 9–20 days).

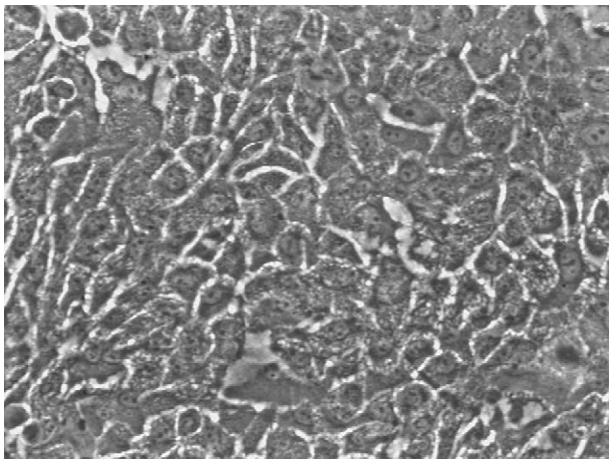

Fig. 3. Cell culture of nasal epithelial cells at confluence (non-CF infant) (×20).

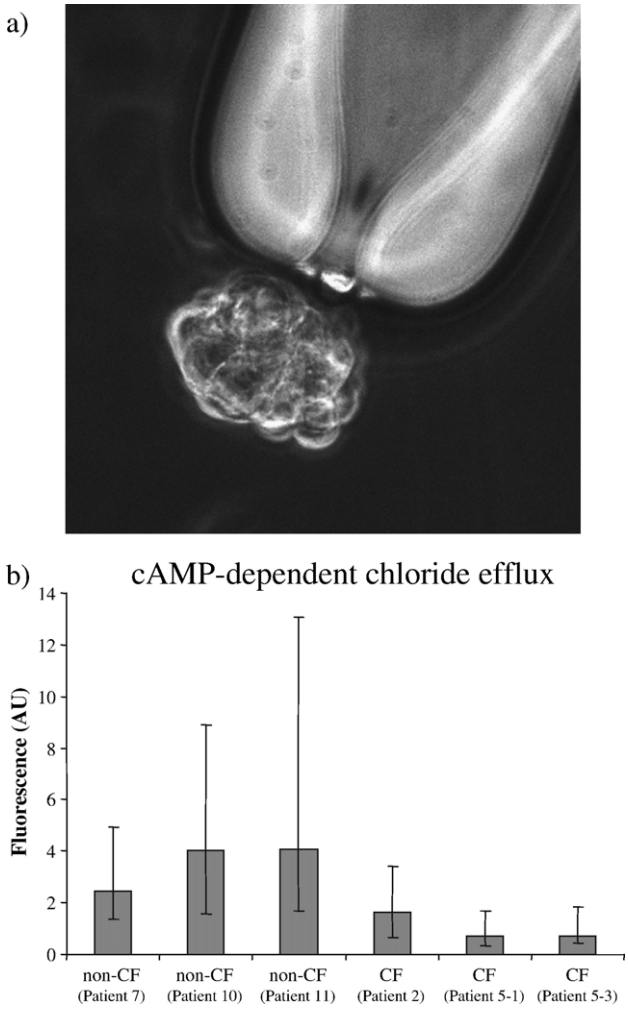

Fig. 2. cAMP-dependent chloride efflux measures in epithelial cells freshly obtained in infants by means of nasal brushing. a) A cell sheet is held by means of a micro-capillary, b) change in fluorescence during the first minute after addition of forskolin (AU for arbitrary units). For each sample, three experiments were performed. Results are presented as median values; error bars indicate range.

3.8. Inflammatory mediators expression and secretion

Only as examples of functional studies that can be performed on cell cultures obtained from nasal brushing, 2 non-CF and 1 CF cell cultures at confluence (seeding density range: 50×10^3–79×10^3 cells/cm^2, time to confluence range: 9–10 days) were used for observation of IL-8, IL-6 and TNF-α expression at basal condition and after a 24 h incubation with *P. aeruginosa* LPS. By RNA extraction, a median yield of 76 ng/μl RNA (range 27–115 ng/μl) was obtained.

