

Modulation of Airway Inflammation in Cystic Fibrosis

In Vivo Suppression of Interleukin-8 Levels on the Respiratory Epithelial Surface by Aerosolization of Recombinant Secretory Leukoprotease Inhibitor

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Abstract

Based on the knowledge that neutrophil elastase (NE) in cystic fibrosis (CF) epithelial lining fluid (ELF) can induce human bronchial epithelial cells to express the gene for interleukin 8 (IL-8), an 8.5-kD neutrophil chemoattractant, we have evaluated CF ELF for the presence of IL-8, and investigated the ability of aerosolized recombinant secretory leukoprotease inhibitor (rSLPI) to suppress NE, and hence IL-8, levels on the respiratory epithelial surface in CF. Enzyme-linked immunoassay revealed 21.9 ± 4.8 nM IL-8 in CF ELF compared with none in normals. Active NE was detectable in ELF of all individuals with CF and was significantly decreased ($P < 0.03$) after aerosolization of rSLPI. Human bronchial epithelial cells exposed to CF ELF recovered before rSLPI therapy expressed IL-8 mRNA transcripts, but ELF recovered after rSLPI therapy induced far less bronchial epithelial cell IL-8 gene expression. Consistent with this, rSLPI aerosol therapy caused a marked reduction in CF ELF IL-8 levels ($P < 0.05$) and neutrophil number ($P < 0.02$). There was also a clear association between CF ELF active NE and IL-8 levels ($r = 0.94$). These data suggest that rSLPI therapy not only suppresses respiratory epithelial NE levels, but also breaks a cycle of inflammation on the CF epithelial surface. (*J. Clin. Invest.* 1992. 90:1296–1301.) Key words: aerosol therapy • antiprotease • cytokine • neutrophil chemoattractant • neutrophil elastase

Introduction

Cystic fibrosis (CF),¹ a chronic disorder caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, has its major clinical manifestations in the lung, with accumulation of purulent mucus, bacterial infections, and airway inflammation and obstruction leading to respiratory failure and death usually by the third decade (1–4). The exact mechanisms by which mutations of the CFTR gene result in these respiratory manifestations are not clear, but it is recognized that epithelial inflammation dominated by neutrophils plays a major role in the respiratory derangements associated with this disease (4–6). Although the neutrophils that accumu-

late on the airway epithelial surface may act appropriately and help in the clearance of microorganisms, they also have a deleterious effect on the epithelium itself (7–11). To a large extent, this damage is mediated by neutrophil elastase (NE), a proteolytic enzyme which directly damages epithelial cells, and interferes with normal host defense (5, 7, 9–14). Although the lung normally has sufficient amounts of serine antiproteases on the epithelial surface to protect against NE, the burden of neutrophils and hence NE is so great in CF that the serine antiprotease defensive shield is overwhelmed (5, 6, 15, 16).

The mechanisms by which neutrophils are attracted to the epithelial surface in CF are complex, and likely involve a variety of neutrophil chemoattractants generated in the airways (17, 18). One neutrophil chemoattractant, interleukin 8 (IL-8), may play a significant role in this process. IL-8, an 8.5-kD protein with potent neutrophil chemoattractive and activating properties, can be produced by bronchial epithelial cells after exposure to a variety of inflammatory mediators (19–23). In the context of CF, we have recently observed that respiratory epithelial lining fluid (ELF) of individuals with CF will induce cultured human bronchial cells to express the IL-8 gene and secrete IL-8, and that the mediator in CF ELF responsible for inducing IL-8 gene expression in the epithelial cells was NE (24). Further, the ability of CF ELF to induce bronchial cells to express the IL-8 gene could be suppressed in vitro with inhibitors of NE. Based on these observations, it is reasonable to postulate that in the CF lung, there is a cycle of respiratory inflammation in which neutrophils release large amounts of NE onto the respiratory epithelial surface which, in response, produces IL-8 with subsequent attraction of more neutrophils to that surface.

