Reduced Interleukin-8 Production by Cystic Fibrosis Airway Epithelial Cells

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The acquisition of *Pseudomonas aeruginosa* in the airways of patients with cystic fibrosis (CF) is the initial event leading to bronchiectasis and lung disease. Although the host factors that permit initial airway colonization are largely unknown, recent studies suggest that secretion of interleukin (IL)-8 by airway epithelia and local recruitment of neutrophils is the final pathway in a pulmonary cytokine network. To determine whether differences in cytokine production exist between normal and CF airway epithelia, secretion of immunoreactive IL-8 and IL-10 as well as specific messenger RNA (mRNA) abundance were compared in airway epithelia expressing normal and mutant CF transmembrane regulator. After induction with IL-1β, a CF airway cell line engineered to express the wild-type CF gene (CFT1-LCFSN) secreted significantly more immunoreactive IL-8 than did its isogenic parent that expressed the mutant CF gene (CFT1) or an isogenic vector control line (CFT1-LC3). Further studies with the three related cell lines demonstrated that expression of CFT1-LCFSN was associated with a significant increase in uninduced secretion of immunoreactive IL-8 as well as a 10- to 20-fold increase in IL-8 mRNA abundance when compared with the isogenic lines expressing the mutant gene. IL-1β induction and intracellular accumulation of IL-8 appeared to be unaffected by CF genotype. These studies suggest that IL-8 secretion by CF airway epithelial cells is defective and may contribute to *Pseudomonas* persistence in the CF airway. Further studies are needed to confirm this difference in other cell lines and determine the linkage between IL-8 production and CF gene expression. **Massengale, A. R. D., F. Quinn, Jr., J. Yankaskas, D. Weissman, W. T. McClellan, C. Cuff, and S. C. Aronoff. 1999. Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. Am. J. Respir. Cell Mol. Biol. 20:1073–1080.**

Cystic fibrosis (CF) is the leading inherited cause of chronic pulmonary disease among American Caucasian children (1). In its most common form (ΔF508/ΔF508), CF is characterized by the production of a defective chloride channel, CF transmembrane regulator (CFTR), as well as diminished expression of CFTR on the surface of epithelial cells (2, 3). It is well established that CF is associated with bacterial airway infection and subsequent colonization (4). Long-standing endobronchial colonization with *Pseudomonas aeruginosa* is the primary cause of morbidity and mortality in CF (5, 6). After *Pseudomonas* colonization, CF airway secretions become rich in proinflammatory cytokines and polymorphonuclear leukocytes, and variably deficient in interleukin (IL)-10 (7–10). Lung injury and loss of pulmonary function follow the release of cytotoxic neutrophil products such as elastase and oxygen radicals into the lung parenchyma (6).

The host factors that permit initial endobronchial colonization of the CF lung by *Pseudomonas* are poorly understood. One hypothesis suggests that defective chloride ion transport by CFTR results in dehydrated mucous secretions and subsequent airway obstruction, thus providing an opportune environment for bacterial infection and subsequent inflammation (9). Another hypothesis suggests that sustained pulmonary inflammation may actually precede airway obstruction, causing early airway damage that predisposes the local environment to bacterial infection (11, 12).

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Abbreviations: bronchoalveolar lavage fluid, BALF; β-galactosidase, βgal; complementary DNA, cDNA; cystic fibrosis, CF; CF transmembrane regulator, CFTR; deoxynucleotide triphosphate, dNTP; enzyme-linked immunosorbent assay, ELISA; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; human β-defensin-1, hBD-1; interleukin, IL; lipopolysaccharide, LPS; messenger RNA, mRNA; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; reverse transcriptase, RT; tumor necrosis factor, TNF.

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Recent investigations concerning the role of pulmonary inflammation in the development of CF lung disease have shown that: (*1*) normal and CF airway epithelial cells can express IL-8 with and without tumor necrosis factor (TNF)-α or IL-1β induction; (*2*) IL-8 produced by these cells causes transmigration of neutrophils across the epithelium into the airway in a basolateral-to-apical direction; and (*3*) airway epithelial cell–mediated neutrophil attraction is blocked, for the most part, by antibody against human IL-8 (13–17). These observations have led to the development of a cytokine network model of initial infection in which airway epithelial cells recruit neutrophils locally via directed IL-8 secretion (13, 14, 16).

IL-10 is an anti-inflammatory cytokine that reduces production of TNF-α and IL-1 by macrophages (18). This cytokine is produced by respiratory epithelial cells and alveolar macrophages in addition to other cell types (7, 8, 18). IL-10 levels have been variably reported as either reduced or normal in CF bronchoalveolar lavage fluid (BALF) when compared with BALF from control subjects (7, 8). Also, production of IL-10 by CD4+ T-lymphocyte clones from patients with CF was significantly reduced when compared with clones from normal patients (19). A reduction in respiratory IL-10 levels could potentially contribute to airway inflammation by allowing increased TNF-α and IL-1β production by alveolar macrophages.

