

Altered respiratory epithelial cell cytokine production in cystic fibrosis

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Background: Reports that lung inflammation in patients with cystic fibrosis (CF) might precede infection raise the possibility that the excessive inflammatory response in lungs of patients with CF might be directly related to defects in epithelial cell cystic fibrosis transmembrane regulator.

Objective: We sought to determine the relationship of epithelial cell cytokine production to CF lung disease.

Methods: Immunofluorescence and cultures of freshly obtained bronchial epithelial cells and ELISA for IL-10, IL-8, and IL-6 were used to study alterations in epithelial cell cytokine production.

Results: Fresh bronchial epithelial cells from healthy control subjects (HCs) secreted 98 ± 20 pg/mL of the anti-inflammatory cytokine IL-10 when placed in primary culture in vitro but little or no IL-8 or IL-6. In contrast, fresh epithelial cells from patients with CF did not secrete detectable IL-10 but produced 38 ± 17 pg/mL IL-8 and 40 ± 17 pg/mL IL-6. These data correlated very well with the immunofluorescence data. The correlation between the immunofluorescent staining of fresh bronchial epithelial cells from both the HCs and patients with CF and the concentrations of cytokines in epithelial lining fluid suggests a reciprocal relationship between anti-inflammatory (IL-10) and proinflammatory (IL-6 and IL-8) cytokine production by the epithelial cells in HCs versus patients with CF.

Conclusions: Alterations in epithelial cell cytokine production in the lungs of patients with CF may contribute to the excessive local inflammation. (*J Allergy Clin Immunol* 1999;104:72-8.)

Key words: Epithelial cells, cystic fibrosis, cytokines, inflammation, lung disease

Proinflammatory cytokines, including IL-1 β , TNF- α , IL-6, and IL-8 can mediate cachexia, hyperglobulinemia, and airway infiltration by neutrophils¹⁻⁴ and may have a pathogenic role in cystic fibrosis (CF) lung disease.⁵ We have previously shown that lung epithelial lining fluid (ELF) obtained from patients with CF contains elevated levels of IL-1 β , TNF- α , IL-6, and IL-8 as compared with ELF from healthy control subjects (HCs). We have also shown that ELF from HCs contains 3.5 ± 1.3 ng/mL of IL-10, whereas ELF from patients with CF had only $1.3 \pm$

Abbreviations used

BAL: Bronchoalveolar lavage
BEC: Bronchial epithelial cell
CF: Cystic Fibrosis
ELF: Epithelial lining fluid
HC: Healthy control subject

0.8 ng/mL. One half of these patients with CF had no detectable IL-10.⁶ IL-10 has been shown to decrease the production of TNF- α , IL-1 β , IL-6, and IL-8 by macrophages^{7,8} and to inhibit immune complex-induced lung injury in experimental models.^{9,10} Furthermore, IL-10 decreases TNF- α production and death after endotoxin injection in animals.¹¹ These observations suggest that IL-10 may have potent effects in vivo. We have also demonstrated that bronchial epithelial cells (BECs) recovered from brushings of healthy airways express IL-10 as indicated by specific immunofluorescent staining, suggesting that these cells may be a constitutive source of IL-10 in the healthy lung.⁶ In contrast, BECs obtained from patients with CF contained little IL-10, suggesting that decreased IL-10 production might contribute to the excessive inflammation that characterizes CF lung disease.⁶ In this article, we demonstrate an inverse relationship between the production of IL-10 and the production of IL-8 and IL-6 by BECs from patients with CF as compared with HCs. We hypothesize that in the healthy lung, BECs produce IL-10, which can downregulate the production of proinflammatory cytokines. In the diseased lungs of patients with CF, decreased epithelial cell IL-10 production may permit increased and/or persistent epithelial cell and macrophage production of proinflammatory cytokines, which leads to excessive inflammation and lung destruction.

METHODS

Subjects

Nine patients with CF (6 female patients and 3 male patients; aged 17 to 27 years) who were clinically stable without pulmonary exacerbation and who had not been hospitalized within 3 months of this study were recruited from our CF center for immunofluorescence studies. Lung secretions from all of these patients contained *Pseudomonas aeruginosa* on culture. Nine healthy nonsmoking adult volunteers (2 female volunteers and 7 male volunteers; aged 21 to 28 years) served as control subjects. For the in vitro secretion data, an additional 6 HCs (2 female subjects and 4 male subjects) and 4 patients with CF (3 female patients and 1 male patient) were studied. None were taking corticosteroids, ibuprofen, or other anti-inflammatory medications. In addition to these patients 2 infants

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Supported in part by National Institutes of Health grants HL 07415, DK 27651, HL 37117, HL 46396, and RR-00080 and by the Cystic Fibrosis Foundation.

