Interleukin (IL)-1 β in Tracheal Aspirates from Premature Infants Induces Airway Epithelial Cell IL-8 Expression via an NF- κ B Dependent Pathway

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ABSTRACT

Tracheal aspirate IL-8 concentration and airway epithelial cell IL-8 expression are each increased in premature infants undergoing mechanical ventilation. We sought to determine the cytokines responsible for IL-8 expression in this context. Tracheal aspirates were collected from 18 mechanically ventilated premature infants. IL-8 protein abundance was high in tracheal aspirates from ventilated premature infants (mean, 5806 $\pm$ 4923 pg/mL). IL-1 α (mean, 20 $\pm$ 6 pg/mL), IL-1 β (mean 67 $\pm$ 46 pg/mL), and tumor necrosis factor (TNF)- α (mean, 8 $\pm$ 2 pg/mL) were also found. Incubation of tracheal aspirates with 16HBE14o- human bronchial epithelial cells increased IL-8 protein in both cell lysates and supernatants, as well as transcription from the IL-8 promoter. Aspirates also induced nuclear factor (NF)- κ B activation. Mutation of the IL-8 promoter NF- κ B site abolished aspirate-induced IL-8 transcription. Endotoxin concentrations in the tracheal aspirates were negligible and incapable of

inducing IL-8 promoter activity. Finally, incubation of tracheal aspirates with a neutralizing antibody against IL-1 β reduced epithelial cell IL-8 production, whereas neutralizing antibodies against IL-1 α and TNF- α had no effect. We conclude that airway fluid from mechanically ventilated premature infants contains soluble factors capable of inducing airway epithelial cell IL-8 expression *via* a NF- κ B-dependent pathway, and that IL-1 β plays a specific role in this process. (*Pediatr Res* 56: 907–913, 2004)

Abbreviations

AP-1, activator protein-1
BPD, bronchopulmonary dysplasia
NF- κ B, nuclear factor- κ B
TNF- α , tumor necrosis factor- α

Despite advances in neonatal care, BPD continues to be a major cause of morbidity and mortality in premature neonates. Accordingly, interest remains high in the pathogenesis of this chronic lung disease. Recent evidence suggests the importance of inflammation in the pathogenesis of BPD. During the first days of postnatal life, an influx of polymorphonuclear leukocytes and macrophages is seen in the tracheal aspirates of mechanically ventilated premature infants who later develop BPD (1). Elevations of pro-inflammatory cytokines such as IL-8, IL-6, IL-1 β , and TNF- α are also seen (2–7). Similar findings are present in animal models of BPD (8) and hyper-

oxic exposure (9). Of the cytokines implicated in BPD, IL-8 may be of special importance, as it is the most potent neutrophil chemotactic factor.

IL-8 may be elaborated by macrophages, T-lymphocytes, neutrophils, and respiratory epithelial cells (4). In a recent study examining IL-8 expression in the lung tissue of neonates with hyaline membrane disease, the majority of cases demonstrated IL-8 immunoreactivity in fetal and neonatal neutrophils, and in almost half of these cases, airway epithelial cell IL-8 expression (10). An earlier study in ventilated preterm infants detected IL-8 in bronchoalveolar macrophages, neutrophils, and exfoliated epithelial cells (6). In newborn rats, hyperoxic exposure induced the expression of cytokine-induced neutrophil chemoattractant-1, an IL-8 homologue, in alveolar macrophages and epithelial cells (9). Numerous studies have shown that cultured airway epithelial cells may produce IL-8 in response to a variety of stimuli (11–14). We therefore tested the hypothesis that there are factors in the

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tracheal aspirates of mechanically ventilated premature infants capable of eliciting IL-8 expression in cultured airway epithelial cells. Further, using ELISA and neutralizing antibody experiments, we attempted to identify the substance(s) responsible for the observed IL-8 production. Finally, we examined the role of NF- κ B in this process.