Previous studies concerning IL-8 have indicated that IL-8 levels are elevated in CF BALF compared with BALF from normal patients, and *Pseudomonas*-induced IL-8 levels were 4-fold higher in a CF cell line than in its corrected cell line (7, 8, 20, 21). However, these studies did not compare uninduced IL-8 levels by CF and normal respiratory epithelial cells in the absence of bacteria or other inflammatory mediators. Given the importance of IL-8 and IL-10 secretion by airway epithelial cells in early respiratory tract protection and the failure of lung defense in CF, we hypothesized that there may be differences in uninduced cytokine production between CF and normal airway epithelial cells. To test this hypothesis, secretion of immunoreactive IL-8 and IL-10 as well as specific messenger RNA (mRNA) abundance were compared in CF and normal airway epithelia. These cytokines were further analyzed in an isogenic series of transformed airway epithelial cells derived from a ΔF508/ΔF508 homozygotic individual expressing either mutant or corrected CFTR.

Materials and Methods

Reagent Preparation

Lipopolysaccharide (LPS) was prepared according to the method of Pier and colleagues (22). Briefly, *P. aeruginosa* CFM5 was grown in 100 ml Luria-Bertaini (LB) broth overnight at 37°C in a shaker incubator, then pelleted and resuspended in 10% sodium lauryl sarcosine to lyse the cells. The cells were then centrifuged, ethyl alcohol was added to the supernatant, and the mix was incubated for 18 h at 4°C. The precipitate was collected by centrifugation, resuspended in phosphate-buffered saline (PBS), and ultracentrifuged. The pellet containing LPS was first treated with a mixture containing DNase I (0.1 mg/ml; GIBCO-BRL, Grand Island, NY), RNase (0.1 mg/ml; GIBCO-BRL), $CaCl_2$ (4 μM), and $MgCl_2$ (4 μM) overnight at 37°C, and then treated with proteinase K (0.1 mg/ml; GIBCO-BRL) for 2 h at 56°C. The LPS was then recovered by ultracentrifugation, redissolved in distilled water, and lyophilized. For LPS induction assays, purified LPS was dissolved in Ham's F-12 culture medium (GIBCO-BRL).

IL-1β (Genzyme, Cambridge, MA) was diluted to a stock concentration of 1 μg/ml with PBS (GIBCO-BRL) containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO).

Epithelial Cell Cultures

The immortalized airway epithelial cell lines used in this study are listed in Table 1. The HBE4-E6/E7-C1 (NHB) cell line was grown in keratinocyte serum-free medium (Collaborative Research, Inc., Bedford, MA) supplemented with 50 μg/ml bovine pituitary extract (Collaborative Research), 5 ng/ml recombinant human epidermal growth factor (EGF) (Collaborative Research), and 10 ng/ml cholera toxin (GIBCO-BRL). The CF/T43 cell line was cultivated in keratinocyte growth medium supplemented with 30 μg/ml bovine pituitary extract, 10 ng/ml EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, and gentamicin–amphotericin (50 μg/ml, 50 ng/ml; Clonetics, Walkersville, MD) (complete medium).

CFT1 was a human bronchial epithelial cell line expressing mutant CFTR recovered from a patient with ΔF508/ΔF508 CF and immortalized with human papillomavirus 18/E6/E7 (23). The vector control strain, CFT1-LC3, expressing mutant CFTR, was derived from CFT1 and

TABLE 1

Descriptions and definitions of airway epithelial cell lines

Cell Line	Description	Source
HBE4-E6/E7-C1 (ATCC CRL-2079) (NHB)	Normal human bronchial epithelial cells immortalized with HPV-16 E6/E7 (31)	American Type Culture Collection*
CF/T43	Human airway epithelial cell line from ΔF508/ΔF508 CF patient immortalized with pZIPneoSV (X) 1/SV40T (32)	Yankaskas (32)
CFT1	Human airway epithelial cell line from ΔF508/ΔF508 CF patient immortalized with HPV-18/E6/E7 (23)	Yankaskas and associates (23)
CFT1-LC3	CFT1 transfected with a retroviral vector encoding βgal (22)	Yankaskas and associates (23)
CFT1-LCFSN	CFT1 transfected with a retroviral vector encoding normal CFTR (24)	Yankaskas and associates (23)

*Rockville, MD.

was transfected with a retroviral vector encoding β-galactosidase (Bgal) (22). CFT1-LCFSN was derived from CFT1 and was transfected with a retroviral vector encoding wild-type CFTR (24). The CFT1 cell lines were grown in Ham's F12K medium supplemented with 10 μg/ml insulin (Collaborative Research), 0.5 μg/ml hydrocortisone (Collaborative Research), 3.75 μg/ml endothelial cell growth supplement (Collaborative Research), 25 ng/ml EGF, 3×10^{-8} M triiodothyronine (Sigma), 5 μg/ml transferrin (Collaborative Research), and 10 ng/ml cholera toxin (22–24). Cell densities were determined by counting trypan blue–stained, trypsinized aliquots from each well and were corrected for final volume.

