ARTICLES

***Burkholderia cepacia*–Induced IL-8 Gene Expression in an Alveolar Epithelial Cell Line: Signaling Through CD14 and Mitogen-Activated Protein Kinase**

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ABSTRACT

Burkholderia cepacia is a prevalent pulmonary pathogen in patients with cystic fibrosis (CF). The lung pathology observed in patients with CF is postulated to be due to an overexpression of chemokines. This study investigated the induction of the neutrophil chemoattractant chemokine IL-8 and the signaling pathways activated by *B. cepacia*–infected human lung epithelial A549 (HLE) cells. Cells were infected with *B. cepacia* (genomovar III of the *B. cepacia* complex), and reverse transcriptase–PCR and ELISA for the cytokines were performed. *B. cepacia* (multiplicity of infection $\geq 4:1$) induced HLE cells to significantly secrete IL-8 in a more potent manner than the predominant CF pathogen *Pseudomonas aeruginosa* (multiplicity of infection $\geq 64:1$). IL-8 secretion by *B. cepacia*–infected HLE cells was abrogated by the gene transcription inhibitor actinomycin D and the protein translation inhibitor cycloheximide, confirming that *B. cepacia*–induced IL-8 secretion was mediated through *de novo* protein synthesis. Treatment of *B. cepacia* with proteinase K failed to down-regulate IL-8 secretion; furthermore, IL-8 secretion by *B. cepacia*–infected HLE cells was abrogated by $\geq 80\%$ in the presence of anti-CD14 [specific lipopolysaccharide (LPS) receptor] antibody, thus suggesting that the IL-8–inducing component of *B. cepacia* was LPS and therefore dependent on CD14. The p38 mitogen-activated protein kinase (MAPK) inhibitor and the

extracellular signal-regulated kinase MAPK inhibitor significantly abrogated IL-8 secretion by *B. cepacia*–infected HLE cells (SB203580, $\geq 80\%$ inhibition; PD98059, $\geq 30\%$ inhibition). In conclusion, *B. cepacia*–induced IL-8 secretion in A549 airway epithelial cells is more potent than *P. aeruginosa*; is mediated through LPS, which is CD14 dependent; and involves activation of the p38 and ERK MAPK pathways. (*Pediatr Res* 54: 297–305, 2003)

Abbreviations

α CD14, anti-CD14 receptor antibody
CF, cystic fibrosis
ERK, extracellular signal-regulated kinase
HLE, human lung epithelial
JNK, c-Jun NH₂-terminal kinase
LBP, lipopolysaccharide-binding protein
LPS, lipopolysaccharide
MAPK, mitogen activated protein kinase
MOI, multiplicity of infection
PK, proteinase K
PKC, protein kinase C
PMSF, phenylmethylsulfonyl fluoride
TNF- α , tumor necrosis factor- α

Cystic fibrosis (CF) is the most common lethal genetic disease of white individuals (1). Exaggerated and persistent

chronic inflammation caused by microbial colonization of the major airways is thought to be one of the major causes of morbidity and mortality in patients with CF (2). One of the major organisms to infect patients with CF is *Pseudomonas aeruginosa*. However, in recent years, the multidrug-resistant pulmonary pathogen *Burkholderia cepacia* has become increasingly prevalent in these patients (3). Approximately 20% of *B. cepacia*–infected patients eventually experience “*B. ce*–

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pacia syndrome.” The symptoms of this syndrome include bacteremia and leukocytosis that result in rapid and fatal clinical deterioration (4) associated with increased levels of cytokines in the blood and sputa (5, 6).

