Increased elastase release by CF neutrophils is mediated by tumor necrosis factor-α and interleukin-8

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Increased elastase release by CF neutrophils is mediated by tumor necrosis factor-α and interleukin-8. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L33–L41, 2000.—Cystic fibrosis (CF) is a lethal, hereditary disorder characterized by a neutrophil-dominated inflammation of the lung. We sought to determine whether neutrophils from individuals with CF release more neutrophil elastase (NE) than neutrophils from normal subjects. Our results showed that peripheral blood neutrophils (PBNs) from normal subjects and individuals with CF contained similar amounts of NE, but after preincubation with CF bronchoalveolar lavage (BAL) fluid, significantly more NE was released by CF PBNs, a release that was amplified further by incubation with opsonized Escherichia coli. To determine which components of CF BAL fluid stimulated this excessive NE release from CF PBNs, we repeated the experiments after neutralization or immunoprecipitation of tumor necrosis factor (TNF)-α and interleukin (IL)-8 in CF BAL fluid. We found that subsequent NE release from CF PBNs was reduced significantly when TNF-α and IL-8 were removed from CF BAL fluid. When TNF-α and IL-8 were used as activating stimuli, CF PBNs released significantly greater amounts of NE compared with PBNs from control subjects and individuals with bronchiectasis. These results indicate that CF PBNs respond abnormally to TNF-α and IL-8 in CF BAL fluid and react to opsonized bacteria by releasing more NE. This may help explain the increased NE burden seen in this condition.

secretion; inflammation; proteases; cytokines

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations of the CF transmembrane conductance regulator (CFTR) gene, a 27-exon, 250-kb segment of chromosome 7 at q31 (17, 25, 26). The major cause of mortality and morbidity in patients with CF is lung disease from chronic pulmonary insufficiency, characterized by a neutrophil-dominated inflammation on the respiratory epithelial surface (5, 20). Extensive research has shown that elevated levels of proteases released from neutrophils, most significantly neutrophil elastase (NE), overwhelm the antiprotease defenses of the lung, thus rendering the epithelium susceptible to proteolytic attack and destruction (3, 23).

The enormous NE burden in the CF lung may be due to infection caused by microorganisms such Staphylococcus aureus or Pseudomonas aeruginosa. However, active NE has been detected in the lungs of very young infants with CF even before the onset of bacterial colonization or infection (2, 4, 19). Although these elevated levels of NE may be due to the increased NE burden on the CF epithelial surface, there are also data to suggest that CF neutrophils differ from normal neutrophils. Stimulated neutrophils from individuals with CF have been shown to release significantly more oxidants (32) and shed significantly less L-selectin compared with those from control subjects (27). This raises the question as to whether the increased NE on the respiratory epithelial surface in CF is due to exaggerated NE secretion by the CF neutrophil. Furthermore, correlation between the CFTR mutation and lung inflammation has been suggested, with dysregulation of cytokine production by CF epithelial cells postulated as causal factors for the sustained inflammation associated with CF (6, 7). These elevated levels of proinflammatory cytokines may act to exaggerate NE secretion from CF neutrophils.

To evaluate this hypothesis, we compared NE release from peripheral blood neutrophils (PBNs) of CF individuals and control subjects. We exposed these cells to the various stimuli found in the milieu of the CF lung. After this, we examined the role of proinflammatory cytokines, shown by these experiments to be centrally involved in NE secretion from CF neutrophils, and compared these effects with those observed for control neutrophils. We also evaluated PBNs from individuals with long-term, non-CF bronchiectasis to ensure that any changes we found were not due to a chronic pulmonary inflammatory stimulus.

MATERIALS AND METHODS

Study Population

Ten children with CF and ten age- and sex-matched control subjects were evaluated for the study, and, in addition, 10 non-CF bronchiectatic patients served as inflammatory controls to the CF population for some of the experiments. The CF individuals attended Our Lady's Hospital for Sick Children (OLHSC; Dublin, Ireland). The mean age of the children...
was 8 ± 4 yr (range 4–12 yr), and the mean forced expiratory volume in 1 s was >50% of the predicted value. CF was diagnosed by standard criteria including sweat tests and genotyping. All the CF patients studied were ΔF508 homozygotes and were colonized with Pseudomonas species. All received standard CF therapy, but none had an active exacerbation at the time of the study. The patients with moderately severe pulmonary bronchiectasis were selected from outpatient clinics. They were all negative for the ΔF508 deletion and had normal sweat tests. Childhood infection was the etiology in all cases. The mean forced expiratory volume in 1 s was 50 ± 5% of the predicted value. All were clinically stable and free of infective exacerbation for at least 6 wk at the time of the study. All patients had normal arterial blood gases. This study was performed under a protocol approved by the Institutional Review Board (OLHSC).

