Generation of complement C3 and expression of cell membrane complement inhibitory proteins by human bronchial epithelium cell line

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Abstract

***Background*—The interrelationship between human airway epithelium and complement proteins may affect airway defence, airway function, and airway epithelial integrity. A study was undertaken to determine (1) whether unstimulated human bronchial epithelium generates complement proteins and expresses cell membrane complement inhibitory proteins (CIP) and (2) whether stimulation by proinflammatory cytokines affects the generation of complement and expression of cell membrane CIP by these cells.**

***Methods*—Human bronchial epithelium cell line BEAS-2B was cultured in a serum-free medium. Cells were incubated with and without proinflammatory cytokines to assess unstimulated and stimulated generation of complement C3, C1q and C5 (by ELISA), and to examine the expression of cell membrane CIP decay accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46), and CD59 (protectin) by flow cytometry analysis.**

***Results*—Unstimulated human bronchial epithelial cell line BEAS-2B in serum-free medium generates complement C3 (mean 32 ng/10^6 cells/72 h, range 18–52) but not C1q and C5, and expresses cell membrane DAF, MCP, and CD59. Interleukin (IL)-1α (100 U/ml/72 h) and tumour necrosis factor (TNF-α; 1000 U/ml/72 h) increased generation of C3 up to a mean of 78% and 138%, respectively, above C3 generation by unstimulated cells. DAF was the only cell membrane CIP affected by cytokine stimulation. Interferon (IFN)-γ (10 U/ml/72 h) and TNF-α (1000 U/ml/72 h) increased DAF expression up to a mean of 116% and 45%, respectively, above that in unstimulated cells. MCP and CD59 expression was not consistently affected by IL-1α, TNF-α, or IFN-γ.**

***Conclusions*—Local generation of complement C3 and expression of cell membrane CIP by human bronchial epithelium and its modulation by proinflammatory cytokines might be an additional regulatory mechanism of local airway defence and may affect airway function and epithelial integrity in health and disease.**

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The respiratory tract is unique in being the only internal organ in the body that is continuously exposed to the external environment. About 10 000 litres of air flow through the respiratory tract every day. This air contains numerous foreign agents, many of which are potentially pathogenic.

The serum complement system is one of the most important mechanisms for protecting the body against these potentially pathogenic agents. Proteins of the complement system are present in the lining fluid of the respiratory tract.[1] These proteins protect the host from invading microorganisms and from other inhaled insults by opsonisation that facilitates phagocytosis and by direct lysis of microorganisms that activate complement. Opsonisation and direct lysis can happen immediately by the alternative complement pathway, before specific antibodies are produced, or later by the classic complement pathway when the specific antibodies are already present.[2] In addition to the protection that complement offers against microorganisms, complement fragments C3a, C4a, and C5a also have important immunomodulatory and inflammatory effects such as cell activation, chemotaxis, bronchoconstriction, increase of endothelial permeability, and production of proinflammatory mediators.[3 4]

Many of the foreign agents inhaled by humans—for example, microorganisms, allergens, organic and inorganic dusts, chemicals, and cigarette smoke—can act as complement activators.[5] In fact, active complement proteins are found within the bronchoalveolar spaces and nose in different diseases of the human respiratory tract.[6–9] Active complement proteins protect the respiratory tract from foreign insults but at the same time these proteins also have the potential to injure the host's respiratory tissue by inducing cell lysis via the formation of complement membrane attack complex.[10] Surprisingly, relatively little is known about interrelationship between the human respiratory tract and the complement system and most of the available information has been obtained from animal models.[11]

We recently described some new aspects of the interrelationship that may exist between the epithelium of the human respiratory tract and the complement system. We have shown that human respiratory epithelium ex vivo from nose to alveoli expresses cell membrane complement inhibitory proteins (CIP) that are known from other cell systems to inhibit complement activation upon the cell surface.[12] These proteins are membrane cofactor protein

(MCP; CD46), decay accelerating factor (DAF; CD55), and CD59 (protectin). These cell membrane CIP molecules are expressed in healthy subjects and their expression is increased in various lung diseases characterised by inflammation or malignant transformation[12] and also in human lung cancer cell lines, both unstimulated and stimulated with proinflammatory cytokines.[13 14] We also found that these molecules are essential for protecting cultured human nasal respiratory epithelial cells from being lysed by human serum complement.[15] However, it is not known whether human respiratory epithelial cells express cell membrane CIP molecules constantly or only in response to stimuli such as proinflammatory cytokines.

