

Culture and comparison of human bronchial and nasal epithelial cells *in vitro*

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Human nasal and bronchial epithelial cells were cultured *in vitro* and compared morphologically and functionally. Morphologic assessment by both light and electron microscope and indirect immunoperoxidase staining techniques confirmed the identity of the two cell types as being epithelial. Light microscopy of confluent cultures revealed tightly packed cell monolayers, whilst electron microscopy showed that cells were linked by tight junctions. Estimation of cell size by planimetry found these cells to have a mean width of $10.6 \pm 1.1 \mu\text{m}$ for nasal cells and a mean width of $10.2 \pm 1.0 \mu\text{m}$ for bronchial cells. A high proportion of both the nasal and the bronchial cells exhibited features of the mature ciliated cell types, and constituted between 50 and 76% of the total cells at the earlier stages of culture although this decreased to between 16 and 23% of the total by 4 weeks in culture. The ciliary beat frequencies of the nasal and bronchial cells were found to be similar at $10.8 \pm 0.7 \text{ Hz}$ and $11.8 \pm 2.3 \text{ Hz}$, respectively. The ciliary beat on adjacent cells was synchronous, suggesting the presence of inter-cellular communication between the neighbouring cells. These studies demonstrated that there was little difference between the cultured nasal and bronchial epithelial cells with respect to either their morphology or ciliary activity.

Introduction

The airway epithelium is thought to play an important physicochemical role in the aetiology of airways disease. Pathological examination of airway tissue of chronic asthmatic patients (1) and studies of respiratory viral and bacterial infections (2–4) have suggested that the frequently observed increase in airway hyperresponsiveness in these conditions may be associated with epithelial damage. Similarly, examination of asthmatic tissue, both post-mortem and in specimens obtained at fibre optic bronchoscopy, has demonstrated that there may be loss of epithelial cells and infiltration of the epithelium by increased numbers of eosinophils and neutrophils (5–7). Recently, studies by Hunter and colleagues (8) and more recently Churchill and colleagues (9) have demonstrated that cultured human airway epithelial cells are capable of synthesizing a variety of inflammatory mediators, which could presumably be released following damage and possibly contribute to airway hyperresponsiveness, *in vivo*. Inspite of increasing evidence for an important physicochemical role of the airway epithelium, it has not, however, proved easy to

assign a specific pathogenic role to the airway epithelium *in vivo*, due to the presence of other cell types and the underlying tissues.

Human airway epithelial cell cultures offer an ideal *in vitro* model system for the study of the epithelium in the aetiology of airway diseases and the underlying mechanism/s of inflammation and hyperresponsiveness. Several authors have demonstrated that nasal epithelial cells can be cultured *in vitro* from surgical specimens of both turbinate (10–12) and polyp tissue (12,13). Similarly, Stoner and colleagues (14) and Lechner and colleagues (15) have demonstrated that epithelial cells can also be cultured, from both surgical or 'immediate autopsy' specimens of the bronchus. Although, using elaborate study protocols and specific culture conditions, it has been possible to culture both nasal and bronchial epithelial cells *in vitro*, it has often proved difficult to consistently grow both confluent primary cultures and fully differentiated ciliated cells in these cultures. Furthermore, to date there are no reports of any form of a comparison between the two cell types, *in vitro*.

In view of this, the purpose of this study was to develop a technique which was suitable for the culture of both ciliated bronchial and nasal epithelial cells to confluence under similar culture conditions, and then to compare the two cell types both morphologically and functionally.

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Materials and Methods

All chemicals and reagents were of tissue culture grade and unless otherwise stated were obtained from Sigma Chemical Co (Dorset, U.K.). Antibody preparations 5B5, M616, M722, M724, M732 and P260 were obtained from Dako Ltd, High Wycombe, U.K. Preparations MCA480 and MCA483 were obtained from Serotec, Oxford, U.K. and CAM 5.2 was obtained from Becton Dickinson Ltd, Oxford, U.K. PR1A3 was prepared as described by Richman and Bodmer (16).

(I) NASAL AND BRONCHIAL TISSUE

Nasal and bronchial tissue was obtained from patients presenting for turbinectomy and lung surgery, respectively. Following resection, only bronchial tissue which was free of tumour and which appeared to be 'normal', as assessed by the surgeon carrying out the resection, was used. The tissues were placed in ice-cold Medium 199 (Northumbria Biologicals Ltd, Northumberland, U.K.) and returned to the laboratory within 30 min to 1 h of resection.

