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Primary airway epithelial cell culture from lung transplant recipients

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ABSTRACT: Long-term survival in lung transplantation is limited by the development of obliterative bronchiolitis, a condition characterised by inflammation, epithelial injury, fibroproliferation and obliteration of bronchioles leading to airflow obstruction. To investigate the role of the bronchial epithelium in the pathogenesis of obliterative bronchiolitis the current study aimed to establish primary bronchial epithelial cell cultures (PBEC) from lung allografts.

Four to six bronchial brushings were obtained from sub-segmental bronchi of lung allografts. Cells were seeded onto collagen-coated plates and grown to confluence in bronchial epithelial growth medium.

Bronchial brushings (n=33) were obtained from 27 patients. PBECs were grown to confluence from 12 out of 33 (39%) brushings. Failure to reach confluence was due to early innate infection. Bacteria were usually isolated from both bronchoalveolar lavage and culture media, but a separate population was identified in culture media only.

Primary culture of bronchial epithelial cells from lung transplant recipients is feasible, despite a high rate of early, patient-derived infection. Latent infection of the allograft, identified only by bronchial brushings, may itself be a persistent stimulus for epithelial injury. This technique facilitates future mechanistic studies of airway epithelial responses in the pathogenesis of obliterative bronchiolitis.

KEYWORDS: Bronchial epithelium, cell culture, lung transplantation

Lung transplantation is an accepted strategy for the management of end-stage lung disease in carefully selected patients [1]. Although the early outcomes following lung transplantation have improved, the long-term survival of lung transplant recipients is limited by the development of the bronchiolitis obliterans syndrome (BOS). BOS presents clinically as progressive airflow obstruction leading to irreversible, fixed, small airways occlusion and premature death, despite the application of current therapeutic strategies [2, 3]. BOS currently affects >50% of patients surviving >5 yrs post lung transplant, and limits 7 yr survival to only 31% [1, 3, 4].

The histological lesion of BOS is bronchiolitis obliterans (BO), which is characterised by peribronchiolar leukocyte infiltration, associated with a later abnormal, exuberant epithelial-mesenchymal (fibroblastic) repair response with fibroproliferation. This leads to luminal obliteration of respiratory bronchioles by the deposition of collagen matrix [1, 3, 5, 6].

Recently, there has been a major paradigm shift in the pathogenesis of lung disease. This has placed the epithelium in a critical position orchestrating airway remodelling and scarring in the development of pulmonary fibrosis (*i.e.* epithelial origin of fibroblastic foci) [7, 8] and in asthma (epithelial–mesenchymal interactions in the pathogenesis of sub-epithelial fibrosis and airway remodelling of asthma) [9, 10]. In lung transplantation, it is increasingly recognised that a common-pathway response to bronchial epithelial injury, *via* a combination of allo-immune dependent and independent mechanisms, leads to epithelial activation, an excessive epithelial–mesenchymal fibroblastic repair response and the later development of BOS [11].

Epithelial activation is noted in BOS, as reflected by increased expression of human leukocyte antigen (HLA) class II antigens (HLA-DR and HLA-DP) [12–14] along with increased expression of mRNA transcripts for the co-stimulatory molecules, CD80 and CD86, on bronchial epithelial cells from patients with BOS [15]. An increased expression of airway epithelium inducible nitric oxide (NO) synthase, associated with elevated exhaled NO levels has been described in subjects with BOS, which were correlated with airway neutrophilia [16]. Neutrophilia and

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elevated neutrophil chemoattractants, such as interleukin-8 and RANTES (regulated on activation, normal T-cell expressed and secreted), are a characteristic feature of BO [16–18]. The bronchial epithelium is a well recognised source of cytokines, chemokines and growth factors which are likely to contribute to inflammatory cell recruitment and activation, and the airway remodelling process of BO [5, 6, 15, 17, 19]. The close spatial relationship between the epithelium and the underlying mesenchymal layer forms an integrated unit known as the epithelial mesenchymal trophic unit (EMTU) [10], which plays a pivotal role in branching morphogenesis in foetal lung development [20] and involves the release of growth factors and cytokines by epithelial cells and myo-fibroblasts [10].

The evidence of increased epithelial activation in BO associated with increased deposition of sub-epithelial collagens and other matrix proteins in the lamina reticularis suggests that the EMTU may be reactivated in this disease, leading to reactivation of morphogenic mechanisms, dysregulated epithelial–mesenchymal signalling and the induction of structural changes in the airway wall [10, 20].

