Nasal Epithelial Cells as Surrogates for Bronchial Epithelial Cells in Airway Inflammation Studies

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The nose is an attractive source of airway epithelial cells, particularly in populations in which bronchoscopy may not be possible. However, substituting nasal cells for bronchial epithelial cells in the study of airway inflammation depends upon comparability of responses, and evidence for this is lacking. Our objective was to determine whether nasal epithelial cell inflammatory mediator release and receptor expression reflect those of bronchial epithelial cells. Paired cultures of undifferentiated nasal and bronchial epithelial cells were obtained from brushings from 35 subjects, including 5 children. Cells were subject to morphologic and immunocytochemical assessment. Mediator release from resting and cytokine-stimulated cell monolayers was determined, as was cell surface receptor expression. Nasal and bronchial cells had identical epithelial morphology and uniform expression of cytokeratin 19. There were no differences in constitutive expression of CD44, intercellular adhesion molecule-1, $\alpha\text{v}\beta 3$, and $\alpha\text{v}\beta 5$. Despite significantly higher constitutive release of IL-8, IL-6, RANTES (regulated on activation, normal T cell expressed and secreted), and matrix metalloproteinase (MMP)-9 from nasal compared with bronchial cells, the increments in release of all studied mediators in response to stimulation with IL-1 β and TNF- α were similar, and there were significant positive correlations between nasal and bronchial cell secretion of IL-6, RANTES, vascular endothelial growth factor, monocyte chemoattractant protein-1, MMP-9, and tissue inhibitor of metalloproteinase-1. Despite differences in absolute mediator levels, the responses of nasal and bronchial epithelial cells to cytokine stimulation were similar, expression of relevant surface receptors was comparable, and there were significant correlations between nasal and bronchial cell mediator release. Therefore, nasal epithelial cultures constitute an accessible surrogate for studying lower airway inflammation.

Keywords: airway epithelium; cultured cells; mediator release; adult; child

The airway epithelium plays a pivotal role in controlling many airway functions, including regulation of inflammatory responses in conditions such as asthma (1, 2). Airway epithelial cells (AEC) from the lower airways can be obtained by bronchial brushing or isolated from bronchial biopsies or resected lung tissue (3). Studies using monolayer cultures have shown distinct patterns of cytokine release from AEC from both adults and children with atopic asthma compared with normal control subjects (4–7). However, investigation of AEC function in childhood, in partic-

CLINICAL RELEVANCE

The airway epithelium has many important functions. Study of bronchial epithelial cells is hampered by difficulties obtaining suitable samples. Evidence that inflammatory responses of nasal epithelial cells reflect those of bronchial cells is limited. Nasal epithelial cells can be used as surrogates for lower airway cells, facilitating more detailed study of the epithelium in airway inflammation in hitherto largely inaccessible populations, including children.

ular, is hampered by ethical and practical difficulties associated with obtaining suitable samples. Although primary cultures of AEC can be established from nonbronchoscopic bronchial brushings via endotracheal tube from children undergoing elective general anesthesia (8, 9), this technique has become less applicable due to increasing use of the laryngeal mask. The nose is a particularly attractive source of AEC because of the greater ease of access compared with the tracheobronchial epithelium and the potential for repeated isolation from the same individual. Primary cultures of nasal AEC have been established from nasal biopsies (10) and brushings (11).

However, the use of nasal AEC to study airway inflammation depends upon the inflammatory responses of nasal AEC correlating with those of bronchial AEC. Although the upper and lower airways are united by many factors, including epidemiology, anatomy, physiology, immunopathology, and pharmacology (12), and the concept of "united airways disease" has been proposed in relation to allergic rhinitis and asthma (13), the assumption that nasal AEC can be used as a surrogate for bronchial AEC is, as yet, unproven. Devalia and coworkers (10) reported that cultured human nasal and bronchial AEC were similar in size, shape, growth characteristics, and ciliary activity. However, the samples were not paired and no functional studies of cytokine release were performed. In exposure studies with ozone (14, 15), swine dust (16), and experimental rhinovirus infection (17), changes in nasal lavage fluid reflected, to some extent, inflammation in the lower airway, but no correlations between upper and lower airway samples were reported. Two pediatric studies demonstrated significant correlations between nasal lavage and bronchoalveolar lavage cytokine levels in disease states: IL-2 in infants ventilated with respiratory syncytial virus-positive bronchiolitis (18), and IL-8 in children with cystic fibrosis (19). However, lavage fluid findings do not necessarily reflect AEC function.

