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Regulation of cytokine secretion by cystic fibrosis airway epithelial cells

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ABSTRACT: Chronic airway inflammation is an important feature of cystic fibrosis (CF), markedly influencing morbidity and mortality. We wanted to assess the contribution of the respiratory epithelium in the mediation of local inflammatory events, and, more particularly, its regulating role through cytokine secretion.

We have studied the regulation of interleukin-6 and 8 (IL-6 and IL-8) production by the SV40 transformed airway epithelial cell line JME/CF15 (homozygous for the deletion of Phe 508).

We show that unstimulated JME/CF15 cells secrete IL-6 and IL-8. Neutrophil chemotactic activity (NCA) is detected in supernatants. The secretion of IL-6 and IL-8 is increased following stimulation of the JME/CF15 cells by IL-1β and neutrophil elastase. Lipopolysaccharide and granulocyte macrophage colony stimulating factor (GM-CSF) have no effect on secretion of IL-6 or IL-8. Neutrophil elastase inactivates recombinant human IL-6 at 37°C *in vitro*, but has no effect at 4°C, suggesting a proteolytic effect of elastase on IL-6. IL-8 activity remains preserved, even after prolonged exposure to elastase.

Our data suggest that the airway epithelium may play an active role in the mediation of neutrophil chemotaxis. Local production of IL-8 in response to elastase and IL-1β, together with the inactivation of the anti-inflammatory protein IL-6, may result in a significant upregulation of airway inflammation in cystic fibrosis.

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Cystic fibrosis (CF) is an autosomal recessive disorder [1], caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) [2]. The principal manifestations of CF include a chronic, progressively destructive bronchitis. Characteristically, the airways of these patients are colonized with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The presence of large numbers of bacteria is associated with a marked inflammatory response, characterized by migration of neutrophils into the airway lumen [3]. The neutrophil proteases, elastase and cathepsin G, as well as other neutrophil products, are released locally in large amounts and are thought to be important mediators of tissue destruction [4]. Recent evidence suggests that biologically active anti-proteases, especially α_1-antitrypsin (α_1-AT) and secretory-leucocyte protease inhibitor (SLPI) are present in insufficient amounts to inhibit neutrophil proteases, or are themselves inactivated by host proteases, such as granulocyte elastase [5]. In addition to the local inflammation of the airways, a systemic inflammatory response is observed in CF patients, as manifested by increased plasma tumour necrosis factor (TNF) levels [6], which decrease after antibiotic treatment of pulmonary exacerbations [7].

In order to prevent the progressive destruction of airways and lung tissue, a better understanding of the pathogenesis of airway inflammation is needed. The airway epithelium plays a central role in CF lung disease, through interactions with bacteria and their exoproducts, as well as through interactions with neutrophils (transepithelial migration, attachment, effects of neutrophil proteases on epithelial cell function). It has been shown, that exposure of mucosal surfaces to bacteria results in a marked increase of interleukin-6 (IL-6) production [8]. Epithelial cells of urinary tract and intestinal origin secrete IL-6 in response to stimulation with Gram-negative bacteria [9]. Mucosal derived IL-6 may contribute to host defence mechanisms on a local level *via* stimulation of submucosal B-cells [10], or once it gains access to the circulation by inducing the acute phase response [8]. Bacterial products such as formylmethionyl peptides are probably involved *in vivo* as chemoattractants for neutrophils in the airway microenvironment [11, 12]. The recent description of neutrophil-activating peptide-1/interleukin-8 (IL-8) as a tissue-derived chemotactic cytokine for neutrophils [13] suggests that neutrophil accumulation in tissue during inflammation may also, in part, be mediated by local tissue derived factors. Although initially described as a monocyte-derived neutrophil chemotactic factor [14], the secretion of IL-8 has been reported for cells of endothelial and epithelial origin, as well as fibroblasts and hepatoma cells (reviewed in [13]). Pulmonary type II-like epithelial cells secrete IL-8 in response to alveolar macrophage derived TNF and interleukin-1 (IL-1) *in vitro* [15].

Based on the accumulating evidence for a role of epithelial cells in the mediation of neutrophil chemotaxis and IL-6 production, we decided to study the contribution of the cystic fibrosis airway epithelium to the local immune response, using an immortalized CF respiratory epithelial cell line [16]. Although we cannot exclude that transformation of cells by the large T-antigen of SV40 may influence their secretory function, the advantages of a homogeneous culture, and the fact that comparison of the results with similar models [15] is possible, outweigh this potential problem. We show in this study that CF airway epithelial cells secrete the cytokines IL-6 and IL-8, and discuss the regulation of cytokine production and bioactivity by bacterial products, cytokines and neutrophil elastase.

Materials and methods

Materials

Culture reagents (Dulbecco's modified Eagle's medium (DMEM), Ham F12, RPMI 1640), phosphate buffered saline (PBS), keratinocyte growth medium (KGM), and foetal calf serum (FCS) were purchased from Gibco (Geneva, Switzerland) and 2-ME from BioRad (Richmond, CA, USA). The following reagents were obtained from Sigma (Buchs, Switzerland): adenine, insulin, transferrin, tri-iodothyronine hydrocortisone, epinephrine, murine epidermal growth factor (EGF), trypsin ethylene diomine tetra-acetic acid (EDTA), lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, LPS from *Pseudomonas aeruginosa* serotype 10, LPS *Salmonella minnesota* Re595.