In the context that excessive amounts of neutrophils, and their product NE, play a major role in the pathogenesis of the respiratory manifestations of CF, and that free NE in CF ELF can induce bronchial epithelial IL-8 gene expression, the present study is directed toward evaluating this pathogenic scenario in vivo. In this regard, we have asked: (a) is there evidence of increased amounts of IL-8 on the respiratory epithelial surface in CF; and (b) if so, will in vivo suppression of the levels of active NE on the respiratory epithelial surface with a serine antiprotease suppress the ability of CF ELF to induce IL-8 gene expression in bronchial epithelial cells in vitro and suppress the levels of IL-8 on the respiratory epithelial surface in vivo?

Methods

Study population. The diagnosis of CF was made in 20 individuals (13 males, 7 females; aged 27 ± 2 yr)² using standard criteria, including a sweat chloride test (4). All were nonsmokers. Pulmonary function

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Received for publication 1 October 1991 and in revised form 16 March 1992.

1. Abbreviations used in this paper: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ELF, epithelial lining fluid; NE, neutrophil elastase; SLPI, secretory leukoprotease inhibitor; rSLPI, recombinant SLPI.

2. All data are expressed as mean \pm SEM; all statistical comparisons were done with the paired two-tailed Student's *t* test.

(25) tests showed (as percent predicted) forced expired volume in 1 s (FEV_1) 50 ± 4 , forced vital capacity (FVC) 68 ± 5 , FEV_1/FVC 72 ± 3 , and diffusing capacity 74 ± 4 . Baseline bronchoalveolar lavage (26, 27) in the CF group revealed $134 \pm 18 \times 10^3$ total cells/ μ l of ELF including $73 \pm 5\%$ neutrophils, $26 \pm 5\%$ macrophages, $< 1\%$ lymphocytes, and $< 1\%$ eosinophils.

To establish the status of IL-8 in normal ELF, 10 normals (29 ± 2 yr; four males, six females, all nonsmokers) were evaluated. All had normal histories, physical examinations, chest roentgenograms, and lung function tests. Bronchoalveolar lavage in the normals revealed $30 \pm 3 \times 10^3$ total cells/ μ l of ELF, including $1 \pm 1\%$ neutrophils, $93 \pm 1\%$ alveolar macrophages, $4 \pm 1\%$ lymphocytes and $< 1\%$ eosinophils.

IL-8, NE, and secretory leukoprotease inhibitor (SLPI) in ELF. ELF was recovered by bronchoalveolar lavage, as previously described (28). The presence and form of IL-8 in respiratory ELF was evaluated using Western analysis of 50-fold concentrated ELF (Centricon 3, Amicon Inc., Beverly, MA) with 15% sodium dodecyl sulphate (SDS) polyacrylamide gels and a polyclonal anti-human IL-8 antibody (Bio-source International, Westlake Village, CA). Levels of IL-8 in ELF were quantified by an enzyme-linked immunoassay (ELISA) using a pair of monoclonal antibodies against IL-8 (Genentech, Inc., South San Francisco, CA). Samples were serially diluted and a mean value determined from four replicates. This monoclonal-based IL-8 ELISA is very sensitive (assay range 15–560 pM) and highly specific (no detectable cross-reactivity with recombinant human interleukin-1 (IL-1), IL-2, IL-4, IL-6, interferon- γ , tumor necrosis factor- α , or granulocyte monocyte colony-stimulating factor). The amount of active NE in ELF was determined using the NE-specific substrate methoxy-succinyl-alanyl-alanyl-prolyl-valyl-nitroanilide in comparison to a NE standard of known activity (28). The levels of SLPI in ELF were quantified using a double-sandwich ELISA (29). The volume of ELF was measured by the urea method (27). NE activity, SLPI, and ELF measures were all performed in triplicate and the mean determined.