We therefore sought to test the hypothesis that the inflammatory responses of nasal AEC reflect those of bronchial AEC, using paired cultures of AEC obtained by brushing from both adults and children. This study represents the first detailed comparison of paired nasal and bronchial AEC, sampled on the same occasion

(Received in original form September 5, 2007 and in final form May 14, 2008)

This study was supported by a Medical Research Council (UK) Clinical Research Training Fellowship (to C.M.McD.) and a grant from NHS Grampian.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 39, pp 560–568, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0325OC on May 15, 2008

Internet address: www.atsjournals.org

from the same subjects, with respect to inflammatory mediator release and receptor expression. Our findings provide evidence that nasal AEC can be used as surrogates for lower airway AEC. Some of the results have previously been published in the form of an abstract (20, 21).

MATERIALS AND METHODS

Please refer to the online supplement for full details of methods.

Subjects and Establishment of AEC Cultures

Patients attending for elective bronchoscopy under general anesthetic were recruited, with written informed consent and the approval of the Grampian Local Research Ethics Committee. A detailed respiratory questionnaire was administered by a physician.

After induction of anesthesia, bilateral nasal brushing was performed to obtain cells from the medial aspect of the inferior turbinate and, during bronchoscopy, a single bronchial brushing was performed to obtain cells from a second- or third-generation bronchus. Undifferentiated cultures of nasal and bronchial AEC were established in an identical fashion and cells were subcultured by trypsinization at 70 to 90% confluence. All experiments were performed on cells at second passage.

Immunocytochemistry

Cells were grown to confluence on chamber slides, and immunocytochemistry was performed using the Vectastain Universal Elite ABC kit (Vector Laboratories, Peterborough, UK), following the manufacturer's instructions. Primary antibodies included cytokeratin 19, cytokeratin 13, CD45, fibroblast-specific and von Willebrand factor.

Electron Microscopy

For transmission electron microscopy, cultured cells were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide then 1% uranyl acetate, dehydrated through graded ethanol, and embedded in TAAB 812 epoxy resin. Sections (70 nm) were stained with uranyl acetate and lead citrate for examination at 80 kV using a Philips CM10 transmission electron microscope (FEI Company, Eindhoven, The Netherlands).

Cell Phenotyping for Surface Receptors

AEC expression of the surface receptors CD44, intercellular adhesion molecule-1 (ICAM-1), $\alpha v\beta 3$, and $\alpha v\beta 5$ was analyzed by flow cytometry. Cells were nonenzymatically removed from culture monolayers and immunostained with saturating concentrations of specific primary antibody or isotype-matched control for 40 minutes at 4°C. After washing and staining with an allophycocyanin-conjugated secondary antibody, the cells were analyzed on a BD LSR flow cytometer.

Analysis of Inflammatory Mediator Release

Eighty percent confluent AEC monolayers were stimulated with IL-1 β and TNF- α (both at 10 ng/ml) for 24 hours or left unstimulated. Concentrations of IL-8, IL-6, granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), RANTES (regulated upon activation, normal T cell expressed and secreted), and monocyte chemoattractant protein-1 (MCP-1) were measured in the culture supernatants by cytometric bead array (CBA) assay (BD Biosciences, Oxford, UK), as per the manufacturer's instructions. Measurements of total matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were performed using commercially available ELISA kits (DuoSet; R&D, Abingdon, UK). Results were normalized to cellular protein content.

Statistical Analysis

All data were tested for skewness and then analyzed further using nonparametric statistical tests. The Wilcoxon matched pairs test was used to compare results for paired nasal and bronchial AEC cultures and for resting and stimulated cultures, and correlations were examined using Spearman's rank correlation (ρ). *P* values of less than 0.05 were considered to be significant. Tests were performed using SPSS v14.0 for Windows (Chicago, IL).

RESULTS

Characteristics of Study Subjects

Forty-two adults (33–80 yr, 23 male) and 10 children (0.7–6.8 yr, 6 male), undergoing bronchoscopy for a variety of indications, were recruited (Table 1). Twenty-five (48%) subjects had a history of wheeze, six of whom had a diagnosis of atopic asthma and seven of whom had physician-diagnosed chronic obstructive pulmonary disease. Nine (17%) subjects had a history of allergic rhinitis. Nineteen (27%) subjects were taking inhaled corticosteroids. None of the participants was taking intranasal corticosteroids.