Recombinant human IL-6 (rhIL-6) and the anti-IL-6 antibody H6f4/3.17 (murine mAb IgG) were kind gifts of E. Alderman (Genetics Institute, Cambridge, MA, USA). Recombinant human IL-1 (rhIL-1) and recombinant TNF (rhTNF) were purchased from Endogen (Bioreba AG, Switzerland). Recombinant human GM-CSF was a gift from Ciba Geigy Co. (Basel, Switzerland) and rhIL-8 was a kind gift of I. Lindley, (Sandoz, Vienna, Austria).

Human neutrophil elastase was purified by H.P. Schnebli (Ciba-Geigy Research, Basel, Switzerland) as described previously [15]. Its elastolytic activity was determined using ^{3}H-elastin and the elastase specific substrate Meo-Succ-Ala-Ala-Prol-Val-7-amino-4-methyl-coumarin (Calbiochem, Luzern, Switzerland) as described previously [5]. All culture reagents were free of endotoxin contamination as assessed by the Limulus assay (Sigma, Buchs, Switzerland).

Cell Culture

The SV40 transformed nasal epithelial cell line JME/CF15 from a patient with cystic fibrosis was cultured, using lethally irradiated 3T3 fibroblasts as feeder layer as described previously [16]. The culture medium (DMEM/nutrient mixture F-12 Ham [3:1]) was supplemented with defined concentrations of adenine (1.8×10^{-4} M), insulin 5 ($\mu g\cdot ml^{-1}$), transferrin (5 $\mu g\cdot ml^{-1}$), tri-iodothyronine (2×10^{-9} M), hydrocortisone (1.1×10^{-6} M), epinephrine (5.5×10^{-6} M), epidermal growth factor (1.64×10^{-9} M) and 10% FCS. The JME/CF15 cells are airway epithelial cells, which maintain their electrophysical properties in long-term culture. We repeatedly measured their capacity to form an electrical barrier when cultured on a permeable support, and consistently found evidence for the formation of tight junctions as the cell layer became confluent (data not shown). This confirms previously published observations [16].

Cytokine secretion and regulation of cytokine secretion by CF airway epithelial cells

Confluent monolayers of JME/CF15 cells were trypsinized after washing with Ca^{2+}-free PBS. Single cell suspensions of JME/CF15 cells in complete medium DMEM/F12 were added to 24 well plates (3×10^5 cells$\cdot$well^{-1}). Following adherence, the medium was removed and the adherent cell layer washed with PBS to remove traces of FCS. To ensure that cells used for experiments did not contain any fibroblasts, sequential trypsinization of the maintenance cultures was used. With this procedure, fibroblasts are readily detached after brief incubation with trypsin/EDTA and are discarded. Only cells detached by a second incubation with trypsin/EDTA were used for experiments. In addition, the antibody used to block IL-6 bioactivity was able to block 100% of IL-6 activity in the supernatants (see results). This served as an additional control to exclude contamination of the epithelial cells by murine fibroblasts, since the anti-human IL-6 antibody does not block bioactivity of murine IL-6. Experiments were performed under serum-free conditions, using KGM supplemented with epidermal growth factor and bovine pituitary extract as culture medium. Cells maintain their morphological characteristics for several days under these culture conditions. Electrophysiological properties are also maintained (see below). The epithelial identity of the cells was confirmed by immunostaining of cells grown on Lab-Tek Chamberslides (Nunc, Naperville, Illinois, USA) using a mouse monoclonal anti-cytokeratin peptide 18 antibody (Sigma, Buchs, Switzerland).

To study the baseline production of cytokines or of chemotactic activity by JME/CF15 cells, 1 ml of KGM was added to the monolayers at time 0, and aliquots of 100 µl were removed at defined intervals during a 24 h period. Aliquots were diluted 1:4 with DMEM/0.5% FCS and stored at -20°C until assay of IL-6 activity, chemotactic activity or IL-8 enzyme-linked immunosorbent assay (ELISA). To study the regulation of cytokine secretion by JME/CF15 cells, monolayers were stimulated with various concentrations of LPS, IL-1β, TNF-α, GM-CSF, or elastase dissolved in KGM. Cells in control wells were exposed to KGM. Harvesting of aliquots and further analysis was performed as described for baseline production. In order to exclude differences in cell number between wells, the monolayers were detached by incubation with trypsin-EDTA at the end of the experiment, and the cell number determined by counting in a Coulter counter. The results of cell counting were incorporated in the analysis of cytokine production when significant differences in cell number would have influenced the data.

Electrophysiological measurements

The electrophysiological properties of the cell line were periodically evaluated, using measurements of transepithelial potential and current as described previously [16]. These measurements allow the transepithelial resistance to be calculated as indicator of the formation of tight junctions and an electrical barrier. For these measurements 1.5×10^6 JME/CF15 cells were seeded onto the upper side of a Transwell filter (Costar, Cambridge, MA, USA) with a polycarbonate membrane (pore size 0.4 μm; growth area 1.0 cm^2). In order to measure transepithelial potential and current, the filter was placed into a modified Ussing chamber with apical and basolateral agar bridges connected to electrodes of a current-voltage clamp apparatus (Physiologic Instrument VCC 600, San Diego, CA, USA).