Several studies have reported a marked increase in proinflammatory cytokines in patients with CF (7), in particular IL-8. As IL-8 is a potent chemokine for the attraction of neutrophils to the lung, an excess of this chemokine leads to a large influx of neutrophils that ultimately contribute to the lung damage observed in patients with CF. The signaling pathways activated during bacterial induction of chemokine gene expression have not been fully elucidated. However, the mitogen-activated protein kinases (MAPKs) have been demonstrated to play a role in regulating intracellular signal transduction during mononuclear cell cytokine production in response to a variety of stimuli (8). Members of the MAPK family include extracellular signal-regulated kinase (ERK) MAPK, c-Jun NH₂-terminal kinases (JNKs), and p38 MAPK. Activation of p38 MAPK before tumor necrosis- α (TNF- α) synthesis is known to occur in human neutrophils poststimulation with lipopolysaccharide (LPS) (9). However, the signaling pathways activated by *B. cepacia* to induce IL-8 synthesis remain unclear.

Respiratory epithelial cells are one of the main cell types encountered by *B. cepacia* infecting the CF lung (10, 11). A recent report by Keig *et al.* (12) demonstrated that *B. cepacia* is capable of invading and surviving within primary type II pneumocytes, and they and others have concluded that the transformed lung epithelial cell line A549 can be used as a valid surrogate for type II pneumocytes (10, 12–15). Hence, in this study, we used A549 cells as a surrogate for type II pneumocytes to evaluate the IL-8-inducing ability of *B. cepacia*.

Several studies have suggested that *P. aeruginosa* is stimulatory for cytokine synthesis in epithelial cells. In this study, we provide novel evidence that a clinical isolate of *B. cepacia* (ATCC 700070, genomovar III of the *B. cepacia* complex) is more potent than *P. aeruginosa* at inducing IL-8 in airway epithelial cells. We also report that *B. cepacia*-induced IL-8 secretion in airway epithelial cells is CD14 dependent and involves the p38 and ERK MAPK pathway.

METHODS

Bacterial strains and culture conditions. *B. cepacia* was obtained from the American Tissue Culture Collection (ATCC) 700070, a clinical isolate from a patient with CF that has been reported to be resistant to chloramphenicol, trimethoprim, and ciprofloxacin (16). *B. cepacia* ATCC strain 700070 was confirmed as belonging to genomovar III by rDNA and recA gene analyses (J. Lipuma, personal communication). *B. cepacia* was routinely cultured on nutrient agar (Sigma Chemical Co., St. Louis, MO, U.S.A.) without any supplements and incubated at 37°C for 48 h. The IL-8 inducing abilities of both *B. cepacia* and the laboratory strain of *P. aeruginosa* PAO1 (ATCC) were compared. *P. aeruginosa* was cultured in a similar manner to *B. cepacia* as described above. One day before experiments, the organisms were cultured overnight in nutrient broth at 37°C, and the number of organisms/mL was calculated by standard optical density measurements. All optical density

calculations were checked by performing direct counts of the organisms by the use of a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA, U.S.A.).

Cell culture. Human lung epithelial (HLE) cells A549 (ATCC CCL185) were cultured in RPMI 1640 medium (Sigma Chemical Co.) containing 10% heat-inactivated fetal bovine serum (HI-FBS) and 50 IU/mL of penicillin and 50 μ g/mL of streptomycin. Before the cytokine assays, the cells were passaged once in the absence of antibiotics and used throughout the assay without antibiotics. For all experiments, the cells were plated at a cell density of 70,000 cells/mL in their respective medium containing 2% HI-FBS and incubated for 18 h at 37°C in 5% CO₂/95% air before infecting with various multiplicity of infections (MOIs) of *B. cepacia* or *P. aeruginosa*. An MOI of 2:1 refers to two bacteria per one HLE cell.

IL-8 and IL-1 β assays. ELISA 24-well plates (Corning, New York, NY, U.S.A.) were cultured with HLE cells at the density mentioned above and incubated overnight (see above). The confluent monolayer was washed three times with PBS, and then either *B. cepacia* or *P. aeruginosa* was added at the MOI stated in the figure legends. Controls consisted of RPMI 1640 plus 2% HI-FBS as a negative control and IL-1 β (500 pg/mL) as a positive control. The plates were incubated for 18 h, and the viability of the cells was determined by trypan blue stain by standard methods. The cell medium was removed, centrifuged at 13,000 $\times$ g for 15 min to remove bacteria and cell debris, and then stored at –80°C until assayed for IL-8 and IL-1 β by ELISA as described by Steffen *et al.* (17).