METHODS

Tracheal aspirate collection. Aspirates were collected during routine tracheal suctioning of mechanically ventilated newborns (15). Infants were positioned in a supine posture with head midline. One milliliter of sterile nonbacteriostatic saline was instilled into the endotracheal tube, four breaths delivered, and the airway suctioned with a sterile catheter into a Leuken's trap. This procedure was repeated before the catheter was flushed with 1 mL saline and placed on ice. Specimens were centrifuged (13,000 rpm for 4 min at 4°C) and supernatants stored at -80°C within 1 h of collection. This study was approved by the University of Chicago Institutional Review Board.

Patients. Tracheal aspirates were collected from a total of 18 premature infants. All study infants were premature (gestational age at birth <32 wk), mechanically ventilated for respiratory distress, and ≤ 14 d of age. The average gestational age was 26.9 ± 0.6 wk (mean $\pm$ SEM). The average day of life was 5.7 ± 1.1 d. The average birth weight was 971 ± 98 g. Due to the small volume of tracheal aspirate collected from any single infant (<2 mL), samples from the same patients could not be used for all experiments. However, aspirates from the same eight patients were used for analyses of tracheal aspirate cytokine concentration, tracheal aspirate-induced IL-8 protein secretion, and neutralizing antibody experiments. Infants were excluded if there was evidence of acute sepsis or postnatal exposure to systemic glucocorticoid therapy. For comparison, aspirates were also collected from two fullterm newborns without respiratory disease who were intubated for surgical procedures (gestational ages at birth, 39 and 40 wks; days of life, 2 and 13 d; birth weights, 2975 and 3080 g).

Cell culture. A derivative of 16HBE14o- human bronchial epithelial cells, provided by S. White (University of Chicago), was studied (16). The line was originally established from human bronchial epithelial tissue by transfection with pSVori-, which contains the origin-defective SV40 genome (17). Unlike the parental line, cells do not grow in distinct clusters and demonstrate improved transfection efficiency. Cultures show specific immunostaining with pan-cytokeratin, bind galactose, or galactosamine-specific lectins particular to basal epithelial cells (18) and express $\beta 1$ -, $\alpha 2$ -, $\alpha 3$ -, and $\alpha 6$ -integrin subunits on their cell surface (19). Cells were grown on coated plates (fibronectin, 10 μ g/mL; collagen, 30 μ g/mL; BSA, 100 μ g/mL) in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 200 mM of L-glutamine. Incubation was at 37°C and 5% CO₂.

Measurement of IL-8, IL-1 α , IL-1 β , and TNF- α protein abundance. IL-8, IL-1 α , IL-1 β , or TNF- α protein abundance was measured by ELISA (Endogen Corporation, Woburn, MA). Measurements from tracheal aspirates and cell superna-

tants were straightforward. To determine intracellular IL-8 concentration, cells were lysed in homogenization buffer (20) and lysates collected and centrifuged to remove debris before assay.

Transient transfection of human airway epithelial cells. The -162/+44 fragment of the human IL-8 promoter was subcloned into a luciferase reporter (-162/+44 hIL-8/Luc) (11, 21). Mutations of the NF- κ B and AP-1 sites in the context of -162/+44 hIL-8/Luc were introduced by polymerase chain reaction with mutagenic primers to obtain Δ NF- κ B 162/+44 hIL-8/Luc and Δ AP-1 162/+44 hIL-8/Luc (11). NF- κ B-TATA/luc was purchased from Stratagene (La Jolla, CA). Cells were co-transfected with pCMV-LacZ (provided by M. Rosner, University of Chicago) to normalize for transfection efficiency. Cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) (20). Approximately 0.5 μ g total plasmid DNA per 35 mm dish was used. After treatment, cells were harvested for analysis of luciferase activity using lysis buffer provided with the Promega Luciferase Assay system (Madison, WI). Luciferase activity was measured at room temperature using a luminometer (Turner Designs, Sunnyvale, CA). β -galactosidase activity was assessed by colorimetric assay using o-nitrophenyl- β -D-galactoside as a substrate (22).

Electrophoretic mobility shift assays. Nuclear extracts were prepared by the method of Dignam *et al.* (23) with modifications, and electrophoretic mobility shift assays performed as described (24). In some instances, antibodies against either p65 RelA, an NF- κ B family binding protein, Jun, or Fos were added (10 min at room temperature; Santa Cruz Biotechnology, Santa Cruz, CA). An oligonucleotide probe encoding the NF- κ B consensus sequence was purchased from Promega (Madison, WI).