Another aspect of complement biology that is not known is whether human airway epithelium can generate complement proteins and, if so, under what conditions.

The origin of complement proteins present in the respiratory tract lining fluid in disease is thought to be mainly from plasma that exudes into the bronchoalveolar space during the inflammatory process. However, complement proteins are found in the lungs of healthy nonsmoking volunteers[1] as well as in the bronchoalveolar lavage fluid of the diseased lung.[6–9] This finding raises questions regarding the origin of complement proteins found in the respiratory tract lining fluid in healthy subjects. Since the epithelium of the upper and lower airways is the first to encounter invading microorganisms and other inhaled insults, we hypothesised that local and constant generation of complement by airway epithelium may facilitate rapid opsonisation and phagocytosis or direct lysis of potentially pathogenic invaders even before exudation of plasma containing complement proteins.

A study was undertaken to examine three questions: (1) whether human bronchial epithelium under basal conditions can generate complement proteins C3 (alternative complement pathway), C1q (classic pathway), and C5 (common pathway); (2) whether it can express cell membrane CIP under in vitro conditions; and (3) whether proinflammatory cytokines can modulate complement protein generation and CIP expression in these cells. To answer these questions we studied generation of complement and expression of cell membrane CIP in human bronchial epithelium in a model of BEAS-2B cell line in a serum-free medium and studied the effects of the proinflammatory cytokines interleukin (IL)-1α, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ, some of the most important cytokines involved in lung defence.

Methods

BRONCHIAL EPITHELIUM CELL CULTURES

To study cells in serum-free culture conditions we used human bronchial epithelium cell line BEAS-2B in all experiments. This cell line was purchased from American Type Culture Collection (ATCC), Rockville, Maryland, USA.[16] Epithelial cells were grown in 25 ml culture flasks (NuNc, Denmark) precoated with a coating medium containing 30 μg/ml purified collagen (Vitrogen-100; Collagen Corporation, Palo Alto, California, USA), fibronectin 10 μg/ml, and BSA 10 μg/ml in a culture medium. The culture flasks were incubated with this coating medium (5 ml per flask) for 24–48 hours in a humidified CO_2 incubator at 37°C. The coating medium was then aspirated and BEAS-2B cells were seeded at 0.5–1.0 × 10^6 cells per flask.

To assess the ability of these cells to generate complement C3, C1Q and C5 and to express cell membrane CIP molecules in the presence or absence of proinflammatory cytokines, the cells were cultured in a keratinocyte serum-free medium containing the manufacturer's supplements rhEGF and bovine pituitary extract (GIBCO BRL, Life Technologies, Grand Island, New York, USA). Cells were incubated in a cell culture humidified incubator (90% humidity at 37°C, 5% CO_2). The culture medium was changed every 3–4 days (6 ml per flask) and the cells reached a near confluence after 5–6 days. To diminish the number of cell passages the original cell stock purchased from ATCC was first recultured and then propagated a few times. The cells were then divided into aliquots and frozen in 10% DMSO in culture medium at –70°C or in nitrogen dioxide for future use. The aliquots of frozen cells were used for a maximum of two or three cell passages.

To perform a cell passage the cell culture medium was aspirated from near confluent cell cultures and 0.5 ml of trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA) was introduced into each flask and immediately aspirated without a following wash. The flask was then incubated for 5–7 minutes at 37°C in a cell incubator. The cells were washed out from the flask using culture medium, centrifuged and the cell pellet was resuspended in a fresh culture medium and reseeded in coated flasks as described above.

CYTOKINE STIMULATION

BEAS-2B human bronchial epithelial cells were incubated with and without cytokines to assess unstimulated and stimulated generation of complement C3, C1q, C5 and expression of cell membrane MCP, DAF, and CD59. The cytokines studied were rhIL-1α (1 μg = 1.6 × 10^5 U), rhTNF-α (1 μg = 1.1 × 10^5 U), and rhIFN-γ (1 μg = 10^4 U). All cytokines were purchased from R & D Systems (Minneapolis, Minnesota, USA). Cytokines in a fresh culture medium were added to cell cultures 48 hours after cell seeding. In unstimulated control cell cultures fresh medium alone was added in parallel. In a preliminary experiment we studied the effect of a given cytokine concentration with time (24, 48, and 72 hours). The maximal cytokine effect was always noted after 48–72 hours and we therefore studied the effect of different concentrations of a given cytokine for 72 hours. Once the cytokines were added the culture medium was not changed further in either the cytokine stimulated or the unstimulated control cell cultures.