In vivo administration of recombinant SLPI (rSLPI). rSLPI (Syn-ergen, Boulder, CO) is a 12-kD single-chain nonglycosylated protein identical in structure and function as an inhibitor of NE to normal human SLPI (30). rSLPI, produced in *Escherichia coli* transformed with an SLPI expression vector as previously described (31), retains its structure and function when aerosolized in vitro or to experimental animals (32).

Aerosolization of rSLPI to individuals with CF was carried out in two phases: an initial dose escalation study to evaluate safety and to provide an initial estimate of in vivo pharmacokinetics, followed by a 1-wk study with a fixed dose for 7 d. Two individuals participated in both phases with an interval of > 1 mo between the two phases. The aerosol was generated with a compressed air-driven nebulizer (32). In vitro studies demonstrated the generated aerosol had a mass median diameter of 2.8 μ m, similar to the characteristics of the aerosol used to deliver rSLPI to the respiratory epithelial surface of large animals (32). For the initial dose escalating study, five individuals with CF received rSLPI at an escalating once daily dose of 1, 12.5, 25, 50, and 100 mg, immediately followed by a twice daily dose of 100 mg for 2 d. All were evaluated by bronchoalveolar lavage before therapy and 12 h after the last aerosol. For the 1-wk study 17 individuals with CF received an aerosol of rSLPI at a dose of 100 mg twice daily for 7 d and were evaluated by lavage pretherapy and at 4 h ($n = 8$) or 12 h ($n = 8$) after their 13th and 14th aerosol, respectively. One individual was removed from protocol because of inability to use the nebulizer device secondary to a preexisting debilitating extrapyramidal tremor; these results are not included.

IL-8 expression by bronchial epithelial cells. To evaluate the role of neutrophil elastase in CF ELF in inducing IL-8 gene expression in bronchial epithelium, the BET-1A human bronchial epithelial cell line (33) was exposed to ELF from individuals with CF. The cells were cultured in serum-free LHC-9 medium with 25 μ g/ml fungizone, 25 U/ml penicillin, and 25 μ g/ml streptomycin (all from Biofluids Inc., Rockville, MD) (34). All studies were carried out when the cells were 70–80% confluent. To these cells were added: 10 nM NE alone, 10 nM

NE plus 500 nM rSLPI, 1 μ l ELF (per milliliter of media) from normal individuals, 1 μ l ELF (per milliliter of media) from CF patients pretherapy, 1 μ l ELF (per milliliter of media) from the same CF patients pretherapy with 500 nM rSLPI added in vitro and 1 μ l ELF (per milliliter media) from the same CF patients after in vivo rSLPI aerosol therapy. After incubation (3 h, 37 $^\circ$), IL-8 mRNA transcripts were evaluated in the BET-1A cells by Northern analysis (35). Total cellular RNA was isolated by the guanidium thiocyanate-CsCl gradient method (36). The RNA (10 μ g per lane) was evaluated by formaldehyde-agarose gel electrophoresis, transfer to nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, NH), and hybridization with a 32 P-labeled IL-8 (pPB 248) or, as a control, β -actin cDNA (pHF β A-1) probe generated by the random priming method, and autoradiography (23, 37, 38). The IL-8 cDNA probe was constructed using polymerase chain reaction amplification of RNA (after conversion to cDNA) from lipopolysaccharide-stimulated human blood monocytes (23).

Results

Although IL-8 was undetectable in ELF from normal individuals, it was clearly present in ELF of individuals with CF (Fig. 1). Western analysis demonstrated no IL-8 in ELF of normals, but the 8.5-kD IL-8 molecule was detectable in CF ELF (Fig. 1A). The ELISA for IL-8 sufficiently sensitive to detect as little as 15 pM IL-8 did not detect IL-8 in normal ELF. However, in ELF from CF individuals, high levels of IL-8 are detected ranging from 1.7 to 98.1 nM, with an average of 21.9 ± 4.8 nM (Fig. 1B). As previously observed (29), SLPI was present in ELF of individuals with CF (range 0.02–4.80 μ M, Fig. 2A), as was active NE (range 1.2–85.7 μ M, Fig. 2B) i.e., despite the presence of SLPI, the burden of NE is so great in CF ELF that it far outweighs the anti-NE defenses, permitting active NE to be present in ELF.

