Adherence of airway neutrophils and inflammatory response are increased in CF airway epithelial cell-neutrophil interactions

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Am J Physiol Lung Cell Mol Physiol 290: L588–L596, 2006. First published November 4, 2005; doi:10.1152/ajplung.00013.2005.—Persistent presence of PMN in airways is the hallmark of CF. Our aim was to assess PMN adherence, percentage of apoptotic airway PMN (aPMN), and IL-6 and IL-8 production when aPMN are in contact with airway epithelial cells. Before coculture, freshly isolated CF aPMN have greater spontaneous and TNF-α-induced apoptosis compared with blood PMN from the same CF patients and from aPMN of non-CF patients. We then examined cocultures of PMN isolated from CF and non-CF airways with bronchial epithelial cells bearing mutated cftr compared with cftr-corrected bronchial epithelial cells. After 18-h coculture, the number of CF aPMN adhered on cftr-deficient bronchial epithelial cells was 2.3-fold higher compared with the coculture of non-CF aPMN adhered on cftr-corrected bronchial epithelial cells. The percentage of CF apoptotic aPMN (9.5 ± 0.2%) adhered on cftr-deficient bronchial epithelial cells was similar to the percentage of non-CF apoptotic aPMN adhered on cftr-corrected bronchial epithelial cells (10.3 ± 0.7%). IL-6 and IL-8 levels were enhanced 6.5- and 2.9-fold, respectively, in coculture of CF aPMN adhered on cftr-deficient bronchial epithelial cells compared with coculture of non-CF aPMN adhered on cftr-corrected bronchial epithelial cells. Moreover, blocking surface adhesion molecules ICAM-1, VCAM-1, and E-selectin on cftr-deficient bronchial epithelial cells with specific MAbs inhibited the adherence of CF aPMN by 64, 51, and 50%, respectively. Our data suggest that in CF patients a high number of nonapoptotic PMN adhered on airway epithelium associated with elevated IL-6 and IL-8 levels may contribute to sustained and exaggerated inflammatory response in CF airways.

THE MAJOR CAUSE OF MORTALITY and morbidity in CF patients is lung disease with the development of progressive chronic respiratory insufficiency, characterized by a polymorphonuclear neutrophil (PMN)-dominated airway inflammation and endobronchial infection (3, 7, 12, 31, 34). A growing body of evidence has emerged supporting the view that the basic genetic defect in the CF transmembrane conductance regulator (CFTR) protein may contribute, not only to a depletion of periciliary liquid volume in airways (5) and an increased propensity to pulmonary bacterial infection (44), but also to defective regulation of the inflammatory response in the CF lung (17, 39, 40, 42, 65, 68).

In CF disease, it is still a debatable research topic whether the pathogenesis of lung inflammation is due to the basic defect of CFTR or is secondary to the result of prior infection (4, 7, 24, 40). In human normal lung, the CFTR expression is mainly found both in airway ciliated epithelial cells (35) and submucosal gland cells (18, 27). The epithelial expression of proinflammatory cytokines after bacterial challenge has been shown to be disproportionate in cells with mutant CFTR, a finding observed in both primary and CF respiratory cell lines (17, 36) and in lungs of cftr−/− mice compared with wild-type mice (8, 20, 23). High levels of IL-8 mRNA and protein have been recently reported from bronchial epithelial cells obtained by brushing and in bronchoalveolar lavage (BAL) fluids of young patients with CF even when amounts of IL-8 were normalized to the bacterial burden (41). However, clinical data have also described that some CF infants have no pulmonary inflammatory response in the absence of detectable bacterial infection (2, 48). Another study (4) has shown that stimulation of primary human tracheobronchial epithelial cells with Pseudomonas aeruginosa filtrates elicited a greater IL-8 response in CF cells compared with normal cells. A recent work (29) found greater activation of NF-κB in unstimulated primary airway epithelial cells from patients with CF (ΔF508/ΔF508) compared with non-CF patients, a finding consistent with other previously reported studies (16, 57–59, 65, 68). Overall these data suggest that defective CFTR might be involved, by a yet unknown mechanism, in the excessive lung inflammatory response of patients with CF.