Chemotaxis assay

A Boyden chamber assay with modifications was used [17]. Neutrophils, collected by venipuncture from normal healthy donors, were separated by density gradient centrifugation (Hypaque-Ficoll), washed in Hank's balanced salt solution (HBSS), counted and resuspended at a concentration of 10^7 cells·ml^{-1} in DMEM containing 0.5% FCS. Serial dilutions 1:25, 1:125, 1:625) of samples in DMEM/0.5% FCS were placed in the lower chamber (400 μl·$well^{-1}$) of Transwell culture chambers. Neutrophils (10^6 in 100 μl) were added to the upper chamber, which is separated from the lower chamber by a polycarbonate membrane with 3.0 μm pores. L-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (10^{-7} M) in DMEM/0.5% or DMEM/0.5% was used as positive and negative control. Chemotaxis was allowed to proceed for 60 min at 37°C/5% CO_2. Neutrophil migration was stopped by removing the filter from the wells after thorough rinsing of the lower side of the filter with medium of the lower chamber. Aliquots of the lower chamber (200 μl) were added to 20 ml of Isoton solution II (Coultronics France, Margency, France) and the number of neutrophils was determined by automated counting (Coulter counter). Chemotactic activity of samples is expressed as a chemotactic index (CI).

$$CI = \frac{\text{number of neutrophils (test sample)}}{\text{number of neutrophils (negative control)}} \times \text{fold dilution of test sample}$$

Interleukin 6 assay

IL-6 bioactivity of samples was measured using the IL-6-dependent murine hybridoma cell line B9 [18]. Proliferation of the B9 cells was determined by MTT-assay as described by MOSMANN [19]. In brief 5×10^3 B9 cells·$well^{-1}$ were added to serial dilutions of test conditions in triplicate, up to a volume of 0.1 ml·$well^{-1}$. MTT (3-[4,5-dimethylthiazol (-2-yl]-2,5-diphenyltetrazoliumbromide; 20 μl, 2.5 mg·ml^{-1}; Sigma) was added to each well during the last 4 h of a 96 h incubation period at 37°C, 5% CO_2. After addition of 150 μl 0.04 N HCl in 2-propanol to each well, the plates were wrapped in Saran wrap and kept at room temperature overnight to allow dissolution of crystals formed. Absorbance was measured on an automated ELISA plate reader, at 550 nm, and results compared with a standard curve, using recombinant IL-6 as stimulus for B9 cell proliferation. Data are represented as the mean (±SD) of triplicate determinations.

Interleukin 8 assay

The presence of interleukin-8 in medium conditioned by JME/CF15 cells was determined using the Quantikine IL-8 ELISA kit (R&D systems, Minneapolis, MN, USA). The ELISA was performed exactly as described in the manufacturers instructions.

Statistical analysis

Student's t-test was used to determine the statistical significance between means of at least three experiments.

Results

Regulation of IL-6 production by cytokines

Supernatants of confluent monolayers of JME/CF15 cells contained biological activity, which stimulated the proliferation of B9 cells in a dose-dependent manner (fig. 1a). B9 cells have been described as dependent on IL-6, and responding to IL-6, but not to other known cytokines and growth factors, with the exception of IL-4. In order to confirm the identity of the JME/CF15 cell derived B9 proliferative factor, we incubated serial dilutions of the conditioned medium with the anti-IL-6 antibody H6f4/3.17.1 or with control antibody (anti-TNF) overnight at 4°C. Following preincubation, B9 assay was performed on all dilutions. Supernatants of JME/CF15 cells preincubated with carrier medium (PBS) alone stimulated B9 cell proliferation even after 240 fold dilution (fig. 1b). This B9 stimulatory effect was completely abolished after preincubation with anti-IL-6 antibody. Preincubation with anti-TNF antibody did not affect the capacity of the supernatant to stimulate B9 cell proliferation (not shown). Based on these results we conclude that cystic fibrosis airway epithelial cells secrete IL-6.

Secretion of IL-6 by JME/CF15 cells gradually increased during the first 8 h, and reached a plateau between 8–24 h following a change of culture medium. Stimulation of the cell layer with IL-1β (10 ng·ml^{-1}) results in a marked increase of IL-6 secretion. This effect was already apparent 2 h after addition of IL-1β, but IL-6 bioactivity continued to increase within the first 8 h of the experiment, after stimulation by the cytokine. In contrast to IL-1β, stimulation with LPS from *E. coli* (0111:B4) at concentrations ranging from 10 ng·ml^{-1} to 1 μg·ml^{-1} did not increase IL-6 bioactivity in the supernatant (shown in figure 2: LPS 1 μg·ml^{-1}). Similarly, GM-CSF at 100 U·ml^{-1} had no effect on IL-6