Culture Establishment and AEC Morphology

Microscopy of nasal and bronchial AEC retrieved by brushing, before culturing, demonstrated both ciliated epithelial cells and a subpopulation of nonciliated cuboidal cells consistent with basal epithelial cells. Immunostaining of cytospin preparations revealed that all cells were positive for the epithelium-specific protein cytokeratin 19. The characteristics of the nasal and bronchial AEC cultures are compared in Table 2. Successful cultures, with the culture reaching 70 to 90% confluence and being successfully subcultured, were established from 82% of nasal brushings and 90% of bronchial brushings. Thirteen of the unsuccessful nasal cultures and one of the bronchial cultures succumbed to infection. In the remaining cases, no obvious reason for the failure could be identified. In all successful cultures, there was evidence of cell attachment within 24 hours of seeding. The median time to first passage was 7 days for nasal cultures and 8 days for bronchial cultures. Bronchial cultures took significantly longer to reach 70 to 90% confluence over 8.8 cm² than the corresponding nasal cultures. However, the calculated generation time for nasal and bronchial AEC was similar. The mean cell number recovered at first passage was significantly higher from nasal cultures than bronchial cultures but, in all cases, was sufficient for functional studies to be performed. The viability of cells at passage one, assessed by trypan blue exclusion, was consistently high in both nasal and bronchial cultures. Subcultured cells from the two nasal AEC cultures from each subject were generally pooled after first passage for subsequent comparison with bronchial AEC. Paired nasal and bronchial AEC cultures reaching passage 2 were established from 30 adults (71%) and 5 children (50%).

Cultured nasal and bronchial AEC were morphologically indistinguishable by light and electron microscopy (Figure 1). Both had a flattened appearance with clearly identified nuclei and mitochondria, endoplasmic reticulum, and electron-dense granules within the cytoplasm. Regular microvilli were present on the apical border of the cells. Both nasal and bronchial AEC showed uniform positive immunostaining for cytokeratin 19, and the proportion of basal epithelial cells (cytokeratin 13 positive) was similar (32.8% of nasal AEC, 32.0% of bronchial AEC). Immunostaining with specific antibodies against other cell types did not show any indication of contamination of cultures by fibroblasts, endothelial cells, or white blood cells.

Receptor Expression on Nasal and Bronchial AEC

The expression of the adhesion molecules CD44 and ICAM-1 and the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ on paired nasal and bronchial AEC was compared in eight subjects. There was significant constitutive expression (i.e., statistical differences from control mean fluorescence intensity) of CD44, ICAM-1, and $\alpha v\beta 5$ on both nasal and bronchial AEC, with negligible expression of $\alpha v\beta 3$ (Figure 2). There were no differences in expression of

TABLE 1. CLINICAL CHARACTERISTICS OF STUDY SUBJECTS

Subject	Age (yr)	Sex	Indication for Bronchoscopy	Wheeze	Current Smoker*	Pack-Years	ICS	Both Nasal and Bronchial AEC Cultures Successful
Adults (n = 42)								
A1	77	F	Tumor	N	Y	60	N	Y
A2	48	F	Hemoptysis	Y	Y	12	N	Y
A3	63	M	Tumor	N	Y	50	N	Y
A4	66	M	Tumor	N	N	36	N	Y
A5	74	F	Tumor	N	N	24	N	Y
A6	51	M	Tumor	Y	Y	34	N	Y
A7	78	M	Tumor	N	N	60	N	Y
A8	53	F	Tumor	Y	Y	48	N	Y
A9	58	M	Tumor	Y	Y	66	N	Y
A10	57	F	?Sarcoidosis	Y	N	0	Y	Y
A11	63	M	?Interstitial lung disease	N	N	0	N	Y
A12	57	M	?Sarcoidosis	Y	N	0	N	Y
A13	61	M	Hemoptysis	Y	N	50	Y	Y
A14	78	F	Tumor	N	Y	62	Y	N
A15	46	F	Chronic cough	Y	N	0	Y	Y
A16	45	M	?Sarcoidosis	N	N	0	N	Y
A17	50	F	Persistent CXR changes	N	N	0	N	N
A18	61	F	Hemoptysis	N	Y	30	N	Y
A19	33	M	?Sarcoidosis	Y	N	12	Y	Y
A20	71	F	Tumor	N	N	16	N	Y
A21	66	M	Persistent CXR changes	Y	N	75	Y	N
A22	61	F	Hemoptysis	N	N	21	N	Y
A23	62	M	?Interstitial lung disease	N	N	30	N	Y
A24	68	M	Tumor	N	N	34	N	N
A25	73	M	Tumor	Y	N	42	Y	Y
A26	60	M	Tumor	Y	N	60	N	N
A27	68	F	Persistent CXR changes	Y	N	1	Y	N
A28	73	F	Tumor	Y	N	0	Y	Y
A29	66	M	Tumor	N	N	21	Y	N
A30	68	M	Hemoptysis†	Y	Y	55	Y	N
A31	75	F	Tumor	N	N	0	N	N
A32	75	F	Tumor	N	N	15	N	Y
A33	60	F	Chronic cough	N	N	14	Y	Y
A34	64	F	Dyspnea, chest pain	N	N	21	N	N
A35	57	F	?Sarcoidosis	N	N	0	N	N
A36	70	M	Tumor	N	Y	52	N	Y
A37	69	M	Tumor	N	Y	25	N	Y
A38	57	M	Persistent CXR changes	Y	Y	132	Y	Y
A39	46	M	Persistent CXR changes	Y	Y	85	Y	Y
A40	72	M	Tumor	N	N	0	N	N
A41	76	M	Tumor	N	Y	64	N	Y
A42	80	F	Tumor	Y	Y	64	N	Y
Children (n = 10)								
C1	3.8	F	Recurrent stridor‡	N	Y	—	N	N
C2	3.4	M	Severe asthma, persistent CXR changes	Y	N	—	Y	N
C3	2.4	M	Recurrent cough/wheeze	Y	Y	—	Y	Y
C4	0.7	M	Recurrent cough/wheeze	Y	N	—	N	N
C5	2.1	M	BPD, oxygen dependent	Y	Y	—	N	N
C6	3.1	M	Persistent CXR changes	Y	N	—	Y	Y
C7	6.1	F	Chronic cough	N	Y	—	N	N
C8	6.8	F	Chronic cough	Y	Y	—	N	Y
C9	6.0	F	Chronic cough	N	N	—	Y	Y
C10	4.5	M	Persistent CXR changes	Y	N	—	Y	Y