In CF patients, one striking feature that can be immediately brought to notice is the early massive infiltration of PMN in airways even in the absence of bacteria (2, 31, 40). High numbers of inflammatory cells and elevated levels of IL-8 have been reported in naive human CF fetal large and small airway xenografts compared with matched non-CF control grafts (61, 62). Furthermore, we have reported that PMN isolated from airways of patients with CF spontaneously released higher IL-8 levels compared with those observed for both blood and airway PMN isolated from patients with non-CF bronchiectasis disease (11). Because the removal of inflammatory cells is a key step in the resolution of inflammation, failure of this process may have proinflammatory consequences in CF airways.
recent study suggests that the accumulation of apoptotic PMN in sputa of CF patients is due to a defective airway clearance and, therefore, may contribute to ongoing airway inflammation (64). However, this work assessed the number and percentage of apoptotic PMN already present in the sputa for an unknown period. Without knowing how long these PMN have been resident in airways, it is difficult to conclude whether the survival of PMN in airways has been shortened or prolonged and whether the airway epithelial cells modulate the percentage of adherence and apoptotic cell death of PMN. Thus we hypothesized that airway epithelial cells might influence the adherence of PMN, the percentage of apoptotic PMN, and inflammatory responses in the context of CF. To date, there are no reports on the percentage of adherent and apoptotic PMN when CF PMN directly interact with cftr-deficient airway epithelial cells compared with the interaction of non-CF PMN with either cftr-corrected airway epithelial cells or normal airway epithelial cells.

The aim of the current proposal was to investigate the effects of CFTR expression status on the adherence and the percentage of apoptotic airway PMN collected from CF and non-CF patients, but also to examine the contribution of the airway PMN/epithelial cell cross talk in the production of cytokines IL-6, IL-8, granulocyte-monocyte colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF)-α. Given the importance of adhesion in the localization of PMN to airway tissues, we also investigated the relative contributions of surface adhesion molecules intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM), and E-selectin expressed on cftr-deficient, cftr-corrected, and normal bronchial epithelial cells that regulate the airway PMN adhesive interactions.

In the present study, we clearly demonstrate a greater number of CF airway PMN adhered in the CF PMN/cftr-deficient epithelial cell coculture compared with the non-CF airway PMN/normal epithelial cell coculture. No significant differences were found between levels of CF and non-CF apoptotic airway PMN adhered in both CF and non-CF epithelial cocultures. By contrast, the ratios of IL-6 and IL-8 to adhered airway PMN are greatly increased in the CF airway PMN/cftr-deficient epithelial cell coculture compared with either non-CF airway PMN/cftr-corrected or non-CF airway PMN/normal epithelial cell cocultures. These new findings provide important clues regarding mechanisms by which cftr-deficient airway epithelial cells may contribute to increased adherence of PMN and enhanced inflammatory responses in airways of CF patients.

MATERIALS AND METHODS

Study subjects. The CF group included 16 children, 9 boys and 7 girls (mean age: 15 ± 2 yr) who visited our CF center. In all patients, the diagnosis of CF was confirmed by a sweat chloride concentration of >60 meq/l and by CFTR gene mutations (49). Results of physical examination, chest radiographs, Shwachman-Kulczycki score, pulmonary function tests with determination of forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1), oxygen saturation, and sputum quantitative bacterial cultures were recorded at the time of the study. For pulmonary function tests, the mean values expressed as percentage of predicted values were 51.7 ± 12.5% for FVC and 39.6 ± 14.1% for FEV1. Thirteen out of 15 CF children were colonized with P. aeruginosa (Table 1).

The non-CF disease group included five children, four boys and one girl (mean age: 13 ± 4 yr), with a mild form of primary ciliary dyskinesia as previously described (11). The volume of sputum that can be collected in those children was limited, and the number of neutrophils was reduced compared with the sputum of CF children. In all patients, diagnosis of primary ciliary dyskinesia was confirmed by electron microscopy. Similar to the group of CF children, all children with primary ciliary dyskinesia who visited the outpatient department over a 3-mo period were invited to participate in the study. The criterion for eligibility was the absence of pulmonary exacerbation at the time of the study. For pulmonary function tests, the mean values expressed as percentages of predicted values were 94 ± 2% for FVC and 99 ± 1% for FEV1. All these patients were colonized with Haemophilus influenzae but not with P. aeruginosa. Written informed consent was obtained in all CF patients and non-CF subjects. The study was approved by the Ethics Committees of St. Antoine University Hospital (Paris, France).