Neutrophil Isolation

Neutrophils were isolated from heparinized (10 U/ml; Sarstedt) venous blood. Briefly, density gradient centrifugation was carried out in Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) to separate the red cell pellet containing the neutrophil population from the lymphocytes. The neutrophils were separated from the erythrocytes by sedimentation in a 3% dextran solution. Residual erythrocytes were lysed by treating the cell pellet with hypotonic saline solution followed by addition of an equal volume of hypertonic saline and finally by washing in Hanks’ balanced salt solution (HBSS; Sigma Aldrich, Poole, UK). The isolated neutrophils were resuspended in RPMI 1640 medium (Sigma Aldrich) and counted. Cell viability was confirmed by trypan blue dye exclusion.

Priming and Activation of Neutrophils

Neutrophils were resuspended in RPMI 1640 medium at 1 × 10⁶ cells/ml, and a sample of 250 µl of cells was used for each experiment unless stated otherwise.

Activation with various stimuli. The cells were incubated with phorbol 12-myristate 13-acetate (PMA; 500 nM), formylmethionyl-leucyl-phenylalanine (fMLP; 400 nM), or opsonized Escherichia coli (20 µl; prepared for Becton Dickinson by Orpegen Pharma, Heidelberg, Germany) for 30 min at 37°C. The stimuli were chosen because PMA is a protein kinase C agonist and therefore mimics the actions of a number of inflammatory cytokines. fMLP is a secreted bactericidal product capable of activating neutrophils, and opsonized E. coli is a phagocytosable organism representative of the opsonized microorganisms almost invariably present in the lungs of individuals with CF. The samples were centrifuged at 200 g for 10 min at 4°C, and the supernatant was removed for determination of NE. The cell pellet was resuspended in 0.1% Triton X-100 in PBS and kept for NE determination.

Incubation with CF bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid (BAL) fluid was obtained from CF individuals with the standard guidelines set out by Klech and Polli (18). CF BAL fluid (50 µl) was added to each cell suspension and incubated for 45 min at 37°C. The cells were then centrifuged at 200 g for 10 min at 4°C, and the supernatant was removed and discarded. The neutrophils were washed twice in HBSS to remove all remnants of NE activity already present in the CF BAL fluid. The cells were then resuspended in medium or medium containing 20 µl of opsonized E. coli for 30 min at 37°C. An incubation period of 30 min with suitable stimuli has been previously shown to be optimal for NE release from neutrophils (8, 11). The samples were centrifuged as before, the supernatants were retained for measurement of NE, and the lysates were resuspended in PBS-0.1% Triton X-100 for NE determination. Because NE may have been released from both sets of neutrophils during incubation with CF BAL fluid, neutrophil cell lysates were retained after incubation with CF BAL fluid to determine how much NE was released. The cells were lysed with 0.2% Triton X-100 in PBS, and NE content was estimated by ELISA.

The neutrophils were also incubated with CF BAL fluid to which neutralizing antibodies to tumor necrosis factor (TNF)-α and interleukin (IL)-8 (R&D Systems, Abingdon, UK) had been added. For this experiment, mouse anti-human TNF-α IgG and mouse anti-human IL-8 IgG were added separately to 50 µl of CF BAL fluid at a final concentration of 12.5 µg/ml for 30 min at room temperature. This BAL fluid sample was then added to the CF and control neutrophils followed by washing and incubation with opsonized E. coli as outlined above. An isotype control IgG was also added to the CF BAL fluid at a final concentration of 12.5 µg/ml and was then added to the CF and control neutrophils followed by washing and incubation with opsonized E. coli. The supernatants were retained for measurement of NE release.

Finally, the neutrophils were incubated with anti-TNF-α IgG and anti-IL-8 IgG either separately or together as described above. The antibody-antigen complexes were then removed by immunoprecipitation with protein A/G (30 µl; Calbiochem-Novabiochem, Nottingham, UK) for 2 h at 4°C. CF BAL fluid was also treated with protein A/G in the same way. After this time, the protein A/G beads containing antibody-antigen complexes were removed by centrifugation at 13,000 rpm for 2 min. The remaining BAL fluid supernatant was then added to CF and control neutrophils followed by washing and incubation with opsonized E. coli as outlined above. The supernatants were retained for measurement of NE release.