ASSESSMENT OF COMPLEMENT C3, C1Q AND C5 GENERATION

The culture medium of cells, both unstimulated and stimulated by cytokines, was aspirated, frozen at –70°C and kept until needed (less than one week).

Complement C3 was assayed by a direct ELISA technique and complement C1q and C5 were assayed by an indirect ELISA technique. Complement C3, C1q and C5 standards were purchased from Sigma Co. (St Louis, Missouri, USA).

C3 ELISA

ELISA 96 well plates were coated overnight at 4°C with goat anti-human C3 (Atlantic Antibody, USA) at 10 μg/ml in a coating buffer, 100 μl per well. The wells were then washed three times with a washing solution (0.05% Tween in PBS, pH 7.4) and incubated with 1% BSA in PBS for one hour. The wells were washed again three times in Tween-PBS and cell culture medium samples were introduced at different dilutions in triplicate for 1.5 hours. The wells were then washed three times and a second antibody, rabbit anti-human C3c peroxidase conjugated (7 mg/ml stock antibody titre; Dako, Denmark), was introduced. The second antibody was used at a dilution of 1:5000 and incubation was continued for 1.5 hours at room temperature. At the end of the incubation time the wells were washed three times and O-phenylendiamine (OPD) substrate (Sigma Co) in a peroxidase buffer was introduced for 4–6 minutes. The reaction was stopped with 1N H_2SO_4, 100 μl per well. The plates were read in an ELISA reader (Sorin Biomedika, Italy) at an optical density (OD) of 492 nm. The lower limit of complement detection in these ELISA assays was 2 ng/ml.

C1q, C5 ELISA

Complements C1q and C5 were assessed by an indirect ELISA. ELISA 96 well plates were coated overnight at 4°C by goat anti-human C5 (stock 1 mg/ml) and C1q (stock 0.5 mg/ml) at a concentration of 10 μg/ml each (Sigma Co) in a coating buffer, 100 μl per well. The assay continued as described above for C3 ELISA except that the second antibodies were unconjugated rabbit anti-human C5 and anti-human C1q (Dako, Denmark) at a dilution of 1:5000 (2.2 mg/ml and 4.9 mg/ml stock protein concentrations, respectively). At the end of incubation with the second antibodies and after three washes a third antibody was introduced, goat anti-rabbit IgG, HRP conjugated (Dako) at a dilution of 1:2000 for one hour at room temperature. The wells were then washed three times and the assay continued as described above for C3 ELISA. The lower limit of detection for both C5 and C1q was 1 ng/ml.

ASSESSMENT OF CELL MEMBRANE CIP EXPRESSION

Cell membrane expression of CIP molecules MCP, DAF, and CD59 was assessed by flow cytometry. Monoclonal antibodies were used to assess expression of the specific cell membrane CIP molecule. Mouse anti-human MCP monoclonal antibody J4-48 (IgG_1, 1 mg/ml),[17] mouse anti-human DAF monoclonal antibody BRIC 216 (IgG_1, 1 mg/ml),[18] and rat anti-human CD59 monoclonal antibody YTH 53.1 (IgG_{2b}, 0.5 mg/ml)[10] were all purchased from Serotec (Oxford, UK).

Constitutive expression was assayed in unstimulated cells and the effect of the cytokines was assayed in stimulated cells.