Measurement of endotoxin levels. Tracheal aspirate endotoxin concentration was measured by *Limulus* Amebocyte Lysate assay (BioWhittaker, Walkersville, MD).

IL-8 response to tracheal aspirates. After serum starvation (8 h for transfection experiments, 24 h for all others), cells were treated with tracheal aspirates (1:10 dilution with MEM). Selected cultures were treated with TNF- α (R & D Systems, Minneapolis, MN) IL-1 α or IL-1 β (Calbiochem, La Jolla, CA). Cells were then incubated overnight (16 h) at 37°C and 5% CO₂. Cell lysates and supernatants were then analyzed for IL-8 protein abundance or luciferase activity, as described above. In selected experiments, tracheal aspirates were preincubated with monoclonal rabbit anti-human IL-1 α (5 μ g/mL, 1 h at 24°C), IL-1 β (5 μ g/mL), or TNF- α neutralizing antibody (1.2 μ g/mL; all antibodies from Endogen, Woburn, MA) or rabbit IgG control (5 μ g/mL; Jackson ImmunoResearch, West Grove, PA). Antibody concentrations were selected by measuring bronchial epithelial cell IL-8 expression in response to various doses of cytokines and determining the minimum concentration of antibody needed to block the maximal observed IL-8 response. After treatment with tracheal aspirates or TNF- α as described above, IL-8 secretion was determined by ELISA. To determine whether neutralizing antibodies interfere with the IL-8 ELISA, neutralizing antibodies against IL-1 α , IL-1 β (5 μ g/mL), and TNF- α (1.2 μ g/mL) were preincubated with recombinant IL-8 protein at 100 pg/mL, 1000 pg/mL, and 2500

pg/mL concentrations, and OD readings determined by ELISA. Preincubation with neutralizing antibodies did not appreciably reduce the OD of standard IL-8 samples (data not shown).

Data analysis. Each experiment was performed at least three times (*i.e.* with samples from at least three separate individuals). Data were described as the mean $\pm$ SEM. For reporter assays, changes in promoter activity were calculated as arbitrary light units/ β -galactosidase calorimetric units/h. The significance of changes in luciferase activity and protein abundance was assessed by paired *t* test or one-way ANOVA with repeated measures. Differences identified by ANOVA were pinpointed by Student-Newman-Keuls multiple range test.

RESULTS

Tracheal aspirate IL-8, IL-1 α , IL-1 β , and TNF- α protein abundance. We measured tracheal aspirate IL-8, IL-1 α , IL-1 β , and TNF- α protein abundance by ELISA. Consistent with previous reports (3, 5, 7), IL-8 protein levels were high (5806 ± 4923 pg/mL, $n = 8$ for each cytokine), whereas concentrations of IL-1 α (20 ± 6 pg/mL), IL-1 β (67 ± 46 pg/mL), and TNF- α (8 ± 2 pg/mL) in the tracheal aspirates were relatively modest.

Tracheal aspirates from mechanically ventilated premature infants increase IL-8 expression in cultured airway epithelial cells. 16HBE14o- cells were serum starved for 24 h and incubated with tracheal aspirates (1:10 dilution) overnight. Incubation of cell cultures with tracheal aspirates from premature infants with respiratory disease increased intracellular IL-8 protein approximately 3-fold (unstimulated, 78 ± 36 pg/mL; tracheal aspirates, 213 ± 40 pg/mL; $n = 5$; $p = 0.028$, paired *t* test; Fig. 1a).

To determine whether tracheal aspirate-induced IL-8 protein expression is transcriptionally regulated, cells were transiently transfected with the -162/+44 hIL-8/Luc reporter plasmid and incubated with tracheal aspirates (1:10 dilution). Tracheal aspirates significantly increased transcription from the IL-8 promoter (fold increase, 4.07 ± 0.97 ; $n = 9$; $p = 0.005$; paired *t* test; Fig. 1b), consistent with the notion that airway epithelial cells are a source of airway fluid IL-8. Tracheal aspirates from two newborn full-term infants without respiratory disease had no significant effect on IL-8 promoter activity (mean fold increase, 1.38).