Measurement of TNF-α and IL-8 in CF BAL Fluid

Levels of TNF-α and IL-8 in CF BAL fluid were measured with commercially available quantitative ELISA kits (R&D Systems).

Activation of NE Release by Dual-Cytokine Stimulation

Neutrophils from normal, bronchiectatic, and CF individuals were activated with TNF-α (10 ng/ml) for 5 min followed by IL-8 (100 ng/ml) for 30 min at 37°C. After this time, the cells were spun down, and the supernatants and cell lysates were kept as before for the measurement of NE.

Measurement of NE Activity in Cell Supernatants

NE activity in neutrophil supernatants was determined with the NE-specific substrate N-methoxyxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (Sigma). Liberation of p-nitroaniline was measured at a 405 nm over a 5-min time period. NE activity in the supernatants was compared with active NE standard (Sigma).

Measurement of NE Concentration by ELISA

Sheep anti-human NE IgG (Serotech, Kidlington, UK) was diluted 1:1,000 in 0.1 M carbonate buffer, pH 9.6, and 100-µl
aliquots were loaded onto a 96-well plate (Immulon 2, Dynatech, Chantilly, VA) and left overnight at 4°C in a humidified chamber. The plates were washed with 200-μl aliquots of PBS-0.05% Tween (PBS-T) three times, and at the end, 100-μl aliquots of PBS-T were pipetted onto the plate. Samples were preincubated with phenylmethylsulfonyl fluoride (Sigma Aldrich) to prevent active NE in the samples from degrading the NE-specific antibodies used in the ELISA and applied to the plate in duplicate 100-μl aliquots. NE standard (250 ng/ml), also inactivated with phenylmethylsulfonyl fluoride, was applied to the plate in duplicate 100-μl aliquots. The standard and samples were then diluted 1:2 across the plate and left at room temperature for 2 h. After this time, the plates were washed as before, and rabbit anti-NE IgG (Calbiochem-Novabiochem) diluted 1:1,000 in PBS-T was loaded onto the plate in 100-μl aliquots and left at room temperature for 1 h. Finally, goat anti-rabbit horseradish peroxidase IgG (DAKO, High Wycombe, UK) diluted 1:2,000 in PBS-T was pipetted onto the plate in 100-μl aliquots and left at room temperature for 1 h. After a final wash, 100 μl of the peroxidase substrate o-phenylenediamine was loaded into each well, and the color was allowed to develop. After development, the reaction was stopped with 50 μl of 2MH₂SO₄ and read at 410 nm with a microtiter plate reader (Bio-Tek, Southhampton, UK). Absorbance values were converted to actual NE concentrations by four-parametric logistic fit of the data with the LYSIS II software (Becton Dickinson, Fullerton, CA).

Measurement of Fc Receptor Type IIa and CD11b/18 Receptor Densities on Neutrophils

A sample (100 μl) of cells resuspended in RPMI 1640 medium was preincubated with 20 μl of CF BAL fluid for 45 min at 37°C. The neutrophils were then washed with HBSS twice and resuspended in medium. BAL fluid-treated neutrophils were then incubated with 5 μl of anti-CD11b/18-PE (Becton Dickinson, Mountain View, CA), 1 μl of anti-Fc receptor type IIa (FcrIIa; Medarex, Annandale, NJ), or 5 μl of isotype control IgG for 30 min at 4°C. After two washes in wash buffer, the cells incubated with anti-CD11b/18-PE were fixed with Cell-Fix (Becton Dickinson). Those neutrophils incubated with anti-FcR IIa were resuspended in RPMI 1640 medium and probed with FITC-labeled goat anti-mouse IgG (DAKO) for 30 min at 4°C. After this time, the cells were washed and fixed as before. Receptor binding of the respective antibodies was quantified by flow cytometry. Flow cytometry analysis was performed on a FACSScan flow cytometer (Becton Dickinson). Receptor binding of the respective antibodies was quantified by flow cytometry. Flow cytometry analysis was performed on a FACSScan flow cytometer (Becton Dickinson) with a 488-nm air-cooled argon laser. A total of 10,000 gated neutrophils were discriminated from lymphocytes with forward versus side (90°) light-scatter characteristics. Fluorescence light emission was collected with a 520-nm band-pass filter. Data were stored and subsequently analyzed with LYSIS II software (Becton Dickinson).