(II) ISOLATION AND TISSUE CULTURE OF HUMAN NASAL AND BRONCHIAL EPITHELIAL CELLS

Nasal and bronchial epithelial cells were cultured by a modification of the explant cell culture technique described by Steele and Arnold (11), for nasal epithelial cells. The epithelium was carefully dissected away from the underlying tissue and further dissected into smaller sections approximately 1–2 mm³ in size. All the sections were 'sterilized' by gently washing three-times, in prewarmed and pregassed Medium 199, containing 1% (v/v) antibiotics/antimycotic solution (Sigma, U.K.) and then 3–4 sections transferred to untreated 6 cm diameter Falcon 'Primaria' culture dishes (Becton Dickinson Ltd, Oxford, U.K.). The explants were incubated, at 37°C in a 5% CO₂ in air atmosphere, in 2.5 ml of a complete culture medium sterilized by passing through 0.2 mm FlowPore D syringe filters (Flow Laboratories, U.K.). Prior to use, the medium was prepared freshly in 100 ml quantities and contained 2.5 ml Nu-serum IV (Flow Laboratories, U.K.), 250 µg bovine pancreatic insulin, 250 µg of human transferrin, 36 µg of hydrocortisone, 3 mg L-glutamine and 1 ml of the 1% (v/v) antibiotics/antimycotic solution, in Medium 199. All explants were observed for cell outgrowth after 2–3 days and the medium was changed 48 hourly until the cells had grown to confluence, normally by 3–4 weeks.

(III) CONFIRMATION OF EPITHELIAL CELL IDENTITY

The identity of both nasal and bronchial epithelial cells was confirmed by several techniques, including (i)

light microscopy, (ii) electron microscopy and (iii) indirect immunoperoxidase staining techniques using monoclonal antibodies with specificity towards cytokeratin and specific ciliated epithelial cell antigens. Cells were initially observed by phase contrast light microscopy using an Olympus IMT-2 inverted microscope (Olympus Optical Co., U.K.), which was modified at a latter date to incorporate the Hoffman Modulation Contrast optical system (Modulation Optics Inc., Greenvale, NY, U.S.A.). This allowed highly detailed three-dimensional visualization of the topographic features of the cells.

Cells to be studied by electron microscopy and indirect immunoperoxidase staining, were cultured on both plastic culture dishes and on 0.5 mm thick cover glasses. For electron microscopy, cultures were fixed by immersion in 3% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The cells were then fixed in 1% osmium tetroxide in cacodylate buffer for 20 min and then dehydrated through graded ethanol, prior to being embedded in 'TAAB Premix' medium grade resin (TAAB Laboratories Ltd, Reading, U.K.). After polymerization of the resin, transfer of the cells onto the resin was accomplished by a modification of the 'pop off' technique of Bretschneider and colleagues (17). Sections (70 nm) were cut both horizontally and vertically, and following staining with uranyl acetate and lead citrate were viewed in a JEOL 100SX.

For the purpose of comparison of the cultured cells with those *in vivo*, at the electron microscopic level, both nasal and bronchial biopsies were obtained from normal subjects and were fixed in 3% gluteraldehyde in 0.1 M sodium cacodylate buffer. The biopsies were processed, as above, and prior to staining and analysis, were sectioned vertically.

For immunostaining techniques, cultures were fixed in a solution of absolute methanol and 3% hydrogen peroxide (4:1, v/v) for 10 min, rinsed in Tris buffered saline and then incubated for 60 min at room temperature in the presence of specific primary monoclonal antibodies. Epithelial cells were determined by incubation with either CAM 5.2, an anticytokeratin monoclonal antibody preparation or else with PR1A3, an anticiliated epithelial cell antigen monoclonal antibody, described by Richman and Bodmer (16). To determine contamination by other cell types, cultures were incubated with monoclonal antibodies: 5B5, directed against 4-proline hydroxylase found predominantly in fibroblasts; M616, directed against Factor VIII in endothelial cells; M724, directed against desmin in muscle cells; M732, directed against antigen CD11c in macrophages; and, MCA480 and MCA483, directed against antigens CD3 and CD37, in T lymphocytes and B lymphocytes, respectively.