Protease treatment of *B. cepacia*. To determine whether a protein component of *B. cepacia* was responsible for IL-8 secretion in HLE cells, we treated *B. cepacia* (MOI 50:1) with increasing concentrations of proteinase K (PK; 0.1–0.5 mg/mL; Sigma Chemical Co.) and incubated it at 37°C for 1 h as described by Weinberg *et al.* (18). Control bacterial suspensions without the PK were incubated under identical conditions. The reaction was then terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) and incubated for an additional 30 min at 37°C (18). The bacterial suspensions were then washed, and the resulting bacterial pellets suspended in PBS to their original volumes. HLE cells were then exposed to either PK-treated or untreated *B. cepacia* (MOI 50:1) incubated for 18 h and IL-8 secreted into the culture medium quantified by ELISA. Controls included PK at the highest concentration tested plus PMSF in the absence of *B. cepacia* and medium only. For confirming that PK treatment had completely digested the bacterial proteins, a sample of the PK-treated *B. cepacia* was run on a SDS-PAGE gel.

Reverse transcriptase-PCR. HLE cells were grown to confluence in six-well dishes (Falcon, NJ) and incubated in RPMI 1640 with 2% HI-FBS for 18 h in the presence of *B. cepacia* (MOI 50:1). Cell viability was checked at the conclusion of experiments by trypan blue exclusion. All cells had a viability of >95%. Total RNA was extracted from the cells using TRIZOL reagent as described by the manufacturer (GIBCO/BRL, Carlsbad, NY, U.S.A.). Single-stranded cDNA was generated as described by Jung *et al.* (19). Amplification of cDNA by PCR was performed using oligonucleotide primer pairs for IL-8 or IL-1 β as described by Jung *et al.* (19). β -Actin was

used to control for loading density. The PCR reactions were carried out in a thermal cycler (Perkin-Elmer) in a 28- μ L reaction volume, containing reaction buffer [20 mM of Tris/HCL (pH 8.4), 50 mM of KCl], 1.5 mM of $MgCl_2$, 0.2 mM of dNTP mix (dATP, dCTP, dGTP, dTTP), 1.5 U of Taq polymerase (GIBCO/BRL), and 2 μ L of first-strand reaction mix. Each primer was added to a final concentration of 0.1 μ M. PCR was for 35 cycles, each cycle consisting of 1 min at 95°C and annealing/extension at 60°C for 2 min and 30 s. The PCR products were separated on 1% agarose gels containing 50 μ g/mL of ethidium bromide, visualized, and photographed under UV light. Densitometry readings of the bands were performed using the Scion Image for Windows program.

Involvement of CD14 receptor. To assess whether CD14 receptors are involved during *B. cepacia*-mediated IL-8 synthesis in HLE cells, we preincubated these cells with either anti-CD14 antibody (MY4; Beckman Coulter, Brea, CA, U.S.A.) or an isotypic control antibody (IgG2b; Beckman Coulter) 1 h before infecting with *B. cepacia* (MOI 50:1) or LPS from *P. aeruginosa* (serotype 10, 100 μ g/mL; Sigma Chemical Co.). After incubating for 18 h, the IL-8 secreted into the culture medium was quantified by ELISA. Controls consisted of either media alone or *B. cepacia* or *P. aeruginosa* LPS in the absence of antibodies.