Measurement of TNF-α Receptor Types I and II and IL-8 Receptor Types A and B Densities on Neutrophils

A sample (100 μl) of cells resuspended in RPMI 1640 medium was preincubated with 20 μl of CF BAL fluid for 45 min at 37°C. The neutrophils were then washed with HBSS twice and resuspended in medium. BAL fluid-treated neutrophils were then incubated with 10 μl of anti-TNF-α receptor types I and II (TNFRI/II)-FITC or anti-IL-8 receptor types A and B (IL-8RA/B)-FITC (R&D Systems) for 30 min at 4°C. After two washes in wash buffer, the cells were fixed with Cell-Fix (Becton Dickinson). Receptor binding of the respective antibodies was quantified by flow cytometry. Flow cytometry analysis was performed on a FACSScan flow cytometer (Becton Dickinson) with a 488-nm air-cooled argon laser. A total of 10,000 gated neutrophils were discriminated from lymphocytes with forward versus side (90°) light-scatter characteristics. Fluorescence light emission was collected with a 520-nm band-pass filter. Data were stored and subsequently analyzed with LYSIS II software (Becton Dickinson).

Statistical Evaluation

Data were analyzed with the GraphPad Prism software package (GraphPad Software, San Diego, CA). Results are expressed as means ± SE and were compared with ANOVA, Student’s two-tailed t-test (paired or unpaired), or nonparametric tests such as Kruskal-Wallis with Dunn’s post hoc analysis as indicated. Differences were considered significant when the P value was 0.05 or less.

RESULTS

Measurement of TNF-α and IL-8 in CF BAL Fluid

Quantitation of TNF-α in CF BAL fluid revealed that this protein was present at 8.5 ng/ml. IL-8 was present at 67 ng/ml CF BAL fluid.

Total NE and NE Release From Control and CF PBNs

The total NE for PBNs isolated from control subjects and individuals with CF is shown in Fig. 1. The mean NE value for control PBNs was 220 ± 33 ng compared with 221 ± 41 ng for individuals with CF (p = 0.33). To evaluate NE release from stimulated cells, three stimuli were used to activate the CF and control neutrophils. PBNs were activated with fMLP, PMA, and opsonized E. coli. Stimulation resulted in extremely low NE

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**Fig. 1.** Neutrophil elastase (NE) in lysates of normal (Norm) and cystic fibrosis (CF) neutrophils and in normal and CF supernatants after stimulation with opsonized Escherichia coli. To determine total cellular NE, normal and CF neutrophils were lysed (PBS-Triton X-100) and analyzed for NE content by ELISA. To determine effect of opsonized bacteria on NE secretion, an equal number of normal and CF neutrophils were incubated with 20 μl of opsonized E. coli for 30 min at 37°C. Cells were centrifuged, and supernatant was retained for analysis of NE content by ELISA. Total NE content was the same in CF and normal neutrophils. Addition of opsonized E. coli to CF and normal neutrophils resulted in little increase in NE secretion over baseline.
release from both the CF and control neutrophils with fMLP (CF, 0.41 ± 0.09 ng; control, 0.85 ± 0.41 ng) and PMA (CF, 2.85 ± 0.72 ng; control, 0.97 ± 0.3 ng). Activation with the strongest stimulant, opsonized E. coli, resulted in <2% release of the total neutrophil NE complement (CF, 3.37 ± 1.32 ng; control, 3.22 ± 1.12 ng; Fig. 1). This suggests that PBNs are not primed for activation before they enter the lung in either individuals with CF or control subjects.

Incubation With CF BAL Fluid

To evaluate NE release in conditions similar to those encountered in the CF lung, CF and control PBNs were primed in CF BAL fluid. This was followed by washing and resuspension in medium and measurement of spontaneous NE release. The results shown in Fig. 2 show NE release from CF BAL fluid-primed cells for both CF individuals and control subjects. This result is higher than that obtained for NE release with fMLP, PMA, or opsonized E. coli. However, NE release for the CF group (33.2 ± 5.1 ng) was significantly higher than that in the control group (23.9 ± 6.1 ng; P < 0.05), suggesting that although the CF BAL fluid may markedly enhance NE release from both the CF and control neutrophils, the CF cells have an intrinsic propensity to secrete more NE. NE release from both sets of neutrophils during the incubation period in CF BAL fluid was determined by measuring the NE content of the cell lysates after this period (and subtracting it from the mean NE content of both sets of cells as shown in Fig. 1). This revealed that only a small amount of NE (<20 ng/250,000 cells) was released from the cells during incubation with CF BAL fluid. Therefore, it is unlikely that a greater amount of NE was released from the normal neutrophils compared with that from CF neutrophils during incubation with CF BAL fluid.