Received for publication July 30, 1998; revised Feb 25, 1999; accepted for publication Feb 25, 1999.

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0091-6749/99 \$8.00 + 0 1/1/98180

with CF (1 infant was 5 months old and the other infant was 8 months old) were also studied. This study was approved by the Institutional Review Board of University Hospitals of Cleveland; written informed consent was obtained from all study subjects and the parents of the infants.

BECs and cell lines

Fresh BECs were obtained from patients with CF and HCs by bronchial brushing; bronchoalveolar lavage (BAL) was performed as previously described.^{5,6} Cytospin preparations were prepared as described below, or cells were cultured in Lab-Tek chambers (Nunc, Inc, Naperville, Ill) at 5×10^4 cells in 0.5 mL of specialized media (referred to here as DF, consisting of a mixture of equal parts of DMEM and Ham's F12 media [Gibco] supplemented with insulin, transferrin, hydrocortisone, and retinoic acid as described elsewhere).^{12,13} After 2 hours the media was gently removed, centrifuged, and stored at -70°C until ELISA assays could be performed for IL-10, IL-8, and IL-6 with kits from Biosource International (Camarillo, Calif). The sensitivity of the assays was between 5 and 10 pg/mL. All of the samples were run in parallel with the supernatants of LPS-stimulated peripheral blood monocytes and purified recombinant IL-10 (rIL-10; Biosource International and Pharmingen) as controls.

Immunocytochemistry of fresh BECs

Fresh BECs from patients with CF and HCs were washed twice in cold HBSS (Gibco, Grand Island, NY) containing 100 $\mu\text{mol/L}$ phenylmethylsulfonyl fluoride, 5 mmol/L EDTA, and 0.1% BSA (HBSS⁺). Cytospin preparations of 2.0×10^4 fresh epithelial cells were prepared as previously described^{5,6,14} and evaluated for the presence of intracellular IL-10, IL-8, and IL-6 by immunofluorescence. Slides were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 for 1.5 minutes in PBS. Non-specific binding was minimized by incubation in 5% normal goat and/or rabbit serum (Atlanta Biologicals, Norcross, Ga) followed by incubation with rat anti-human IL-10 (Pharmingen), rabbit anti-human IL-8 (R&D Systems), or rabbit anti-human IL-6 (R&D Systems), as described previously.^{5,6,14} Most of the slides were also incubated with mouse anti-human cytokeratin (Boehringer Mannheim, Indianapolis, Ind) to identify epithelial cells, although orientation and polarity define the epithelial cells as well. After washes, phycoerythrin-conjugated F(ab')₂ rabbit anti-rat IgG or goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) or FITC-conjugated F(ab')₂ goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc) were added to detect IL-10, IL-8, or IL-6 and cytokeratin, respectively, as described in detail previously.^{5,6} Slides were evaluated for intensity and percent of epithelial cells staining positive for IL-10, IL-8, or IL-6 by a microscopist who was unaware of their origin by comparison to slides treated with FITC antibody alone. Four fields were evaluated, and at least 100 epithelial cells were counted. Specimens from every patient and HC were stained for all 3 cytokines.

Statistical analysis

Comparisons between samples from patients with CF and HCs were performed with the Student *t* test.

RESULTS

Fresh BECs from HCs and patients with CF were examined by specific immunofluorescence for expression of IL-10, IL-8, and IL-6 (Fig 1). Epithelial cells from HCs expressed IL-10 but little or no IL-8 or IL-6, although epithelial cells from patients with CF expressed