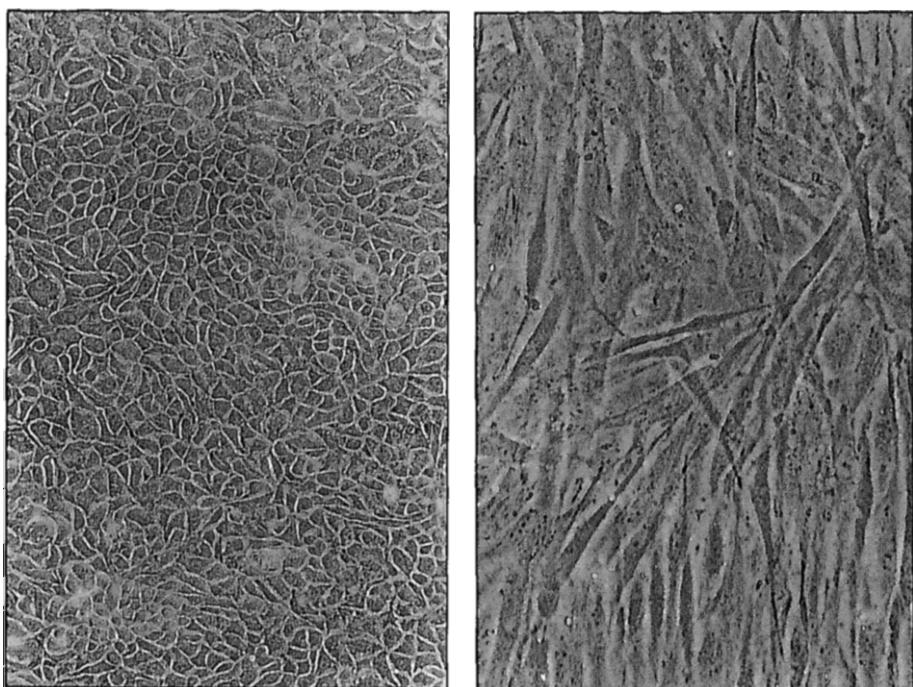


Plate 1 Phase contrast light micrographs of (a) bronchial epithelial cells after 3 weeks in culture and (b) bronchial fibroblasts after 3 weeks in culture. Bronchial epithelial cells are indistinguishable from nasal epithelial cells which are not shown ($\times 300$).

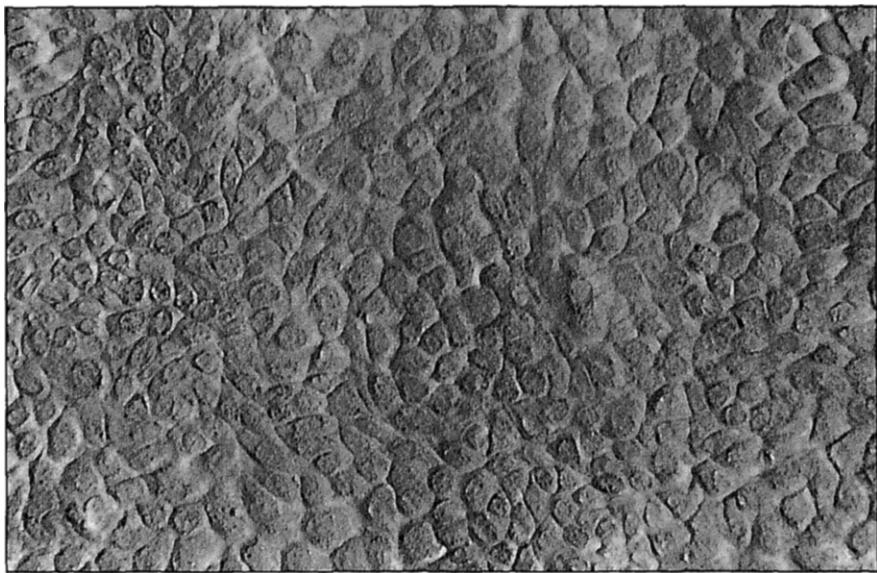


Plate 2 Hoffman Modulation contrast light micrograph of bronchial epithelial cells after 3 weeks in culture. Bronchial and nasal epithelial cells viewed by this method are indistinguishable from one another ($\times 300$).

Following incubation in the presence of any one of these primary antibodies, the cells were washed three-times for 2 min in Tris buffered saline, and then further

incubated for 30 min in the presence of P260, a secondary rabbit antimouse peroxidase conjugated polyclonal antibody preparation. The cells were again

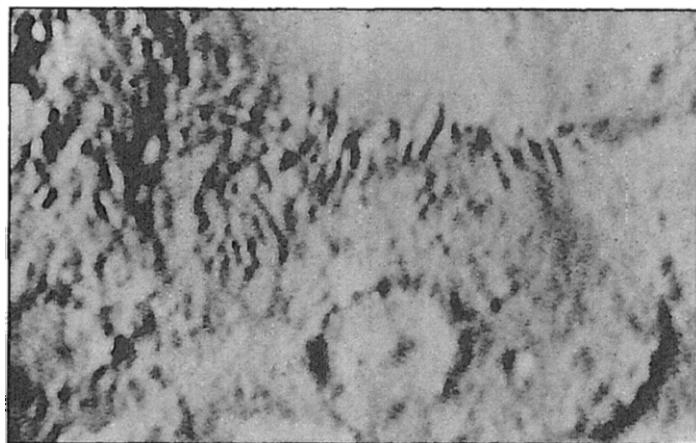


Plate 3 Hoffman Modulation contrast light micrograph of a bronchial epithelial cell showing many cilia, as seen on a television monitor screen. Bronchial and nasal epithelial cells viewed by this method are indistinguishable from one another.

washed with Tris buffered saline as before, treated with a solution of 0·06% 3,3 diamino-benzidine tetrahydrochloride (DAB) and 0·03% hydrogen peroxide in 0·05 M Tris buffer, pH 7·6, for 20 min and counter-stained with Mayer's haematoxylin stain. The stained cells were dehydrated in graded ethanol and finally mounted either directly on culture dishes or else mounted onto microscope slides, when grown on coverslips. The cells were viewed by light microscopy and photographed, prior to analysis.

Following examination of these cells, all subsequent morphological studies by electron microscopy and immunoperoxidase staining were carried out on cells cultured on cover glasses, since these were easier to handle than cells cultured on culture dishes.