Definition of abbreviations: AEC, airway epithelial cell; BPD, bronchopulmonary dysplasia; CXR, chest x-ray; ICS, inhaled corticosteroids.

* Y indicates current smoker for adult subjects and parental smoking for children.

† Right nasal brushing inadvertently discarded.

‡ No bronchial brushing performed due to finding of tight laryngeal stenosis at bronchoscopy.

these receptors between nasal and bronchial AEC (Figures 2 and 3).

Comparison of Nasal and Bronchial AEC Mediator Production

Paired data on nasal and bronchial AEC mediator release were obtained for 30 adults and 5 children. Data are presented for all 35 subjects. However, similar results were obtained when the data for children and adult subjects were analyzed separately. There were no associations between mediator release from

either nasal or bronchial AEC and sex, smoking status, lung cancer, medication use, or underlying lung disease (data not shown).

Differential Constitutive Release of Mediators from Nasal and Bronchial AEC

The constitutive production of the studied mediators from unstimulated cells is shown in Figure 4. Constitutive release of IL-8, IL-6, RANTES, and MMP-9 was significantly higher from

TABLE 2. COMPARISON OF CHARACTERISTICS OF PAIRED NASAL AND BRONCHIAL AIRWAY EPITHELIAL CELL CULTURES

	Nasal Cultures	Bronchial Cultures
Success rate	84/103 (81.6%)	46/51 (90.2%)
Median time (range) to P1, d	7 (5–12)	8 (6–21)*
Mean (SD) cell no. at P1	4.3 (1.7) × 10 ⁵	2.8 (1.7) × 10 ^{5†}
Mean (SD) viability at P1	96.2% (1.3%)	95.4% (2.3%)
Mean (SD) generation time, h‡	26.8 (4.2)	26.6 (4.6)

Paired cultures of nasal and bronchial airway epithelial cells were established in an identical fashion.

* $P < 0.001$ (Wilcoxon matched pairs test), bronchial versus nasal cultures.

† $P < 0.001$ (Student's paired t test) bronchial versus nasal cultures.

‡ Generation time calculated for four pairs of cultures (P1 = first passage).

nasal than bronchial AEC, and constitutive release of TIMP-1 was significantly lower from nasal than bronchial AEC. No significant differences were observed in the amount of G-CSF, VEGF, and MCP-1 produced by nasal or bronchial AEC.