much less IL-10 but stained brightly for IL-8 and IL-6. A microscopist who was unaware of the source of the samples evaluated similar slides from each of the 9 HCs and 9 patients with CF and recorded the percent of epithelial cells that stained positive for each cytokine and the intensity with which the cells were stained. Each patient and HC is represented by an individual symbol. Epithelial cells from HCs had higher numbers of IL-10⁺ cells ($n = 9$; $79\% \pm 5\%$; Fig 2) and increased intensity of staining, 3 ± 0.3 , as compared with epithelial cells from CF subjects, $12\% \pm 4\%$ and 1 ± 0.3 , respectively ($n = 9$; $P < .001$, for both intensity and positivity). In contrast, an increased percentage of the epithelial cells from patients with CF stained brightly for IL-8 ($78\% \pm 6\%$; Fig 2) and IL-6 ($78\% \pm 4\%$; Fig 2) as compared with $24\% \pm 8\%$ ($n = 9$; $P < .001$) and $24\% \pm 7\%$ ($n = 9$; $P < .001$) for HCs, respectively. The intensity of IL-8 and IL-6 in CF epithelial cells was also greater than that observed for the HCs. These data are summarized in Table I.

To determine whether the BECs were actually secreting these cytokines, extensively washed epithelial cells were cultured in vitro for 2 hours. Because of the limited numbers of epithelial cells obtained by brushing, we studied 6 additional HCs and 4 additional patients with CF for the concentrations of IL-10, IL-6, and IL-8 secreted into the culture supernatants. The fresh epithelial cells obtained from the HCs spontaneously secreted IL-10, which was maximal at 2 hours in vitro, but they did not secrete any detectable IL-8 or IL-6 (Fig 3). CF epithelial cells cultured under identical conditions (Fig 3) secreted only minimal amounts of IL-10 during the first 2 hours in culture ($P = .001$) but spontaneously secreted IL-8 ($P = .013$) and IL-6 ($P = .040$).

The likely physiologic significance of these in vitro findings is illustrated by serial studies in an infant with CF in whom detectable bacterial infection was eradicated by antibiotic treatment. A 5-month-old infant was diagnosed with CF at the age of 4 months because of failure to thrive. At the age of 5 months, cough developed, but her throat culture was negative. Bronchoscopy with BAL revealed *P aeruginosa*, *Haemophilus influenzae*, and *Klebsiella pneumoniae*. She received a 2-week course of intravenous antibiotics. Repeat bronchoscopy with BAL 2 months later yielded sterile fluid. As shown in Fig 4, the percentage of neutrophils in the ELF decreased from 83% before antibiotic treatment to 36% after antibiotic treatment when the fluid was sterile. This is consistent with the studies by Kahn et al,¹⁵ which show elevated BAL neutrophils in some babies with CF despite a lack of detectable pulmonary infection. The concentrations of IL-8 (Fig 4) and IL-6 were also higher in the pretreatment versus posttreatment ELF, but both IL-8 and IL-6 were still elevated at both time points, as compared with their concentrations in the ELF of HCs. IL-10 was not detectable in either sample.

We also evaluated the in vitro production of proinflammatory cytokines by the cultured BECs obtained from this infant with CF. The production of IL-8 by both the pretreatment and posttreatment specimens was