(IV) ESTIMATION OF CELL SIZE

Nasal and bronchial epithelial cells from ten patients were analysed for size. All cultures were observed by Hoffman Modulation contrast and photographed randomly at weekly intervals for up to 4 weeks. Photographs of a size of 10" x 8" were obtained and individual cells were analysed for area, perimeter and mean width, by planimetry using a Hewlett-Packard Imagan System, incorporating a basic quantitation programme. A specially marked out grid with ten randomly selected areas was placed on each photograph and the cells in only these areas were analysed for size. The magnification factor for the photographed cells was calculated using a microscope stage graticule, which was viewed and photographed at similar magnification as the cells, and was subsequently used for determination of the actual size of the cells.

(V) ESTIMATION OF RATE OF CELL GROWTH IN CULTURE

The rate of cell growth was estimated in both nasal and bronchial cell cultures of at least ten different subjects, over a period of 4 weeks. Cultures were stained by indirect immunoperoxidase staining, using the primary monoclonal antibody M722, which labels the proliferating cells at all stages other than G_0 phase of the cell cycle. The size of each culture was determined by planimetry, as above, and the numbers of cells at each time point in culture were calculated according to the formula:

$$\text{Mean total area of cultures } (\mu\text{m}^2) \\ \text{Total cell number} = \frac{\text{Mean cell area } (\mu\text{m}^2)}{\text{Mean total area of cultures } (\mu\text{m}^2)}$$

(VI) ESTIMATION OF NUMBER OF CILIATED CELLS IN CULTURE

The numbers of ciliated cells present in nasal and bronchial cell cultures of ten patients, was also estimated over a period of 4 weeks. Cultures were established on cover glasses and stained by the indirect immunoperoxidase staining technique, using PR1A3 as the primary monoclonal antibody. Each culture was viewed and photographed serially from the centre to the right edge and the numbers of ciliated cells present in random areas on each photograph, predetermined as above, were expressed as a percentage of the total numbers of cells in those areas.

(VII) ESTIMATION OF CILIARY BEAT FREQUENCY

Ciliary activity of the ciliated cells in ten different cultures of both nasal and bronchial cultures was studied in a time-dependent manner as above. Ciliary beat frequency (CBF) was measured by a modification

of the analogue contrast enhancement technique using the Reece Scientific PCX On Screen Measurement System (Brian Reece Scientific Instruments, Newbury, U.K.). Cultures were routinely viewed by Hoffman Modulation contrast and the image of the ciliated cells observed was transferred onto a television monitor via a COHU solid state video camera and a CE-1 contrast enhancer (Brian Reece Scientific Instruments, Newbury, U.K.) The latter dramatically increased the contrast of the video signal such that it was possible to visualize the cilia and other cellular organelles in much greater detail on the television monitor. The cultures were allowed to equilibrate at room temperature for one minute and the ciliary beat frequency of at least six different ciliated cells in each culture was determined over a period of 1 min. The position of individual cilia to be monitored was 'fixed' on the screen by means of a cross-hair light sensor and differences in light intensity resulting from ciliary motion at the cross point of the sensor was analysed by the use of a microcomputer incorporating a PCX Video Digitiser Card and specifically programmed for this application. The differences in light intensities were computed in units of Hz.

Results

Nasal and bronchial epithelial cells appeared similar morphologically when observed by light microscopy. Examination of the cultures by phase contrast microscopy revealed that the cells grew outwards from the explant and when confluent, presented as a monolayer consisting of homogeneous polygonal cells [Plate 1(a)] which contrasted sharply with fibroblasts [Plate 1(b)]. Use of the Hoffman Modulation contrast system produced a sharper image clearly demonstrating that neighbouring cells were tightly attached to one another (Plate 2). Ciliated cells could also be readily identified and closer examination of the cells that were ciliated revealed that each of these had numerous cilia (Plate 3), which beat synchronously on adjacent cells. Transmission electron microscopy confirmed the epithelial nature of these cells since micro-villi, tight junctions and desmosomes, characteristic of epithelial cells, could readily be identified [Plate 4(a)] similar to those found in biopsy tissue of normal subjects [Plate 4(b)]. This was found to be the case for both nasal and bronchial cell cultures. The epithelial nature of both the cell types was further confirmed by the indirect immunoperoxidase staining with CAM 5.2, which revealed that all the cells in the cultures stained for cytokeratin [Plate 5(a)] and examination of the stained cells at higher magnification demonstrated that there were fine cellular projections extending between

adjacent cells [Plate 5(b)]. Staining of the epithelial cells with this monoclonal antibody was found to be similar for all cells, whether the cells were cultured on plastic or glass surfaces. Cultures stained with the monoclonal antibodies directed specifically against fibroblasts, endothelial cells, muscle cells, macrophages and lymphocytes did not stain positively for any of these cells.