Hence, it is the present authors' belief that the bronchial epithelium plays a pivotal role in the development of BOS both as a target for injury and as a mediator of the disease process through response to injury. The present study aimed to establish and characterise a method for primary bronchial epithelial cell culture from lung allografts, to facilitate the future study of epithelial cell responses *in vitro* during the pathogenesis of BOS.

METHODS

The study was performed with ethical approval from the Local Research Ethics Committee and informed consent was received from all study patients.

Patients

All patients underwent lung function testing and an assessment of BOS status was made, based on standardised criteria [21]. Bronchial brushings (n=33) were obtained from 27 consecutive lung transplant recipients undergoing bronchoscopy. Bronchoalveolar lavage (BAL) of the middle lobe/lingula was performed and sent for routine microbiological assessment and transbronchial biopsies performed to exclude acute vascular rejection based on standard International Society for Heart and Lung Transplantation criteria [22].

The patients included 10 single lung transplants, 16 bilateral lung transplants and 1 heart–lung transplant. The indications for transplantation included, cystic fibrosis (CF) (n=15), emphysema (n=11) and pulmonary fibrosis (n=1). Twelve patients were female and 15 male with a mean age of 39 yrs (range 16–59). Bronchoscopy was carried out at a mean time from transplantation of 27 weeks (range 1–120). Indications for bronchoscopy were symptoms of cough/breathlessness and/or a decline in lung function in four cases and as a routine surveillance procedure in the remaining 29 subjects. Only one patient had established BOS at the time of bronchoscopy. Patient immunosuppressive regimes included prednisolone, cyclosporine and azathioprine as routine, with the substitution of tacrolimus and/or mycophenalate mofetil based on clinical course. No patients in the study were on immunosuppressives with ''anti-proliferative'' properties such as everolimus or rapamycin. Furthermore, no patients were on macrolide antibiotics (azithromycin) at the time of study.

Patient details are summarised in table 1.

Patient microbiological prophylaxis

The preventative strategies using antimicrobials in the transplant programme for the study patients included: 1) Post-transplant flucloxacillin/metronidazole/nebulised colomycin in patients for up to 1 week post-transplant based on pre-transplant and donor microbiology. 2) Nebulised colomycin (2 mega units, *b.i.d.*) for patients colonised with Pseudomonas pre-transplant and/or BAL positivity for Pseudomonas. 3) Cotrimoxazole 960 mg three times per week for *Pneumocystis carinii* prophylaxis after 1 week. 4) Oral gancyclovir for patients with cytomegalovirus (D+/R-) mismatch continued 3 months post-transplant. 5) Fungal prophylaxis comprised of voriconazole in patients with Aspergillus (either pre-transplant or in BAL post-transplant) and fluconazole if Candida was present in either donor or recipient BAL.

Bronchial epithelial cell isolation and culture

Bronchoscopy (using an Olympus FB45.5 bronchoscope; Olympus, Tokyo, Japan) was performed in patients premedicated with intravenous midazolam and topical 4% lignocaine, applied to the vocal cords and tracheal lumen in 1 mL aliquots to a maximum dose of 7 $mg \cdot kg^{-1}$ body weight. Bronchial brushings (n=4–6) were obtained from subsegmental bronchi using a protected specimen single-sheathed nylon cytology brush (5 fr; Wilson-Cook, Winston-Salem, NC, USA) and dispersed in 5 mL of sterile phosphate-buffered saline with later addition of 5 mL of RPMI and 10% foetal calf serum (FCS) based on a method previously described [13]. The suspended samples were centrifuged for 5 min at 1000×*g*. The ensuing cell pellet was re-suspended in 2 mL of basal epithelial growth medium (BEBM^{TM}; Clonetics (Cambrex), San Diego, CA, USA) supplemented with BEGM^{TM} Singlequots (Clonetics), penicillin and streptomycin. Final antimicrobial concentrations in the culture medium throughout the culture process were penicillin 50 $U \cdot mL^{-1}$, streptomycin 50 $\mu g \cdot mL^{-1}$, gentamicin 50 $\mu g \cdot mL^{-1}$ and amphotericin B 50 $ng \cdot mL^{-1}$. A 100 µL aliquot was taken for cell count and differential, the remaining cell suspension was then transferred to a 25 cm^2 plate pre-coated with collagen (Vitrogen 100; Cohesion, Palo Alto, CA, USA) and placed in a CO_2 incubator (37°C/5% CO_2). A further 3 mL of supplemented medium was added after the first 48 h and the medium was subsequently exchanged every 48 h until primary bronchial epithelial cell cultures (PBECs) reached confluence. Once confluent, PBECs were passaged using trypsin, which was neutralised using an equal volume of RPMI supplemented with 10% FCS. PBECs were then transferred in 10 mL of culture medium to Vitrogen (Cohesion) coated 75 cm^2 flasks (5×10^5 $cells \cdot flask^{-1}$) or to eight chamber slides (Lab-Tek, Nunc, Naperville, IL, USA; 2.5×10^4 $cells \cdot chamber^{-1}$) and cultured to confluence.