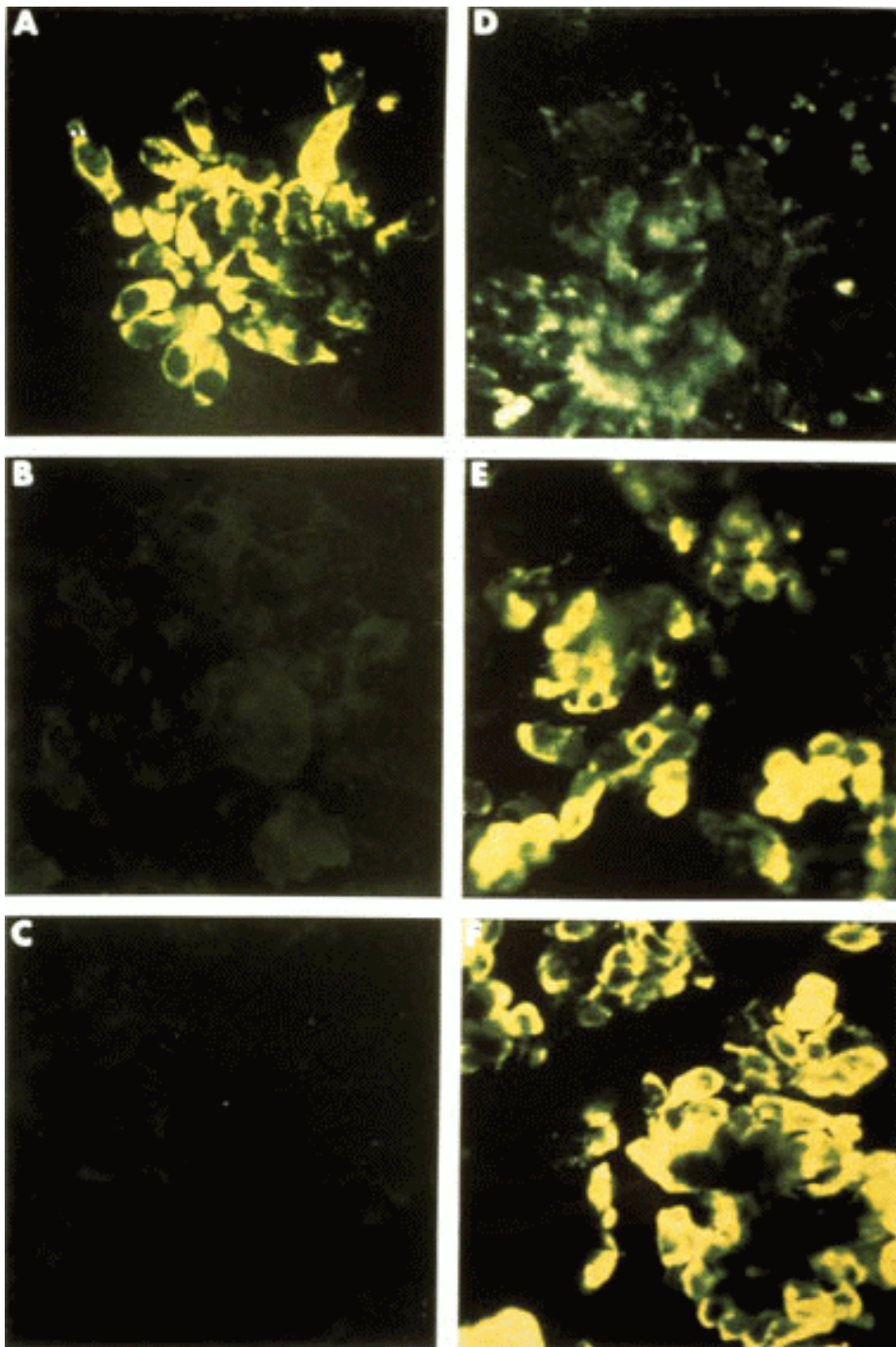


FIG 1. Fresh BECs obtained from a patient with CF and an HC by brushing were fixed, permeabilized, and stained for IL-10, IL-8, and IL-6. Representative immunofluorescent slides of HC (**A-C**) and patient with CF (**D-F**) BECs are shown. Epithelial cells were stained for IL-10 (**A** and **D**), IL-8 (**B** and **E**), and IL-6 (**C** and **F**) as described.

markedly elevated, although the pretreatment cells produced more IL-8 than the posttreatment cells (Fig 4). Similar results were found for IL-6. No IL-10 production was detectable from the pretreatment cells, although a

slight amount of IL-10, just at the limits of the assay, became detectable from the posttreatment cells. Our data (Fig 3) have shown that HC BECs secrete no IL-8 or IL-6 and approximately 100 pg/mL of IL-10 under the same