Analysis of size of the nasal and the bronchial epithelial cells demonstrated that these cells were not significantly different in this respect, as assessed by Student's *t*-test. The size of the cells was not affected by the time in culture and the nasal cells were found to have a mean area, width and perimeter of $487 \mu\text{m}^2$, $10.6 \mu\text{m}$ and $89.5 \mu\text{m}$ respectively, compared with mean area, width and perimeter of $477 \mu\text{m}^2$, $10.2 \mu\text{m}$ and $90.8 \mu\text{m}$, respectively, for bronchial cells (Table 1).

Cultures of both nasal and bronchial cells stained with monoclonal antibody M722, labelling the proliferating cells, demonstrated that these cells were dividing and actively proliferating in culture, over a period of at least 4 weeks. Analysis of colony size and estimation of cell number in each colony demonstrated that both cell types were similar in this respect and had a cell doubling time of one week (Fig. 1).

PR1A3 also stained cells equally well, whether these were cultured on plastic or glass surface. Cultures stained with this antibody revealed that only a proportion of cells were stained (Plate 6). Calculation of the total numbers of ciliated cells in week old nasal and bronchial cultures showed that 51% of nasal cells and 76% of the bronchial cells were ciliated (Table 2). The numbers of ciliated cells gradually decreased as time in culture increased and by 4 weeks in culture 16.9% of nasal cells and 23.0% of bronchial cells were ciliated (Table 2). There was, however, no significant difference in the numbers of ciliated cells in the nasal cultures compared to bronchial cultures. Although the proportion of ciliated cells was affected by time in culture, ciliary activity was not altered. Both cell types were found to be similar regarding their ciliary activity and at the earlier stages of culture, exhibited mean CBF of $10.8 \pm 0.7 \text{ Hz}$ and $11.8 \pm 2.3 \text{ Hz}$ (mean \pm SD), for nasal and bronchial cells, respectively. Although the CBF of both cell types increased as time in culture was extended, this was not significant. Additionally, the CBF of cultured cells was no different from that on the respective explant tissue from which the cells were derived (Table 3).

Discussion

Although there are several reports in the literature detailing different methods for culturing both human

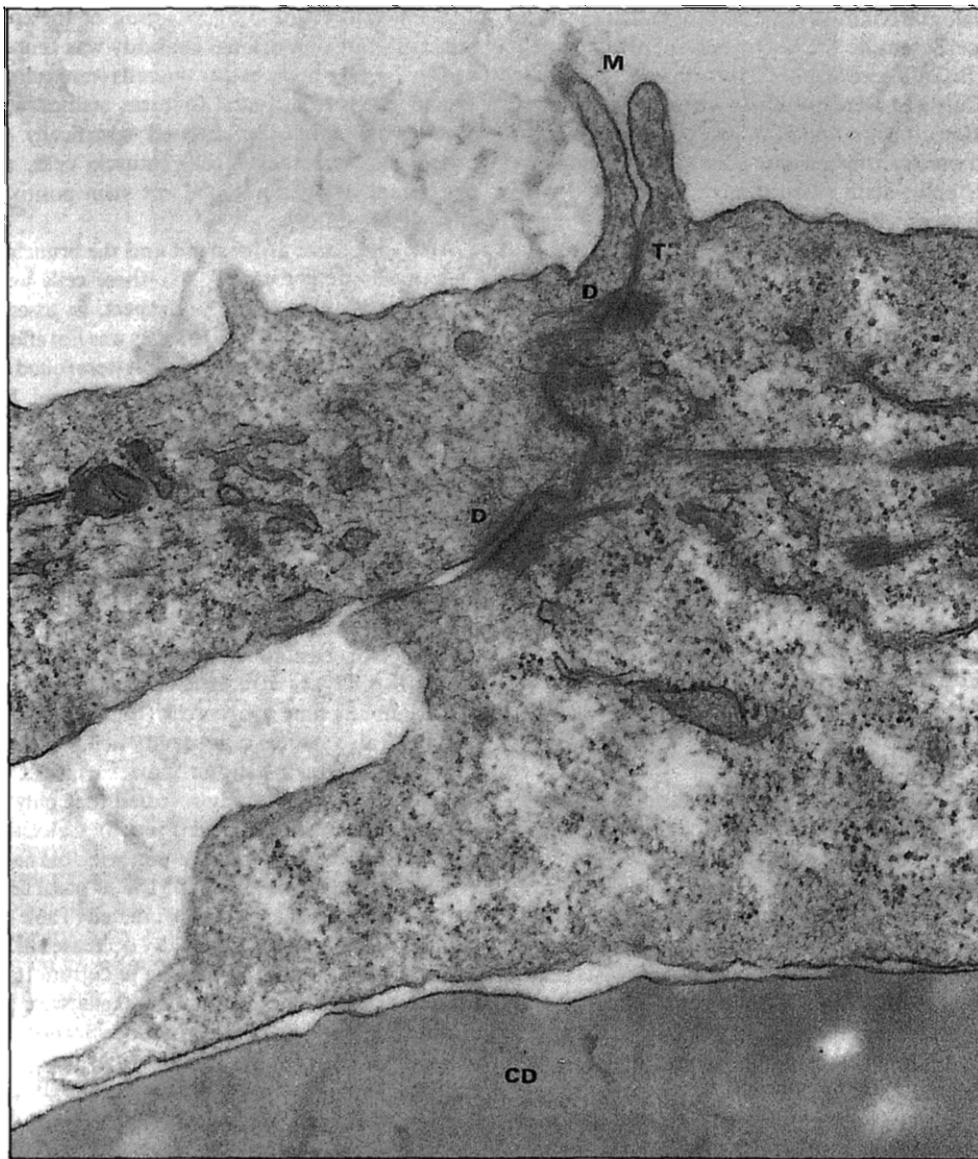


Plate 4a.