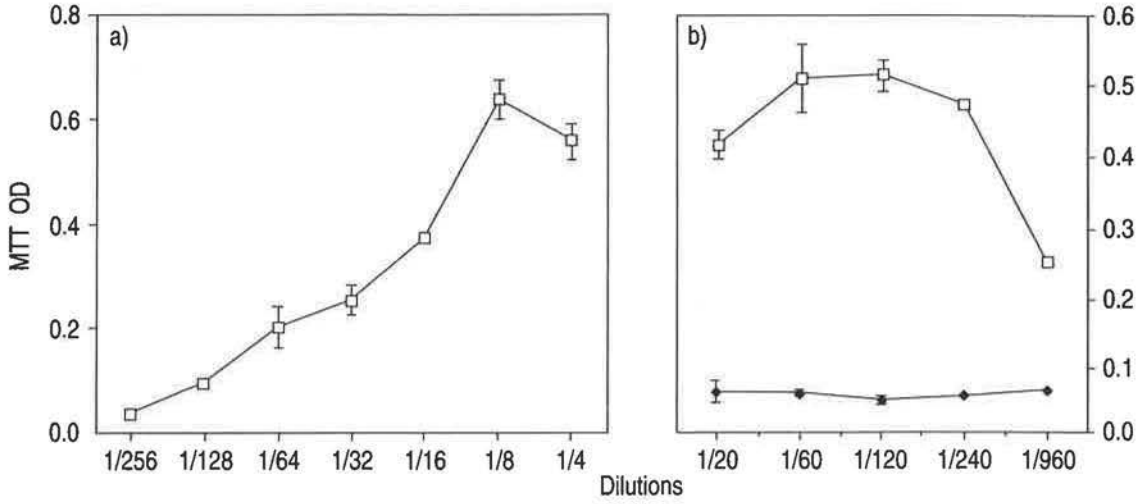

Fig. 1. – Supernatant of JME/CF15 cells stimulates proliferation of B9 cells. B9 cells were cultured with serial dilutions of supernatant harvested after 24 h. a) mean values and standard deviations of triplicate determinations of optical density (550 nm) (OD) after MTT assay of B9 cells. b) anti-IL-6 antibody blocks JME/CF15 derived IL-6 activity. Supernatants derived from JME/CF15 cells were incubated with anti-IL-6 antibody H6f4/3.17 (5.5 μg·ml^{-1}) or PBS (control) and tested in the IL-6 assay (B9 cells). Means and standard deviations of triplicate determinations are shown. —□—: control; —◆— : anti-IL-6 (5.5 μg·ml^{-1}). PBS: phosphate buffered saline; IL-6: interleukin-6.

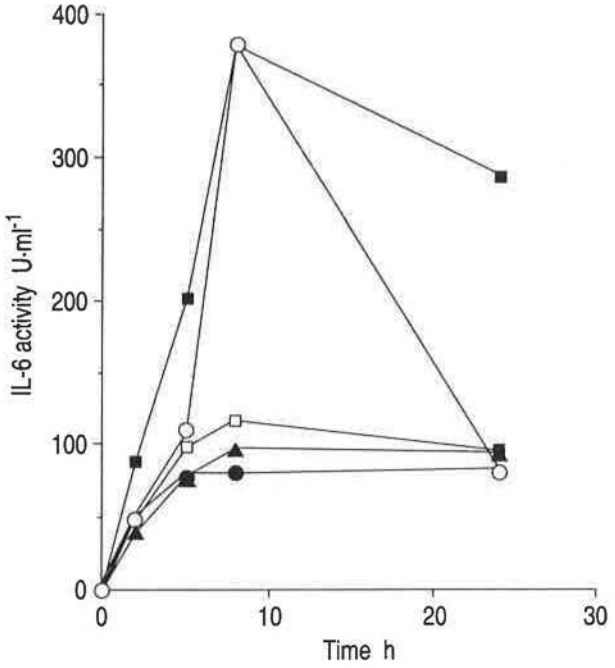

Fig. 2. – Kinetics of IL-production by JME/CF15 cells. Cells were cultures in KGM containing LPS from *E. coli* (1 μg·ml^{-1}), IL-1β (10 ng·ml^{-1}), GM-CSF (100 U·ml^{-1}), elastase (100 nM) or no additive (control). Supernatanats were harvested at 2, 5, 8 or 24 h and tested for IL-6 activity. Mean IL-6 activity (U·ml^{-1}) of supernatants from two wells is shown for each time point and condition. —□—: control; —●— : LPS; —■— : IL-1β; —▲— : GM-CSF; —○— : elastase. IL-6: interleukin-6; LPS: lipopoplysaccharide; GM-CSF: granulocyte macrophage colony stimulating factor; IL-1β: interleukin-1β; KGM: keratinocyte growth medium.

secretion. Figure 2 shows results of one of four experiments. Similar kinetics of cytokine secretion were observed in all four experiments. In additional experiments, we observed a marked stimulatory effect of TNF on IL-6 release, whereas LPS from *P. aeruginosa*, *E. coli* and *S. minnesota* Re595 had no effect (table 1).

Effect of neutrophil elastase on IL-6 production and IL-6 bioactivity

Since CF respiratory epithelial cells are exposed to neutrophil elastase *in vivo* [4], we decided to study the effect of purified granulocyte elastase on IL-6 secretion by JME/CF15 cells *in vitro*. As shown in figure 2, the coincubation of JME/CF15 cells with neutrophil elastase (100 nM) also increased the secretion of IL-6. Lower concentrations of elastase were less effective (not shown). After 8 h, IL-6 activity in response to elastase reached practically identical levels to the levels measured following stimulation with IL-1β. However, IL-6 activity at 24 h returned to the same level as IL-6 activity of unstimulated conditions.