Immunoreactive IL-8 Assay

The secretion of IL-8 by the airway epithelial cell lines was measured at baseline and after induction with 100 pg/ml IL-1β or various concentrations of bacterial LPS. The smooth LPS was purified from the mucoid clinical isolate, *P. aeruginosa* CFM5, as described above (West Virginia University Hospital, Morgantown, WV). Parallel cell cultures of each cell line were grown to confluence in 24-well plastic tissue culture plates (approximately 2×10^5 cells/well). Some monolayers were then induced by replacing the supernatant in each well with cell culture medium containing 100 ng/ml IL-1β or bacterial LPS (1,000, 100, 10, 1, and 0 μg/ml) and incubating the monolayers for 24 h at 37°C in 5% CO_2. In each experiment, three wells were induced with IL-1β or LPS at each concentration. Supernatants were then collected from each well and cell counts performed. IL-8 levels were measured in the supernatant samples by enzyme-linked immunosorbent assay (ELISA) as described below. Experiments were performed in triplicate for each cell line.

Commercially available ELISA kits (DuoKits; Genzyme) were used to determine cytokine levels in cell culture supernatants. Ninety-six–well microtiter plates (VWR, Cleveland, OH) were coated with mouse antihuman IL-8 (2.5 μg/ml in 0.1 M carbonate buffer, pH 9.4) and incubated at 4°C overnight. The plates were then washed five times with PBS containing 0.05% Tween 20 (Bio-Rad, Melville, NY) (wash buffer) and blocked with PBS containing 0.05% Tween 20 and 1% BSA (Sigma). The block buffer was decanted and the wells were blotted dry. One hundred microliters of a recombinant human IL-8 standard or a sample were added to each well, and the plates were incubated for 1 h at 37°C. After washing, the plates were developed with rabbit antihuman IL-8 biotinylated antibody, streptavidin–horseradish peroxidase, tetramethylbenzidine, and hydrogen peroxide, according to manufacturer's instructions. The reaction was stopped with 2 N sulfuric acid and the optical density at 450 nm (OD_{450}) was determined. An IL-8 standard curve (range 81.75 to 872 pg/ml) and blank buffer wells were included in duplicate in each assay. Specific cytokine concentrations were expressed as picograms of IL-8 per 10^5 cells.

Immunoreactive IL-10 Assay

For immunoreactive IL-10 assays, cell cultures were prepared as described for IL-8 assays, and IL-10 was measured by sandwich ELISA. Capture (JES3-9D7) and biotinylated detection (JES3-12G8) antibodies as well as recombinant human IL-10 standard were obtained from Pharmingen, San Diego, CA. Sequential incubations with interval washing steps were performed in microtiter plates using capture antibody, sample or standard biotinylated detection antibody, streptavidin–peroxidase, and, finally, tetramethylbenzidine peroxidase. Sample color development at OD_{450} was assessed relative to standard curves. The lower limit of detection for this assay was 250 picograms recombinant IL-10/ml.

Preparation of Complementary DNA and DNA Probes

To prepare complementary DNA (cDNA) probes for Northern blot analysis, polymerase chain reaction (PCR) primers and cDNA to IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased commercially (Clontech, Palo Alto, CA). These cDNA segments spanned multiple exons of each respective gene. The probes were prepared by PCR (*see below*). Conditions for PCR were similar to those described below. The amplified PCR product was rapidly purified (QIAquick PCR Purification Kit; Qiagen, Chatsworth, CA) and 50 ng of the cDNA product was resuspended in 15 μl double deionized H_2O, boiled for 10 min, and immediately chilled on ice for 3 min. The cDNA was labeled with [α-^{32}P]deoxycytidine triphosphate by the random primer labeling method of Feinberg and Vogelstein (25). The final probe was purified by centrifugation (QIAquick Nucleotide Removal Kit; Qiagen), boiled for 10 min, and immediately chilled on ice for 5 min. A total of 1 μl of the purified probe mixture was counted (Wallace 1410 liquid scintillation counter; Pharmacia, Gaithersburg, MD) to determine incorporation.

Reverse Transcriptase-PCR Analysis of Cytokine mRNA

Total RNA was isolated from approximately 3.5×10^6 cells using RNAzol B (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. After deoxyribonuclease treatment, total cDNA was prepared by first-strand cDNA synthesis from approximately 70 μg total RNA by combining 5 μl (70 μg) total RNA with 1 μl 10× reaction buffer, 2 μl 25 mM $MgCl_2$, 0.5 μl ribonuclease inhibitor (50 U/μl), 0.5 μl deoxynucleotide triphosphate (dNTP) mixture (2.5 mM each dNTP), 0.5 μl random primer $p(dN)_6$ (1.6 μg/μl), and 0.5 μl avian myeloblastis virus reverse transcriptase (RT) (cDNA synthesis kit; Boehringer Mannheim, Indianapolis, IN). The cDNA reaction mixture was then incubated for 1 h at 42°C, mixed with an equivalent amount of Tris–ethylenediaminetetraacetic acid buffer (pH 8.0), and stored at −20°C until use.

The following commercial amplimer sets were used in the subsequent PCR analysis (Clontech): human GAPDH control amplimer set, human IL-8 amplimer set, and human IL-10 amplimer set. For PCR, 2 μl cDNA was reacted with 0.25 μl (1.25 U/μl) Taq DNA polymerase (Promega, Madison, WI), 2 μl dNTP mixture (2.5 mM each dNTP), 2.5 μl 10× PCR buffer (Promega), and 2 μl primer mix (5 mM 5′ and 3′ primer) in a total volume of 25 μl. Positive controls consisted of the reaction mixture containing 1 μl of the control cDNA supplied with the kit instead of sample cDNA. Conditions for PCR consisted of one cycle of 94°C for 4 min, 55°C for 2 min, and 72°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min; and one cycle of 94°C for 45 s, 55°C for 1 min, and 72°C for 7 min. Samples were analyzed by gel electrophoresis on a 1.5% agarose gel containing 1.6×10^{-4} mg/ml ethidium bromide.