nasal and bronchial epithelial cells *in vitro* (8–15), none describes comparison of these two cell types cultured under the same conditions. A major difficulty in these studies has been to obtain confluence of cultured cells such that large numbers of cells can subsequently be harvested. Where this has been achieved, the epithelial cells have usually been isolated by methods involving enzymatic disaggregation, well known to cause detrimental morphological and biochemical changes. In

addition the subsequent cultured cells represent a population most resistant to enzymatic treatment and capable of attaching to culture dishes. In some studies bronchial tissue obtained 8–12 h postmortem has been used for culture. Farber and Young and others (18–19) have demonstrated that anoxic conditions lead to accelerated degradation of membrane phospholipids and consequently cause irreversible cell injury.

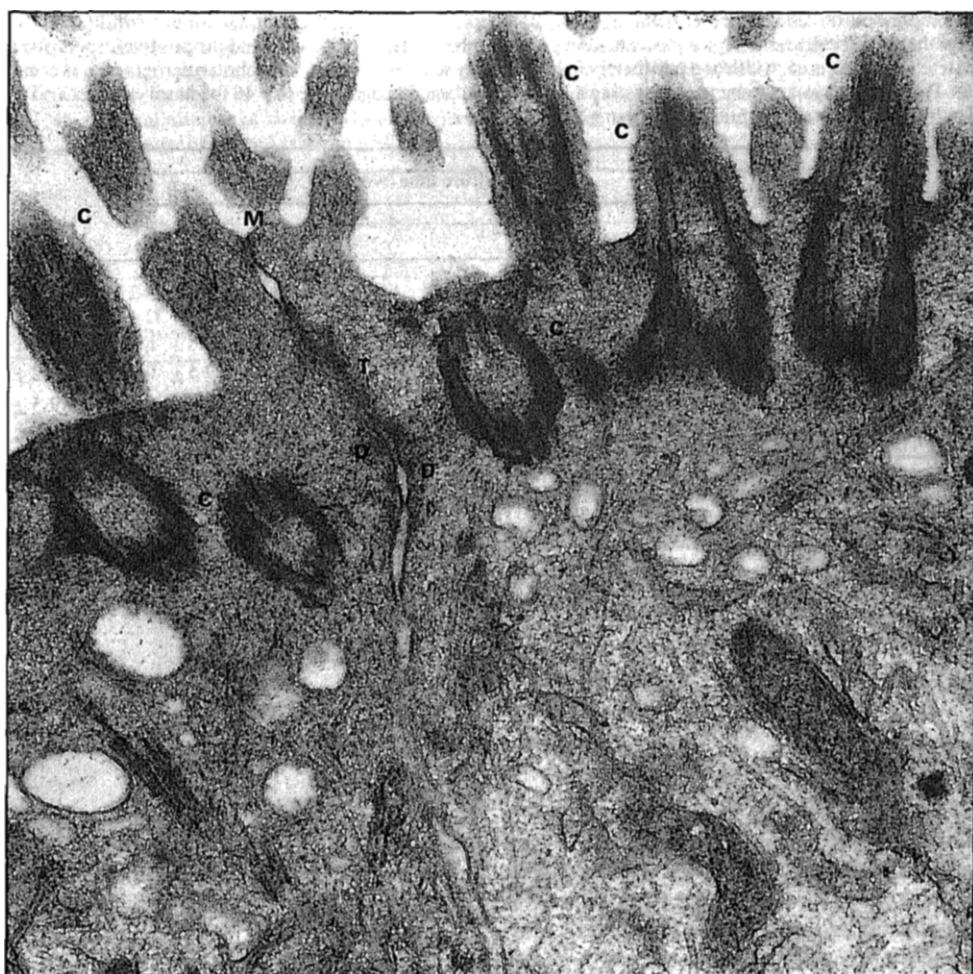


Plate 4b.

Plate 4 Electron micrograph of (a) cultured bronchial epithelial cells and (b) bronchial biopsy of a normal subject, showing cilia (C), microvilli (M), tight junction (T) and desmosomes (D). Bronchial and nasal epithelial cells viewed by this method are indistinguishable from one another (CD = culture dish; magnification of cultured cells $\times 44\,800$ and bronchial biopsy $\times 33\,600$).