Cell count and viability

Cell count and differential was performed on the PBEC cell suspension following brushing using a Nebhauer Haemocytometer and differential cell count performed on

TABLE 1 Characteristics of study patients

Pt	Diagnosis pre-transplant	Op	Weeks	Biopsy#	Immuno-suppression	Clinical Infection	Anti-microbial	BOS+	BAL microbiology	Culture microbiology	Confluent
1	Emphysema	B	24	A0B0	P/C/A	No	Cot	0	Neg.		Yes
2	Emphysema	S	24	A1B2	P/T/A	No	Cot	0p	Neg.		Yes
3	Emphysema	S	52	A0B0	P/T/A	No	Cot	0	Neg.		Yes
4	CF	B	24	A0B0	P/C/A	No	Cot	0	Neg.		Yes
5	CF	B	52	A0BX	P/T/A	No	Col/Cot	0	Neg.		Yes
6	IPF	S	12	A0B1	P/T/A	No	Col/Cot	0	Neg.	*C. lusitanae*	No
			16	A2B2	P/T/A	No¶	Col/Cot	0	Neg.		Yes
7	Emphysema	S	12	A1B1	P/T/A	No	Cot	0	Neg.		Yes
			24	AxBx	P/T/A	No	Cot/Flu	0	*C. albicans*	*C. albicans*	No
8	CF	HL	12	A1B0	P/T/A	No	Col/Cot	0	Neg.		Yes
			24	AXB1	P/T/A	No	Col/Cot	0	Neg.	*C. albicans*	No
9	Emphysema	S	52	A1BX	P/T/A	No	Cot	0	Neg.		Yes
10	CF	B	52	A0BX	P/C/A	No	Cot	1	Neg.		Yes
11	Emphysema	S	40	A2BX	P/C/A	No¶	Cot	0	Neg.	*P. fluorescens*	No
			52	A0B0	P/C/M	No	Cot	0	*A. flavus*		Yes
12	Emphysema	S	12	A1B1	P/T/A	No	Gcv/Cot	0	Neg.		Yes
			24	A0B0	P/T/A	No	Cot		Neg.	*P. fluorescens*	No
13	CF	B	4	A1B1	P/C/A	No	Col/Cot	0	*P. aeruginosa*	*P. aeruginosa*	No
14	CF	B	52	A0B1	P/T/A	No	Col/Cot		*P. aeruginosa*	*P. aeruginosa*	No
15	Emphysema	S	1	A1B0	P/C/A	No	Flx/Met/Col	0	*C. albicans*	*P. aeruginosa*	No
16	CF	B	1	A0BX	P/C/A	No	Flx/Met/Col	0	Neg.	*A. faecalis*	No
17	Emphysema	S	2	A2BX	P/C/A	No¶	Cot/ Flu	0	*Candida* spp.	*Candida* spp.	No
18	Emphysema	B	12	A1BX	P/C/A	No	Gcv/Cot	0	*P. aeruginosa*	*P. aeruginosa*	No
19	CF	B	6	AXBX	P/C/A	No	Col/Cot	0	Neg.	*E. dermatididis*	No
20	CF	B	52	A0B2	P/C/A	Yes	Col/Cot	0	*P. aeruginosa*	*A. fumigatus*	No
21	CF	B	24	A1B1	P/C/A	No	Cot	0	Neg.	*A. xylosoxidans*	No
22	CF	B	52	A1B1	P/T/A	No	Col/Cot		Neg.	*P. Aeruginosa*	No
23	CF	B	2	A2BX	P/C/A	No¶	Col/Cot	0	*S. maltophilia*	*S. maltophilia*	No
24	CF	B	12	A0BX	P/C/A	No	Cot	0	Neg.	*C. albicans*	No
25	CF	B	120	AXBX	P/T/M	Yes	Cot	0	*P. aeruginosa*	*S. maltophilia*	No
26	CF	B	6	A1B1	P/C/A	No	Cot/Flu	0	*C. albicans*, *S. aureus*	*C. albicans*, *S. aureus*	No
			8	A0BX	P/C/A	No	Cot/Flu	0	*C. albicans*, *S. aureus*	*C. albicans*, *S. aureus*	No
27	CF	B	24	A2B1	P/T/A	No	Cot	0	*P. aeruginosa*	*B. cenocepacia*	No