IL-8 mRNA was also quantified by competitive PCR using IL-8 PCR MIMIC (Clontech) according to the manufacturer's recommendations. The IL-8 PCR MIMIC is a nonhomologous DNA fragment derived from the *v-erb* gene to which have been added IL-8 primer templates (26). When serial dilutions of PCR MIMIC are added to PCR reactions containing constant amounts of total cDNA, the PCR MIMIC competes with the target DNA for the same primers. When visualized by gel electrophoresis and ethidium bromide staining, the dilution at which the PCR MIMIC band matches the target DNA band (isoamplification point) is the equimolar concentration at which PCR MIMIC is equivalent to target DNA. Thus, this concentration represents the molar amount of target DNA in a given PCR sample. To quantify the amount of IL-8 present in the CFT1 cell-line series, equal concentrations of total cDNA prepared by RT-PCR of total RNA isolated from each cell line were mixed with 10-fold dilutions of IL-8 PCR MIMIC cDNA to yield identical final volumes. The resulting mixtures were combined with 1.25 U/μl Taq DNA polymerase, 4 μl dNTP mixture (2.5 mM each dNTP), 5 μl 10× PCR buffer, and 4 μl primer mix (5 mM 5′ and 3′ primer) to give a final volume of 50 μl. Positive controls consisted of the reaction mixture containing 1 μl of IL-8 cDNA from each cell line without the addition of PCR MIMIC cDNA. Samples were separated by agarose gel electrophoresis, and the isoamplification endpoints were determined visually after staining with ethidium bromide.

Northern (RNA) Blot Analysis

Total RNA was isolated from confluent monolayers as before and the RNA concentration of each sample was determined by ultraviolet spectrophotometry. Ten micrograms of each RNA sample were placed onto a 1.0% agarose–formaldehyde gel, separated by electrophoresis, and transferred to HiBond-N$^+$ membranes (Amersham, Arlington Heights, IL) by capillary blotting. The RNA blot was then crosslinked, prehybridized, and hybridized with the radiolabeled IL-8 probe by the method of Church and Gilbert (27). After hybridization, the blot was initially exposed to a phosphoimager screen and analyzed (SI phosphoimager; Molecular Dynamics, Sunnyvale, CA). The blot was then exposed to RX X-ray film with an intensifying screen at −70°C, developed, and quantified by densitometry (Optimas Image Analysis System; Optimas, Edmonds, WA). After analysis of the blot, the membrane was stripped, reprobed with GAPDH, developed, and quantified by densitometry. Specific message was expressed as a ratio of IL-8 to GAPDH.

Statistical Analysis

Significant differences among multiple means were determined by analysis of variance. Where significant differences existed, *post hoc* comparisons among means were performed using Duncan's test.

Results

Secretion of Immunoreactive IL-8

To compare the production of IL-8 by normal and respiratory epithelial cells, NHB and CF/T43 cell lines were assayed for uninduced levels of immunoreactive IL-8. Preliminary experiments demonstrated that supernatant IL-8 concentrations were constant beyond 24 h of induction (data not shown). Supernatants from wells containing 2 to 4×10^6 cells were assayed for immunoreactive IL-8 after 24 h incubation with either cell culture medium only or cell culture medium containing 100 pg of human IL-1β/ml. No IL-8 was detected in these two cell lines in the absence of IL-1β. This could have been due to IL-8 levels below the limit of detection of the ELISA or to differences between heterogeneous cell lines. The mean, induced IL-8 concentrations from triplicate experiments of CF/T43 and NHB cell lines were 184 ± 42 and 565 ± 202 pg/10^5 cells, respectively. These values were significantly different ($P = 0.033$).

Because differences in IL-8 secretion between the CF/T43 and NHB cell lines may reflect differences between two heterogeneous cell lines, uninduced and IL-1β–induced levels of IL-8 were measured in a monozygotic ΔF508 CF cell line (CFT1), CFT1 expressing wild-type CFTR (CFT1-LCFSN), and CFT1 transfected with the same vector as CFT1-LCFSN containing a βgal gene (CFT1-LC3). Without IL-1β induction, mean immunoreactive IL-8 concentrations in cell supernatants from triplicate experiments of CFT1, CFT1-LC3, and CFT1-LCFSN were 320 ± 157, 264 ± 122, and 961 ± 152 pg/10^5 cells, respectively (Figure 1).