The cells used for these assays were from the same cell cultures as those used to assess complement generation. After the cell culture medium was aspirated for further assay for complement the cells were detached from the flasks by EDTA-trypsin as described above. Detached cells formed a single cell suspension. The cells were immediately counted by a Coulter machine (Technicon H-1 System, USA), then washed twice in 10% heat inactivated FCS in "encriched" PBS (containing Ca^{2+} and Mg^{2+} (1 mM each), BSA and glucose (0.5% each)) and divided into flow cytometry test tubes (1×10^6 cells/ml, 0.5 ml per test tube). Flow cytometry was performed as described elsewhere.[13] Briefly, cells in flow cytometry tubes were centrifuged, the supernatant was aspirated, and the first antibody against a specific CIP molecule was introduced

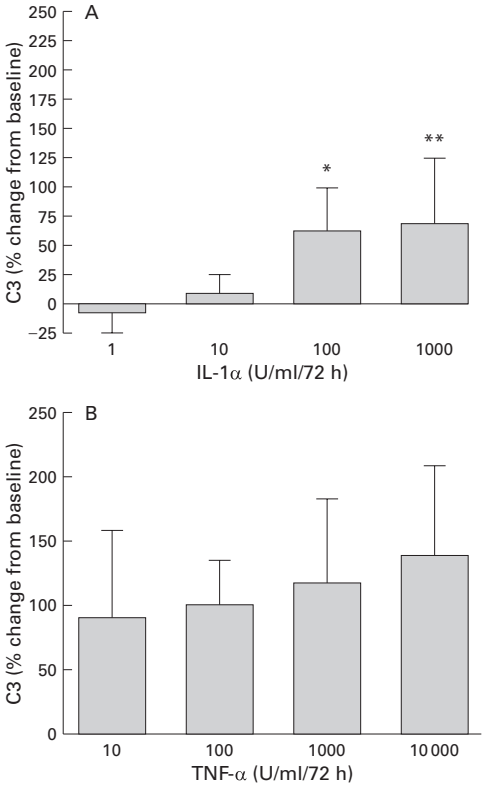

*Figure 1 Effect of IL-1α and TNF-α on complement C3 generation by human bronchial epithelium cell line BEAS-2B. Bronchial epithelial cells in culture were incubated with IL-1α and TNF-α for 72 hours. The cell culture medium was then collected and assayed for C3 by a direct ELISA technique. The absolute amount of C3 detected was expressed as ng/10^6 cells. Mean (SD) C3 generation by unstimulated cells was 32 (11) ng/10^6 cells/72 h (see results section). Values are the mean (SE) percentage change in C3 generation by cytokine stimulated cells compared with that by unstimulated cells otherwise treated in an identical manner (% change from baseline). Six experiments were performed for IL-1α and four for TNF-α. *p<0.03, **p<0.05 in relation to 1 or 10 U/ml.*

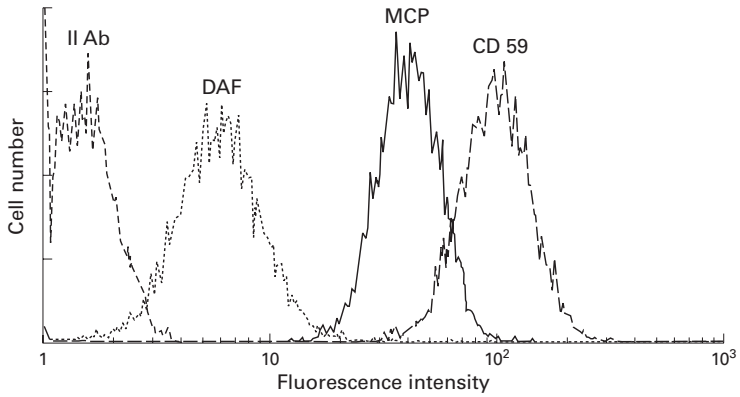

Figure 2 Flow cytometry profiles showing the constitutive expression of cell membrane complement inhibitory proteins MCP (CD46), DAF (CD55), and CD59 in human bronchial epithelium cell line BEAS-2B. Bronchial epithelial cells were grown in serum-free medium.

at a dilution of 1:50 in "enriched" PBS for 30 minutes at 4°C with gentle rotatory shaking. A control test tube was treated in the same way except that the primary antibody was omitted. At the end of incubation with the primary antibody the cells were washed twice and a secondary antibody, FITC conjugated goat F(ab')2 antibody against mouse or rat IgG, was added to the cell pellet at a dilution of 1:50, 100 µl per test tube for 30 minutes at 4°C in the dark with gentle shaking. The appropriate secondary antibody was similarly introduced into the negative control test tubes. After incubation with the secondary antibody the cells were washed twice in "enriched" PBS and 400 µl of ice cold calcium- and magnesium-free PBS were added to the cell pellet. Cell fluorescence was assayed by a flow cytometer (Epics Profile II Flow Cytometer, Coulter Electronics, Luton, UK). The voltage used was 1000 V. Cell suspensions constituted single cell suspensions, as shown by the low variability in cell size presented in the linear forward scatter histograms.