Assessment of signaling proteins: ELISA and Western analysis. For determining whether the MAPK pathways are activated by HLE cells in response to *B. cepacia* to secrete IL-8, HLE cells were preincubated with either the p38 MAPK inhibitor SB203580 (1–3 μ M; Calbiochem, La Jolla, CA, U.S.A.) or the ERK MAPK inhibitor PD98059 (10 μ M; Calbiochem) for 1 h. These cells were then exposed to *B. cepacia* for 18 h, and the IL-8 secreted into the culture medium was quantified by ELISA. Controls consisted of either media only or *B. cepacia* in the absence of inhibitors. Western analysis was used to assess whether there was an increase in the protein for phosphorylated p38 MAPK postexposure to *B. cepacia* in HLE cells. Confluent HLE cells were cultured in six-well plates and exposed to *B. cepacia* (MOI 50:1) in the absence and presence of 3 μ M of SB203580 for 10 min. The cells were then lysed with 200 μ L of RIPA lysis buffer (containing 60 μ M of PMSF, 30 U/mL of aprotinin, and 1 mM of sodium orthovanadate; Sigma Chemical Co.). Before loading onto 10% SDS polyacrylamide gels (Invitrogen, Carlsbad, CA, U.S.A.), the samples were treated with sample buffer and then denatured by boiling for 5 min. Gels were run at 200 mA for 1.5 h at room temperature. Proteins were transferred onto Hybond-ECL nitrocellulose paper (Millipore, Bedford, MA, U.S.A.) in blotting buffer (20 mM of Tris-base, 192 mM of glycine, 20% methanol) at 400 mA for 1.5 h at room temperature. Membranes were blocked overnight with 5% (wt/vol) nonfat dry milk in Tris-buffered saline (10 mM of Tris-base, 150 mM of NaCl). For probing for phosphorylated p38 MAPK, the membranes were then incubated for 1 h with mouse monoclonal anti-human phosphorylated p38 IgM (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Membranes were then washed five times with PBS-Tween (0.1%) and incubated for 45 min with anti-mouse IgM–horseradish peroxidase (1:1000 dilution; Santa Cruz Biotechnology). After another five washes, antibody-labeled proteins were detected by Enhanced Chemilumi-

nescence as described by the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were subsequently stripped (as described by the manufacturer, Santa Cruz Biotechnology) and probed for p38 MAPK using goat polyclonal anti-human p38 MAPK IgG (Santa Cruz Biotechnology).

Statistical Analysis

All of the experiments in this study were conducted at least three times. The data shown are representative results. Experimental values are expressed as the means $\pm$ SD. The statistical significance of differences between two means was evaluated by *t* test, and $p \leq 0.05$ was considered significant.

RESULTS

B. cepacia Induces HLE Cells to Secrete IL-8 in a More Potent Manner than *P. aeruginosa*

To compare the potency of IL-8 induction between *B. cepacia* and *P. aeruginosa*, we exposed HLE cells to either pathogen at various MOIs ranging between 1:1 and 1000:1 for 4 h. Both *B. cepacia* and *P. aeruginosa* induced HLE cells to secrete IL-8 in a MOI-dependent manner after an exposure time of 4 h (Fig. 1). However, at each MOI tested, IL-8 secreted by *B. cepacia*-infected HLE cells was significantly greater compared with the IL-8 secreted in response to *P. aeruginosa* over 4 h. Furthermore, significant induction of IL-8 secretion by *B. cepacia* required an MOI of only >4:1 (within 4 h; Fig. 1) compared with *P. aeruginosa*, which was several-fold higher (MOI >64:1).