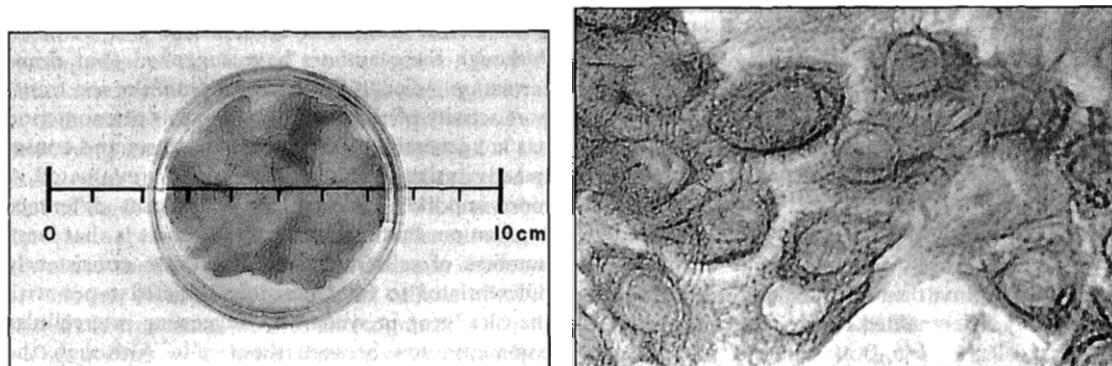


Plate 5 (a) A typical bronchial epithelial cell culture stained for cytokeratin, with monoclonal antibody CAM 5.2, and (b) cytokeratin stained cells demonstrating inter-cellular processes between neighbouring cells. Nasal cells stain similarly with CAM 5.2. (magnification $\times 400$).

Table 1 Comparison of cellular size of nasal and bronchial epithelial cells cultured under similar conditions. Nasal and bronchial epithelial cell cultures from ten patients were photographed weekly for 4 weeks and the photomicrographs analysed by planimetry. The mean area, width and perimeter of ten randomly selected areas on each photomicrograph was computed by the Hewlett-Packard Imagen system, incorporating a basic quantitation programme ($n=46$ for nasal cultures and $n=49$ for bronchial cultures). Results are expressed as mean \pm SD.

Epithelial cell type	Culture time (weeks)				Mean \pm SD	
	1	2	3	4		
Area (μm^2)	Nasal	527.9 \pm 147.2	438.6 \pm 32.3	642.0 \pm 166.0	340.0 \pm 7.2	487.0 \pm 111.4
	Bronchial	479.1 \pm 42.0	406.0 \pm 63.6	631.7 \pm 48.4	394.3 \pm 33.4	477.0 \pm 106.0
Width (μm)	Nasal	11.0 \pm 1.3	10.9 \pm 0.5	11.9 \pm 1.4	8.7 \pm 0.3	10.6 \pm 1.1
	Bronchial	10.4 \pm 0.9	9.6 \pm 0.9	11.4 \pm 0.2	9.5 \pm 0.4	10.2 \pm 1.0
Perimeter (μm)	Nasal	94.3 \pm 14.8	86.3 \pm 2.4	101.6 \pm 14.8	75.8 \pm 0.3	89.5 \pm 9.6
	Bronchial	95.5 \pm 7.3	83.0 \pm 5.8	106.2 \pm 9.1	81.1 \pm 2.8	90.8 \pm 11.7

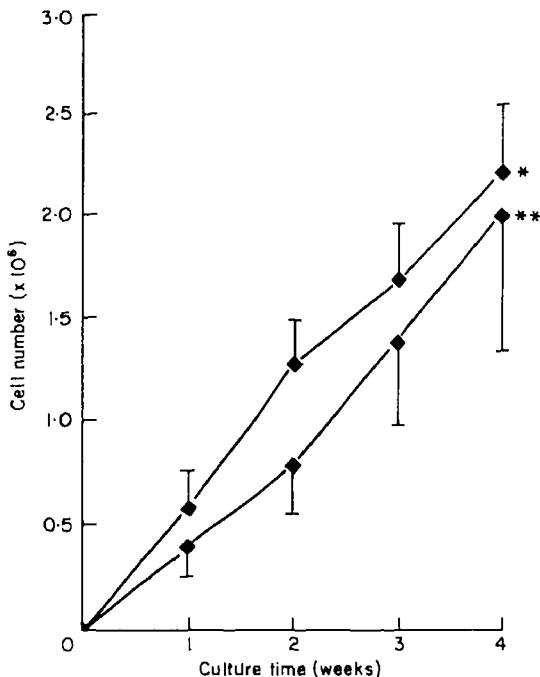


Fig. 1 Estimation of cell number in nasal (*) and bronchial (**) epithelial cell cultures over a period of 4 weeks. Nasal and bronchial epithelial cell cultures from ten patients were stained, with the monoclonal antibody M722, labelling proliferating cells, and each culture analysed by planimetry. The number of cells in each culture was estimated as described in the text and expressed as mean \pm SD.