Isolation of blood PMN. The PMN from venous blood (blood PMN) of CF patients were obtained as described previously (11). In brief, blood PMN from 8 out of 16 CF patients were isolated by the glucose dextran and Ficoll (Amersham Pharmacia Biotech, Les Ulis, France) method. Thereafter, the blood PMN were purified after being incubated with the pan anti-human leukocyte antigen (HLA) class II-coated magnetic beads (procured from Dynabeads M450;
Dynal, Oslo, Norway) (11, 47). The purity and viability of blood PMN were assessed by trypan blue dye and May-Grünewald-Giemsa staining. For ethical reasons, it was not possible to obtain blood samples from non-CF disease control children with dyskinetic cilia syndrome.

Isolation of airway PMN. The spontaneous sputa were collected in sterile cups from either CF and five non-CF disease control patients and processed immediately. The PMN in sputa were isolated in accordance with a previously adopted procedure (11). In brief, the sputum was incubated with the trypsin-EDTA, the mixture was shaken vigorously at 37°C, and the reaction was stopped with trypsin inhibitor and then washed with cold phosphate-buffered saline (PBS). Sputum was not from healthy subjects since we had found in a previous study (11) that induced sputum from healthy subjects does not contain enough PMN for isolation and culture. Similar to the procedure described for the blood PMN, airway PMN were purified after incubation with the pan anti-human HLA class II-coated magnetic beads. We followed a similar cell counting procedure and assessment of viability as was carried out for the blood PMN. The purity of the airway PMN suspension was found higher than 99%, as assessed by May-Grünewald-Giemsa staining.

Epithelial cell culture and coculture with airway PMN. Three different types of transformed human bronchial epithelial cells were used in the present study: IB3-1 cells that express mutant CFTR (genotype AF508/W1282X); C38 cells, the “corrected” CFTR-complemented cell line with a functional CFTR that is derived from IB3-1 cells and stably transfected with an episomal, truncated of CFTR; and BEAS-2B cells that express wild-type CFTR (46, 71). BEAS-2B are hereafter referred to as normal human bronchial epithelial cells. IB3-1, C38, and BEAS-2B cells were purchased from American Type Culture Collection (Rockville, MD). IB3-1, C38, and BEAS-2B cell lines are well characterized (26, 71) and widely used as a model drug treatment system in the CF context (56, 66). The experiments were performed in nonpolarizing cell types grown on impermeable support system in the CF context (56, 66). The experiments were performed during the coculture and assays of cytokine in supernatants of cocultures. We analyzed 10,000 cells for each sample.

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Measurement of active caspase-3. The amount of the intracellular active caspase-3 in airway PMN or blood PMN was quantified by use of a biotin-ZV KD-fluoromethylketone (FMK) substrate according to the instructions of the manufacturer (R&D Systems, Lille, France). Airway and blood PMN were incubated with 10 μM of biotin-conjugated caspase inhibitor-ZV KD-biotin-FMK substrate added to the medium for 1 h. This inhibitor of caspase, a cell-permeable covalent inhibitor, enters into the cells and irreversibly binds the active site of activated proteases to form a stable thio-ether bond with the cysteine on the active site. Inhibitor does not covalently modify inactive caspase-3, which is the basis for discrimination between active and inactive caspase-3. In brief, proteins of airway PMN or blood PMN were extracted in presence of protease inhibitors (25 μg/ml leupeptin, 25 μg/ml pepstatin, 25 μg/ml PMPSF, and 3 μg/ml aprotinin), and caspase-3 specificity was achieved by using a caspase-3-specific monoclonal antibody coated on a microplate. Detection was realized with horseradish peroxidase-streptavidin that binds the biotin on the inhibitor attached on the caspase-3 large subunit. The ELISA read the relative amount of caspase-3 modified with biotin-ZV KD-FMK. The plates were read at 450 and 540 nm on a microplate reader spectrophotometer (Dynex Technologies, Paris, France). Values are means ± SD of 3 cocultures of five different CF patients and five non-CF disease control patients. Each assay was performed in duplicate. All these results were confirmed by a modified in situ terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay (data not shown).

Analysis of airway PMN adherence and apoptosis in coculture. The number of airway PMN adhered to the cultures of BEAS-2B, IB3-1, and C38 cells was counted after fixation of the coculture in methanol assay (ELISA) method. In these experimental conditions of coculture, cell viability of BEAS-2B, IB3-1, and C38 cells exposed to airway PMN exceeded 97%, as determined by trypan blue exclusion.