Pt: patient; Op: operation type; BOS: bronchiolitis obliterans syndrome; CF: cystic fibrosis; IPF: idiopathic pulmonary fibrosis; B: bilateral lung transplant; S: single lung transplant; HL: heart–lung transplant; A0: no significant abnormality; A1: minimal; A2: mild; B0: no active airway damage; B1: minimal lymphocytic bronchiolitis; B2: mild lymphocytic bronchiolitis; AX/BX: insufficient material for assessment. P: prednisolone; C: cyclosporine; A: azathioprine; T: tacrolimus; M: mycophenalate mofetil; Cot: co-trimoxazole; Col: nebulised colomycin; Flx: flucloxacillin; Flu: fluconazole; Met: metronidazole; Gcv: gancyclovir. BOS 0: forced expiratory volume in one second (FEV_1)>90% baseline; BOS 0p: FEV_1 81–90% baseline; BOS1: FEV_1 66–80% baseline; *C. albicans*: *Candida albicans*; *A. flavus*: *Aspergillus flavus*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S. maltophilia*: *Stenotrophomonas maltophilia*; *S. aureus*: *Staphylococcus aureus*; *C. lusitanae*: *Candida lusitanae*; *P. fluorescens*: *Pseudomonas fluorescens*; *A. faecalis*: *Alcaligenes faecalis*; *E. dermatididis*: *Exophiala dermatididis*; *A. fumigatus*: *Aspergillus fumigatus*; *A. xylosoxidans*: *Alcaligenes xylosoxidans*; *B. cenocepacia*: *Burkholderia cenocepacia*. #: transbronchial biopsy was evaluated for acute rejection as per International Society for Heart and Lung Transplantation (ISHLT) criteria [22]. ¶: patient bronchoscoped because of new symptoms (cough, breathlessness, fever). +: based on ISHLT criteria [21].

Giemsa-stained cytospin preparations, counting a minimum of 500 cells. Viability of cultured cells was assessed by the exclusion of trypan blue dye (0.4%; Sigma, Poole, UK).

Immunocytochemistry

To confirm epithelial characteristics, PBECs were grown on eight-chamber slides and stained for cytokeratin using monoclonal mouse anti-human cytokeratin antibodies (LP34 and MNF116; DakoCytomation, Ely, UK) with fluorescein isothiocyanate-conjugated secondary reagents, mounted in fluorescence mounting medium (DakoCytomation) and examined using confocal microscopy. Identification of resident inflammatory cells within the monolayers was performed using antibodies to leukocyte common antigen, CD3 and CD68 (KP-1 macrophage; DakoCytomation). To visualise immunoreactivity a modified immunoperoxidase staining

method was used with an indirect reporter system (Envision; DakoCytomation). Isotype matched immunoglobulins were employed as negative controls.

Microbiological assessment of bronchoalveolar lavage and culture supernatant

Specimens of BAL fluid were processed in the Dept of Medical Microbiology in a standardised fashion which included the use of selective agars and extended culture for bacterial, fungal, and *Legionella* spp. Cell culture supernatants that became infected, based on daily visual inspection, were assessed for microbiological growth in the same laboratory as per BAL fluid. Briefly, culture media was centrifuged at 1,200×*g* for 10 min and the ensuing pellet was inoculated onto blood agar and chocolate bacitacin agar and incubated for 48 h at 37°C/5% CO_2 and examined for growth. Bacterial isolates were identified using standard methods and extended sensitivity testing. Gram stain or quantitative culture was not performed routinely in either BAL or culture medium.

Statistical analysis

Comparison between successful culture and cultures that failed to grow was made using the Chi-squared test with Fisher's exact test for categorical data and Mann-Whitney U-test for noncategorical data. Significance was assumed at $p \leqslant 0.05$.