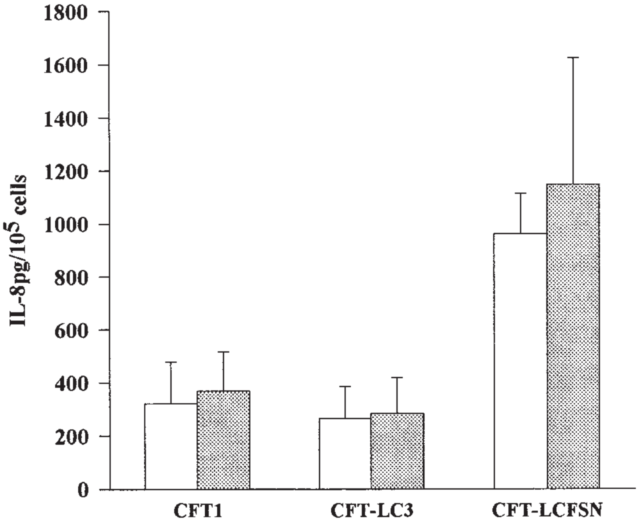

Figure 1. IL-8 secretion by airway epithelial cells with and without IL-1β induction. Parallel cell cultures of the isogenic cell lines CFT1, CFT1-LC3, and CFT1-LCFSN were grown to confluence in quadruplicate wells of 24-well tissue culture plates. Monolayers were then induced for 24 h with either cell culture medium alone or cell culture medium containing 100 ng/ml IL-1β. Supernatants were then collected and assayed by ELISA for the presence of IL-8. Each *bar* represents the mean (± SD) IL-8 concentration per 10^5 epithelial cells from three separate experiments. *Clear bars*, uninduced; *colored bars*, IL-1β–induced.

These values were significantly different ($F = 21.5$; $P = 0.0018$), and the supernatant from the CFTR-corrected cell line expressing wild-type CFTR (CFT1-LCFSN) contained significantly more IL-8 than did the other cell lines ($P < 0.05$). IL-1β induction had no effect on these differences ($F = 7.59$; $P = 0.023$). The similar IL-8 levels in the CFT1 cell line and its vector control cell line, CFT1-LC3, indicate that differences in IL-8 production were not due to the retroviral vector used in transfection of the cell lines with wild-type CFTR.

IL-10 was not detected in the supernatant of any cell line, regardless of IL-1β induction. This could have been due to IL-10 levels below the limit of detection of the ELISA.

Effect of Heat-Killed *P. aeruginosa* and LPS on IL-8 Secretion

In the above experiments, IL-8 secretion was significantly increased in the LCFSN cell line with or without induction by IL-1β. To determine whether this increase in IL-8 secretion was a broad-based phenomenon, IL-8 was induced in CFT1, CFT1-LC3, and CFT1-LCFSN with heat-killed, mucoid *P. aeruginosa* isolated from a patient with CF or from purified LPS. In a representative experiment, increasing bacterial inocula produced dose-dependent IL-8 secretion by all of the cell lines, although this increase was not significant in LCFSN (Figure 2A). The CFTR-corrected cell line, CFT1-LCFSN, secreted almost 10-fold more IL-8 than did the mutant CFTR lines without induction and at both bacterial inocula tested, confirming that the differences in IL-8 secretion exist with and without induction. When CFT1, CFT1-LC3, and CFT1-LCFSN were induced with varying concentrations of purified *P. aeruginosa* LPS, no significant increase in IL-8 secretion was noted in any cell line tested (Figure 2B).

To determine whether CFTR phenotype affected the process of secretion of IL-8 and IL-10 in airway epithelia, immunoreactive cytokine concentrations were measured in cell supernatants and corresponding cell lysates. IL-10 was not detected in the supernatant or cell lysate of any cell line. IL-8 was not detected in CFT1 and CFT1-LC3 cell lysates, whereas lysates of CFT1-LCFSN contained 28.0 ± 17.8% of the supernatant IL-8 concentrations. These observations suggest that reduced IL-8 secretion by CFT1 and CFT1-LC3 was not due to intracellular accumulation of the cytokine.

Abundance of IL-8 and IL-10 mRNA

To determine whether CFTR phenotype affected IL-8 mRNA abundance, specific message was semiquantified by RT-PCR, by competitive RT-PCR, and by Northern blotting in CFT1, CFT1-LC3, and CFT1-LCFSN. IL-10 mRNA was assayed by RT-PCR only.

The results of the RT-PCR assay for IL-8 are shown in Figure 3A. CFT1-LCFSN appeared to produce significantly more IL-8 message than did the mutant CFTR cell lines. There was no significant difference between CFT1 and CFT1-LC3 mRNA levels, indicating that the differences in IL-8 mRNA were not due to the retroviral vector used in transfection of the cell lines with wild-type CFTR. The differences in IL-8 cDNA concentration were confirmed by competitive PCR using the same template dilutions as in the previous experiment and decreasing concentrations of competitor (Figure 3B). Isoamplification occurred at 10^{-3} amol of competitor/μl for CFT1 and CFT1-LC3 and 10^{-2} amol of competitor/μl for CFT1-