Five thousand cells were analysed by gating a uniform cell population on a two parameter histogram of forward versus side scatter. The fluroscence histograms were overlaid to determine significant differences from negative control antibodies. The effect of cytokines on CIP molecules was expressed as the percentage change in mean fluorescence intensity (MFI) compared with unstimulated control cells, as follows:

$$\% \text{ change in MFI} = \frac{\text{MFI of stimulated cells} \times 100}{\text{MFI of unstimulated cells}} - 100$$

DATA ANALYSIS

Mean values of the various treatment groups were compared by using a two way analysis of variance (ANOVA) test.

Results

COMPLEMENT C3, C1Q, AND C5 GENERATION

Unstimulated human bronchial epithelium cell line BEAS-2B generated complement C3 in serum-free medium (mean (SD) for nine experiments 32 (11.45) ng/10^6 cells/72 h, range 18–52). Of the three cytokines studied, only IL-1α had a statistically significant modulating effect, increasing the generation of C3 up to a mean of 78% compared with unstimulated cells (fig 1). TNF-α increased C3 generation up to 138% above the level of unstimulated C3 generation (fig 1) and IFN-γ at concentrations

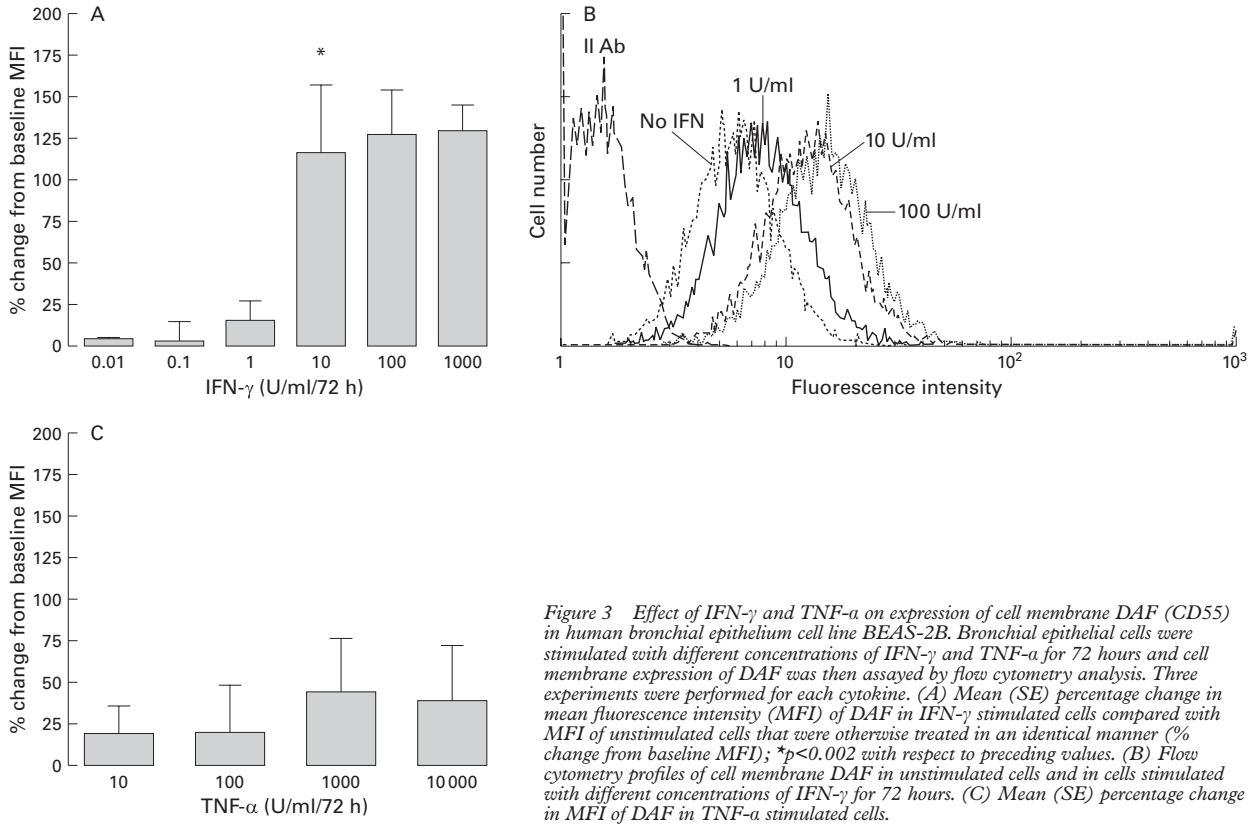

*Figure 3 Effect of IFN-γ and TNF-α on expression of cell membrane DAF (CD55) in human bronchial epithelium cell line BEAS-2B. Bronchial epithelial cells were stimulated with different concentrations of IFN-γ and TNF-α for 72 hours and cell membrane expression of DAF was then assayed by flow cytometry analysis. Three experiments were performed for each cytokine. (A) Mean (SE) percentage change in mean fluorescence intensity (MFI) of DAF in IFN-γ stimulated cells compared with MFI of unstimulated cells that were otherwise treated in an identical manner (% change from baseline MFI); *p<0.002 with respect to preceding values. (B) Flow cytometry profiles of cell membrane DAF in unstimulated cells and in cells stimulated with different concentrations of IFN-γ for 72 hours. (C) Mean (SE) percentage change in MFI of DAF in TNF-α stimulated cells.*

of 0.01–1000 U/ml had an inconsistent effect (data not shown). Complement C1q and C5 were not detected in unstimulated cells nor after cytokine stimulation (data not shown).

CELL MEMBRANE CIP EXPRESSION

Unstimulated human bronchial epithelium cell line BEAS-2B expressed MCP, DAF, and CD59 (fig 2). Of the three cytokines studied only IFN-γ (10 U/ml/72 h) had a statistically significant modulating effect on CIP expression, and only on DAF, increasing its expression after 72 h up to a mean of 130% above that in unstimulated cells (fig 3). TNF-α (10–1000 U/ml/72 h) increased DAF expression after 72 h up to a mean of 45% above that in unstimulated cells (fig 3). Expression of MCP and CD59 was not affected by IFN-γ or TNF-α. IL-1α had no effect on any of the studied cell membrane CIP molecules (data not shown).