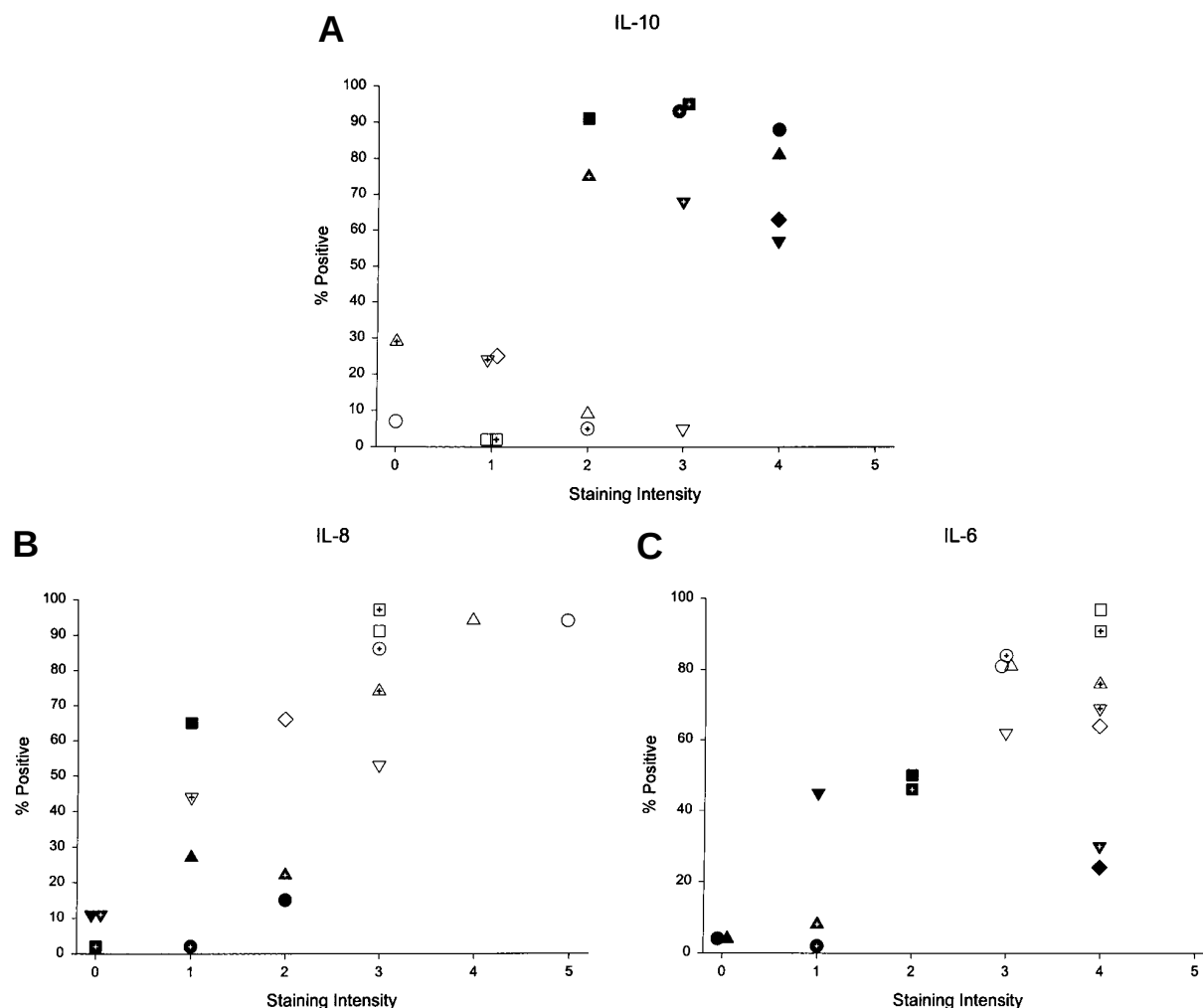


FIG 2. Immunofluorescent staining for intracellular cytokines in CF (n = 9) and HC (n = 9) BECs. BECs were gently removed from brushes by light vortexing. Cytospin slides were fixed, permeabilized, and stained for IL-10 (**A**), IL-8 (**B**), and IL-6 (**C**) as in Fig 1. Results shown are for the intensity of fluorescence as compared with the positive control of LPS-stimulated monocytes (5+) and a negative control (0) with no first antibody. Fluorescent and total cells within each microscope field were evaluated, and the percentage of fluorescent cells was determined by counting 100 ciliated, cytokeratin positive-staining epithelial cells in each specimen. Each symbol represents a single patient or control subject; each patient and control subject was evaluated for each of the cytokines. Closed symbols are for HCs; open symbols are for CF patients.

conditions. The results from a second infant with a similar course of disease were comparable. This infant was evaluated at 8 months and again at 11 months after antibiotic treatment. Again, the initial BAL had positive bacterial cultures, but repeat bronchoscopy yielded sterile fluid, and the BAL neutrophils were decreased from 41% to 5%. The same was observed for IL-8 in the BAL, which decreased from 10 ng/mL ELF to 1 ng/mL ELF after antibiotic treatment. There was 0.5 ng/mL ELF IL-6 in the BAL before the treatment but none after the treatment. The epithelial cells obtained from this patient before the treatment secreted 100 pg/mL ELF IL-8 when cultured in vitro, but no detectable IL-6 or IL-10. After antibiotic treatment, the IL-8 secreted by the epithelial cells decreased to 70 pg/mL, which was still higher than

HCs. There was no detectable IL-6 secreted by the specimens before or after treatment, and we did not detect any IL-10 either secreted by the epithelial cells or in the BAL fluid from either specimen. Thus we speculate that the downregulated IL-10 secretion by the epithelial cells in CF may facilitate persistent IL-8 and/or IL-6 production, even when infection is no longer detectable.