Aerosol therapy with rSLPI was well tolerated, patients taking ~ 25 min to inhale a complete 100-mg dose of rSLPI. The initial study with an escalating dose of rSLPI suggested that a dose of 100 mg every 12 h would significantly raise ELF SLPI levels and suppress ELF active NE levels. After aerosolization of rSLPI, ELF levels of SLPI increased significantly, compared to pretherapy levels ($P < 0.01$) (Fig. 2A). Western blot analysis of ELF pretherapy showed SLPI in a cleaved or complexed form, but after aerosolization of rSLPI, normal molecular size SLPI was now evident in ELF (not shown). The augmentation

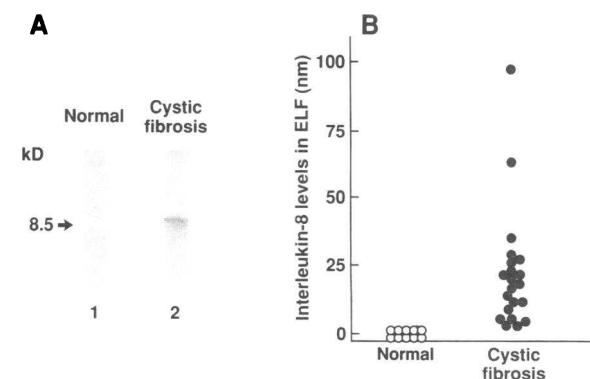


Figure 1. Form and levels of IL-8 in respiratory ELF. (A) Western analysis in respiratory ELF for the presence of IL-8. Lane 1, normal individual; lane 2, individual with cystic fibrosis (CF). The migration of an 8.5-kD IL-8 standard is indicated. (B) Levels of IL-8 in respiratory ELF from normals and individuals with CF. Each data point represents the average of four determinations for each individual.