Neutrophils were also incubated with opsonized bacteria after preincubation with CF BAL fluid. This was to determine how CF and control cells respond to phagocytosable particles in an environment similar to that encountered by neutrophils in the CF lung exposed to opsonized microorganisms. Once again, NE release was higher in both groups (Fig. 2) compared with the results obtained with stimuli alone or cells pretreated with CF BAL fluid only. NE release was markedly increased in the CF group compared with control group (CF, 91.6 ± 11.7 ng; control, 48.1 ± 1.6 ng; P < 0.01) and represented ~42% of the total NE complement compared with only 22% for the control neutrophils. These results show that in conditions similar to those found in the CF lung, neutrophils from individuals with CF can release up to twice as much NE as control neutrophils.

In all experiments, the NE activity in each supernatant was also measured with a NE-specific substrate. The results obtained revealed that more active NE was present in CF samples than in normal samples and correlated very closely with the results obtained for antigenic NE levels in each supernatant (r = 0.8).

Evaluation of FcRIIa and CD11b/18 Receptor Expression on Neutrophils After Incubation in CF BAL Fluid

To evaluate whether the increased E. coli-stimulated NE release, noted in CF neutrophils after incubation with CF BAL fluid, was due to increased expression of receptors involved in the binding and internalization of opsonized bacteria, we measured FcRIIa and CD11b/18 receptor expression on control and CF neutrophils. The data shown in Fig. 3 show no differences in CD11b/18 receptor [control, 1,005 ± 131 mean channel fluorescence (MCF); CF, 962 ± 96 MCF; P = 0.42] or FcRIIa (control, 1,129 ± 38 MCF; CF, 1,029 ± 90 MCF; P = 0.27) expression after incubation with CF BAL fluid. This suggests that exposure to CF BAL fluid does not increase receptor binding or ingestion of opsonized E. coli by neutrophils from CF patients compared with those from control subjects.

Evaluation of Factors in CF BAL Fluid Involved in Stimulating NE Release

To ascertain the role of proinflammatory cytokines in CF BAL fluid in exaggerating the response of CF neutrophils to opsonized E. coli, neutralizing antibodies to these stimuli were incubated with CF BAL fluid to abolish their activity. The results shown in Fig. 4 show that NE release from normal (A) or CF (B) neutrophils is not affected when neutralizing antibodies to either TNF-α or IL-8 are added separately to CF BAL fluid followed by incubation with opsonized E. coli (for anti-TNF-α IgG, effect on NE release was 93.8 ± 15.4 to 95.1 ± 16.0 ng for CF and 51.3 ± 6.4 to 50.8 ± 10.7 ng for normal neutrophils; for anti-IL-8 IgG, effect on NE release was 93.8 ± 15.4 to 95.0 ± 8.3 ng for CF and 51.3 ± 6.4 to 51.2 ± 12.5 ng for normal neutrophils compared with that from CF neutrophils during incubation with CF BAL fluid.)
phils). The addition of an isotype control antibody to CF BAL fluid did not reduce NE release from CF (96.11 ± 8.17 ng) or control (51.28 ± 3.93 ng) neutrophils. However, the addition of anti-TNF-α and IL-8 IgGs together to CF BAL fluid followed by incubation with opsonized E. coli had the effect of reducing NE release from CF (93.8 ± 15.4 to 43.9 ± 6.0 ng; P < 0.05) and normal (51.3 ± 6.4 to 38.6 ± 7.2 ng) neutrophils. Intriguingly, the reduction in NE release is more pronounced from CF neutrophils (~53%) compared with that from control neutrophils (~25%). This indicates that the TNF-α and IL-8 components of CF BAL fluid act together to have a greater effect on the subsequent NE release from CF neutrophils when these cells are incubated with opsonized E. coli in comparison to the effect on NE release observed for control neutrophils treated in the same manner.