Table 1. – Stimulation of IL-6 release by JME/CF15 cells

	IL-6 activity U·ml^{-1}		
Stimulant	KGM[a]	DMEM/F12[b]	KGM[c]
0	7.2 (0.7)	16.0 (1.0)	12.4 (0.3)
Elastase 100 nM	26.4 (9.4)	36.4 (1.7)	ND
LPS* from *P. aeruginosa*	-	-	10.1 (1.7)
LPS from *S. minnesota* Re 595	-	-	12.2 (0.8)
LPS from *E. coli* 0111:B4	-	-	13.2 (0.4)
IL-1β 20 ng·ml^{-1}	-	-	32.5 (3.4)
TNF-α 20 ng·ml^{-1}	-	-	30.6 (7.2)

Data are presented as mean and (SD) in parenthesis. [a]: the cells were stimulated with reagents diluted in KGM (serum-free condition) in parallel with [b]. Cell numbers were not significantly different between KGM and DMEM/F12. [b]: the cells were stimulated with reagents diluted in DMEM/F12 with 10% FCS in parallel with [a]. [c]: the data are from a separate experiment. *: all types of LPS were used at 1 μg·ml^{-1}. Data of experiment using concentration of 500 ng·ml^{-1} were similar (not shown). IL: interleukin; KGM: keratinocyte growth medium; DMEM: Dulbecco's modified Eagle's medium; LPS: lipopolysaccharide; TNF-α: tumour necrosis factor-α; ND: not determined; FCS: foetal calf serum.

This suggests that a significant proportion of IL-6 bioactivity was lost during the time period between 8–24 h. The rise of IL-6 levels in the supernatants following stimulation with elastase was not due to proteolytic cleavage of IL-6 located on the surface of the epithelial cells, since the effect of elastase on IL-6 secretion was similar when the experiment was performed in medium containing 10% FCS (table 1), which effectively abrogates the enzymatic activity of elastase (data not shown).

Based on the result described above documenting a "loss" of IL-6 activity between 8 and 24 h in the presence of neutrophil elastase, we decided to study the possibility that neutrophil elastase may inactivate rhIL-6 directly. Recombinant human IL-6 was exposed to various concentrations of neutrophil elastase (0.1–100 nM) at 37°C for 24 h. IL-6 activity was then measured in the B9 assay, using RPMI medium containing 10% FCS to prevent any carry over effect of elastase on the B9 cells. In addition, rhIL-6 mixed with elastase immediately prior to the B9 assay retained its full biological activity on the B9 cells. These observations virtually exclude any interference of elastase with the bioassay used. As shown in figure 3, exposure of rhIL-6 to concentrations of neutrophil elastase between 1.0–100 nM resulted in a dose-dependent reduction of IL-6 bioactivity. This reduction was not observed or was less marked, following preincubation at 4 or 20°C, instead of 37°C (table 2), suggesting that enzymatic activity of neutrophil elastase is responsible for the loss of IL-6 bioactivity. In contrast to IL-6, preincubation of rhIL-8 with elastase at the same temperatures did not affect the chemotactic activity of this peptide (table 2).

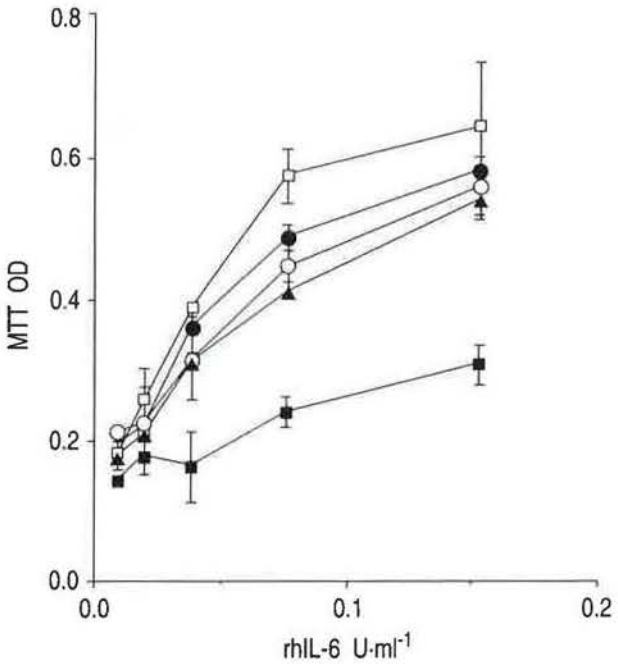

Fig. 3. – Effect of elastase on IL-6 bioactivity after *in vitro* incubation. Recombinant interleukin-6 (rhIL-6) was incubated at 37°C with various concentrations of elastase (0.1–100 nM), or control (KGM), in the absence of serum. After 24 h serial dilutions of each condition were tested for their activity in the IL-6 assay (MTT of B9 cells). Mean and standard deviations of triplicate conditions are shown. —□— : control; —●— : elastase (0.1 nM) —○— : elastase (1 nM); —▲— : elastase (10 nM); —■— : elastase (100 nM). For abbreviations see legends to figures 1 and 2.

Table 2. – Temperature-dependent effect of elastase* on bioactivity** of IL-6 and IL-8

Temperature °C	Reduction† of IL-6 bioactivity %	Reduction of IL-8 bioactivity %
4	0	0
20	2.2	0
37	70	0

*: recombinant human IL-6 (5 U·ml^{-1}) and IL-8 (5 ng·ml^{-1}) were incubated at the temperatures indicated over 24 h, in the absence or presence of elastase (10^{-7} M). **: at the end of the incubation period, bioactivity of the cytokines was tested in the B9 assay (IL-6), and the neutrophil chemotaxis assay (IL-8), as described in the methods. †: results of the bioassay were compared to the results obtained with cytokines incubated in the absence of elastase (control). The results in table indicate percentage reduction of bioactivity compared to control. IL: interleukin.