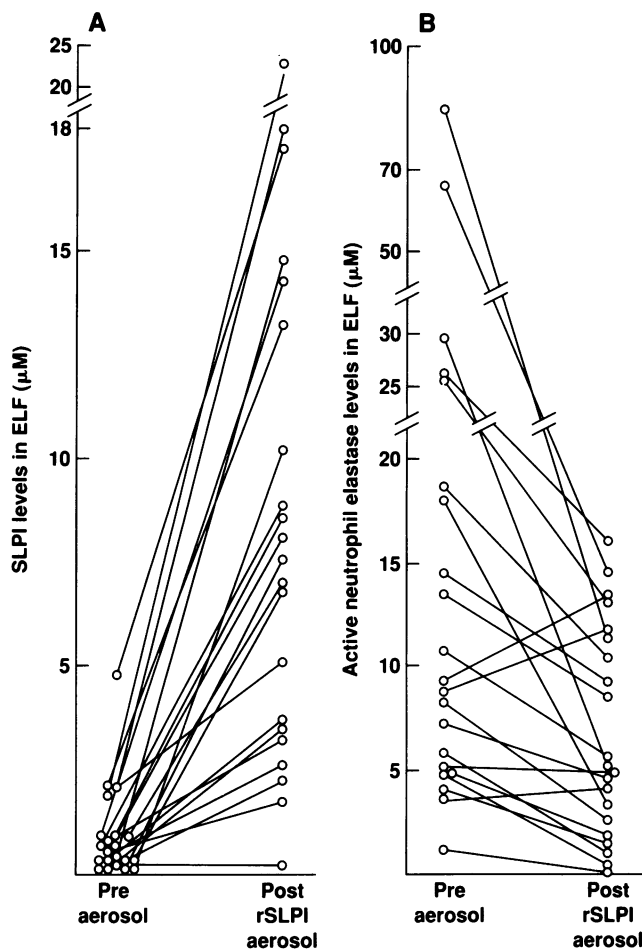


Figure 2. Effect of rSLPI aerosol therapy on SLPI and active NE levels in ELF of individuals with CF. (A) SLPI levels in CF ELF before and after rSLPI aerosol therapy. (B) NE levels in CF ELF before and after rSLPI aerosol therapy. Each datapoint represents the average of three determinations for each individual.

in ELF SLPI levels was associated with a concomitant fall in ELF active NE levels in almost all individuals (Fig. 2B). In this regard, for 18 of 21 individuals, there was a significant suppression of ELF NE. For the entire group, the pretherapy active NE levels decreased an average of 68% ($P < 0.03$).

In the context that NE was present on the CF respiratory epithelial surface, and that NE in CF ELF is capable of inducing bronchial epithelial cells to express the IL-8 gene (24), CF ELF was assessed *in vitro* for its effect on IL-8 gene expression in bronchial epithelial cells before and after the individuals with CF received aerosol therapy with rSLPI. Resting BET-1A cells demonstrated no detectable IL-8 mRNA transcripts (Fig. 3, lane 1), but after exposure to active NE, 1.8-kb IL-8 gene transcripts were present (lane 2). The IL-8 gene expression by NE was obviated by the addition of rSLPI *in vitro* (lane 3). ELF from normal individuals added to BET-1A cells did not induce IL-8 gene expression (lane 4). However, ELF from CF individuals caused a marked expression of the IL-8 gene as evidenced by easily detectable IL-8 mRNA transcripts (lane 5), which was prevented by the *in vitro* addition of rSLPI to the CF ELF (lane 6). Strikingly, in contrast to CF ELF pretherapy (lane 5), ELF from CF patients post-rSLPI aerosol therapy

caused markedly decreased IL-8 gene expression in bronchial epithelial cells (lane 7).

Consistent with these *in vitro* studies, levels of IL-8 in CF ELF fell dramatically with rSLPI therapy (Fig. 4). In this regard, for 15 of 21 CF individuals receiving the rSLPI aerosol, ELF IL-8 levels decreased; for the group as a whole there was a 50% reduction in ELF IL-8 levels (before therapy 21.7 ± 4.8 nM, after therapy 10.7 ± 1.4 nM; $P < 0.05$). Concomitant with this was a fall in the number of neutrophils in ELF (Fig. 5; before therapy $10.6 \pm 1.8 \times 10^4/\mu\text{L}$ of ELF, after therapy $5.8 \pm 0.8 \times 10^4/\mu\text{L}$ of ELF; $P < 0.02$). Further, as evidence that NE in CF ELF induced IL-8 gene expression *in vivo*, the relationship of ELF IL-8 and active NE in CF ELF (all data combined, including before and after therapy) demonstrated a remarkable correlation ($r = 0.94$, $P < 0.01$; Fig. 6).

Discussion

IL-8, an 8.5-kD potent chemoattractant and activator of neutrophils, is released by bronchial epithelial cells in response to a variety of inflammatory stimuli, including NE (19–24). The present study demonstrates that IL-8 is not detectable in normal respiratory epithelial lining fluid, but is present in large quantities in CF ELF, a disorder characterized by neutrophil-dominated chronic respiratory tract inflammation (4–6). *In vitro* studies have demonstrated that NE will induce bronchial epithelial cells to express the IL-8 gene and release IL-8, and that the active NE in respiratory ELF of individuals with CF has the same effect (24). The induction of the expression of the IL-8 gene is blocked by the addition of inhibitors of NE such as phenylmethylsulfonyl-fluoride, methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone, plasma purified α_1 -antitrypsin, or rSLPI. We therefore hypothesized that suppression of NE *in vivo* on the respiratory epithelial surface in CF would prevent respiratory epithelial cell IL-8 gene expression, consequently lowering CF ELF IL-8 levels and thereby decrease the neutrophil-dominated inflammation on the respiratory epithelial surface in CF. This is what was observed. When rSLPI was aerosolized to individuals with CF, respiratory ELF