In the two non-CF cultures, a 24 h incubation with *P. aeruginosa* LPS resulted in no or slight increase in IL-8 protein release (from 813 to 798 pg/μg total RNA and from 598 to 728 pg/μg total RNA), no or slight decrease in IL-8 mRNA expression (from 1.13 to 1.14 and from 2.11 to 1.63 arbitrary units (AU)), and increases in IL-6 protein expression (from 80 to 105 and from 53 to 181 pg/μg total RNA), IL-6 mRNA expression (from 5.02 to 6.77 AU and from 6.45 to 8.63 AU), TNF-α protein release (from 0.27 to 0.37 and from 0.10 to 0.20 pg/μg total RNA), and TNF-α mRNA expression (from 1.27 to 1.34 and from 1.76 to 1.91 AU).

In one CF culture, a 24 h incubation with *P. aeruginosa* LPS resulted in a major increase in IL-8 protein release (from 388 to 1028 pg/μg total RNA), no change in IL-8 mRNA expression (from 0.42 to 0.47 AU), an increase in IL-6 protein expression (from 14 to 39 pg/μg total RNA), and decreases in IL-6 mRNA expression (from 1.26 to 0.60 AU), TNF-α protein release (from 0.28 to 0.18 pg/μg total RNA), and TNF-α mRNA expression (from 0.84 to 0.60 AU).

4. Discussion

A better understanding of the physiopathological mechanisms which are involved in the first stages of CF lung disease development, requires the analysis of respiratory epithelium samples obtained from young asymptomatic CF infants. For this purpose, broncho-alveolar lavage and bronchial brushing have been proposed [12]. However, these techniques requiring tracheal intubation under general anesthesia are rather aggressive for asymptomatic babies and we believe that less invasive techniques might be used. Nasal epithelium is generally considered to be representative of the lower airway epithelium [16–18,21]. The present pilot study shows that, by means of a simple nasal brushing technique easily performed and well tolerated, it is possible in infants to harvest respiratory cells in sufficient amounts to study the airway epithelium using a broad range of techniques.

As far as we know, Rutland et al. were the first to report in 1981 the use of nasal brushing for the study of ciliary beat frequency and ultrastructure, in adults and in children [27,34]. Nasal brushing has also been used for different purposes such as, in adults, the study of rhinitis [28,35] and, in children, the study of ciliary function in chronic bronchopneumopathies [25,26] and studies of chronic and allergic rhinitis [24,36]. Nasal brushing in infants has been recently reported by Nuesslin et al. for ciliary function analysis in children with chronic bronchitis [25].

Studies using nasal brushing technique in CF are scarce [21,29,30]. In 1997, Bridges et al. were the first to be able to obtain cultured nasal epithelial multicellular spheroids from nasal brushings performed in adults [29,37]. More recently, Harris et al. have described a technique for immunolocalization of CFTR in freshly isolated native airway epithelial cells obtained by means of nasal brushing in CF patients [21]. To our knowledge, the study from Black et al. published in 1998 is the only one to have reported the use of nasal brushing in CF and non-CF infants. However the patients enrolled in their study were older than those included in our present one (Range: 5–52 vs 1–18 months; median age: 11 vs 6 months). The aim of the present study was to show the feasibility of nasal brushing in a few very young infants to harvest respiratory cells in sufficient amounts for cell functions assays and cell culture.

Different tools have been used to perform nasal brushing, i.e. interdental brushes, cytology brushes and nasal curettes [21,22]. As advocated by the Cardiff team, we used sterile cytology brushes which are soft and probably easier for subjects to tolerate [21]. Several authors perform nasal brushing under direct visualization of the inferior nasal turbinate [27,29,30,34,38]. However, as the Lisboa group [21], we did not use rhinoscopy which we felt more aggressive to the infant than simply introducing the brush into the nostril. Under antalgic and hypnotic premedication, nasal brushing was well tolerated, without any incident except a minor self-limited nasal bleeding in one infant. Nasal brushing was repeated in one patient in order to increase the number of CF samples and as an example that it will be possible to follow-up patients using this minimally invasive technique. One limitation of performing nasal brushing is that although the amount of mucus was already reduced by doing a nasal lavage prior to the brushing procedure, some mucus still present in collected samples impeded evaluation of secretory cells number after Alcian blue–PAS staining.