DISCUSSION

Respiratory epithelial cells may participate in local immune responses and cytokine networks by producing mediators, including IL-1 β , IL-6, IL-8, GM-CSF, and arachidonic acid metabolites that can modulate the activities of other cells.¹⁶⁻¹⁸ Because of its anatomic position,

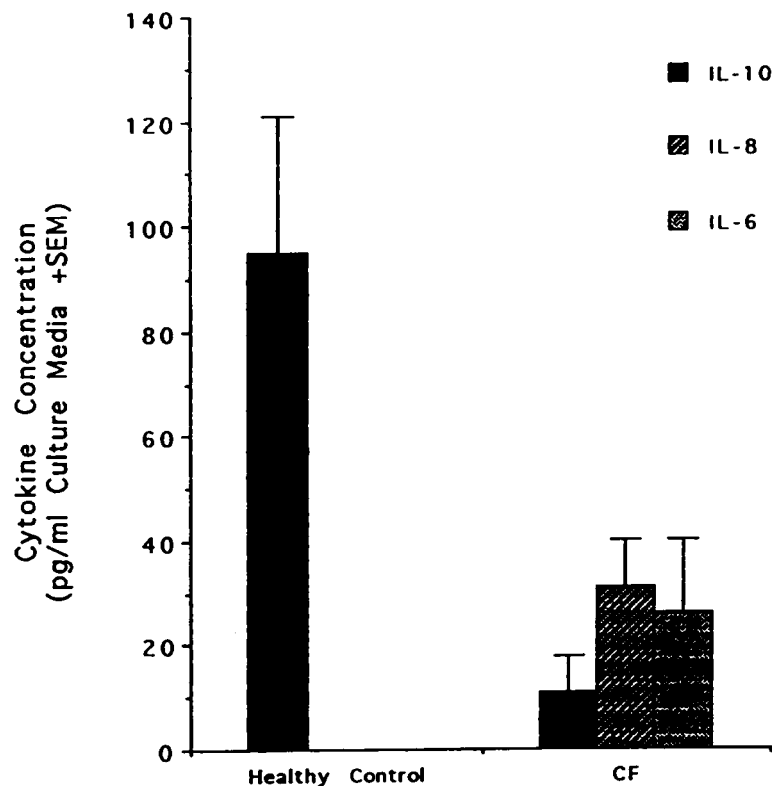


FIG 3. Secretion of IL-10, IL-8, and IL-6 by freshly obtained HC and CF BECs cultured for 2 hours. Cells were eluted from brushes and washed, then 5×10^4 epithelial cells were plated in 500 μ L of media. After 2 hours, the media was removed and assayed for IL-10, IL-8, and IL-6 by ELISA. There were 6 for HCs and 4 for patients with CF (all of the latter cultured positive for *P aeruginosa*). Note that there was no detectable IL-8 or IL-6 in cultures from HCs. $P < .01$ for differences between HCs and patients with CF for both IL-10 and IL-8, whereas $P < .05$ for IL-6.

TABLE I. Percent positivity and immunofluorescent staining intensity of IL-10, IL-8 and IL-6 in fresh BECs from patients with CF and HCs

	CF	HC	P
Patients (n)	9	9	—
Sex (M/F)	3/6	7/2	—
Epithelial cell staining			
IL-10 (% positive)*	12 $\pm$ 4.0	79.0 $\pm$ 5.0	<.001
IL-10 (intensity)†	1 $\pm$ 0.3	3.0 $\pm$ 0.3	<.001
IL-8 (% positive)	78 $\pm$ 6.0	24.0 $\pm$ 8.0	<.001
IL-8 (intensity)	3 $\pm$ 0.4	0.9 $\pm$ 0.3	<.001
IL-6 (% positive)	78 $\pm$ 4.0	24.0 $\pm$ 7.0	<.001
IL-6 (intensity)	4 $\pm$ 0.6	2.0 $\pm$ 0.5	.021

*Percent of the epithelial cells staining positive for the respective cytokine.