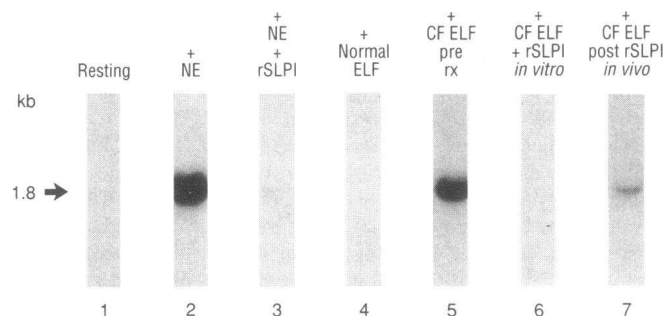


Figure 3. Northern analysis of IL-8 mRNA transcripts in BET-1A bronchial epithelial cells. All lanes contained 10 µg of total cellular RNA. Lane 1, cells at rest; lane 2, cells exposed to NE; lane 3, cells exposed to NE + rSLPI; lane 4, cells exposed to ELF from a normal individual; lane 5, cells exposed to ELF from an individual with CF before rSLPI aerosol therapy; lane 6, as in lane 5, but with rSLPI added *in vitro*; and lane 7, ELF from the same individual as lanes 5 and 6, but after *in vivo* rSLPI aerosol administration. The 1.8-kb IL-8 mRNA transcripts are indicated. Parallel analysis with a γ -actin probe showed γ -actin transcripts of equal intensity in all lanes (not shown).

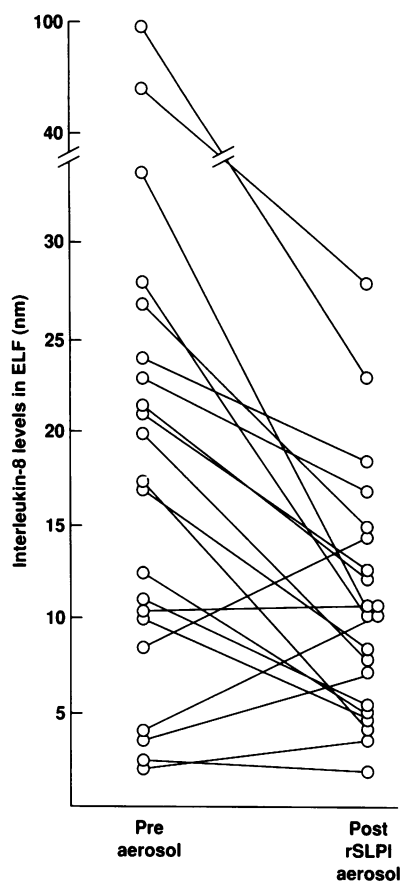


Figure 4. Effect of rSLPI aerosol therapy on IL-8 levels in CF ELF. IL-8 levels were determined before and after rSLPI aerosol. Each data point represents the average of four determinations from each individual; a line connects each individual before and after therapy.

SLPI levels increased, ELF active NE levels decreased, CF ELF had markedly diminished ability to induce IL-8 gene expression in bronchial epithelial cells in vitro, ELF IL-8 levels were markedly suppressed in vivo, and the number of neutrophils in ELF decreased. Although it is not possible to examine the bronchial epithelium per se for IL-8 gene expression in vivo, the fact that there was an excellent correlation between CF ELF IL-8 and active NE levels strongly supports the link between NE and respiratory epithelial cell IL-8 gene expression observed in vitro.