The number of cells we collected (median numbers of 2.3×10^5 in CF infants and 3.4×10^5 in non-CF infants) is in the range reported by Bridges et al. [29] who used a cytology brush (from 10^4 to 10^6), and only slightly lower than the cell number reported by Lopez-Souza et al. who used surgical plastic or metallic curettes (mean numbers of 2.9×10^5 and 1.4×10^6 respectively) for nasal scrapings in healthy adults [22].

Airway epithelial cultures are generally derived from tracheal cells collected postmortem or obtained after dissociation of nasal polyps, turbinates or surgical specimens such as airways obtained at time of open thoracotomy for lung transplantation. Apart from the paper from Bridges et al. in 1997 [37], nasal brushings have been little used as starting material for cultures [23,30,39]. Recently, Lopez-Souza et al. have reported that human airway epithelial cells obtained from nasal scrapings or bronchial brushings in healthy volunteers can be grown in culture to produce polarized cell sheets suitable for studies of vectorial transport [22]. Black et al. obtained primary cell cultures from nasal brush biopsies performed in infants and children undergoing flexible bronchoscopy, and used these cultures to study IL-8 production by CF nasal epithelial cells, under basal conditions and after stimulation with TNF-α and respiratory syncytial virus [30].

In the present study, nasal brushing technique was able to harvest useful quantities of epithelial cells with a median viability of 67% which favorably compares with previously reported data. Mean viability of respiratory epithelial cells was 17.3% in the study of Lane et al. using non-bronchoscopic brushing in normal and asthmatic children [15], and between 25% and 30% in studies using bronchial brushing under direct vision in asthmatic adults [40–42]. Interestingly, we found similar numbers of viable cells in CF and non-CF samples.

Limitations of the cell culture conditions we used (on plastic 24-well-plates) are that they do not allow ciliated cells redifferenciation and do not permit complete studies needed to confirm that the cells are truly polarized monolayers. The trans-epithelial resistance measurement implies that cells be cultured on permeable supports and, for ciliated cell differentiation, at the air–liquid interface. Another limitation of our study is that we experienced a significant drop off in cell culture success. Although cell culture confluence was attained in 6 out of 7 non-contaminated cultures when seeding concentration was greater than 50 000 viable cells/cm2, 10 cultures failed, either because of the limited number of seeded cells or because bacterial or fungal contamination. This points out that, because of the limited samples obtained in small infants, studies performed in this group of patients are very challenging.

Several techniques could be applied to the respiratory epithelium samples we collected by means of nasal brushing in infants. The present results demonstrate that it is feasible to

study ciliary beating frequency and cAMP dependant chloride efflux from samples freshly obtained, and that cell cultures can be used to study the inflammatory cytokine release in response to stimulation by bacterial LPS. The experiments were done on a very small number of samples, as examples of techniques potentially useful in the field of CF research, which precludes any definite conclusion and comparison between CF and non-CF infants. It is likely that, due to the limited samples that can be obtained in small infants, it will not be always possible to undertake on each sample all the techniques we present here. CBF measurements are done on freshly obtained epithelial sheets without any alteration of cells which can then be used for other studies. As CBF analysis, halide efflux studies do not need cell culture and can be done on epithelial sheets. Halide efflux studies, easier to perform than techniques needing cell culture, are of obvious interest in CF disease and should probably be attempted before studies of inflammatory mediators expression and secretion. Based on the measurements variability within patients and the estimated difference between CF and non-CF subjects, we have calculated that at least 12 subjects should be included in each group to draw statistically significant conclusions when comparing halide efflux in CF and non-CF infants.