De novo protein synthesis is required for IL-8 secretion by *B. cepacia*-infected HLE cells. To determine the optimum dose of *B. cepacia* required to induce IL-8 secretion from HLE cells over 18 h, we infected these cells with increasing MOIs of *B. cepacia* (1:1–500:1) for 18 h. *B. cepacia* was able potently to induce HLE cells to secrete IL-8 in a dose-dependent manner (Fig. 2A). The optimum dose of *B. cepacia* over 18 h was at MOI $\geq$ 40:1. Hence, in future experiments, we used an MOI of 50:1 that was near the optimal dose of *B. cepacia* over an 18-h period. The reason for using an 18-h time point was that as the experiment was continued for 18 h, the IL-8 signal became much greater, making it easier to quantify the levels of IL-8 secreted by ELISA. The increase in signal for IL-8 secretion also facilitated easier interpretation of inhibition of IL-8 when the inhibitors were used in future studies. It was not possible to incubate A549 cells with intact *P. aeruginosa* for >4 h as we found that the viability of the cells was compromised at time points >4 h in the presence of this organism. However, *B. cepacia* did not alter the viability of the cells over 18 h as indicated in the figure legends.

To investigate whether *de novo* protein synthesis is required for IL-8 secretion by HLE cells exposed to *B. cepacia*, we preincubated HLE cells either with the gene transcription inhibitor actinomycin D (5 μ g/mL) or with the protein translation inhibitor cycloheximide (10 μ g/mL) for 1 h before exposure to *B. cepacia*. After 18 h, the culture medium was collected and IL-8 secretion was quantified by ELISA. In the presence of actinomycin D, *B. cepacia*-induced IL-8 secretion

Figure 1. *B. cepacia* induces HLE cells to secrete IL-8 in a more potent manner than *P. aeruginosa*. HLE cells were infected for 4 h with increasing MOI (1:1–1000:1) of either *B. cepacia* (■) or *P. aeruginosa* (●). The culture medium was then removed and centrifuged to remove the bacteria, and ELISAs were performed to detect the level of IL-8. Both *B. cepacia* and *P. aeruginosa* induced HLE cells to secrete IL-8 in an MOI-dependent manner after an exposure time of 4 h. However, *B. cepacia* induced HLE cells to secrete IL-8 in a more potent manner (MOI $\geq$ 4:1) compared with *P. aeruginosa* (MOI $\geq$ 64:1). The data are expressed as the mean $\pm$ SD. The results obtained for the test samples were compared with that of cells exposed to media only by the *t* test with a probability value of $p \leq 0.05$ considered to be significant. * $p \leq 0.05$.

by HLE cells was completely inhibited (Fig. 2B). Similarly, there was almost complete inhibition of IL-8 secretion by HLE cells exposed to *B. cepacia* in the presence of cycloheximide (Fig. 2B). These results suggest that *B. cepacia*-induced IL-8 secretion in HLE cells involves *de novo* IL-8 protein synthesis that occurs at the level of gene transcription.

***B. cepacia* ATCC strain 700070 induces A549 cells to secrete IL-8 in a similar manner to *B. cepacia* strain J2315.** To elucidate whether *B. cepacia* ATCC strain 700070 induced A549 cells to secrete IL-8 in a similar manner to the highly transmissible strain of *B. cepacia* strain J2315, we incubated HLE cells with either strain for 18 h and then tested for IL-8 secretion as previously described. We found that both strains of *B. cepacia* induced HLE cells to secrete IL-8 in a similar manner (Fig. 3). Both of these strains belong to genomovar III of the *B. cepacia* complex.

***B. cepacia*-Induced IL-8 Secretion by HLE Cells Is CD14 Dependent and Is Not Down-Regulated Posttreatment with PK**

To elucidate whether IL-8 secretion by *B. cepacia*-infected HLE cells was being mediated by a protein component on the