Differential Release of Mediators from Nasal and Bronchial AEC Stimulated with Proinflammatory Cytokines

Paired cultures of nasal and bronchial AEC were stimulated with the proinflammatory cytokines IL-1 β and TNF- α (both at 10 ng/ml) for 24 hours. The release of mediators from these stimulated cells is shown in Figure 5. Again, release of IL-8, IL-6, RANTES, and MMP-9 was significantly higher from nasal than bronchial AEC, and release of TIMP-1 was significantly lower from nasal than bronchial AEC. Stimulated production of

G-CSF, VEGF, and MCP-1 was not significantly different between nasal and bronchial AEC.

Responses of Nasal and Bronchial AEC to Stimulation with Proinflammatory Cytokines

Compared with constitutive mediator production, stimulation with IL-1 β and TNF- α for 24 hours produced significant increases in release of IL-8, IL-6, G-CSF, MMP-9, RANTES, and MCP-1 from both nasal AEC and bronchial AEC. The release of VEGF and TIMP-1 was not significantly enhanced by proinflammatory cytokine stimulation in either cell type.

Although the absolute levels of constitutive and stimulated mediator production differed between nasal and bronchial AEC, the magnitudes of the responses of nasal and bronchial AEC to cytokine stimulation were similar. There were no significant differences between nasal and bronchial AEC in the increments in release of each mediator with stimulation, expressed as a percentage of the constitutive level (Table 3).

Correlations between Nasal and Bronchial AEC Mediator Release

There were significant positive correlations between nasal and bronchial AEC secretion of IL-6, RANTES, VEGF, MCP-1, MMP-9, and TIMP-1, from both resting and cytokine-stimulated cultures (Table 4 and Figure 6).

Exclusion of Current Viral Infection of Nasal and Bronchial AEC

It is well established that infection of AEC with viruses alters their cytokine production (22–25). To determine whether this

Figure 1. Appearance of cultured nasal and bronchial epithelial cells. (A) Phase contrast light micrographs showing typical "cobblestone" morphology of epithelial cells (*original magnification: ×200*). (B) Uniform positive immunostaining for cytokeratin 19 (Vectastain Universal Elite ABC Kit; Vector Laboratories) (*original magnification: ×200*). (C) Transmission electron micrographs showing cell ultrastructure, including mitochondria (M), endoplasmic reticulum (ER), and microvilli (MV) (size marker = 0.5 μ m).

Figure 2. Receptor expression on nasal and bronchial airway epithelial cells (AEC). Nasal and bronchial AEC were seeded at 3,500 cells/cm² in 75 cm² tissue culture flasks and grown to confluence. Cells were detached using nonenzymatic cell dissociation solution and labeled with specific monoclonal antibodies. Receptor expression was quantified using flow cytometry and expressed as mean fluorescence intensity (MFI). The box plots show the median and interquartile range and the bars show the range ($n = 8$). * $P < 0.05$ compared with control (Mann Whitney test). There were no significant differences between nasal and bronchial AEC by Wilcoxon matched pairs test.

was a potential explanation for differences in mediator release from nasal and bronchial AEC, paired cultures of nasal and bronchial AEC from three subjects were screened by PCR for 11 respiratory viruses. All samples were negative.

DISCUSSION

This study addressed the hypothesis that the inflammatory responses of nasal AEC reflect those of bronchial AEC. To our knowledge, this is the first report comparing inflammatory mediator release, under resting and cytokine-stimulated conditions, and receptor expression in paired cultures of human nasal and bronchial AEC.

AEC are clearly a potent source of inflammatory mediators, and we have shown that a broad range of mediators, including the CXC chemokine IL-8, the CC chemokines RANTES and MCP-1, the pleiotropic cytokine IL-6, the colony-stimulating factor G-CSF, the growth factor VEGF, and the extracellular matrix proteins MMP-9 and TIMP-1, are all constitutively released by both nasal and bronchial AEC. IL-1 β and TNF- α are proinflammatory cytokines that activate the same set of transcription factors, particularly nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) (26), and are both implicated in the pathogenesis of many acute and chronic infectious and noninfectious inflammatory diseases of the lung, often acting synergistically (27). Both nasal and bronchial AEC responded to stimulation with a combination of IL-1 β and TNF- α with significant increases in release of IL-8, IL-6, G-CSF, RANTES, MCP-1, and MMP-9 compared with baseline.