Beyond the present preliminary report of nasal brushing feasibility to study airway epithelial function in very young infants, it will be necessary to investigate a larger number of patients to know whether or not the present data are in accordance with current knowledge about the airway epithelium in CF. The CBF is normal in CF [43]. The cAMP-dependent chloride efflux from nasal epithelial cells is decreased in CF patients [44], and there is a dysregulation of IL-8 secretion by CF epithelial cells in response to *P. aeruginosa* [11,45–47]. Eventually, data obtained from nasal brushings in infants will have to be compared to those from bronchial brushings and BALs in order to assess the value of this minimally invasive technique for the study of the airway epithelium in CF infants.

5. Conclusions

The present study shows that by means of a simple nasal brushing technique easily performed and well tolerated, it is possible, in very young infants, to harvest respiratory cells in sufficient amounts to study the airway epithelium using a broad range of techniques including primary epithelial cell culture. Although the finding of the present preliminary study have to be confirmed in a larger one, we believe that the techniques we describe here might offer the opportunity to study the airway epithelium morphology and functions at the very early stages of CF, in infants, before the occurrence of clinical symptoms.

Acknowledgements

Katharina Mosler was supported by the German Association Mukoviszidose e.V. and by a grant from Chiron (Chiron New Technology Award 2003). The present study was also supported by AVLM (Association Vaincre La Mucoviscidose) and AESE (Association pour l'Etude de la Santé de l'Enfant).

References

[1] Mosler K, Coraux C, Fragaki K, Zahm JM, Bajolet O, Bessaci-Kabouya K, et al. Nasal epithelial brushing: a valuable method to study airway epithelial cells in CF and non-CF infants. J Cyst Fibros 2006;5 (suppl1):S10.
[2] Kreda SM, Mall M, Mengos A, Rochelle L, Yankaskas J, Riordan JR, et al. Characterization of wild-type and deltaF508 cystic fibrosis transmembrane regulator in human respiratory epithelia. Mol Biol Cell 2005;16(5):2154–67.
[3] Bronsveld I, Mekus F, Bijman J, Ballmann M, de Jonge HR, Laabs U, et al. Chloride conductance and genetic background modulate the cystic fibrosis phenotype of Delta F508 homozygous twins and siblings. J Clin Invest 2001;108(11): 1705–15.
[4] Sermet-Gaudelus I, Vallee B, Urbin I, Torossi T, Marianovski R, Fajac A, et al. Normal function of the cystic fibrosis conductance regulator protein can be associated with homozygous (Delta)F508 mutation. Pediatr Res 2002;52(5):628–35.
[5] Dupuit F, Kalin N, Brezillon S, Hinnrasky J, Tummler B, Puchelle E. CFTR and differentiation markers expression in non-CF and delta F 508 homozygous CF nasal epithelium. J Clin Invest 1995;96 (3):1601–11.
[6] Bonfield TL, Panuska JR, Konstan MW, Hilliard KA, Hilliard JB, Ghnaim H, et al. Inflammatory cytokines in cystic fibrosis lungs. Am J Respir Crit Care Med 1995;152(6 Pt 1):2111–8.
[7] Noah TL, Black HR, Cheng PW, Wood RE, Leigh MW. Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. J Infect Dis 1997;175(3):638–47.
[8] Zahm JM, Gaillard D, Dupuit F, Hinnrasky J, Porteous D, Dorin JR, et al. Early alterations in airway mucociliary clearance and inflammation of the lamina propria in CF mice. Am J Physiol 1997;272(3 Pt 1): C853–9.
[9] Høiby N. Inflammation and infection in cystic fibrosis—hen or egg. Eur Respir J 2001;17(1):4–5.
[10] Puchelle E. Early bronchial inflammation in cystic fibrosis. J Soc Biol 2002;196(1):29–35.
[11] Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DW. Early pulmonary inflammation in infants with cystic fibrosis. Am J Respir Crit Care Med 1995;151(4):1075–82.
[12] Armstrong DS, Grimwood K, Carzino R, Carlin JB, Olinsky A, Phelan PD. Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis. BMJ 1995;310(6994):1571–2.
[13] Armstrong DS, Hook SM, Jamsen KM, Nixon GM, Carzino R, Carlin JB, et al. Lower airway inflammation in infants with cystic fibrosis detected by newborn screening. Pediatr Pulmonol 2005;40(6):500–10.
[14] Doherty GM, Christie SN, Skibinski G, Puddicombe SM, Warke TJ, de Courcey F, et al. Non-bronchoscopic sampling and culture of bronchial epithelial cells in children. Clin Exp Allergy 2003;33(9):1221–5.
[15] Lane C, Burgess S, Kicic A, Knight D, Stick S. The use of non-bronchoscopic brushings to study the paediatric airway. Respir Res 2005;6(1):53.
[16] Knowles M, Gatzy J, Boucher R. Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. N Engl J Med 1981;305(25):1489–95.
[17] Gaga M, Vignola AM, Chanez P. Upper and lower airways: similarities and differences. Eur Respir Monogr 2001;6(18):1–15.
[18] Roxo-Rosa M, da Costa G, Luider TM, Scholte BJ, Coelho AV, Amaral MD, et al. Proteomic analysis of nasal cells from cystic fibrosis patients and non-cystic fibrosis control individuals: search for novel biomarkers of cystic fibrosis lung disease. Proteomics 2006;6 (7):2314–25.