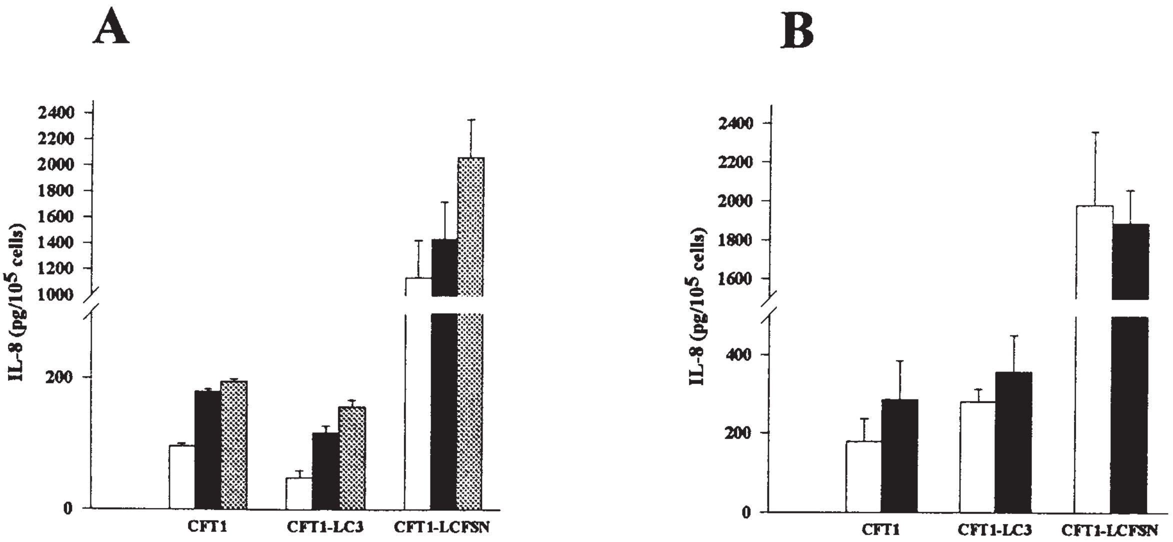

Figure 2. (*A*) IL-8 secretion by airway epithelial cells induced with heat-killed *P. aeruginosa.* CFT1, CFT1-LC3, and CFT1-LCFSN were grown to confluence in quadruplicate wells of 24-well tissue culture plates. The monolayers were then induced with the indicated concentration of *P. aeruginosa* and assayed as described in Figure 1. *Clear bars,* 0 cfu *P. aeruginosa*; *black bars,* 10^7 cfu/ml; *gray bars,* 10^8 cfu/ml. Each *bar* represents the mean (± SD) IL-8 concentration per 10^5 epithelial cells from three separate experiments. (*B*) IL-8 secretion by airway epithelial cells induced with *P. aeruginosa* LPS. After induction with either 0 or 1,000 μg/ml LPS, cell cultures were grown and assayed as described in Figure 1. *Clear bars,* 0 μg/ml LPS; *black bars,* 1,000 μg/ml LPS.

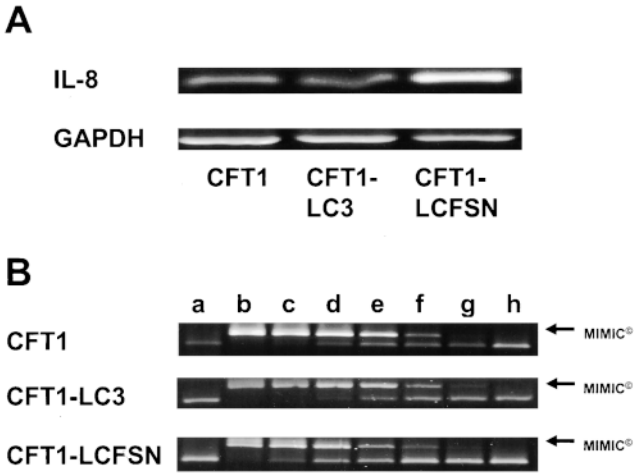

Figure 3. (*A*) Measurement of GAPDH and IL-8 by RT-PCR using total cDNA prepared from three isogenic airway epithelial cell lines. This experiment was performed twice and the data shown are from a representative experiment. (*B*) Measurement of IL-8 concentrations in three isogenic airway epithelial cell lines using competitive RT-PCR. Identical concentrations of total cDNA from CFT1, CFT1-LC3, and CFT1-LCFSN, and decreasing concentrations of competitor (MIMIC) were added to each RT-PCR reaction. Isoamplification of target cDNA and IL-8 MIMIC occurred at concentrations of 10^{-3} amol/μl for CFT1 and CFT1-LC3 and 10^{-2} amol/μl for CFT1-LCFSN. MIMIC/μl, in amol: *lane a*: 0; *lane b*: 10; *lane c*: 1; *lane d*: 10^{-1}; *lane e*: 10^{-2}; *lane f*: 10^{-3}; *lane g*: 10^{-4}; *lane h*: 10^{-5}.

LCFSN. This 10-fold difference in IL-8 mRNA is consistent with the IL-8:GAPDH ratios and ELISA results.

Direct measurement of IL-8 mRNA from CFT1, CFT1-LC3, and CFT1-LCFSN by Northern blotting is shown in Figure 4. When 10 μg of each sample was loaded onto the agarose gels and blotted, specific IL-8 mRNA was detected only for CFT1-LCFSN.

In RT-PCR experiments measuring IL-10, only IL-10 positive control and GAPDH bands were present; no IL-10 amplicon was detected in samples from CFT1, CFT1-LC3, or CFT1-LCFSN (data not shown).