As before, NE activity in each sample was also measured and found to correlate well with the antigenic levels of NE determined (r = 0.73). This revealed that NE activity was decreased only in the cells treated with anti-TNF-α and anti-IL-8 IgGs together.

**Immunoprecipitation of TNF-α and IL-8 From CF BAL Fluid**

To ensure that the reduction in NE release from CF and normal neutrophils observed in the presence of both anti-TNF-α and anti-IL-8 IgGs was not due to an immunosuppressive effect of the antibody-antigen complexes on neutrophil function, we immunoprecipitated TNF-α and IL-8 from CF BAL fluid separately and together. To do this, the same antibodies used in the blocking experiments were used again. However, on this occasion, the antibody-antigen complexes formed were removed from the CF BAL fluid with protein A/G (depletion of TNF-α and IL-8 was confirmed by measuring the levels of both cytokines with ELISA). Incubation of neutrophils with CF BAL fluid treated in this manner gave similar results as those observed in the blocking experiments. The results in Fig. 5 show that NE release from normal (A) and CF (B) neutrophils after exposure to opsonized E. coli was reduced by 10.22 ± 0.33 ng on October 19, 2017 http://ajplung.physiology.org/ Downloaded from 0.53% (* P < 0.05).
79 ± 8.1 ng for CF and 46.75 ± 10.4 to 43 ± 7.6 ng for normal neutrophils). However, the immunoprecipitation of TNF-α and IL-8 together from CF BAL fluid followed by incubation with opsonized E. coli had the effect of reducing NE release from CF (83.1 ± 7.7 to 37.8 ± 4.4 ng; P < 0.05) and normal (46.75 ± 10.4 to 35.8 ± 6.4 ng) neutrophils as observed in the blocking experiments. As before, the reduction in NE release is more pronounced from CF neutrophils (~55%) compared with that from control neutrophils (~24%). These results confirm those of the blocking experiments that showed that the TNF-α and IL-8 components of CF BAL fluid act together to have a greater effect on the subsequent NE release from CF neutrophils. Once again, NE activity was higher for the CF cell supernatants than for the normal supernatants and correlated closely with antigenic NE amounts (r = 0.76).

Evaluation of TNFRI/II and IL-8RA/B Expression on Neutrophils Before and After Incubation With CF BAL Fluid

To evaluate whether the increased NE release noted in CF neutrophils after incubation with CF BAL fluid was due to increased expression of TNF-α and IL-8 receptors, we measured the cell surface expression of TNFRI/II and IL-8RA/B on control and CF neutrophils. The data in Fig. 6 show no differences in TNFRI (control, 8.60 ± 0.29 MCF; CF, 8.34 ± 0.47 MCF; P = 0.31), TNFRII (control, 9.96 ± 0.28 MCF; CF, 10.12 ± 0.22 MCF; P = 0.31), IL-8RA (control, 60.67 ± 0.89 MCF; CF, 59.49 ± 1.11 MCF; P = 0.5), or IL-8RB (control, 98.66 ± 1.21 MCF; CF, 100.67 ± 0.98 MCF; P = 0.31) after incubation with CF BAL fluid. This suggests that exposure to CF BAL fluid does not increase cell surface receptor expression of TNF-α and IL-8 receptors.
From Neutrophils can decrease NE release from these cells in the presence of two cytokines, TNF-α and IL-8, neutrophils. Furthermore, by inhibiting the actions of TNF-α and IL-8 on NE release from CF neutrophils is the same as that in normal individuals. This is despite the fact that the total complement of NE released from CF neutrophils exposed to these cytokines is greater than that in control neutrophils and greater than NE release from neutrophils of bronchiectatic patients. The latter finding suggests that increased NE release from CF neutrophils is not due purely to changes induced by chronic airway inflammation.

It has been assumed that elevated levels of NE in the CF lung are due to increased neutrophil number rather than any inherent abnormality in the CF neutrophil (1, 20). However, increased myeloperoxidase and oxidant release from CF neutrophils has previously been described (30), and it has recently been shown that stimulated neutrophils from CF patients shed less L-selectin than neutrophils from control and bronchiectatic individuals (27). Furthermore, analysis of BAL fluid from individuals with CF after lung transplantation has shown that NE and IL-8 remain significantly elevated compared with those in BAL fluid from non-CF transplant patients (14). Thus although the CFTR defect in bronchial epithelial cells may be “cured” as a result of transplantation, NE levels remain elevated, perhaps due to excess NE secretion. In addition, neutrophil-stimulating factors, including cytokines, have been associated with the CFTR defect in epithelial cells. Basal cell expression of IL-6 and IL-8 is significantly higher in cultured human tracheal gland serous cells from individuals with CF compared with that from control subjects, and on stimulation with Pseudomonas aeruginosa lipopolysaccharide (LPS), CF cells express even more IL-6 and IL-8 than their control counterparts (16). Clearly, the ability of unstimulated CF epithelial cells to produce large amounts of proinflammatory cytokines (13, 30) in conjunction with the hyperactive secretory response of CF neutrophils demonstrated in this study can both initiate and propagate a severe cycle of inflammation on the epithelial surface.