Release of neutrophil chemotactic activity by airway epithelial cells

In order to study whether CF airway epithelial cells might contribute to the neutrophil chemotactic activity found in airway secretions, we measured chemotactic activity for neutrophils of medium conditioned by unstimulated monolayers of JME/CF15 cells. Fourfold diluted medium placed in the lower compartment of a Transwell system elicited a marked chemotactic response by human neutrophils. As illustrated by figure 4a, which shows a representative experiment, the effect of fourfold diluted conditioned medium on neutrophil chemotaxis was substantially more pronounced than the effect of the positive control formyl-methionyl-leucyl-phenylalanine (FMLP) (1.0–100 nM). The release of chemotactic activity by unstimulated JME/CF15 cells into the supernatant occurred in a time-dependent manner, and gradually increased over the observed 24 h (fig. 4b).

In parallel to the release of IL-6 following stimulation of the JME/CF15 cells by cytokines, LPS or elastase, we determined the chemotactic activity in the same supernatants. We found spontaneous production of chemotactic activity in all experiments (n=3), and observed a gradual rise of chemotactic activity in response to IL-1β, which was moderately higher at 5 and 24 h than chemotactic activity of control conditions. The differences between chemotactic index of unstimulated control and IL-1β stimulated cells were, however, not statistically significant (data not shown). The chemotactic index following stimulation with elastase was consistently lower than the chemotactic index of supernatants from control at all time points tested. Resembling the regulation of IL-6 release, production of chemotactic activity was not increased after stimulation of the epithelial cells with LPS and GM-CSF.

Regulation of IL-6 secretion in airway epithelial cells

In light of our data documenting the release of chemotactic activity by JME/CF15 cells and of published reports

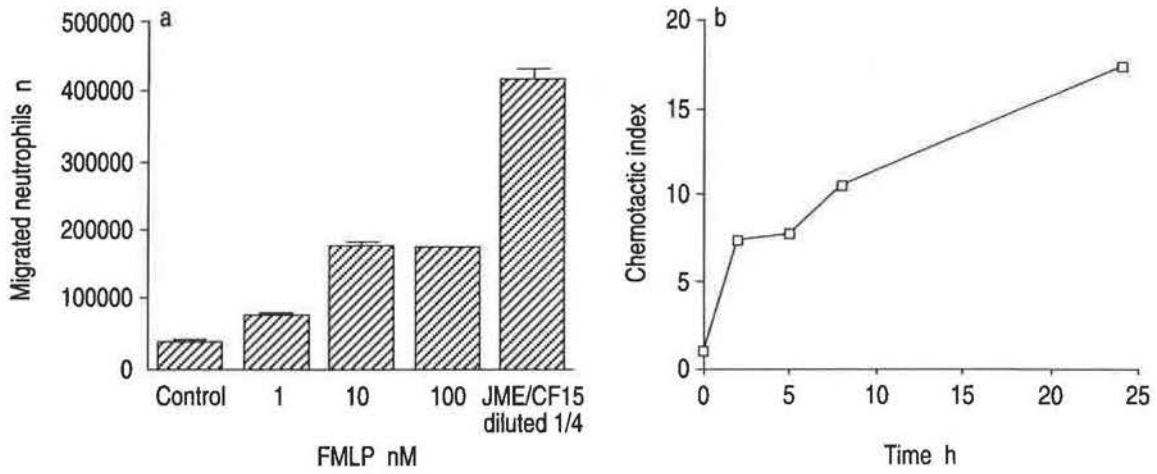

Fig. 4. – a) neutrophil chemotaxis in response to FMLP or supernatant of JME/CF15 cells. Neutrophils (10^6) were allowed to migrate across a transwell filter membrane in the presence of control (DMEM/F12, 0.5% FCS), FMLP (1–100 nM) or four fold diluted supernatant of JME/CF15 cells. Neutrophils, which had migrated into the lower chamber were counted after 60 min (Coulter counter) in triplicate. Mean and SD are shown. b) Kinetics of spontaneous release of neutrophil chemotactic activity by JME/CF15 cells. Cells were cultured in KGM. Supernatants were harvested at 2, 5, 8 or 24 h and tested for neutrophil chemotactic activity. The mean chemotactic index of each time point of duplicate cultures is shown. FMLP: formyl-methionyl-leucyl-phenylalanine; FCS: foetal calf serum; DMEM; Dulbecco's modified Eagles medium; KGM: keratinocyte growth medium.