Fluorescence-activated cell sorting analysis. Suspensions of purified 10³ airway PMN and 10⁶ blood PMN collected from the same CF patient (n = 5) and suspensions of 10³ airway PMN collected from non-CF disease control patients (n = 5) were incubated in RPMI 1640 supplemented with antibiotics and 10% of normal fetal bovine serum in a 5% CO₂ incubator for a period of 2 h at 37°C. Compared with the spontaneous apoptosis of freshly isolated airway and blood PMN, the inducible apoptosis of airway and blood PMN was also characterized by addition of 20 ng/ml of purified TNF-α (Calbiochem, Meudon, France) to the culture medium. After a 2-h culture period, the proportion of both spontaneous and TNF-α-mediated apoptotic airway PMN and blood PMN was assessed with a Becton-Dickinson FACSCalibur flow cytometer (Becton-Dickinson, Oxford, UK). The percentage of apoptotic airway PMN and blood PMN was assayed by FITC-conjugated annexin V (AnV) and propidium iodide (PI) following the manufacturer’s instructions (Roche Diagnostics, Meylan, France). Double labeling of airway and blood PMN with AnV, which binds to the phosphatidylserine (PS) on the surface of apoptotic cells, and PI, which allows the assessment of membrane integrity, enables differentiation between viable nonapoptotic cells (AnV⁻, PI⁻) and viable apoptotic cells (AnV⁺, PI⁻) and late necrotic cells (AnV⁺, PI⁺). The data analysis was undertaken by using the CellQuest software (Becton-Dickinson, Oxford, UK).

To characterize the expression of surface adhesion molecules of three BEAS-2B, IB3-1, and C38 cell types, cells were detached with PBS without calcium and magnesium with 10 μM of EDTA, centrifuged, and resuspended at 0.5 × 10⁶ cell/ml in labeling buffer (PBS, 1% BSA). Cells were incubated on ice during 30 min with specific antibodies to either ICAM-1 (diluted 1:50) or VCAM-1 (diluted 1:20) or E-selectin (diluted 1:20) from Serotec (Cergy Saint-Christophe, France) and with a nonimmune IgG as a negative control. After PBS washes and fixation with 4% of paraformaldehyde in 0.1 M PBS, each cell type was analyzed by fluorescence-activated cell sorter (FACS) flow cytometry (FacCalibur, Becton-Dickinson) with standard settings. We analyzed 10,000 cells for each sample.

Measurement of active caspase-3. The amount of the intracellular active caspase-3 in airway PMN or blood PMN was quantified by use of a biotin-ZV KD-fluoromethylketone (FMK) substrate according to the instructions of the manufacturer (R&D Systems, Lille, France). Airway and blood PMN were incubated with 10 μM of biotin-conjugated caspase inhibitor-ZV KD-biotin-FMK substrate added to the medium for 1 h. This inhibitor of caspase, a cell-permeable covalent inhibitor, enters into the cells and irreversibly binds the active site of activated proteases to form a stable thio-ether bond with the cysteine on the active site. Inhibitor does not covalently modify inactive caspase-3, which is the basis for discrimination between active and inactive caspase-3. In brief, proteins of airway PMN or blood PMN were extracted in presence of protease inhibitors (25 μg/ml leupeptin, 25 μg/ml pepstatin, 25 μg/ml PMPSF, and 3 μg/ml aprotinin), and caspase-3 specificity was achieved by using a caspase-3-specific monoclonal antibody coated on a microplate. Detection was realized with horseradish peroxidase-streptavidin that binds the biotin on the inhibitor attached on the caspase-3 large subunit. The ELISA evaluated the relative amount of caspase-3 modified with biotin-ZV KD-FMK. The plates were read at 450 and 540 nm on a microplate reader spectrophotometer (Dynex Technologies, Paris, France). Values are means ± SD of 3 cocultures of five different CF patients and five non-CF disease control patients. Each assay was performed in duplicate. All these results were confirmed by a modified in situ terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay (data not shown).

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for a period of 10 min maintained at −20°C, air-dried, and rehydrated in 0.1 M PBS. Then, a light microscope at ×200 magnification was employed to read five randomly selected fields. At least 100 of the BEAS-2B, IB3-1, and C38 epithelial cells by field were analyzed by Nomarski differential interference. This was followed by the counting of the number of the adhered airway PMN on each cell line and was expressed for each 10² epithelial cells.