Both constitutive and cytokine-stimulated release of IL-8, IL-6, RANTES, and MMP-9 were significantly higher from nasal AEC than bronchial AEC. This may reflect greater exposure of nasal AEC to "inflammatory stress." Respirable particles generally enter the airways via the nasal passages, even though some

people are mouth breathers. The nose plays a pivotal role in the defense mechanisms of the respiratory apparatus, protecting the more sensitive lower airways. The nasal epithelium is thus exposed first and to a greater extent than the bronchial epithelium to all environmental agents, including infectious agents, allergens, and air pollutants. Many environmental agents are known to provoke airway inflammation *in vivo* and to induce cytokine release from AEC *in vitro*. Viruses, including rhinoviruses (22), influenza viruses (23), parainfluenza viruses (24) and respiratory syncytial virus (25); dust mite proteolytic allergens (4, 28); cigarette smoke (29); and air pollutants, including ozone (30), nitrogen dioxide (30), diesel exhaust particles (5), and residual oil fly ash (31), have been demonstrated to stimulate AEC release of cytokines, notably IL-8, IL-6, RANTES, and GM-CSF. In addition, these insults can have co-operative effects, exaggerating the inflammatory responses. For example, parainfluenza virus type 4 enhances AEC IL-8 and IL-6 production by both transcriptional and post-transcriptional mechanisms and, importantly, during the phase of reduced mRNA degradation, heightens the cells' responsiveness to a secondary stimulus (24). In view of the documented effects of viruses on AEC responses, we excluded current viral infection of our AEC cultures as an explanation for the differential mediator release between nasal and bronchial AEC, but this does not address previous exposure of the epithelium to viruses. It is, therefore, tempting to speculate that constant exposure to such environmental stimuli leads to up-regulation of cytokine production in nasal AEC relative to bronchial AEC. Whether this up-regulation is due to increased gene transcription, decreased mRNA degradation, altered post-translational processing, increased release of preformed mediators, or a combination of these factors is unknown. Studies of specific mRNA and intracellular cytokine levels in paired nasal and bronchial AEC cultures would be illuminating.

It is important to note that our reported findings are from experiments performed on cells at passage 2. Although it is possible that, despite the length of time in culture, nasal AEC mediator release was still influenced by the milieu of the nose at the time of brushing, it seems more likely that the maintenance of phenotypic differences through serial passages reflects an intrinsic difference between nasal and bronchial AEC. Whether this difference is present at birth or arises postnatally is uncertain. The differential mediator release was evident in our youngest subjects (aged 2–6 yr), suggesting that, if the AEC differences arise postnatally, they do so early in life. A study of paired nasal and bronchial AEC from newborn infants would be required to address this question fully. The mechanisms by which intrinsic differences between nasal and bronchial AEC arise are also unknown. It is possible that *in vivo* exposure of nasal AEC to proinflammatory stimuli results in greater survival, and thus selection, of those cells most able to mount an inflammatory response, leading to the nasal epithelium containing a population of cells able to release higher levels of cytokines than the bronchial epithelium. Another possibility is that differential gene expression between nasal and bronchial AEC arises through epigenetic mechanisms, which remodel chromatin and are passed down with each subsequent cell division (32). As well as allowing phenotypic plasticity during development, the "epigenome" is an important target of environmental modification (33). For example, toxins such as heavy metals disrupt DNA methylation and chromatin and diet has been shown to influence DNA methylation. Although such processes have not been explored in respiratory epithelium, it is conceivable that environmental exposures early in life could alter the expression of genes involved in inflammatory mediator production in nasal AEC.

It is important to be aware of absolute differences in mediator release between nasal and bronchial AEC. However, in terms of

Figure 3. Representative expression data from flow cytometric analysis of AEC receptor expression. Confluent AEC were nonenzymatically removed from culture monolayers and stained with the indicated primary antibody. Nasal AEC expression is shown by the *dashed line* and bronchial AEC expression by the *solid line*.

using nasal AEC as a surrogate for lower AEC, it is more important that differences in bronchial AEC mediator secretion between individuals are reflected by differences in nasal AEC secretion and that the behavior of the cells in response to exogenous stimuli is similar. We have demonstrated significant

correlations between nasal and bronchial AEC secretion of IL-6, RANTES, VEGF, MCP-1, MMP-9, and TIMP-1. Furthermore, the incremental responses of paired nasal and bronchial AEC to IL-1β/TNF-α stimulation were similar for all of the studied mediators. Although our findings cannot necessarily be extrapolate

Figure 4. Constitutive mediator release from nasal and bronchial AEC. Nasal and bronchial AEC were seeded at 3,500 cells/cm² in 4-cm tissue culture plates and grown to 80% confluence. Cells were then exposed to fresh bronchial epithelial growth medium for 24 hours. Mediator concentrations in culture supernatants were determined by CBA assay or ELISA and protein content of cell monolayers by Bradford assay. The *box plots* show the median and interquartile range and the *bars* show the range (*n* = 35, with 2 replicates). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 bronchial versus nasal cells (Wilcoxon matched pairs test).