Discussion

This study has shown that human bronchial epithelium cell line BEAS-2B generates complement C3 and expresses cell membrane CIP. C3 generation and CIP expression in these cells is a constant process that responds to stimulation by proinflammatory cytokines. These findings suggest that complement C3 may have a role in local airway defence, and it may be important for maintaining local homeostasis before exudation of plasma proteins into the air spaces. Complement C3 is a critical protein in the activation cascade of both complement pathways. The alternative complement pathway is initiated by C3 activation and acts immediately against offending microorganisms, before specific antibodies are produced.[2] C3 derivatives such as C3b, iC3b, and C3d are essential for opsonisation and phagocytosis of various microorganisms and for cell to cell adhesion.[19 20] They also have immunomodulatory effects.[3] Local generation of C3 may also affect airway function in disease. C3a elicits histamine release, smooth muscle contraction, and production of arachidonate metabolites.[4]

Our results showed that both IL-1α and TNF-α had an upregulatory effect on C3 generation by BEAS-2B bronchial epithelium while IFN-γ had an inconsistent effect. IL-1α and TNF-α are present in the human respiratory tract where they are mainly generated by active resident pulmonary macrophages.[21 22] Human eosinophils may also generate these cytokines.[23] Both cytokines are considered to have a prominent role in augmenting local airway immune defence mechanisms and it is very possible that these cytokines may also affect local generation of complement C3. Our findings are supported by Rothman *et al*[24] who found that the human type II pneumocyte cell line A549 generated C3 in response to IL-1α while IFN-γ had no effect.

The generation of complement proteins by human bronchial epithelium has not been previously reported. However, generation of complement C3 in BEAS-2B human airway epithelium is probably not limited to cells originating from the lower airways. We have found that human nasal epithelium in primary cell cultures, supplemented with 5% fetal calf serum and grown as described elsewhere,[13 15] also generates complement C3 (unpublished personal observations). Other human lung cells such as type II alveolar cells[25] and lung fibroblasts[26] have also been found to generate complement, although this occurred in a serum-containing medium.