The number of apoptotic PMN adhered to BEAS-2B, the IB3-1, or the C38 epithelial cocultures was evaluated blindly by two different methods 1) visualization of the positive caspase-3 immunoreactive of PMN and 2) modified TUNEL staining assay according to the manufacturer’s recommendations (Promega, Madison, WI). The rabbit antiserum to the human active caspase-3 (R&D Systems) was used with a donkey anti-sheep FITC-conjugated to immunostain the cocultures. Negative controls were obtained by using either the nonspecific IgG as the primary antibody or the FITC-conjugated antibody alone. The cocultures were counterstained with Harris hematoxylin solution, mounted in citifluor antifading solution (Agar Scientific, Stansted, UK), and subsequently observed under a Leica Med microscope (Leica Microsystem, Rueil Malmaison, France). All the images were digitized, and the caspase-3-positive airway PMN adhered to epithelial cells were detected and counted in epifluorescence and Nomarski differential interference.

Assays of cytokines. The supernatants of the cocultures of BEAS-2B, IB3-1, and C38 cells with CF and non-CF airway PMN were collected, centrifuged for a period of 10 min at 300 g, and kept at −80°C, before the assessment of the cytokines was carried out. The ELISA assays for IL-8, IL-6, TNF-α, and GM-CSF were carried out according to the manufacturer’s instructions in commercially available ELISA kits (Biosource International, Camarillo, CA; and Oxford Biomedical Research, Oxford, MI). Concentrations of cytokines were expressed in pg/ml normalized to the number of non-CF and CF airway PMN adhered in each epithelial cell coculture.

Statistical analyses. Results are expressed as means ± SE. Statistical analyses using Sigma Stat for Windows, version 3.0 (SPSS, Chicago, IL), were performed by one-way ANOVA Student’s t-tests. Probability values of $P < 0.05$ were interpreted to denote statistical significance. A $P$ value of $<0.05$ was considered significant.

RESULTS

**Spontaneous and inducible apoptosis of airway and blood PMN.** We first compared the spontaneous and TNF-α-induced apoptosis of airway PMN between the CF patients and the non-CF disease control patients. The percentage of apoptotic airway PMN was quantified either by the loss of the DNA content (Fig. 1, A and B) or by enzymatic measurement of active caspase-3 in airway PMN (Fig. 1C). Figure 1A shows data from a representative experiment of the spontaneous apoptosis of airway PMN from CF and non-CF disease control patients, as assessed by FACS analysis. The mean percentage of spontaneous CF apoptotic airway PMN (53.2 ± 19.4%, $n = 8$) was significantly ($P < 0.001$) greater compared with airway PMN from non-CF patients (20.3 ± 0.5%, $n = 5$) and from blood PMN collected from the CF patients (6.1 ± 0.8%, $n = 8$), as shown in Fig. 1B. We further confirmed our data by evaluating the amount of the active caspase-3 produced by airway PMN of CF patients and non-CF disease controls, as shown in Fig. 1C. Significantly higher amounts of the active caspase-3 were found in resting and TNF-α-stimulated airway PMN of CF patients ($P < 0.001$) compared with that observed in airway PMN of non-CF patients and in blood PMN from CF patients (Fig. 1D), respectively. Thus these results show that CF airway PMN have greater spontaneous and TNF-α-induced apoptosis compared with blood PMN from the same CF patients and from airway PMN of non-CF disease control patients.

**Adherence of airway PMN to cftr-deficient and corrected epithelial cells.** To further investigate the airway PMN adherence in a condition more relevant to the clinical situation, we

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**Fig. 1.** Airway polymorphonuclear neutrophil (aPMN) apoptosis. Apoptosis of freshly isolated non-CF and CF aPMN was estimated by the percentage of nuclei with hypodiploid DNA content evaluated by FACS analysis. Representative FACS analysis of spontaneous apoptosis of CF PMN and non-CF PMN (A); mean levels of spontaneous apoptosis of CF airway PMN, non-CF airway PMN, and CF blood PMN (bPMN) collected from 8 CF patients and 5 non-CF disease control patients (B); mean levels of PMN active caspase-3 in CF and non-CF aPMN (C) and CF blood PMN (D) were calculated after 2 h of culture in the presence or the absence of TNF-α (20 ng/ml). Histograms (means ± SD) are representative of values of 2 different airway PMN cultures from 5 non-CF disease control patients and from aPMN and bPMN cultures from 5 patients with CF. Each assay was performed in triplicate for each non-CF and CF patient. ***$P < 0.001$.**
first compared the adherence of CF airway PMN cocultured to the IB3-1 parental cell line (cftr-deficient epithelial cell) to the adherence of airway PMN collected from non-CF disease control patients cocultured with the matched cftr-complemented cell line (C38 cells). As shown in Fig. 2A, the number of CF PMN adhered to cftr-deficient cells was significantly higher (a 2.3-fold increase, \( P < 0.001 \)) compared with the number of non-CF PMN adhered to corrected epithelial cells. However, we have observed that the number of CF airway PMN adhered to cftr-deficient cells was only 1.7-fold increased compared with CF airway PMN/cftr-corrected epithelial cell cocultures (data not shown).