showing that the neutrophil chemotactic peptide IL-8 is secreted by other epithelial cells stimulated by IL-1β, we tested supernatants of unstimulated, as well as stimulated, JME/CF15 cells for the presence of IL-8. Using a specific double-sandwich ELISA, we detected IL-8 in supernatants of unstimulated cells. Whilst the effect of IL-1β on chemotactic activity was only modest, its effect on IL-8 secretion was marked (fig. 5). IL-8 levels increased rapidly, and remained clearly higher than in supernatants of unstimulated cells. At 24 h the IL-8 concentration was 34.8 ng·ml^{-1} in supernatants of cells stimulated by IL-1β, compared to 23.8 ng·ml^{-1} in supernatants of unstimulated cells. This difference was statistically significant (p=0.010; Student's t-test). Reflecting a pattern similar to the one seen in the chemotaxis assay, stimulation of JME/CF15 cells with LPS did not increase IL-8 release. Neither did elastase at a concentration of 10 nM. However, elastase at 100 nM resulted in higher levels of IL-8 at 5 and 8 h than control conditions. At 24 h, the IL-8 level in supernatants of cells stimulated with elastase (100 nM) was not higher than in supernatants from controls. In another experiment, the mean IL-8 concentration in supernatants of unstimulated cells was 16.5 ng·ml^{-1}, compared to 33.3 ng·ml^{-1} in supernatants of cells stimulated by elastase (100 nM). The concentrations of IL-8 measured in supernatants of JME/CF15 cells are sufficient to elicit a chemotactic response. In parallel experiments using recombinant IL-8, we found a dose-dependent stimulation of neutrophil chemotaxis in a range of 5–50 ng·ml^{-1} rhIL-8 (data not shown). Our inability to detect significant differences between the chemotactic index of unstimulated and stimulated cells may possibly be explained by the fact that IL-8 levels in supernatants of both unstimulated and stimulated cells were in a concentration range of 10–25 ng·ml^{-1}, where increases of IL-8 concentration do not necessarily result in further augmentation of chemotactic activity.

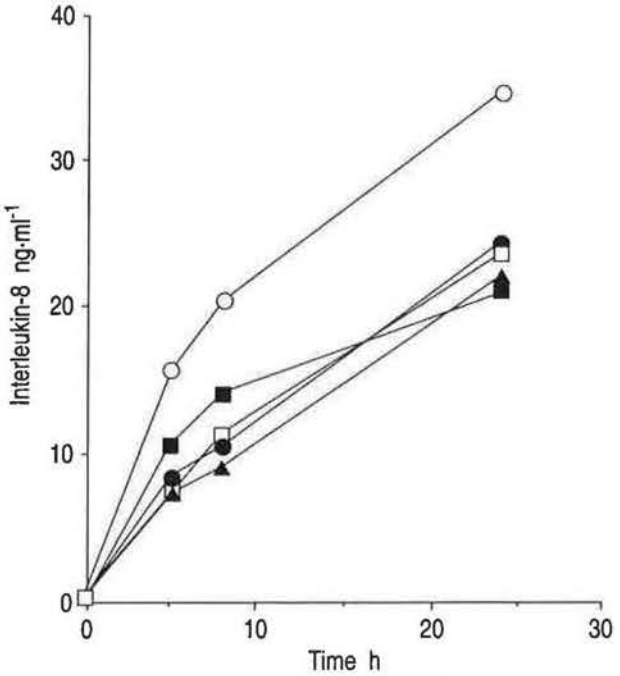

Fig. 5. – Regulation of IL-8 secretion by JME/CF15 cells. Cells were stimulated with IL-1β (10 ng·ml^{-1}), LPS from *E. coli* (1 μg·ml^{-1}), elastase (10, 100 nM) and control. Supernatant was harvested after 5, 8 and 24 h. IL-8 levels were measured by ELISA. Means of duplicate determinations are shown. —□— : control; —○— : IL-1β; —■— : elastase (100 nM); —▲— : elastase (10 nM); —●— : LPS (1 μg·ml^{-1}). ELISA: enzyme-linked immunosorbant assay. For further abbreviations see legend to figure 2.

Discussion

Chronic airway inflammation dominated by neutrophils and their secretory products is an important manifestation of CF. The chronicity of bacterial colonization is probably an important cause of this process, which results in progressive destruction of the integrity of the airways. Our present study suggests that the cystic fibrosis airway epithelium

participates in local host defence mechanisms, and may play a role in perpetuating local inflammation.

We have shown that the cystic fibrosis airway epithelial cell line JME/CF15 secretes the cytokines IL-6 and IL-8. IL-6 secreted by the CF airway epithelial cell (CF-AEC) line was identified, based on the stimulation of the IL-6 dependent cell line B9 in response to serial dilutions of medium conditioned by JME/CF15 cells. The specific blocking of B9 proliferation following preincubation of the supernatant by anti-IL-6 antibody further confirmed the identity of this B9 proliferative factor with IL-6. IL-6 production was upregulated following stimulation with IL-1β and TNF-α, whereas LPS and GM-CSF had no effect.

Granulocyte elastase serves as an additional signal to regulate production of IL-6 by CF-AEC. As illustrated by figure 2, release of IL-6 into the supernatant occurred just as rapidly, and in similar quantities, after exposure of the CF-AEC to elastase as after exposure to IL-1β. The effect of elastase on IL-6 levels in supernatants during the first 8 h of the experiment was similar under serum-free conditions and conditions including 10% FCS (table 1), which abrogate the enzymatic effect of elastase. This suggests that elastase induces *de novo* synthesis of IL-6 by CF-AEC, rather than cleavage of cell surface bound IL-6. The study by NAKAMURA *et al.* [20], who recently documented that neutrophil elastase induces IL-8 gene expression in a human bronchial epithelial cell line, clearly establishes the role of elastase as an inducer of cytokine production in airway epithelial cells.

Our kinetic experiments have shown a dual effect of elastase on IL-6. The observed marked reduction of IL-6 bioactivity after the initial rise of IL-6 suggested that elastase degrades IL-6. Coincubation of rhIL-6 with elastase *in vitro* resulted in a temperature and concentration (elastase) dependent loss of IL-6-bioactivity, suggesting that enzymatic activity is responsible for the reduction of IL-6 bioactivity. Similar observations have been made for the action of Pseudomonas elastase on TNF-α [21].