Endotoxin levels in tracheal aspirates. Endotoxin has been reported to increase IL-8 expression in airway epithelial cells (25, 26). Endotoxin concentrations of tracheal aspirates from three premature infants with respiratory disease were measured by *Limulus* Amebocyte Lysate assay. The mean endotoxin concentration was 2.33 EU/mL, equivalent to 0.23 ng/mL of lipopolysaccharide (LPS). To test whether endotoxin in tracheal aspirates could be responsible for the observed transcription from the IL-8 promoter, cells were transiently transfected with the IL-8 reporter plasmid and then incubated with *Escherichia coli* endotoxin (BioWhittaker). Concentrations of endotoxin up to 1 μ g/mL failed to increase IL-8 promoter activity, whereas as incubation of cells with TNF- α (10 ng/mL) increased transcription more than 10-fold. Together, these data

Figure 1. (A) Tracheal aspirates from mechanically ventilated premature infants with respiratory disease increase cultured airway epithelial cell IL-8 protein abundance. Intracellular IL-8 protein was measured by ELISA ($n = 5$, $*p = 0.028$). (B) Tracheal aspirates from mechanically ventilated premature infants with respiratory disease increase transcription from the IL-8 promoter in cultured airway epithelial cells. Cells were transfected with -162/+44 hIL-8/Luc, serum starved, and incubated with tracheal aspirates ($n = 9$) for 16 h. Luciferase and β -galactosidase activities were measured with a luminometer and by colorimetric assay, respectively ($*p = 0.005$).

suggest that endotoxin is not responsible for tracheal aspirate-mediated increments in IL-8 expression.

Effect of cytokines and tracheal aspirates on airway epithelial cell IL-8 secretion. We tested whether IL-1 α , IL-1 β , or TNF- α were responsible for tracheal aspirate-induced IL-8 expression in airway epithelial cells. First, in pilot studies, we examined whether the concentrations of these cytokines found in tracheal aspirates could induce airway epithelial cell IL-8 expression. Serum-starved 16HBE14o- cells were incubated overnight (16 h) with known concentrations of cytokines. Supernatant IL-8 levels were measured by ELISA. Concentration-response studies (Fig. 2) showed that 10 pg/mL IL-1 α increased IL-8 protein secretion from 43 to 239 pg/mL, and 30 pg/mL IL-1 α increased IL-8 protein secretion to 889 pg/mL, consistent with the notion that levels of IL-1 α present in the tracheal aspirate (mean, 20 pg/mL) could be responsible for the

Figure 2. IL-1α, IL-1β, and TNF-α concentration response curves for airway epithelial cell IL-8 protein secretion. IL-1β was the most potent activator of airway IL-8 expression, followed by IL-1α and TNF-α.

observed IL-8 expression. Pilot studies showed a more vigorous response to IL-1β, with 30 pg/mL increasing IL-8 protein secretion to 2737 pg/mL (mean IL-1β concentration in tracheal aspirate, 67 pg/mL). Finally, 10 pg/mL TNF-α increased IL-8 expression only to 72 pg/mL (mean TNF-α concentration in tracheal aspirate, 8 pg/mL). Together, these data suggest that concentrations of IL-1β and IL-1α (but not TNF-α) in the tracheal aspirates of premature infants undergoing mechanical ventilation for respiratory distress within the first 2 wk of life are capable of inducing the level of airway epithelial cell IL-8 expression observed.

Second, we examined the effect of neutralizing antibodies on tracheal aspirate-induced airway epithelial cell IL-8 secretion. Tracheal aspirates from mechanically ventilated premature infants (1:10 dilution) increased supernatant (extracellular) IL-8 protein abundance from 21 ± 1 to 2052 ± 714 pg/mL (*n* = 8, Fig. 3). This response was substantially more vigorous than the change in intracellular IL-8 (Fig. 1a), suggesting that most of the induced IL-8 protein was secreted. Given the 1:10 dilution of tracheal aspirates, this increase in extracellular IL-8 protein cannot be accounted for by IL-8 in the tracheal aspi-