Based on previous reports that the three best-characterized static adhesion molecules for PMN are ICAM-1, VCAM-1, and E-selectin, we first assessed by FACS analysis the percentage of IB3-1, C38, and BEAS-2B cells expressing ICAM-1, VCAM-1, and E-selectin. As depicted in Fig. 2C, the percentage of IB3-1, C38, and BEAS-2B cells expressing ICAM-1 was higher compared with the expression of VCAM-1 and E-selectin, respectively. The percentage of IB3-1 cells expressing ICAM-1 and VCAM-1 was greater compared with that observed in C38 and BEAS-2B cells. To further investigate the molecular basis for adherence of CF PMN to cftr-deficient cells, cultured IB3-1 cells were pretreated for 30 min with blocking antibodies to ICAM-1, VCAM-1, and E-selectin before the starting of airway PMN coculture. As shown in Fig. 2C, treatment of IB3-1 cells with antibodies against ICAM-1, VCAM-1, and E-selectin resulted in a marked reduction of CF airway PMN adherence by \( \sim 64, 51 \), and 50\%, respectively. Thus ICAM-1 plays an important role in the adherence of CF airway PMN, although other epithelial ligands also contribute.

Apoptosis of airway PMN adhered to cftr-deficient and corrected epithelial cells. To characterize the apoptotic percentage of airway PMN in response to contact with either the cftr-deficient or cftr-corrected epithelial cells, we evaluated the percentage of adhered airway PMN positively immunostained for active caspase-3 in both CF and non-CF PMN/epithelial cell cocultures (Fig. 3, A–C). As shown in Fig. 3A, a similar low percentage of apoptotic PMN was demonstrated in CF PMN of CF PMN/cftr-deficient cell cocultures after 18-h coculture (9.5 ± 0.2\%) compared with non-CF PMN (10.3 ± 0.7\%) in non-CF PMN/cftr-corrected epithelial cell cocultures. In regard to our previous data showing an elevated number of CF airway PMN adhering to cftr-deficient epithelial cells (Fig. 2) and here, we demonstrate a similar low percentage of apoptotic airway PMN in CF and non-CF epithelial cocultures; we therefore made the conclusion that the number of nonapoptotic CF airway PMN adhered to cftr-deficient epithelial cells is greater compared with that observed in the coculture of non-CF airway PMN/cftr-corrected epithelial cells.

It is of interest to note that the apoptotic percentage of non-CF airway PMN adhered to normal bronchial cells (BEAS-2B) was slightly higher (14.6 ± 1.2\%) to that observed with the cftr-corrected cells (data not shown).

Production of IL-8 and IL-6 in CF and non-CF airway PMN/epithelial cell cocultures. The production of cytokines IL-8, IL-6, TNF-\( \alpha \), and GM-CSF was quantified in supernatants of cocultures of CF and non-CF airway PMN/cftr-deficient and cftr-corrected epithelial cells. As shown in Fig. 4A, the IL-8 level in the CF PMN/cftr-deficient epithelial cell coculture was significantly higher (a 2.9-fold increase, \( P < 0.001 \)) compared with that observed in the non-CF PMN/cftr-corrected epithelial cell coculture. We also noted that smaller amount of IL-8 was produced in the non-CF PMN/normal bronchial cell coculture (data not shown).
Similar to the release of IL-8, the IL-6 level was significantly \( (P < 0.001) \) higher in the CF PMN/cftr-deficient epithelial cell coculture (a 6.5-fold increase) compared with the non-CF PMN/cftr-corrected coculture (Fig. 4C). Here also, a smaller amount of IL-6 release was observed in the non-CF PMN/normal bronchial cell coculture (data not shown). The production of the other cytokines, TNF-\( \alpha \) and GM-CSF, was lower than the minimum detectable limit (TNF-\( \alpha \) <5.0 pg/ml, GM-CSF <2.0 pg/ml) in all supernatants of cocultures tested.