Local generation of complement C3 is not limited to the lung. Human renal tubular epithelium was shown by Gerritsma *et al*[27] to generate C3 in a serum-free medium and its generation was increased by IL-1α. Moutabarrik *et al*[28] reported that glomerular epithelium generated C3 in a serum-free medium and its generation was increased especially after stimulation with IL-1β, and less so after stimulation with TNF-α. These findings further support our observations regarding local generation of complement C3 by human airway epithelium and its response to cytokine stimulation.

In the present study we found that human bronchial epithelium expresses cell membrane CIP molecules in a cell culture system in a serum-free medium. The BEAS-2B cell line can now be used for studying the biology of bronchial epithelium CIP. We have shown recently ex vivo in tissue biopsy specimens that cell membrane CIP molecules are expressed along the entire human respiratory tract from nose to alveoli.[12] We have also found that these CIP molecules are expressed in primary cell cultures of human nasal epithelium and in human lung cancer cell lines grown in 5–10% fetal calf serum.[12 14 29] However, expression of bronchial epithelial cell membrane CIP molecules in a serum-free medium, without the presence of added or in vivo cytokines, was examined only in the present study with BEAS-2B cells. Cell membrane CIP expression in BEAS-2B human bronchial epithelial cells is less prominent (personal observations) than that in nasal epithelial cells and significantly less than that in lung cancer cell lines as measured by flow cytometry.[13]

Our observation that cytokines modulate the expression of cell membrane DAF in human bronchial epithelium is also a novel one. Of the three cytokines studied, IFN-γ was the most potent at upregulating DAF expression while TNF-α was much less effective and IL-1α had no effect. IFN-γ is mainly generated by activated T lymphocytes (TH_0, TH_1, CD_8) and natural killer (NK) cells and acts to augment local cellular immune defence in several ways including the induction of cell membrane expression of receptors and antigens such as class I and II MHC antigens.[30] Modulation of the airway epithelium cell membrane DAF by IFN-γ might influence another aspect of local airway defence by increasing airway epithelial cell resistance to complement mediated lysis, affecting airway tissue integrity and susceptibility to injury. This possibility is suggested by our recent study in which cell membrane DAF and CD59 were shown to be critical for preventing complement mediated cell lysis of human nasal epithelial cells in primary cultures.[15] In addition, modulation

of DAF expression may affect the function of airway epithelial cells since DAF has also been found to be involved in intracellular signalling.[31 32]

Based on our recent studies,[14] it appears that the expression of DAF in human lung cancer cell lines responds somewhat differently to cytokine stimulation than in human bronchial epithelium. DAF expression in ChaGo K-1 lung cancer cells was more sensitive and more responsive to TNF-α stimulation than in BEAS-2B cells. DAF expression in NCI-H596 lung cancer cells was more sensitive, and comparably responsive, to IFN-γ stimulation while in ChaGo K-1 cells DAF expression showed a small downregulatory response to IFN-γ. IL-1α stimulated DAF expression in both lung cancer cell lines while it had no effect on DAF expression in BEAS-2B human bronchial epithelium. Whether these differences reflect cell specific inherent differences, or mere differences in cell culture conditions (serum-free versus serum containing media), is not clear.

The modulation of bronchial epithelium cell membrane DAF by cytokines shown in this in vitro study is also supported by our previous observations of increased expression of cell membrane CIP in biopsy specimens of inflamed human respiratory tract tissue.[12] In vivo it is possible that proinflammatory cytokines other than IFN-γ, and perhaps some growth factors, may affect the expression of DAF as well as the expression of MCP and CD59. Our observations of cytokine mediated modulation of bronchial epithelium cell membrane CIP expression are also supported by recent studies of the effect of proinflammatory cytokines on cell membrane CIP expression in human endothelial cells[33] and human glomerular epithelium.[25]

In summary, we have shown that the BEAS-2B cell line of human bronchial epithelium constantly generates complement C3 in a serum-free medium and that this is upregulated by IL-1α and TNF-α. We have also shown that this bronchial epithelial cell line expresses MCP, DAF, and CD59, and that DAF expression significantly increases in response to stimulation with IFN-γ.

These observations may improve our understanding of the interrelationship between human respiratory epithelium and the complement system, and add to our understanding of local inflammatory mechanisms that may affect local airway defence, tissue integrity, and airway function.

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