RESULTS

Bronchial brushings yielded a mean of 4.1×10^4 cells (range $1.8\text{-}8.7\times10^4$). Differential counts confirmed that epithelial cells accounted for 89% of cells (range 77–99) with 6.9% neutrophils (0.2–5.2), 3.7% macrophages (0–26) and a small population of lymphocytes, 0.5% (0–5.4). Ciliated and basal epithelial cells were both identified in the brushed cell suspension (fig. 1a).

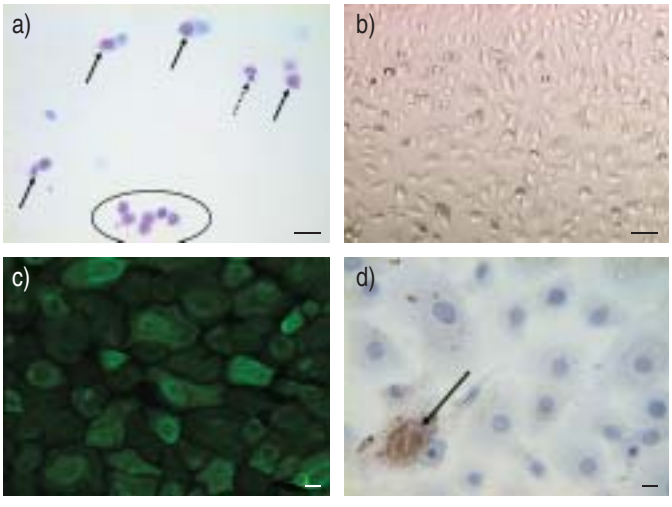

FIGURE 1. a) Giemsa-stained bronchial brushing cytospin demonstrating ciliated epithelial cells (solid arrows), basal cells (ringed) and neutrophil (broken arrow; scale bar=100 μm). b) Brightfield microscopy of confluent primary bronchial epithelial cell cultures (PBEC) monolayer (scale bar=100 μm). c) Cytokeratin immunostained epithelial cells demonstrating immunofluorescence using confocal microscopy (scale bar=100 μm). d) Macrophage (arrow) staining positive for CD68 within PBEC monolayer after passage number 2 (scale bar=100 μm).

Of 33 brushings, 12 reached confluence at a median of 14 days post-bronchoscopic sampling and underwent passage. At confluence cells had the phenotypic appearance of undifferentiated cells, lacking visible cilia or other morphological features of differentiation (fig. 1b). Cells demonstrated cytokeratin positivity (fig. 1c). Immunocytochemistry confirmed the presence of macrophages (<2% of total cell count) in the PBEC monolayer even after passage (fig. 1d).

The remaining 21 brushings failed to reach confluence due to infection of the culture, occurring within the first 72 h post brushing. The organisms isolated from culture media are shown in table 1 together with the corresponding BAL microbiology from the same bronchoscopic procedure. Successful culture was most likely if brushings derived from patients with negative BAL microbiology ($p<0.0001$), indeed only one of the 12 successful cultures derived from a patient with BAL positive microbiology. There was an association between a diagnosis of CF and failure to culture cells compared with patients with a non-CF diagnosis ($p=0.04$), despite CF patients only being slightly more likely to have BAL positive microbiology (eight out of 18, 44%) compared with non-CF patients (five out of 13, 38%; p=nonsignificant). Successful culture tended to derive from brushings taken from patients further out from transplant (median 24 weeks) compared with nonsuccessful culture (median 12 weeks), although this association failed to reach significance ($p=0.08$). No other factors in terms of operation type or acute rejection score appeared to predict culture success. There appeared to be no systematic differences between the four patients who underwent bronchoscopy due to symptoms and the routine surveillance patients (table 1).

DISCUSSION

While primary fibroblast cultures have been established from lung transplant recipients [23], to the best knowledge of the authors', this is the first description of human PBECs derived from lung allografts. This technique allows the study of epithelial cells derived from clinically characterised lung transplant recipients. Whilst animal models of obliterative bronchiolitis are available and support the importance of the bronchial epithelium in murine heterotopic allografts [24], the study of human lung allograft recipients is required to examine the role of epithelial cells in the development of BOS. This is of particular importance given the nonalloimmune insults, such as infection, that the human allograft is subjected to.