**DISCUSSION**

In CF patients, destruction of the airways is caused by repeated bouts of infection and inflammation (7, 12). A chronic PMN inflammation of the airways is felt to be responsible for the majority of the tissue damage and subsequent decline of lung function in CF (13). Despite a great number of studies showing the marked infiltration of PMN into the lung both in CF animal models (8, 21, 23, 51) and in human studies (6, 31, 61), we are unaware of studies on airway PMN collected from CF patients in which attention had been specifically directed to the behavior of airway PMN in close contact with airway epithelial cells. The purpose of this study was to characterize the airway PMN epithelial cell interaction in the context of CF. To our knowledge, the present study is the first regarding specifically the adherence of PMN, the percentage of PMN apoptosis, and the production of cytokines in a model system of CF airway PMN/airway epithelial cell interaction. Our findings clearly demonstrate that airway PMN adherence and levels of IL-6 and IL-8 production were greatly enhanced compared with non-CF PMN/cftr-corrected epithelial cell coculture, IL-6 and IL-8 production were greatly enhanced compared with non-CF PMN/cftr-deficient epithelial cell coculture.
lial coculture. Moreover, correction for the increased number of airway PMN adhered on cftr-deficient epithelial cells did not change our results of high levels of IL-6 and IL-8 found in CF PMN/epithelial cocultures.

**Why was the adherence of CF airway PMN on cftr-deficient epithelial cells greater than that observed with non-CF PMN/cftr-corrected or normal epithelial cell cocultures?** FACS analyses of adhesion molecules ICAM-1, VCAM-1, and E-selectin on cftr-deficient, corrected, and normal bronchial epithelial cells demonstrated that ICAM-1 expression is detected in a high percentage of cftr-deficient airway epithelial cells compared with the expression of VCAM-1 and E-selectin in this cell line and might explain partly the increased adherence of CF PMN, although other surface ligands could be involved. ICAM-1 is one of the acute-phase response genes induced by IL-6 in a variety of tissues (37, 67) and plays a critical role in mediating leukocyte-epithelial adhesion (25). A report by Kide-ney and Proud (32), in agreement with findings of other studies (28, 63), demonstrated that the adherence of blood PMN to cultured bronchial epithelial cells can be differentially induced by a number of proinflammatory mediators and was at least partially dependent on the expression of epithelial cell-ICAM-1, which is the principal counterligand for β2-integrins, primarily αMβ2 (Mac-1) expressed on blood PMN. Furthermore, another study has analyzed the responsiveness of CF PMN from blood and concluded that no differences in Mac-1 adhesion molecule were observed in CF compared with non-CF patients even after stimulation with IL-8 (50). Further studies are needed to characterize the expression of the counterligands of PMN from airways in its adhesion to airway epithelial cells. Our data showing that ICAM-1 is more expressed in cftr-deficient IB3-1 cells compared with C38 and BEAS-2B cells corroborates with other studies showing high ICAM-1 expression in the CFTE29o cell line (ΔF508 homozygote tracheal epithelial cells) (22) and elevated soluble ICAM-1 levels in blood samples from CF patients (14).

There is no obvious answer to the increased adherence of CF airway PMN on cftr-deficient IB3-1 cells, but it might be also related to abnormalities previously described for PMN isolated from CF blood. The CF blood PMN have been shown to shed less l-selectin (50), release significantly more oxidants (70), display abnormal intracellular pH regulation (10) and respond abnormally to TNF-α and IL-8 stimulation resulting in an increased elastase secretion (by a twofold factor) compared with those from non-CF blood PMN (60). Whether or not the elevated number of CF airway PMN adhering on cftr-deficient epithelial cells, as shown in the present study, is due to dysregulation of CF “primed” PMN, as reported for CF blood PMN and/or to different integrin molecules expressed on CF airway PMN, requires future investigations.