Figure 2. *B. cepacia*-induced IL-8 secretion is MOI dependent and requires *de novo* protein synthesis. HLE cells were infected with *B. cepacia* (MOI 1:1–500:1) for 18 h. The culture medium was then removed and centrifuged to remove the bacteria, and ELISAs were performed to detect the level of IL-8. HLE cells exposed to *B. cepacia* significantly released nanogram levels of IL-8 at an MOI from as low as 1:1 up to 500:1 (■) compared with cells that received media alone (□). The optimum dose of *B. cepacia*-induced IL-8 secretion by HLE cells over 18 h was 40:1 (A). HLE cells were preexposed to either actinomycin D (5 μ g/mL) or cycloheximide (10 μ g/mL) 1 h before infecting with *B. cepacia* (MOI 50:1). The culture media was then removed, and IL-8 was quantified by ELISA. Actinomycin D (5 μ g/mL) or cycloheximide (10 μ g/mL) alone failed to induce IL-8 synthesis. Actinomycin D (■) and cycloheximide (▨) inhibited *B. cepacia*-induced IL-8 synthesis by 100% and 80%, respectively (B). The data are expressed as the mean $\pm$ SD. The data obtained for the test samples were compared with that of cells exposed to *B. cepacia* in the absence of inhibitors (■) by the *t* test with a probability value of $p \leq 0.05$ considered to be significant. *** $p < 0.001$.

bacteria, we digested *B. cepacia* with PK as described in “Methods.” After treating *B. cepacia* with 0.1 mg/mL of PK, there was total bacterial protein digestion as monitored by SDS-PAGE (data not shown). There was no significant difference in IL-8 secretion by HLE cells infected with either

Figure 3. *B. cepacia* strain ATCC 700070 induces HLE cells to secrete IL-8 in a similar manner to the highly transmissible strain of *B. cepacia* strain J2315. HLE cells were infected with either *B. cepacia* strain ATCC 700070 or *B. cepacia* J2315 for 18 h. The culture medium was then removed and centrifuged to remove the bacteria, and ELISAs were performed to detect the level of IL-8 secreted. Both strains of *B. cepacia* induced HLE cells to secrete IL-8 in a dose-dependent and similar manner. *B. cepacia* strain ATCC 700070 was confirmed to belong to genomovar III of the *B. cepacia* complex.

PK-treated *B. cepacia* compared with untreated *B. cepacia* (Fig. 4A). These data suggest that the component of *B. cepacia* responsible for IL-8 secretion by HLE cells was not likely to be a protein. To determine whether LPS was the critical component necessary for IL-8 synthesis, we investigated the role of the LPS receptor CD14 as described in "Methods." In the presence of anti-CD14 (α CD14, MY4) receptor antibody, IL-8 secretion by *B. cepacia* was significantly abrogated (Fig. 4B), thus suggesting that *B. cepacia*-induced IL-8 synthesis in HLE cells is CD14 dependent. IL-8 synthesis by HLE cells exposed to the positive control *P. aeruginosa* LPS (serotype 10) was completely abrogated in the presence of α CD14 receptor antibody (Fig. 4B). This abrogation was not due to nonspecific binding by the antibody as the isotypic control antibody failed to inhibit IL-8 secretion by either *B. cepacia* or *P. aeruginosa* LPS. As capturing of LPS by CD14 receptors is facilitated by a serum component known as LPS-binding protein (LBP), we tested whether increasing the percentage of serum (0–2%) increased *B. cepacia*-induced IL-8 synthesis. We found that IL-8 secretion by *B. cepacia*-infected HLE cells was serum sensitive. As the percentage of serum was increased from 0 to 2%, IL-8 secretion by HLE cells infected by *B. cepacia* also increased (data not shown). This provides further evidence to support the observation that *B. cepacia*-LPS may be the component responsible for IL-8 induction in HLE cells. In addition, polymyxin-B inhibited *B. cepacia*-induced IL-8 secretion