[19] Pitrez PM, Brennan S, Turner S, Sly PD. Nasal wash as an alternative to bronchoalveolar lavage in detecting early pulmonary inflammation in children with cystic fibrosis. Respirology 2005;10(2):177–82.
[20] Bergoin C, Gosset P, Lamblin C, Bolard F, Turck D, Tonnel AB, et al. Cell and cytokine profile in nasal secretions in cystic fibrosis. J Cyst Fibros 2002;1(3):110–5.
[21] Harris CM, Mendes F, Dragomir A, Doull IJ, Carvalho-Oliveira I, Bebok Z, et al. Assessment of CFTR localisation in native airway epithelial cells obtained by nasal brushing. J Cyst Fibros 2004;3(Suppl 2):43–8.
[22] Lopez-Souza N, Avila PC, Widdicombe JH. Polarized cultures of human airway epithelium from nasal scrapings and bronchial brushings. In Vitro Cell Dev Biol Anim 2003;39(7):266–9.
[23] Hull J, Harris A. Limitations of cell culture of airway epithelium collected by a nasal brushing technique. In Vitro Cell Dev Biol Anim 1994;30A(8):488–9.
[24] Jean R, Delacourt C, Rufin P, Pfister A, Waernessyckle S, de Blic J, et al. Nasal cytology in rhinitis children: comparison between brushing and blowing the nose. Allergy 1996;51(12): 932–4.
[25] Nuesslein TG, Hufnagel C, Stephan V, Rieger CH. Yield of bronchial forceps biopsies in addition to nasal brushing for ciliary function analyses in children. Klin Padiatr 2004;216(4):238–43.
[26] Cali A, Berni Canani M, De Luca L, Terracciano L, Berni Canani R, Chiummariello S. Results of nasal brushing in the study of ciliary conformation and function in chronic bronchopneumopathies in childhood. Pediatr Med Chir 1991;13(4):351–4.
[27] Rutland J, Dewar A, Cox T, Cole P. Nasal brushing for the study of ciliary ultrastructure. J Clin Pathol 1982;35(3):357–9.
[28] Gilain L, Chapelin C, Boucherat M, Peynegre R, Escudier E. Evaluation of the brushing technique in nasal cytology. Bull Assoc Anat (Nancy) 1994;78(240):5–8.
[29] Bridges MA, Walker DC, Davidson AG. Cystic fibrosis and control nasal epithelial cells harvested by a brushing procedure. In Vitro Cell Dev Biol 1991;27A(9):684–6.
[30] Black HR, Yankaskas JR, Johnson LG, Noah TL. Interleukin-8 production by cystic fibrosis nasal epithelial cells after tumor necrosis factor-alpha and respiratory syncytial virus stimulation. Am J Respir Cell Mol Biol 1998;19(2):210–5.
[31] Zahm JM, Dionisius J-P, Pierrot D, Chevillard M, Puchelle E. Mesure de la fréquence de battement ciliaire sur écran vidéo. Innov Tech en Biol et Méd 1990;2(11):118–29.
[32] Bonnet N, Zahm JM. Analysis of image sequences in fluorescence videomicroscopy of stationary objects. Cytometry 1998;31(3):217–28.
[33] Lechner JF, LaVeck MA. A serum-free method for culturing normal human bronchial epithelial cells at clonal density. J Tissue Cult Methods 1985;9:43–8.