The major clinical manifestations of CF take place on the respiratory epithelial surface (4–6). Although neutrophils in the lung in CF must have a protective value in regards to host defense, the massive chronic neutrophil-dominated inflammatory response in CF is associated with chronic epithelial damage (4–11). There is growing evidence that NE released by the neutrophils plays a major role in this process, not only by its direct assault on the respiratory epithelial surface but also by impairing lung defense by virtue of its ability to adversely affect mucociliary clearance, and to cleave immunoglobulins, complement components, and complement receptors on neutrophils (9–14, 39). The later observation is of particular importance for respiratory host defense, in that many of the neutrophils attracted to the CF lung may be rendered ineffective and thus may cause damage rather than provide antimicrobial protection (5, 12, 13). The fact that NE can act as an abnormal stimulus to IL-8 production on the bronchial epithelial surface suggests it plays a central role in a cycle of inflammation, stimulating IL-8 release leading to more neutrophil accumulation with more NE release. Furthermore, IL-8 promotes NE release

from neutrophils, thereby accentuating this cycle of inflammation (40). In addition to bronchial epithelial cells, other cells in the CF lung may contribute to the IL-8 burden on the respiratory epithelial surface, including macrophages, neutrophils, alveolar epithelial cells, endothelial cells, and fibroblasts (19–24). It is difficult to assess the role of NE in inducing IL-8 gene expression for most of these cells in vivo because of their inaccessibility (alveolar epithelium, endothelium, and fibroblasts) or chronic stimulation secondary to bacteria (macrophages and neutrophils), and thus, for now their relative contribution to NE induced IL-8 expression compared to that of the bronchial epithelium in CF lung is unknown. However, the clear effect of NE on bronchial cells, taken in conjunction with the in vivo relationships between active NE and IL-8 in CF ELF, suggest the bronchial epithelium as a significant contributor to NE-induced IL-8 production in the CF lung.

The major protection of the lung against NE includes α_1 -antitrypsin and SLPI. α_1 -Antitrypsin, a 52-kD glycoprotein, is produced in the liver and secreted into plasma where it is available to diffuse across into tissues including the lung (15). SLPI, a 12-kD, nonglycosylated, disulfide-linked antiprotease secreted by cells of mucosal surfaces including the epithelium of the airways, provides the major anti-NE protection of the large airways (15, 29, 41–43). In CF, the endogenous anti-NE defenses are overwhelmed and inactivated due to the massively increased burden of NE. This is true for both α_1 -antitrypsin and SLPI; both molecules in CF ELF are complexed with NE, proteolytically cleaved, or modified by oxidants released by inflammatory cells (5, 16, 29). Thus, in CF there is a major imbalance on the epithelial surface between the large burden of NE and the ineffective anti-NE defenses. This imbalance can be redressed and the active NE on the epithelial surface suppressed by aerosol augmentation with recombinant SLPI. rSLPI can be placed in aerosol droplets capable of depositing on the respiratory epithelial surface without losing form or function. The molecule is acid stable and thus may retain function in the environment of the neutrophil metabolic burst (44, 45). Further, rSLPI has an isoelectric point similar to that of

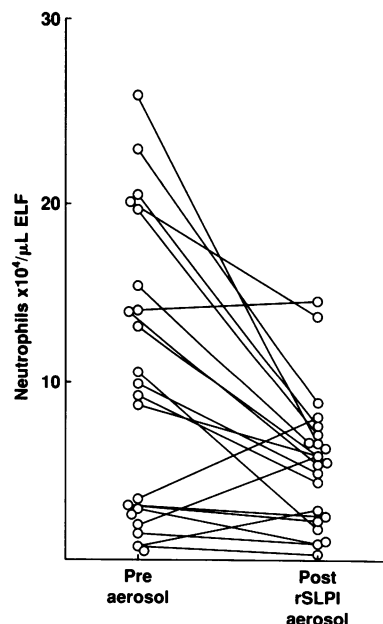


Figure 5. Effect of rSLPI aerosol therapy on the number of neutrophils in CF ELF. Neutrophil numbers were determined before and after rSLPI aerosol. Each data point represents the average of two determinations for each individual.