Figure 4. The IL-8-inducing component of *B. cepacia* is not a protein and is CD14 dependent. *B. cepacia* was treated with PK (0.1 mg/mL) and incubated at 37°C for 1 h. PMSF was then added to stop the reaction. HLE cells were infected with PK-treated *B. cepacia* (MOI 50:1), and the IL-8 released by HLE cells was quantified by ELISA. Controls were untreated *B. cepacia* (■) and PK (□). PK-treated *B. cepacia* (▨) induced similar IL-8 synthesis by HLE cells compared with untreated *B. cepacia* (■), suggesting that the induction of IL-8 is not due to a protein (A). For testing whether IL-8 synthesis was CD14 dependent, HLE cells were preincubated with α CD14 receptor antibody for 1 h before treating with either *B. cepacia* or *P. aeruginosa* (serotype 10) LPS. After 18 h, the culture medium was removed and tested for IL-8 synthesis by ELISA. The isotypic control IgG2b was also used to test for nonspecific binding (▨). In the presence of α CD14 receptor antibody, *B. cepacia*-induced IL-8 secretion was significantly abrogated (B, ▨). Anti-CD14 receptor antibody also abrogated *P. aeruginosa* LPS-induced IL-8 secretion (▨). The data are expressed as the mean $\pm$ SD. The results obtained for the test samples were compared with that of cells exposed to untreated *B. cepacia* (■) or *P. aeruginosa* LPS (▨) by the *t* test with a probability value of $p \leq 0.05$ considered to be significant. *** $p < 0.001$.

(data not shown), providing further confirmation that IL-8 secretion by *B. cepacia*-infected alveolar epithelial cells is via the LPS of this microorganism.

IL-8 Secretion in *B. cepacia*-Infected HLE Cells Does Not Require IL-1 β Synthesis

For determining whether HLE cells expressed an increase in mRNA for IL-1 β or IL-8 upon exposure to *B. cepacia*, HLE cells were infected with *B. cepacia* and incubated for 18 h at 37°C before extraction of the RNA. The mRNA was isolated, and RT-PCR for IL-8 and IL-1 β was performed as described in "Methods." *B. cepacia*-infected HLE cells displayed a significant (1.3-fold) increase in IL-8 mRNA compared with basal levels (Fig. 5) as quantified by densitometry. However, in contrast, mRNA expression for IL-1 β in HLE cells did not significantly change after exposure to *B. cepacia* (Fig. 5). We also failed to detect any IL-1 β secreted into the culture medium by HLE cells 18 h after exposure to *B. cepacia* by ELISA. In contrast, HLE cells infected by *B. cepacia* in the same manner secreted at least 5 ng/mL of IL-8 (Fig. 2A). These data suggest that IL-8 gene transcription by HLE cells in the presence of *B. cepacia* may be occurring independent of IL-1 β synthesis.

Inhibition of p38 and ERK MAPK activation down-regulates IL-8 secretion by *B. cepacia*-induced HLE cells. The signaling proteins activated by HLE cells to secrete IL-8 by *B. cepacia* are unknown. In general, as the MAPK signaling pathways have been reported to be involved in IL-8 secretion, we preincubated HLE cells with either the p38 MAPK inhibitor SB203580 or the ERK MAPK inhibitor PD98059 as described in "Methods." Pretreatment with SB203580 (3 μ M) inhibited $\geq 80\%$ of the IL-8 secretion in HLE cells infected with *B. cepacia* (Fig. 6A). In contrast, IL-8 secretion in the presence of the ERK MAPK inhibitor PD98059 (10 μ M) was abrogated by only $\geq 30\%$ (Fig. 6A). Western blot analysis was performed to confirm whether resting levels of p38 MAPK were phosphorylated postinfection by *B. cepacia*. There was a marked increase in phosphorylated p38 protein after exposing HLE cells to *B. cepacia* for 10 min (Fig. 6B). This phosphorylation for p38 protein was inhibited in the presence of the p38 MAPK inhibitor SB203580, confirming that the p38 MAPK pathway is an integral pathway during *B. cepacia*-induced IL-8 synthesis in HLE cells. There was little or no difference in the basal levels of phosphorylated p38 protein in cells exposed to media and SB203580 only (data not shown).