Interestingly, our data also demonstrate that the levels of IL-6 and IL-8 normalized to adhered PMN numbers were greater for CF PMN/cftr-deficient epithelial cocultures compared with non-CF PMN/cftr-corrected epithelial cocultures. These data are consistent with in vivo previous studies showing that high levels of IL-6 and IL-8 were found early in nasal and BAL fluids of CF infants and children compared with non-CF disease children (31, 40, 43). In children with CF, neutrophilic infiltration can be detected early in airways, being found in infants as young as 4 wk, and sometimes occurs without detectable bacterial infection (31). From our data of CF PMN/cftr-deficient epithelial cocultures, we suggest that IL-6 along with IL-8 might be possible candidates as first signals following PMN adherence on CF airway epithelium. These could be considered as early signals that participate in the cascade of events leading to the chronic inflammation and promote PMN adherence at inflammatory sites in CF airway tissues before bacterial infection. IL-8 was found to be a major PMN-specific stimulus for adherence of PMN on primary bronchial epithelial cells isolated from human bronchi and trachea (28). IL-6, which is not produced by PMN (1), is produced by macrophages and T cells (55) and is secreted in large amounts by CFTE29o tracheal epithelial cells when stimulated by P. aeruginosa components (22). We thus suggest that the high production of IL-6 that we observed in cocultures of CF PMN/cftr-deficient bronchial epithelial cells may originate from cftr-deficient bronchial epithelial cells.

In airways of CF patients, the marked accumulation of PMN is influenced by their percentage of migration but also by their longevity. A report of Pizurki et al. (45) showed there is no difference in the percentage of blood PMN transmigration across either the cftr-deficient surface airway epithelial cells or corrected or normal airway epithelial cells. The lifespan of PMN is regulated by apoptotic cell death, and the removal of apoptotic inflammatory cells, which is accomplished by resident macrophages via the PS receptor (19), is crucial for the resolution of inflammation (53, 54). In our study, we demonstrate that isolated CF airway PMN have greater spontaneous and TNF-α-induced apoptosis compared with blood PMN from the same CF patients and from airway PMN of non-CF disease control patients. Vandivier et al. (64) also found that patients with CF had higher apoptotic cells in sputa compared with patients with non-CF bronchiectasis and with chronic bronchi-tis. One possible explanation for the high level of apoptotic PMN in sputa collected in our population of CF patients might be the excess presence of a great number of factors (e.g., IL-6, IL-8, TNF-α, GM-CSF, leukotriene B,4), which has been reported in CF sputa capable of modulating the lifespan of PMN (30, 33, 38, 52). In the present study, we show that the percentage of isolated CF apoptotic airway PMN but not airway PMN from non-CF patients was markedly increased following a treatment with TNF-α. We therefore hypothesize that in airway lumen of CF patients, the presence of high level of TNF-α might increase the percentage of PMN apoptosis.

Interestingly, we show that the percentage of CF apoptotic airway PMN is further strongly reduced following the adherence of CF PMN to cftr-deficient epithelial cells and was similar to that observed in non-CF PMN/cftr-corrected epithelial coculture. What are the molecular mechanisms by which the cftr-deficient airway cells could modify PMN apoptosis? A series of molecular events is involved in PMN apoptosis and is dependent on numerous environmental factors (9). Recent reports have demonstrated that intracellular mechanisms, which determine the lifetime of PMN critically involve the differential expression of the Bcl-2 family members Bax-a and Bcl-Xi, that control the apoptotic machinery of PMN in vivo (15, 69). The environmental factors in our coculture model that might be involved in regulating pro- and antiapoptotic genes of PMN in close contact with airway epithelial cells await further attention. As all CF patients in the present study have a severe lung disease, it will be further interesting to analyze whether the degree of severity of lung disease and chronic infection
with P. aeruginosa could influence the PMN/epithelial cross talk in CF patients. We may also interpret our observation obtained with cfrr-deficient and corrected respiratory cell lines with caution for therapy in CF patients. Further studies at the in vivo and in vitro levels are needed to elucidate the mechanism underlying this increased adherence of PMN in CF airway epithelium. We suggest that the present data will therefore serve as a reference database for analyses on human primary and well-differentiated airway epithelial cells obtained from CF and non-CF disease control patients.

Based on the present study and recent clinical research and studies on experimental animal models, we speculate that early inflammation reported in airways of children with CF may be in great part due to an elevated number of nonapoptotic PMN adhering on airway epithelium, which is associated with proinflammatory responses. Therapies directed at decreasing the adherence of airway PMN to airway epithelium early in the course of the patient’s CF might prevent or delay the establishment of the chronic and exaggerated inflammation in airways of CF patients.

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