Figure 5. Mediator release from nasal and bronchial AEC stimulated with proinflammatory cytokines. Nasal and bronchial AEC were seeded at 3,500 cells/cm² in 4-cm tissue culture plates and grown to 80% confluence. Cells were then exposed to fresh bronchial epithelial growth medium containing IL-1 β and TNF- α (both at 10 ng/ml) for 24 hours. Mediator concentrations in culture supernatants were determined by CBA assay or ELISA and protein content of cell monolayers by Bradford assay. The box plots show the median and interquartile range and the bars show the range ($n = 35$, with two replicates). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ bronchial versus nasal cells (Wilcoxon matched pairs test).

lated to other inflammatory markers, we have shown that, across a range of different mediators, nasal AEC reflected bronchial AEC.

A study of this nature is, by necessity, opportunistic, particularly in children, in whom sampling is only ethically acceptable in those being investigated for clinical reasons. We found no association between AEC mediator release and underlying disease state, although the study was not designed to investigate this and the numbers of individuals with any one condition were very small. Furthermore, the pattern of results from nasal and bronchial AEC did not vary between children and adults or between individuals with different respiratory pathologies, emphasizing the utility of nasal AEC as surrogates for bronchial AEC in a wide range of clinical situations.

In addition to inflammatory mediator release, we report significant constitutive expression of CD44, ICAM-1, and $\alpha\text{v}\beta 5$ on AEC, with no differences observed in expression levels of these relevant receptors between nasal and bronchial AEC. CD44 is a transmembrane adhesion receptor with a diverse role in many cellular functions (34) that has been shown to be up-regulated when the airway epithelial surface is damaged in subjects with asthma (35). ICAM-1, an adhesion molecules of the immunoglobulin supergene family, is involved in antigen presentation and in the mobilization of leukocytes to inflammatory sites (36). Of note, it is subverted as a receptor by human pathogens, notably rhinoviruses, of relevance to the study of airway diseases (37). ICAM-1 expression is increased on the surface of bronchial AEC recovered from subjects with asthma, and this increase is correlated with the clinical severity of asthma (38). Integrins were initially identified as receptors for components of the extracellular matrix, but are now known to also function as signaling receptors (39). Previous work in our laboratory has established the importance of $\alpha\text{v}\beta 5$ and $\alpha\text{v}\beta 3$ as recognition receptors in the phagocytosis of apoptotic eosinophils by bronchial AEC (40, 41).

Although we have demonstrated similarities between cultured nasal and bronchial AEC, it could be argued that they are a consequence of the behavior of cultured epithelial cells in general. To address this issue, we also studied other epithelial cell types. There were significant differences in cytokine responses and levels of integrin expression between AEC and both non-respiratory primary epithelial cells (retinal pigmented epithelium) and cell lines (A549 alveolar adenocarcinoma and WiDr colorectal adenocarcinoma) (data not shown), indicating that not all epithelial cells cultured as submerged monolayers behave in the same manner and that our findings were specific to primary respiratory epithelial cultures. Due to difficulties in obtaining relevant target organ tissue, most airway epithelial research has relied heavily on commercially produced cells or transformed cell lines, including A549. Our findings emphasize the need for caution in interpreting the results of studies using cell lines. Primary cultured cells should be used for *in vitro* analyses because they are most similar to cells *in vivo* and it is possible to obtain detailed clinical information about the cell donors. Our method for culturing AEC obtained by minimally invasive nasal brushing should facilitate this and should overcome the ethical and practical difficulties associated with obtaining suitable samples, particularly in children. Each nasal brushing takes only a few seconds to perform and in our experience the procedure has not been associated with any adverse effects. The nasal brushings

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TABLE 3. INCREMENTS IN MEDIATOR RELEASE FROM NASAL AND BRONCHIAL AIRWAY EPITHELIAL CELLS STIMULATED WITH IL-1 β AND TNF- α

Mediator	Median (IQR) % Increase in Mediator Release in Response to Cytokine Stimulation		P Value*
	Nasal AEC	Bronchial AEC	
IL-8	245.9 (335.0)	195.5 (246.4)	0.397
IL-6	178.8 (215.6)	162.7 (192.0)	0.765
G-CSF	397.3 (1,086.5)	656.4 (905.5)	0.695
MCP-1	392.2 (697.0)	395.2 (509.6)	0.694
RANTES	392.9 (991.4)	438.1 (869.9)	0.837
VEGF	12.0 (44.5)	0.0 (32.9)	0.337
MMP-9	37.9 (59.5)	32.0 (53.8)	0.961
TIMP-1	2.6 (26.8)	-9.8 (28.2)	0.128

Definition of abbreviations: AEC, airway epithelial cells; G-CSF, granulocyte colony-stimulating factor; IQR, interquartile range; MCP-1, monocyte chemoattractant protein-1; MMP-9, matrix metalloproteinase-9; RANTES, regulated on activation, normal T cells expressed and secreted; TIMP-1, tissue inhibitor of metalloproteinase-1; VEGF, vascular endothelial growth factor.