Figure 3. Rabbit anti-human IL-1β neutralizing antibody inhibits tracheal aspirate-induced IL-8 protein secretion. Tracheal aspirates (*n* = 8) were preincubated with anti-IL-1β, IL-1α, or TNF-α before treatment of airway epithelial cells (1:10 dilution). Neutralizing antibody significantly attenuated extracellular IL-8. Incubation with anti-IL-1α, anti-TNF-α, or rabbit IgG did not attenuate tracheal aspirate-induced responses **p* < 0.05, ANOVA).

rates alone. We therefore examined the effects of neutralizing antibody on extracellular, rather than intracellular, IL-8 protein abundance. Preincubation with rabbit anti-human neutralizing antibody to IL-1β, but not neutralizing antibody to IL-1α or TNF-α, significantly reduced tracheal aspirate-induced IL-8 protein abundance (*p* < 0.03, ANOVA). Rabbit IgG had no significant effect. These data suggest that IL-1β, but not IL-1α or TNF-α, is in part responsible for the observed increase in IL-8 expression.

Tracheal aspirates increase NF-κB protein binding to DNA and NF-κB transactivation. To test whether tracheal aspirates induce the binding of NF-κB to DNA, we obtained nuclear extracts from airway epithelial cell cultures exposed to tracheal aspirates from three individual premature infants with respiratory disease. Nuclear extracts were then incubated with [γ-32P]-ATP labeled oligonucleotide encoding the consensus NF-κB binding site. Incubation of airway epithelial cells with tracheal aspirates induced significant NF-κB DNA binding (Fig. 4a). Co-incubation of nuclear extracts with an antibody against p65 RelA, but not Jun or Fos, induced supershift of the DNA binding complex, demonstrating the presence of p65 (Fig. 4b).

To confirm that NF-κB binding results in transactivation, 16HBE14o- cells were transfected with a NF-κB reporter plasmid and exposed to tracheal aspirates from premature infants with respiratory disease. IL-1β treatment (0.1 ng/mL for 16 h) was used as a positive control. Aspirates induced significant NF-κB transactivation (Fig. 5). Incubation of 16HBE14o- cells with 0.1 ng/mL IL-1β also increased NF-κB transactivation.

Effects of NF-κB and AP-1 mutations on tracheal aspirate-induced transcription from the IL-8 promoter. To examine the role of the NF-κB transcription factor complex in the induction of IL-8 expression, cells were transfected with cDNAs encoding IL-8 promoter constructs with mutations of either the NF-κB or AP-1 binding sites. Mutation of the NF-κB site abolished the IL-8 promoter responsiveness to tracheal

Figure 4. (A) Tracheal aspirates increase NF-κB protein binding to DNA. Nuclear extracts were incubated with labeled NF-κB oligonucleotide and resolved by electrophoresis through a polyacrylamide gel. Tracheal aspirates from three separate individuals induced the binding of two NF-κB proteins (arrows). Binding was abolished with cold excess NF-κB, but not AP-2 probe. For comparison, the response to TNF-α (5 ng/mL) is shown. (B) Incubation with anti-p65 RelA induced supershift of both NF-κB DNA binding complexes.

Figure 5. Tracheal aspirates and IL-1 β each increase NF- κ B transactivation. Cells were transfected with an NF- κ B reporter plasmid, serum starved, and incubated with tracheal aspirates for 16 h. Selected cultures were treated with 0.1 ng/mL IL-1 β ($n = 11$, *different from control, $p < 0.05$, ANOVA).

aspirates, whereas mutation of the AP-1 site had little effect, *i.e.* there was still a significant increase in response to tracheal aspirates ($n = 4$, $p < 0.05$, ANOVA; Fig. 6). On the other hand, mutation of the AP-1 site decreased both basal and tracheal aspirate-induced promoter activity, suggesting that this site functions as a basal level enhancer. Together, these data suggest that NF- κ B transactivation is required for tracheal aspirate-induced transcription from the IL-8 promoter.