†The brightness of IL-10, IL-8, or IL-6 staining as compared with LPS-stimulated monocytes as a positive control (5+) and epithelial cells incubated with secondary antibody alone as a negative control (0).

the epithelium represents the first line of contact with noxious or infectious agents and irritants. Epithelial cells are thus ideally situated to regulate mucosal defense mechanisms. In this study, we set out to determine the contribution of epithelial cell cytokines to the regulation of inflammation in the lungs of patients with CF.

Our studies of freshly obtained, cytokeratin-positive BECs that were fixed immediately after removal from the airways showed that higher numbers of epithelial cells from HCs expressed intracellular IL-10 than did cells from patients with CF. The intensity of the IL-10 staining was also significantly greater in the cells from HCs than in the specimens from patients with CF. These studies are in addition to and confirm data previously reported by our laboratory.⁹ In contrast, higher percentages of the cells from patients with CF expressed intracellular IL-8 and IL-6 than did cells from HCs. The staining intensity was also greater for both of these proinflammatory cytokines in the cells from patients with CF. Because each patient and HC was evaluated for each cytokine and we observed this reciprocal relationship in each of these patients and control subjects, these results suggest a reciprocal relationship between the production of anti-inflammatory versus proinflammatory cytokines in epithelial cells in vivo in HCs versus in patients with CF. We speculate that the decrease in production of the anti-inflammatory cytokine IL-10 in CF may actually predispose these patients to increased and/or persistent proinflammatory cytokine production.

In parallel studies, the cells of HCs secreted 100 pg/mL IL-10 when placed in culture, but no IL-6 or IL-

8. In contrast, cells from patients with CF secreted little or no IL-10 but 20 to 30 pg/mL of IL-6 and IL-8. The concordance of these results with the immunofluorescence results and the fact that we⁹ have previously documented the presence of IL-10 mRNA in fresh BECs from HCs suggest that the *in vitro* secretion during the initial 2-hour culture period is a good indicator of the actual ongoing secretion *in vivo*. When cultured for longer intervals, such as 8 to 24 hours, cells from HCs decrease the IL-10 production and begin to spontaneously produce IL-8 (data not shown). Because there was little or no IL-8 detectable by immunofluorescence in the freshly fixed specimens from the HCs, we believe that only the results from the initial culture period accurately reflect the true secretory activity of the cells *in vivo*. Dedifferentiation and/or the effects of mechanical disruption¹⁹ and reattachment to the plastic tissue culture plates may be responsible for the spontaneous decrease in IL-10 production and increase in IL-8 production *in vitro*.

The opportunity to perform serial lavages in infants with CF before and after treatment that eradicated bacterial infection allowed us to show that the *in vitro* cytokine secretion data correlated with BAL findings indicative of the inflammatory consequences in the lung. Clearance of the infection correlated somewhat with decreased concentrations of IL-6 and IL-8 in ELF, but the concentrations of both of these proinflammatory cytokines in 1 infant and IL-8 in both infants remained markedly elevated as compared with HCs. There was also a decrease in the secretion of these mediators by BECs from these patients *in vitro* but not to the undetectable levels produced by cells from HCs. Neither infant's cells secreted significant amounts of IL-10 on either occasion. The changes in the *in vitro* secretion and concentrations of IL-6 and IL-8 in the lavage fluid not only correlated with the *in vitro* cytokine secretion by the epithelial cells but also with the inflammatory consequences *in vivo*, as manifested by a decrease in the percentage of neutrophils in the airways after antibiotic treatment, although even in the sterile specimens, the percentage of neutrophils was still much higher than in the airways of HCs.²⁰ Unfortunately, it is not often possible to perform serial studies like these in patients with CF nor to sterilize their airways once chronic *P aeruginosa* infection is established. Determination of the effects of CF transmembrane regulator mutations on the kinetics and persistence of inflammatory cytokine production will require standardized bacterial inoculation and larger numbers of individuals and can be done only in animals.