Our studies have demonstrated that it is possible to culture fully differentiated human nasal and bronchial epithelial cells, *in vitro*. Both cell types are similar in size, shape, growth characteristics and ciliary activity. In accordance with the findings of other authors, we have demonstrated that these cells are of epithelial

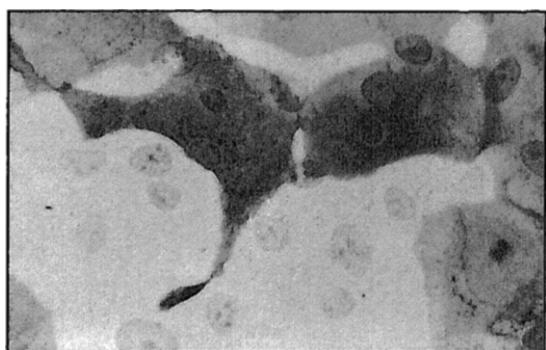


Plate 6 Bronchial epithelial cells stained with monoclonal antibody PR1A3 directed against human bronchial ciliated epithelial cell antigens. Nasal cells stain similarly with PR1A3 (magnification $\times 300$).

nature since they retain vital morphological and histochemical characteristics, as assessed by both light and electron microscope and immunocytochemical techniques. Unlike Wu and colleagues (12), we have not observed the formation of domes in our cultures. Although these authors have suggested that dome formation indicates the preservation of the ion transport activity of these cells *in vitro*, this phenomenon has not generally been reported by others and consequently its significance clearly needs to be evaluated. A more important and possibly significant difference between our findings and those of others is that large numbers of cells in our cultures have consistently differentiated to fully functional ciliated types with the cilia being in synchrony, suggesting intercellular communication between these cells. Although the presence of ciliated cells in human bronchial and nasal epithelial cell cultures has been documented by Stoner and colleagues (14) and Ayars and colleagues

Table 2 Comparison of total numbers of ciliated cells in cultures of nasal and bronchial epithelial cells grown under similar conditions. Nasal and bronchial epithelial cell cultures from ten patients were stained with the monoclonal antibody PR1A3 and photographed weekly for 4 weeks. Ten randomly selected areas on each photomicrograph were counted for the total number of stained cells and expressed as a percentage of the total cells in the areas. Results are expressed as mean \pm SD.

Epithelial cell type	Culture time (weeks)			
	1	2	3	4
Nasal	51.3 \pm 9.5	52.4 \pm 10.8	33.3 \pm 14.5	16.9 \pm 12.1
Bronchial	76.0 \pm 8.0	73.0 \pm 7.1	51.0 \pm 15.9	23.0 \pm 12.4

Table 3 Comparison of ciliary activity of nasal and bronchial epithelial cells cultured under similar conditions. Nasal and bronchial epithelial cell cultures from ten patients were analysed for their CBF weekly for 4 weeks. CBF of six randomly selected ciliated cells from each culture were analysed by the analogue contrast enhancement technique. Results are expressed as mean \pm SD.

	Culture time (days)				
	1	7	14	21	28
Nasal tissue	10.4 \pm 0.6	8.6 \pm 1.1	10.6 \pm 0.8	16.1 \pm 0.7	14.5 \pm 0.6
Nasal cells	-	10.8 \pm 0.7	10.1 \pm 0.7	14.7 \pm 0.3	15.6 \pm 0.3
Bronchial tissue	10.6 \pm 0.1	11.9 \pm 2.3	14.7 \pm 1.5	-	14.5 \pm 0.5
Bronchial cells	-	11.8 \pm 2.3	14.3 \pm 1.3	13.3 \pm 1.0	15.3 \pm 0.6

(10), respectively, these authors have reported that the ciliated cells are only found occasionally in their cultures. In contrast to the findings of Stoner and colleagues (14), we have found that ciliary activity observed on the explanted cells in our cultures is not dependent on the presence of the explant itself and can be maintained for periods of up to minimum of 4 weeks. Our studies have, however, demonstrated that the numbers of ciliated cells decrease as time in culture increases. Whilst it is difficult to explain this phenomenon, one explanation might be that this is a consequence of the lack of mucus *in vitro*, the preponderance of non-ciliated types representing a form of 'ciliated cell redundancy'. None of the techniques employing any form of enzymatic dissociation of the tissue prior to culture have described the presence of ciliated epithelial cells, and consequently it is possible that the inability of epithelial cells to differentiate and mature to the final ciliated form *in vitro* may represent yet another form of the detrimental cellular effect of enzymatic treatment, hitherto unrecognised.

Our studies with ciliated cells have demonstrated that these cells are functionally competent. At the earlier stages of culture, the cilia beat at mean frequencies in the range 8–12 Hz and closely resemble those

observed by other workers employing different model systems and techniques (20–23). The cilia appear to beat faster as time in culture is increased and it is possible that this is an autoregulatory compensatory effect for lowered numbers of ciliated cells.

In conclusion these studies have demonstrated that both bronchial and nasal epithelial cells cultured under similar conditions *in vitro* are similar in their morphology, growth characteristics and ciliary activity. Cultures of either cell type should provide a valuable model for the study of the role of airway epithelium in health and disease. The demonstration that ciliated cells can be cultured consistently using this technique will also provide an important test system for the evaluation of agents which affect ciliary beat *in vivo*.

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