Figure 6. Effects of NF- κ B and AP-1 mutations on tracheal aspirate-induced transcription from the IL-8 promoter. Cells were transfected with Δ NF- κ B 162/+44 hIL-8/Luc or Δ AP-1 162/+44 hIL-8/Luc, serum starved, and incubated with tracheal aspirates ($n = 4$) for 16 h. Data are shown as fold increase relative to control for the full-length promoter. Mutation of the NF- κ B site abolished the response to tracheal aspirates, whereas IL-8 promoter activity still increased significantly after mutation of the AP-1 site (*different from unstimulated control, $p < 0.05$, ANOVA). However, mutation of the AP-1 site substantially reduced basal promoter activity, demonstrating this region to be a basal level enhancer.

DISCUSSION

We have shown that 1) tracheal aspirates from mechanically ventilated premature infants with respiratory disease contain ample amounts of IL-8 protein, as well as modest levels of IL-1 α , IL-1 β , and TNF- α ; 2) tracheal aspirates induce IL-8 promoter activity and protein expression in human bronchial epithelial cells, whereas aspirates from full-term newborn infants have no significant effect on IL-8 promoter activity; 3) neutralizing antibody against IL-1 β , but not IL-1 α or TNF- α , significantly attenuates tracheal aspirate-induced IL-8 protein expression; 4) tracheal aspirates induce NF- κ B DNA binding activity; 5) tracheal aspirates and IL-1 β induce NF- κ B transactivation; and 6) the IL-8 promoter NF- κ B site is required for tracheal aspirate-induced IL-8 transcription. Together, these results suggest that airway fluid from mechanically ventilated premature infants contains factor(s) that are capable of inducing airway epithelial cell IL-8 expression *via* an NF- κ B-dependent pathway, and that IL-1 β appears to play a significant role in this process.

Recent evidence suggests the importance of inflammation in the pathogenesis of BPD. During the first days of postnatal life, an influx of polymorphonuclear leukocytes and macrophages is seen in the tracheal aspirates of mechanically ventilated premature infants who later develop BPD (1). Elevations of pro-inflammatory cytokines such as IL-8, IL-6, IL-1 β , and TNF- α are also seen (3–7). Of the cytokines implicated in BPD, IL-8 may be of special importance, as it is the most potent neutrophil chemotactic factor. We found that tracheal aspirates from mechanically ventilated premature infants induced IL-8 promoter activity and protein expression in human bronchial epithelial cells. Although the difficulty of obtaining tracheal aspirates prevented us from making comprehensive measurements of lung fluid from full-term infants without lung disease, limited studies showed no significant effect on IL-8 promoter activity. In a study of chronic lung disease in premature baboons, Coalson and colleagues (8) found that increased tracheal aspirate IL-8 and neutrophils preceded morphometric changes consistent with human chronic lung disease of prematurity. In addition, Deng *et al.* (9) found that antibodies against rat neutrophil chemokines analogous to IL-8 attenuate hyperoxia-induced inflammatory cellular influx, septal thickening, and mortality in these animals. Together, these data suggest that IL-8 plays a significant role in the pathogenesis of chronic lung disease in premature infants. Accordingly, we speculate that blockade of IL-1 β signaling might attenuate airway inflammation in premature infants undergoing mechanical ventilation, thereby decreasing the incidence or severity of BPD.

Based on transcription from the IL-8 promoter, we conclude that tracheal aspirate-induced IL-8 expression in the airway epithelium is transcriptionally regulated. Other mechanisms for the regulation of IL-8 protein abundance, for example, the regulation of IL-8 translation, mRNA half-life, or protein degradation may also exist (27, 28).

We confirmed that IL-8, IL-1 α , IL-1 β , and TNF- α are present in the tracheal aspirates of premature infants undergoing mechanical ventilation. The amounts of IL-8, IL-1 β , and TNF- α we observed were within the broad range of values

previously reported (2–7). We also found that concentrations of IL-1 β and IL-1 α adequate to induce airway epithelial cell IL-8 expression are present in tracheal aspirates of mechanically ventilated premature infants within the first 2 wk of life. Further, we found that neutralizing antibody to IL-1 β , but not IL-1 α or TNF- α , significantly attenuated tracheal aspirate-induced IL-8 protein abundance. In a recent study examining IL-8 expression in the lung tissue of neonates with hyaline membrane disease, the majority of cases demonstrated IL-8 immunoreactivity in fetal and neonatal neutrophils, and in almost half of these cases, airway epithelial cell immunoreactivity (10). Alveolar IL-8 expression was also present in nearly one-quarter of the cases (10). IL-8 expression has also been observed in exfoliated epithelial cells obtained following tracheal lavage of premature infants undergoing mechanical ventilation (6). Together these findings suggest that IL-1 β -induced bronchial epithelial cell IL-8 expression may contribute to the increased IL-8 levels observed in the airways of mechanically ventilated premature infants.