Successful culture resulted in establishment of phenotypically characteristic epithelial cells though with loss of ciliated differentiation. Maintenance of the ciliated phenotype requires the use of air–liquid interface culture techniques [25]. Whilst the monolayers appeared to be composed of >98% PBECs, there was a persistence of a small population of macrophages in the monolayers, even after one or two passages up to 4–6 weeks after bronchial brushing. The contribution of the macrophage population to subsequent experimental findings requires further investigation. Macrophages in theory could be removed from the initial cell suspension using specific anti-CD14 coated immunomagnetic beads as has been demonstrated in primary fibroblast culture [26]. However, the ''co-culture'' of macrophages with PBECs may actually be more

representative of the biological system *in vivo*. Certainly, the interaction of macrophages and airway epithelial cells has been demonstrated to amplify the response to exogenous stimuli in terms of inflammatory mediators [27].

A high rate of infection was observed in the brushing cultures, occurring within the first 72 h of incubation. The presence of organisms was associated with an absolute failure of culture in all cases. Organisms appeared to be derived from patients who were generally stable and in whom a clinical diagnosis of infection was not made, though who frequently had microbiologically positive BAL. Extended antibiotic sensitivity testing suggested the same organism accounted for all eight patients infected with the same species in both BAL and culture medium, although phage typing was not performed. There was a group of infected cultures (n=9) derived from patients whose BAL microbiology was negative. These organisms included *Candida* spp., *Pseudomonas* spp., *Alcaligenes faecalis* and *Exophiala dermatitidis*, all of these organisms being well recognised in the airways of transplant recipients. *E. dermatitidis* is a black yeast recognised to be a potential pathogen in the airways of CF patients [28] identified for the first time in the culture medium of a CF patient (patient 19; table 1). Additionally, in one patient (patient 27; table 1), *Burkholderia cenocepacia* was identified in culture media and not from BAL. Indeed, whilst this patient had been colonised with *B. cenocepacia* pre-transplant, the organism had not been identified in repeated BAL samples post-transplant. *B. cenocepacia* is associated with a necrotising pneumonia in CF patients and a poor post-transplant outcome [29].

The identification of microbes in the culture medium of patients when BAL microbiology is negative may have a number of explanations. Microbiological growth may simply reflect contamination of the medium in the culture hood/incubator. The current authors feel that this is most unlikely given the nature of the organisms involved and the fact that infection of cultures after 72 h was not seen. Secondly, the culture medium, containing antibiotics and antifungals, may select out organisms present in low concentrations from the brushings and encourage their growth. Thirdly, organisms may derive from the upper airways of patients including the nasopharynx, although the use of protected specimen brush may reduce the chance of spurious contamination. Finally, the process of epithelial brushing may be identifying a discrete population of organisms that may be adherent to the epithelium as a biofilm. The current authors have previously demonstrated the presence of quorum signalling molecules in the BAL of stable lung transplant recipients suggesting the presence of bacterial biofilm formation even in the presence of apparently culture negative BAL [30]. Whilst a recent study in CF patients failed to demonstrate a difference between bronchial brushings, BAL and sputum microbiology in terms of the nature of *Pseudomonas aeruginosa* colonisation [31] there is a paucity of literature regarding biofilm formation in the lung transplant population.

The authors did not employ quantitative culture in this study. Future studies may address questions relating quantitative microbiology along with differences in airway microbiology above/below the bronchial anastamosis, changing biofilms over time and the role of donor-derived infection.

In the current study there was an overall success rate of 39% in reaching confluence and passage of PBECs. The previously published study of primary fibroblast cell culture from lung transplant recipients found a success rate of 54% [23]. Whilst the authors did not specifically comment on the infection rate of the culture medium, the success of fibroblast culture, in contrast to the current study, did not appear to be influenced by BAL microbiology. This difference, the authors suggest, may be due to the difference in sampling methodology (transbronchial biopsy *versus* bronchial brushings).

The present study shows that despite high infection rates, it is possible to establish PBEC cultures from lung allografts. The methodology also highlights the potential injurious role of occult infection in this patient group, supporting the concept of biofilm formation and re-emphasising the difficulties in deciding what represents ''infection'' in the lung allograft airway.

The current authors believe that the use of primary cell cultures from allograft recipients is an important adjunct to the use of commercially available airway epithelial cell lines in understanding the mechanisms of chronic airway dysfunction. Development of a reliable method for primary bronchial epithelial cell culture will facilitate comparison of epithelial cell responses between stable lung allografts and cells from patients with bronchiolitis obliterans syndrome. This will allow dissection of the role of the bronchial epithelium in the pathogenesis of obliterative bronchiolitis.

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