Since sputum of CF patients did not contain measurable IL-6 bioactivity (own unpublished information), we postulate that a similar degrading effect of elastase on IL-6 derived from the airway mucosa may also be taking place *in vivo*. Whether Pseudomonas elastase contributes to the degradation of airway IL-6 *in vivo* is unknown.

In addition to IL-6, the CF airway epithelial cell line JME/CF15 spontaneously produces neutrophil chemotactic activity and IL-8. The production of IL-8 is, at least partially, responsible for the observed neutrophil chemotactic activity in supernatants of JME/CF15 cells, since the levels of IL-8 found in the supernatants are well within the chemotactic range (5–50 $ng \cdot ml^{-1}$). IL-8 release by JME/CF15 cells is stimulated by IL-1β and the neutrophil-derived protease elastase. Stimulation with LPS from *E. coli*, *S. minnesota* Re595 and *P. aeruginosa* did not result in increased production of NCA or IL-8. Unlike IL-6, IL-8 appears to be unaffected by the proteolytic effects of elastase, since incubation of rhIL-8 with elastase at concentrations ranging from 0.1–100 nM at 37°C did not affect its chemotactic effect on neutrophils. Similarly, NAKAMURA *et al.* [20] showed that elastase at concentrations ranging from 10 nm–10 μM did not degrade rhIL-8, as examined by Western blot analysis.

Our results studying the regulation of IL-8 production by JME/CF15 cells are in agreement with the findings of NAKAMURA *et al.* [20], who reported on upregulation of IL-8 gene expression in the SV40 transformed human bronchial epithelial cell line BET-1A by purified neutrophil elastase, TNF-α and epithelial lining fluid from an individual with CF. However, some differences between their results and ours are noteworthy. In contrast to their findings, we did observe a marked release of chemotactic activity and IL-8 by JME/CF15 cells under unstimulated conditions. This may explain our finding of only a modest effect of elastase (100 nM) on the release of IL-8 by the CF-AEC line. Whether the difference in baseline production of IL-8 between BET-1A and JME/CF15 cells reflects true differences between CF and normal epithelial cells, or whether this simply reflects variations among cell lines, could not be established in this study. STANDIFORD *et al.* [15], who documented IL-8 production by the alveolar type II-like epithelial cell line A549, also observed an upregulation of IL-8 release in response to IL-1β and TNF-α, and a lack of response to LPS. Similarly, synovial cells and fibroblasts do not upregulate IL-8 release following LPS stimulation [22, 23]. In contrast to these cell types, mononuclear phagocytes [24], as well as endothelial cells [25], respond to LPS by increasing their IL-8 secretion.

The airway epithelium is certainly exposed to bacterial LPS, and an inflammatory response has been observed *in vivo* after intratracheal instillation of LPS [26]. How is this response generated, if LPS has no direct effect on the epithelial cell? The most likely explanation is the model proposed by STANDIFORD *et al.* [15], who postulated the existence of a local cytokine network in the airways, with interactions between monocyte/macrophages and epithelial cells. The former release IL-β and TNF in response to bacterial LPS. These soluble products, in turn, stimulate the release of IL-8 by epithelial cells. Local release of IL-8 by epithelial cells of the airway mucosa creates a gradient across the mucosa, which promotes neutrophil migration into the airway lumen. In addition, IL-8 has activating effects on neutrophils, resulting in release of elastase and oxygen radicals [13].

Based on the work of STANDIFORD *et al.* [15], NAKAMURA *et al.* [20] and our study, the impression emerges that the regulation of airway inflammation is the result of the interactions of multiple pro- and anti-inflammatory signals. As already pointed out by NAKAMURA *et al.* [20], the signal initiating the recruitment of neutrophils remains unknown. LPS as a consequence of bacterial colonization may be a sufficient signal resulting in IL-8 production *via* induction of the proinflammatory cytokines IL-1β and TNF-α [26]. IL-8, in concert with other chemotactic factors, may then promote the initial influx of neutrophils. Neutrophils present in the airway lumen release elastase, which stimulates IL-8 release by epithelial cells, thus perpetuating a "cycle of inflammation on the respiratory epithelium in CF" (NAKAMURA *et al.* [20]), which results in the migration of more neutrophils into the airway lumen. This cycle is normally interrupted by counter-regulatory mechanisms.

The description of an anti-inflammatory effect of intratracheally injected rhIL-6 by ULICH *et al.* [26] supports the view that this cytokine may downregulate local inflammation. Co-administration of IL-6 and LPS resulted in marked inhibition of the inflammatory response, which was observed after the injection of LPS alone, and consisted of a massive influx of neutrophils into the tracheal lumen [26]. These authors also demonstrated that local whole lung IL-6 messenger ribonucleic acid (mRNA) expression was upregulated after the intratracheal injection of LPS. IL-6 may, therefore, act as an endogenous negative feedback mechanism, to stop the neutrophil accumulation of an acute inflammatory response [26]. However, the level of neutrophil elastase present in the airway lumen of CF patients is sufficient to inactivate IL-6. Thus, this anti-inflammatory protein is eliminated locally, along with the antiprotease α_1-antitrypsin and secretory leucocyte protease inhibitor (SLPI). Based on this scenario, therapeutic interventions, such as local administration of recombinant protease inhibitors as an attempt to block the deleterious effects of neutrophil elastase on the epithelium in CF, appear to be crucial for a better control of the inflammatory aspects of CF.

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