Although neutralizing antibody to IL-1 β significantly reduced tracheal aspirate-induced IL-8 protein abundance, a substantial amount of IL-8 induction remained. Because the tracheal aspirates themselves included IL-8, the blocking effect of the IL-1 β antibody may have been underestimated. On the other hand, it is conceivable that other cytokines play a role. For example, neutralizing antibody to IL-1 α also reduced IL-8 expression, though the effect was not statistically significant.

We obtained data strongly suggesting that endotoxin in the tracheal aspirates was not responsible for the observed IL-8 expression. Endotoxin has been demonstrated to increase airway epithelial cell IL-8 expression cells (25, 26). In the latter studies, LPS concentrations of 10–100 μ g/mL were used. We found endotoxin concentrations of tracheal aspirates to be much lower, on average 2.33 EU/mL, equivalent to 0.23 ng/mL LPS. Finally, overnight incubation of cells with concentrations of LPS up to 1 μ g/mL failed to increase IL-8 promoter activity. On the other hand, we cannot rule out the possibility that bacterial colonization contributes to IL-8 induction *in vivo*.

The basic NF- κ B complex is a dimer of two members of the Rel family of proteins, p50 (NF- κ B1) and p65 (Rel A). In unstimulated cells, NF- κ B is sequestered in the cytoplasm by I- κ B family proteins. I- κ B phosphorylation, with subsequent polyubiquitination, allows unmasking of the NF- κ B nuclear localization sequence, leading to its translocation to the nucleus, where it may regulate gene transcription. In our study, incubation of tracheal aspirates from mechanically ventilated premature infants with cultured bronchial epithelial cells induced the binding of nuclear proteins to NF- κ B oligonucleotide binding sequences. Both tracheal aspirates and IL-1 β induced transactivation of an NF- κ B reporter plasmid. We also identified the presence of p65 RelA in the DNA binding complex. Mutation of the IL-8 promoter NF- κ B site abolished tracheal aspirate-induced IL-8 transcription. Although basal and tracheal aspirate-induced IL-8 promoter activities were reduced after mutation of the AP-1 site, responsiveness to the tracheal aspirates was maintained. Together, these data demonstrate the importance of the IL-8 promoter NF- κ B site for

tracheal aspirate-induced IL-8 transcription, and the role of the AP-1 site as a basal level enhancer.

It should be noted that the content of tracheal aspirates from premature infants may not reflect the airway liquid from distal airways. Nonetheless, a comparison of tracheal aspiration and nonbronchoscopic lung lavage in ventilated infants with lung disease demonstrated that, although bronchoalveolar lavage has more distal origin, analysis of tracheal aspirate fluid may have equal validity in the estimation of indices of pulmonary surfactant (29). Also, we did not normalize tracheal aspirates for dilution. Although various groups have used such indices as urea, albumin, and total protein for this purpose, the concentrations of these molecules may be altered by such factors as dwell time and capillary permeability. A recent European Respiratory Society task force on the use of bronchoalveolar lavage for research in children concluded there are no reliable dilution factors in tracheal aspirates from children, and did not recommend correcting results for dilution (30). Finally, we did not study human airway epithelial cells from premature infants. Nevertheless, we consider the human bronchial epithelial cell line we used to be an adequate indicator of human bronchial epithelial cell response to the cytokine milieu present in tracheal aspirates.

We have established a useful system for determining the functional relevance of various factors that may be found in the aspirates, and for examining the biologic effects of therapeutic interventions on airway inflammation in these infants. Future clinical studies could assess the effects of anti-inflammatory therapy and blockade of IL-1 β signaling on airway epithelial cell cytokine elaboration.

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