Discussion

The data presented suggest that significant differences exist in the secretion of immunoreactive IL-8 *in vitro* by normal, CF, or correct CF airway epithelia. IL-8 production by the CF/T43 cell line (ΔF508/ΔF508) was significantly less than that secreted by the normal airway epithelial cell line, NHB. To eliminate the possibility that this difference might be due to variation between the cell lines, IL-8 expression was measured in an isogenic cell-line series. IL-8 expression by the homozygotic ΔF508 CF cell line, CFT1, was approximately 10-fold less than that secreted by its phenotypically corrected cell line (CFT1-LCFSN). This reduction was not associated with an intracellular accumulation of immunoreactive IL-8 or with a failure to induce IL-8 secretion in response to heat-killed bacteria. Purified LPS did not induce IL-8 secretion and did confirm previous studies measuring IL-8 response to induction by *P. aeruginosa* LPS (13, 21). Finally, the CF airway epithelial cell line (CFT1) contained less IL-8 mRNA than did its corrected cell line. These observations provide preliminary evidence that IL-8 secretion by CF respiratory epithelial cells is defective, and suggests a potential mechanism for early airway colonization with bacteria, notably *P. aeruginosa*.

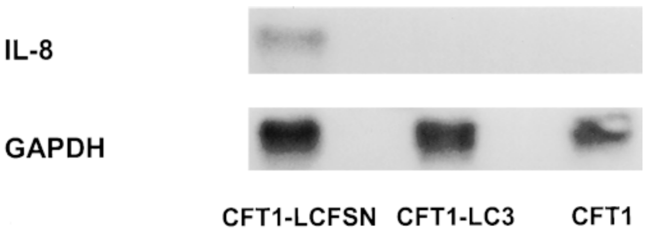

Figure 4. Northern blot analysis of IL-8 and GAPDH mRNA in the isogenic cell lines CFT1, CFT1-LC3, and CFT1-LCFSN. The quantity of 10 μg RNA was isolated from each cell line, separated by agarose gel electrophoresis, and analyzed by Northern blotting using radiolabeled oligonucleotides specific for IL-8 and GAPDH as probes. Probes were detected by exposing the blots to X-ray film and subsequent development and densitometry.

Our results are in contrast to those of DiMango and colleagues, which indicated a 4-fold increase in IL-8 secretion *in vitro* in response to bacterial induction by a CF respiratory epithelial cell line (ΔF508/W1282X) as compared with its CFTR-corrected cell line (21). DiMango and colleagues did not report IL-8 secretion in a vector control cell line, nor did they examine differences in specific message. Therefore, differences between our experiments and this previous study may be due to differences in CF genotype, effects of the vector on IL-8 expression, or differences in CFTR expression. Our finding that CF/T43 also secreted less IL-8 than did NHB further supports our findings.

The host factors that permit initial endobronchial colonization of the CF lung by *Pseudomonas* are poorly understood. Smith and associates noted that primary cell cultures of airway epithelia from unaffected individuals killed a small inoculum of *P. aeruginosa* or *Staphylococcus aureus* placed on the apical surface; airway epithelial cells from patients with CF not only failed to kill the inocula, but also permitted bacterial growth (28). Correction of the CFTR defect in CF epithelia with a recombinant adenoviral vector containing wild-type CFTR restored bactericidal activity to the apical surface. These investigators were also able to show that: (*1*) the cell-associated bactericidal activity resided in the fluid on the apical surface of the cells; (*2*) [Cl$^-$] was significantly higher in the surface fluid of CF cells than in normal cells (182 ± 10 versus 132 ± 3 mM); (*3*) normalization of [Cl$^-$] in the surface fluid of the CF epithelial cells restored bactericidal activity; and (*4*) increasing the [Cl$^-$] in the surface fluid of the normal cells abolished bactericidal activity.

Goldman and coworkers confirmed the presence of salt-sensitive bactericidal activity in normal and CF airway epithelial cells grown as xenografts on denuded rat trachea (29). These authors went on to clone human β-defensin-1 (hBD-1) from primary cultures of normal human bronchial epithelial cells and to demonstrate its presence in CF and non-CF lung tissue by *in situ* hybridization. Synthetic hBD-1 was shown to have intrinsic, salt-inhibitable, antimicrobial activity against a spectrum of organisms, including *P. aeruginosa*. In addition, when hBD-1 expression by normal bronchial cells was ablated by antisense DNA, the antimicrobial activity in cell surface fluid was lost. Both of these studies suggest that in CF, high [Cl^-] in apical airway epithelial cell fluid inactivates hBD-1, permitting survival and replication of *P. aeruginosa* and other endobronchial pathogens. Although these observations explain the initial survival of *P. aeruginosa* in the CF airway at the time of initial colonization, these authors propose that other elements of nonspecific immunity must fail to clear the pathogens from the CF airway.