* Percentage changes from paired nasal and bronchial AEC cultures were compared using Wilcoxon matched pairs test ($n = 35$, with two replicates).

TABLE 4. SPEARMAN RANK CORRELATIONS BETWEEN NASAL AND BRONCHIAL AIRWAY EPITHELIAL CELL MEDIATOR RELEASE

Mediator	Constitutive		Stimulated	
	ρ	P Value	ρ	P Value
IL-8	0.161	0.362	0.288	0.099
IL-6	0.347	0.045	0.389	0.023
RANTES	0.393	0.022	0.477	0.005
MCP-1	0.384	0.025	0.486	0.004
G-CSF	0.162	0.376	0.079	0.658
VEGF	0.662	<0.001	0.728	<0.001
MMP-9	0.394	0.019	0.318	0.062
TIMP-1	0.678	<0.001	0.490	0.003

Definition of abbreviations: G-CSF, granulocyte colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; MMP-9, matrix metalloproteinase-9; RANTES, regulated on activation, normal T cells expressed and secreted; TIMP-1, tissue inhibitor of metalloproteinase-1; VEGF, vascular endothelial growth factor.

consistently yielded successful cultures. Our success rate of 82% compares favorably with other studies in which primary AEC cultures have been established from bronchial or nasal tissue and, in all successful cultures, the number of cells obtained was sufficient for functional studies to be performed. Using our method, nasal AEC can easily be obtained from anesthetized children and adults and also from nonanesthetized co-operative subjects, thus creating the potential for repeated isolation from the same individual over time.

In this study, AEC were collected during general anesthetic using sevoflurane or intravenous propofol for induction and sevoflurane for maintenance. The effects of these agents on

AEC are unknown. However, there was no difference in the rate of establishing successful nasal AEC cultures in the present study compared with an earlier pilot study in nonanesthetized adult volunteers, and no significant difference in IL-8 production from cultured cells from the two studies (our unpublished observations).

It must be highlighted that our results were obtained using submerged monolayer cultures and, therefore, poorly differentiated cells. Most studies published to date of cultured AEC in airway diseases in both adults and children have used submerged monolayer cultures. Therefore, data generated from this study are comparable with the existing literature. However, the characteristics and responses of these cells grown in air-liquid interface cultures could be qualitatively different. This would need to be investigated in a future study. Well-differentiated AEC are almost certainly more representative of the *in vivo* situation than submerged monolayers, although they are not a perfect model, as other resident and infiltrating cell types and matrix structures are absent.

In conclusion, we have demonstrated that an *in vitro* model of airway epithelium, suitable for functional studies, such as determination of mediator release and receptor expression, can be consistently established from minimally invasive nasal brushings. Nasal and bronchial AEC have identical morphology. Despite differences in absolute mediator levels, the responses of nasal and bronchial AEC to cytokine stimulation were similar, and there were significant correlations between nasal and bronchial AEC mediator release. The expression of relevant receptors on nasal and bronchial AEC was similar. Therefore, we conclude that nasal AEC cultures constitute an accessible surrogate for studying lower airway inflammation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Dr. Peter Mackie, Department of Virology, Aberdeen Royal Infirmary, for performing the PCR for respiratory viruses; all the study participants; and the bronchoscopists, nurses, and anesthetists who facilitated subject recruitment and sample collection.

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Figure 6. Scatterplots of constitutive nasal and bronchial AEC mediator release. Nasal and bronchial AEC were seeded at 3,500 cells/cm² in 4-cm tissue culture plates and grown to 80% confluence. Cells were then stimulated with IL-1 β and TNF- α (both at 10 ng/ml) or left unstimulated for 24 hours. Mediator concentrations in culture supernatants were determined by CBA assay or ELISA and protein content of cell monolayers by Bradford assay. There were significant ($P < 0.05$) positive correlations between nasal and bronchial AEC mediator release from resting cells by Spearman rank correlation ($n = 35$, with two replicates) (see Table 3).

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