The question arises as to how preincubation with CF BAL fluid and subsequent incubation with opsonized particles leads to this increase. We initially investigated the ability of CF BAL fluid to upregulate the CD11b receptors and FcRIIa, which are involved in binding and ingestion of opsonized particles. However, analysis of these receptors revealed that their number did not differ between CF and control neutrophils after incubation with CF BAL fluid, suggesting that increased ingestion of opsonized particles by CF neutrophils does not occur. Due to the fact that NE release was markedly higher after preincubation with CF BAL fluid, we also investigated the components of CF BAL fluid that might be responsible for priming CF neutrophils. Various proinflammatory modulators in CF BAL fluid were inhibited by the use of neutralizing antibodies or inhibitors. Inhibitors of NE and LPS, both of which are present in CF BAL fluid and have been shown to be proinflammatory (3, 21, 31), did not reduce the effect of a dual stimulus of TNF-α and IL-8 on NE release from activated neutrophils (11). To assess the effect of a dual stimulus of TNF-α and IL-8 on NE release from CF compared with control neutrophils, the cells were incubated with both cytokines and NE release was measured in the supernatant. We also isolated neutrophils from non-CF bronchiectatic patients and incubated these with TNF-α and IL-8 to ascertain NE release. The rationale for enlisting non-CF bronchiectatic patients was to determine whether chronic pulmonary inflammation per se could result in increased NE release from PBNs isolated from CF individuals.

As in the case of preincubation with CF BAL fluid, PBNs from CF patients released more NE than those from normal subjects, and normal subjects had a profile similar to that of bronchiectatic patients (control, 29.6 ± 4.6 ng; bronchiectatic, 35.1 ± 7.1 ng; CF, 61.3 ± 6.4 ng; P < 0.01; Fig. 7). NE activity also correlated very closely to the antigenic NE values that were determined (r = 0.88).

**DISCUSSION**

This study shows that neutrophils from individuals with CF secrete supranormal levels of NE when exposed to stimuli similar to those found in the CF lung. This is despite the fact that the total complement of NE in the CF neutrophil is the same as that in normal neutrophils. Furthermore, by inhibiting the actions of two cytokines, TNF-α and IL-8, in CF BAL fluid, one can decrease NE release from these cells in the presence of opsonized particles to levels observed for control neutrophils treated in a similar manner. This suggests that the increased NE secretion observed in the lungs of individuals with CF is due, in part, to the combined actions of TNF-α and IL-8 in enhancing the response of CF neutrophils to opsonized particles. NE release from CF neutrophils exposed to these cytokines is greater than that in control neutrophils and greater than NE release from neutrophils of bronchiectatic patients. The latter finding suggests that increased NE release from CF neutrophils is not due purely to changes induced by chronic airway inflammation.

**Fig. 7. Effect of TNF-α and IL-8 on NE release from activated normal, bronchiectatic, and CF neutrophils.** Neutrophils from normal, CF, and bronchiectatic individuals were activated to release NE with TNF-α (10 ng/ml) for 5 min followed by IL-8 (100 ng/ml) for 30 min at 37°C. Cells were then pelleted, and supernatant was evaluated for NE content by ELISA. CF neutrophils released significantly more NE than neutrophils from normal and bronchiectatic individuals (⁎ P < 0.01), suggesting that dual action of these cytokines in CF BAL fluid is important in stimulating CF neutrophils to release more NE than in normal or bronchiectatic individuals.
NE release from CF or control neutrophils after incubation with opsonized E. coli (data not shown). Initial efforts to neutralize either TNF-α or IL-8 in CF BAL fluid, both of which were present at elevated levels, followed by incubation with opsonized E. coli had no effect on reducing NE release from either CF or control neutrophils. However, when antibodies to TNF-α and IL-8 were added together to CF BAL fluid followed by incubation with opsonized E. coli, this had the result of decreasing NE release from the control neutrophils by ~25%, but interestingly, NE release from the CF neutrophils was decreased by >50%. This was not related to increased IL-8 and TNF-α-receptor density because there was no difference in TNFRI/II and IL-8RA/B densities on the surface of CF and control neutrophils before and after incubation with CF BAL fluid. Recombinant TNF-α and IL-8 also increased NE release from CF and normal neutrophils, comparable to the results obtained with CF BAL fluid.