[34] Rutland J, Griffin W, Cole P. Nasal brushing and measurement of ciliary beat frequency. An in vitro method for evaluating pharmacologic effects on human cilia. Chest 1981;80(6 Suppl):865–7.
[35] Raulf-Heimsoth M, Wirtz C, Papenfuss F, Baur X. Nasal lavage mediator profile and cellular composition of nasal brushing material during latex challenge tests. Clin Exp Allergy 2000;30(1):110–21.
[36] Sacco O, Tarantino V, Lantero S, Silvestri M, Spallarossa D, Barretta MA, et al. Nasal brushing: a clinically useful procedure in pediatric patients with rhinosinusitis. Int J Pediatr Otorhinolaryngol 1999;50 (1):23–30.
[37] Bridges MA, Walker DC, Harris RA, Wilson BR, Davidson AG. Cultured human nasal epithelial multicellular spheroids: polar cyst-like model tissues. Biochem Cell Biol 1991;69(2–3):102–8.
[38] Bridges MA. Culture of airway epithelial cells collected by a nasal brushing technique. In Vitro Cell Dev Biol Anim 1997;33(2):82–3.
[39] Schmidt MC, Peter H, Lang SR, Ditzinger G, Merkle HP. In vitro cell models to study nasal mucosal permeability and metabolism. Adv Drug Deliv Rev 1998;29(1–2):51–79.
[40] Campbell AM, Chanez P, Vignola AM, Bousquet J, Couret I, Michel FB, et al. Functional characteristics of bronchial epithelium obtained by brushing from asthmatic and normal subjects. Am Rev Respir Dis 1993;147(3):529–34.
[41] Romagnoli M, Vachier I, Vignola AM, Godard P, Bousquet J, Chanez P. Safety and cellular assessment of bronchial brushing in airway diseases. Respir Med 1999;93(7):461–6.
[42] Vignola AM, Campbell AM, Chanez P, Bousquet J, Paul-Lacoste P, Michel FB, et al. HLA-DR and ICAM-1 expression on bronchial epithelial cells in asthma and chronic bronchitis. Am Rev Respir Dis 1993;148(3):689–94.
[43] Adam EC, Schumacher DU, Schumacher U. Cilia from a cystic fibrosis patient react to the ciliotoxic *Pseudomonas aeruginosa* II lectin in a similar manner to normal control cilia—a case report. J Laryngol Otol 1997;111(8):760–2.
[44] Andersson C, Dragomir A, Hjelte L, Roomans GM. Cystic fibrosis transmembrane conductance regulator (CFTR) activity in nasal epithelial cells from cystic fibrosis patients with severe genotypes. Clin Sci (Lond) 2002;103(4):417–24.
[45] Kube D, Sontich U, Fletcher D, Davis PB. Proinflammatory cytokine responses to *P. aeruginosa* infection in human airway epithelial cell lines. Am J Physiol Lung Cell Mol Physiol 2001;280(3):L493–502.
[46] Muhlebach MS, Stewart PW, Leigh MW, Noah TL. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. Am J Respir Crit Care Med 1999;160(1):186–91.
[47] DiMango E, Zar HJ, Bryan R, Prince A. Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. J Clin Invest 1995;96(5):2204–10.