The initial immune response to bacterial infection of the lung includes the recruitment of neutrophils from the peripheral circulation to the local site of infection, antibody-independent cell activation, and subsequent opsonophagocytosis of the trespasser. Using a type II pulmonary epithelial cell line, Standiford and associates demonstrated that cell-derived neutrophil chemotactic activity was present in cell supernatants and that this activity increased dramatically over time after incubation with cytokines (IL-1β or TNF-α) or macrophage-conditioned media (16). Media from LPS-stimulated macrophages produced 5-fold more IL-8 mRNA transcripts in epithelial cells than did media from nonstimulated macrophages. However, LPS induction of the respiratory epithelial cells had no effect on IL-8 supernatant concentrations. These investigators also showed that treatment of epithelial cells with macrophage-conditioned media containing increasing concentrations of rabbit antibody to human IL-8 decreased neutrophil chemotaxis in a dose-dependent fashion. Accumulation of IL-8 mRNA and secretion of immunoreactive IL-8 following cytokine induction but not LPS induction were shown to be dose- and time-dependent in this cell line. By Northern blotting, Nakamura and colleagues demonstrated low levels of IL-8 mRNA transcripts in two human bronchial epithelial cell lines; both lines accumulated IL-8 mRNA after induction with IL-1β or TNF-α (14). Finally, Ruef and associates demonstrated the production and IL-1β induction of immunoreactive IL-8 by an SV40-immortalized, CF airway epithelial cell line (13). Our experiments confirmed the presence of IL-8 mRNA and IL-8 protein in separate CF airway epithelial cell lines, CFTR-corrected cell lines, and normal cell lines. We also confirmed that production of IL-8 by CF airway epithelial cell lines is inducible by IL-1β and heat-killed bacteria, but not by LPS. Together, these observations suggest that both normal and CF airway epithelial cells produce IL-8, that IL-8 is a potent neutrophil chemokine, and that production of this chemokine by airway epithelial cells can be induced by proinflammatory cytokines but not directly induced by LPS. Furthermore, these observations suggest that proinflammatory cytokines may function as intercellular messengers between alveolar macrophages and airway epithelia. The alveolar macrophages produce inflammatory cytokines such as IL-1β in response to induction by LPS and other bacterial components. These cytokines, in turn, upregulate production of IL-8 by airway epithelial cells and other macrophages, which allows neutrophil recruitment into the airway.

Because airway epithelial cells have been shown to produce IL-8 after direct stimulation by proinflammatory cytokines secreted by activated macrophages, and because there are far more epithelial cells in the airway than alveolar macrophages, it has been proposed that the epithelial cell IL-8 response serves to “amplify” the neutrophil recruitment message (14). If this cytokine network model is correct, then IL-8 should be the primary chemokine involved in neutrophil migration across epithelial barriers. Liu and coworkers used radiolabeled neutrophils and endothelial and epithelial cell monolayers grown on filters to examine cytokine-mediated neutrophil migration (15). In normally oriented monolayers, neutrophil movement across endothelia was significantly greater than movement across airway epithelia regardless of chemokine (formylmethionyl leucylphenylalanine, IL-8, or C5a). Whereas endothelial cell orientation did not affect neutrophil migration, basolateral-to-apical neutrophil migration across airway epithelial cells was markedly greater than apical-to-basolateral migration; the greatest difference was with IL-8–mediated migration. The roles of specific chemokines in neutrophil migration across IL-1β–induced, normally oriented endothelial and epithelial cell layers were determined by specific inhibition studies (15). Although antibody against IL-8 and an antagonist of platelet-activating factor receptor were equally effective in blocking transendothelial neutrophil migration, only antibody against IL-8 significantly inhibited transepithelial cell migration. Of even more interest, antibody against intercellular adhesion molecule-1, the main adhesion molecule induced in neutrophils by IL-1β, inhibited transendothelial but not transepithelial cell neutrophil migration. These observations provide additional evidence for the important role of epithelial cell-derived IL-8 in the primary immune response to infection in the lung at the local level of the respiratory epithelium.

Clinical studies examining the contents of BALF from extremely young infants support the importance of IL-8 in airway inflammation. Armstrong and colleagues examined BALF from a cohort of patients with CF with a mean age of 2.6 mo (30). When compared with uninfected CF and control BALF, BALF from infected patients with CF contained significantly more macrophages and neutrophils. Infected CF BALF contained 100-fold more IL-8 than did BALF from noninfected patients with CF or control infants. Of particular interest, these authors failed to find inflammatory cells in some infected infants with CF. Khan and coworkers demonstrated evidence of pulmonary inflammation in CF infants as young as 4 wk (11). IL-8 was found in high concentrations in BALF from the CF infants when compared with nonaffected controls. The abundance of IL-8 mRNA produced by airway macrophages was significantly greater in CF infants than in control subjects. These authors concluded that macrophages were the apparent primary source of IL-8 in CF airways early in life.

In summary, we have demonstrated reduced production of IL-8 by a CF airway epithelial cell line *in vitro* as compared with a normal airway epithelial cell line. We also demonstrated that uninduced IL-8 secretion by a CF airway epithelial cell line is substantially reduced compared with its isogenic CF cell line expressing wild-type CFTR. Similar differences were found in IL-8 mRNA abundance. Even though overall levels of IL-8 as measured in CF BALF are typically elevated, a deficiency in IL-8 production by CF airway epithelial cells could potentially downregulate the local immune response at the airway surface and delay neutrophil chemotaxis across the epithelium. Furthermore, it is proposed that defects in IL-8 secretion at the initial time of bacterial colonization of the airway predispose the CF lung to chronic infection. Variations in IL-8 secretion among heterogeneous cell lines may be due to differences in CF genotype, effects of the vector on IL-8 expression (if any), or differences in CFTR expression. Therefore, additional studies are needed to confirm this defect in IL-8 secretion in other isogenic cell lines and to determine more directly whether defective CFTR production and surface expression alter the secretion of immunoreactive IL-8.

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