With this as background, it can be postulated that the abnormality in NE secretion from the CF neutrophil is most likely intracellular, involving one or more of the complex mechanisms governing degranulation. This might involve a disturbance in the signal transduction pathway leading from TNF-α and/or IL-8 binding to the CF neutrophil through to activation of protein kinase C and influx of extracellular calcium. Chemotacticants such as IL-8 bind to their receptor on neutrophils, and it is thought that this results in activation of phospholipase C/D that, in turn, leads to the generation of inositol trisphosphate and diacylglycerol. Inositol trisphosphate stimulates the release of calcium from intracellular stores, and diacylglycerol activates protein kinase C (12, 15, 34). The culmination of these events is an influx of extracellular calcium and subsequent oxidant burst and degranulation of azurophilic granules. Degranulation of neutrophils has also been shown to be governed by cGMP and cAMP levels, with cGMP promoting degranulation and cAMP preventing it (24, 29). A combination of TNF-α and IL-8 activation of neutrophils has been shown to lead to a decrease in cAMP levels that results in degranulation (8). The balance between cGMP and cAMP levels in activated CF neutrophils might be altered in response to TNF-α and IL-8 that may, in turn, lead to greater degranulation.

Another possible explanation stems from the fact that, on activation, the cytosolic pH of CF neutrophils acidifies to a greater extent than that of normal neutrophils (10). Increased acidification of the cytosolic pH of neutrophils, as occurs during phagocytosis, is thought to lead to an increase in phagosomal pH and increased secretion of proteases including NE (9, 28). This disturbance in pH regulation in CF neutrophils may also provide an explanation as to why NE secretion from CF neutrophils is greater than that from control neutrophils. We have found that although resting cytosolic pH is very similar in CF, bronchiectatic, and control PBNs on stimulation with FMLP and PMA, CF PBNs underwent a significant acidification that was not observed with PBNs from control or bronchiectatic subjects (10). These pH differences were not attenuated by amiloride and bafilomycin. Further experiments with DIDS, which inhibits HCO₃⁻/Cl⁻ exchange, caused alkalinization of activated control but not of CF neutrophils, suggesting abnormal anion transport in CF cells (10). These results are important in CF because neutrophil cytosolic acidification has been previously shown to be associated with increased secretion of azurophilic granule contents (9, 28). We have also shown that experiments with a wide variety of physiological stimuli including CF epithelial lining fluid, Pseudomonas LPS, and secretory products of activated monocytes caused enhanced proton extrusion in normal neutrophils that is in marked contrast to the values of lower cytosolic pHi observed in CF PBNs on activation. The question remains as to why CF BAL fluid and TNF-α and IL-8 might have specific effects on CF neutrophils. At present, there is no obvious answer to this, although a disturbance in the CF degranulation response may be related to CFTR function or some other intrinsic abnormality in these cells. In this regard, it should be noted that although CFTR mRNA has been described in neutrophils, CFTR protein or cAMP-regulated Cl⁻ channel activity has not (33).

In summary, we have shown that CF neutrophils act differently from control neutrophils when exposed to a milieu such as that observed in the CF lung, secreting nearly twice as much NE as its normal counterpart. Blocking of the combined activites of TNF-α and IL-8 in CF BAL fluid returns NE secretion from CF neutrophils to levels observed for normal neutrophils treated in the same way. This suggests that TNF-α and IL-8 in CF BAL fluid play a significant role in the priming and/or activation of CF neutrophils, which, in turn, behave abnormally, resulting in exaggerated NE release and accounting, in part, for the enormous NE burden and lung destruction observed in this condition.

This work was supported by The Royal College of Surgeons in Ireland, The Health Research Board of Ireland, The Charitable Infirmary Charitable Trust, and the Higher Education Authority of Ireland.

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Received 22 April 1999; accepted in final form 31 August 1999.

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