Adherence of *Pseudomonas* and/or products of these organisms and neutrophil elastase can induce BEC cell lines to secrete IL-8 and/or IL-6.^{17,19,21,22} Alveolar macrophage products such as TNF- α and IL-1 β may also augment the production of IL-8 and/or IL-6 by the epithelial cells.⁴ It is thus not surprising that epithelial cells obtained from infected patients with CF secrete IL-8 and IL-6 *in vitro*. It is likely that, in addition to the epithelial cells, macrophages and other inflammatory cells in the airway were also producing these proinflam-

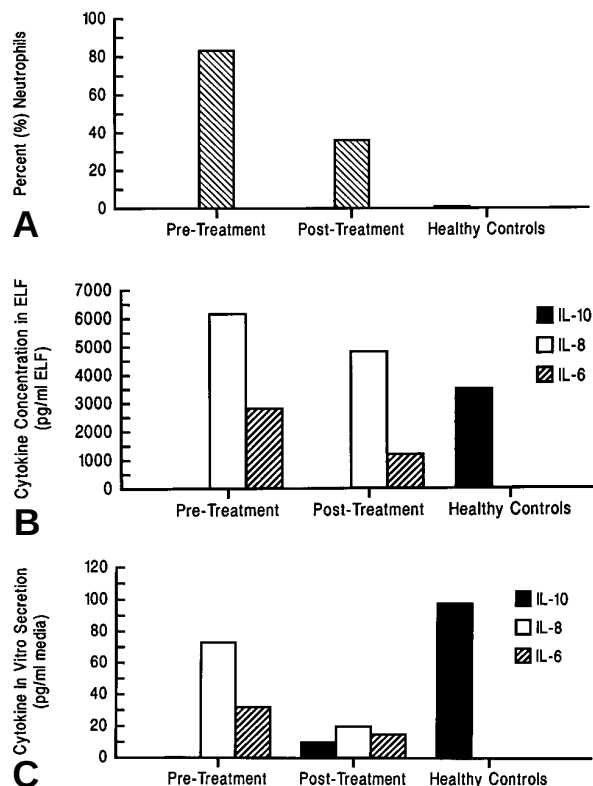


FIG 4. BAL inflammatory cells, soluble cytokines, and *in vitro* cytokine production by epithelial cells from an infant with CF before and after eradication of *P aeruginosa* lung infection. The dilution of ELF in the BAL fluid was determined as previously described.^{5,6,20} **A**, Percentage of neutrophils in the ELF obtained before and after treatment. **B**, Cytokine concentrations in the ELF before and after treatment. **C**, Cytokine production from freshly obtained BECs cultured *in vitro* for 2 hours at 5×10^4 per 500 μ L of media. Although control values shown in this figure are for adults, the neutrophil and IL-8 data are consistent with values published by Kahn et al¹⁵ for HCs with a mean age of 24 months. All cytokine assays on ELF and culture supernatants were performed in duplicate.

matory cytokines in response to the infection.²² Interestingly, no IL-10 could be detected in the BAL samples from the infected adult patients with CF nor in the infected or sterile samples from the infants. No IL-10 was produced *in vitro* by the BECs obtained when the infants were actively infected, and only a slight amount was produced by the posttreatment specimen of 1 infant. We do not yet know whether stimuli associated with the infection downregulate IL-10 production by epithelial cells or whether this is a direct effect of the CF transmembrane regulator mutations, but we speculate that the decrease in IL-10 production that we observed allows the persistent secretion of IL-8 even in the absence of infection. This and similar phenomena may contribute to reports of increased concentrations of IL-8 and neutrophils in BAL samples from infants with CF who had no apparent infection at the time of lavage.¹⁵

In CF, the epithelial cells secrete IL-8 and IL-6, each of which likely contributes directly to the pulmonary

pathologic condition. IL-8 recruits and activates neutrophils,^{23,24} and IL-6 stimulates B cells, contributing to increased antibody production, hyperglobulinemia, and local immune complex deposition.²⁵ Although these cytokines were secreted *in vitro* by freshly cultured BECs from patients with CF, only IL-10 and none of the proinflammatory cytokines were secreted *in vitro* by epithelial cells from HCs. Because IL-10 is known to suppress the production of IL-8, IL-1 β , and TNF α , it is potentially important in the normal lung for limiting local inflammatory responses. In other studies in our laboratory, we have shown that both recombinant and epithelial cell-derived IL-10 cannot only suppress TNF and IL-1 by LPS-stimulated monocytes but also the secretion of IL-8 by epithelial cells themselves. Indeed, it has been shown that neutralization of endogenous IL-10 exacerbates immune complex lung injury in animal models and that the administration of exogenous IL-10 ameliorates this type of injury.^{9,10,26} The decreased IL-10 production in CF is thus likely to contribute to an excessive and persistent inflammatory response to local immune complex deposition and other stimuli produced as a result of the chronic and recurrent infection.

We thank Jay and Kathleen Hilliard for their assistance in these studies.

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