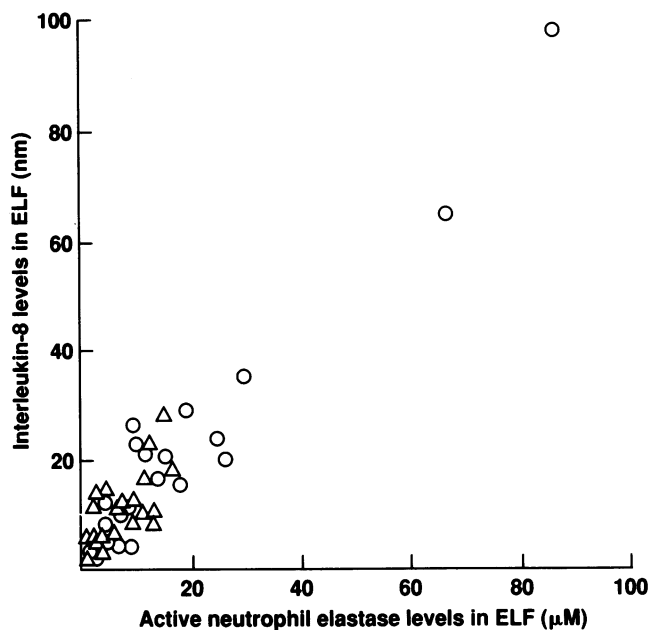


Figure 6. Correlation between IL-8 levels and active NE levels in ELF of individuals with CF. Included are data before therapy (○) and after therapy (△). The data are based on Figs. 2 B and 4.

NE, and thus may be able to track NE to various tissue sites and inhibit tissue-bound NE (15, 29, 45). Finally, whereas α_1 -antitrypsin provides > 90% of the anti-NE defenses of the lower respiratory tract, SLPI is the major anti-NE inhibitor of the bronchi (15, 29), and may be an ideal molecule for protecting the bronchial epithelium in disorders such as CF which are characterized by bronchial epithelial damage.

Aerosolization of rSLPI to individuals with CF suppresses the NE burden on the respiratory epithelial surface and at the same time greatly reduces the IL-8 levels in ELF. Although this may have little effect on other potential IL-8-inducing molecules such as tumor necrosis factor- α (23), the effect of rSLPI in inhibiting NE is of particular importance in CF because the massive NE burden on the epithelial surface is the most likely major local stimulus to IL-8 production (5, 24). The mechanisms by which NE induces IL-8 gene expression and IL-8 release from bronchial epithelium are unknown, but in vitro, NE induces bronchial epithelial cell IL-8 gene transcription, leading to increased IL-8 mRNA levels and secretion of functional IL-8 (24). Although there are many potential neutrophil chemoattractants in CF ELF other than IL-8, the fact that IL-8 mRNA transcript levels in bronchial epithelium can be markedly reduced in vitro and IL-8 protein levels in ELF markedly reduced in vivo by rSLPI administration, and the clear correlation between active NE levels and IL-8 levels in ELF before and after therapy, argue strongly for the role of NE as a major inducer of IL-8 in the CF lung. Furthermore, after aerosolization of rSLPI and the subsequent decrease in active NE and IL-8 levels in CF ELF, there was also a decrease in the number of neutrophils in ELF, further evidence for the role of IL-8 as a major neutrophil chemoattractant in this disorder. In this regard, suppression of the NE burden on the respiratory epithelial surface may remove the NE stimulus to local respiratory IL-8 production with resultant decrease in neutrophil chemoattraction in the lung, leading to less neutrophil chemoat-

traction, less NE release, and thus decreased epithelial damage and reduced impairment of lung host defense in CF.

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