To test whether IL-8 secretion in *B. cepacia*-infected HLE cells was mediated via prostaglandin or protein kinase C (PKC), we preincubated *B. cepacia* with the prostaglandin inhibitor indomethacin (50 μ M; Calbiochem) or the PKC inhibitor GF109203X (5 μ M; Calbiochem). There was no inhibition of IL-8 secretion by *B. cepacia*-infected HLE cells in the presence of either indomethacin or GF109203X. HLE cells treated with either indomethacin (50 μ M) or GF109203X (5 μ M) alone failed to induce IL-8 secretion.

Figure 5. Induction of only IL-8 mRNA and not IL-1 β occurs in HLE cells after exposure to *B. cepacia*. HLE cells were infected with *B. cepacia* (MOI 50:1) for 18 h. The culture medium was then removed, and RT-PCR was performed on the cells to determine increases in mRNA expression for IL-8 or IL-1 β . HLE cells showed significant induction of mRNA levels only for IL-8 but not for IL-1 β after exposure to *B. cepacia*, compared with cells in the absence of *B. cepacia*. The data are expressed as densitometry units of each cytokine band obtained by RT-PCR. *B. cepacia*-infected cytokine levels were compared with basal levels (control, media alone, □; infected by *B. cepacia*, ■). β -Actin was used as a loading control. The data obtained for the test samples were compared with that of cells exposed to media only by the *t* test with a probability value of $p \leq 0.05$ considered to be significant. *** $p < 0.001$.

Inhibition of NF- κ B Down-Regulates IL-8 Secretion by *B. cepacia*-Induced HLE Cells

For investigating whether NF- κ B was involved during *B. cepacia*-induced IL-8 secretion, HLE cells were exposed to the general I κ B inhibitor dexamethasone (1–1000 nM) (20, 21) for 1 h before infection with *B. cepacia*. After 18 h of incubation, the IL-8 secreted into the culture medium was quantified by ELISA. Dexamethasone at a concentration as low as 10 nM was able to inhibit significantly IL-8 secretion by HLE cells exposed to *B. cepacia* (Fig. 7). Furthermore, this inhibition of IL-8 secretion was found to be dose dependent, where 1 μ M of dexamethasone was able to inhibit IL-8 secretion by $> 80\%$.

DISCUSSION

Proinflammatory cytokines have been implicated as playing an essential role in the pathogenesis of CF infection (5, 7), and pathogens such as *B. cepacia* are significantly responsible for this cytokine induction. As *B. cepacia* is a multidrug-resistant microorganism, treatment of patients who have CF and are infected with this bacteria is increasingly difficult (22, 23).

Figure 6. *B. cepacia* induces phosphorylation of p38 MAPK in HLE cells. HLE cells were preincubated with the p38 MAPK inhibitor SB203580 (1–3 μ M), 1 h before infecting with *B. cepacia* (MOI 50:1). The conditioned medium was then collected, and IL-8 release was quantified by ELISA. Western blot analysis was also performed to detect the phosphorylation of p38 MAPK protein. In the presence of 10 μ M of PD98059, 30% of IL-8 secretion was abrogated by *B. cepacia*-induced HLE cells (▨) in comparison with *B. cepacia* in the absence of PD98059 (▩). A total of 3–10 μ M of SB203580 inhibited *B. cepacia*-induced IL-8 synthesis by 80% (▧) compared with *B. cepacia* in the absence of SB203580 (A, ▩). SB203580 or PD98059 alone failed to induce HLE cells to secrete IL-8. A marked increase in the phosphorylated p38 MAPK protein occurred within 10 min of infecting HLE cells with *B. cepacia* as monitored by Western analysis. This phosphorylation of p38 MAPK protein was then inhibited down to basal levels in the presence of 3 μ M of SB203580 (B). The data are expressed as the mean $\pm$ SD. The results obtained for the test samples were compared with that of cells exposed to *B. cepacia* in the absence of inhibitors by the *t* test with a probability value of $p \leq 0.05$ considered to be significant. * $p < 0.05$, *** $p < 0.001$.

Hence, further information on the signaling pathways triggered by *B. cepacia* to induce cytokines is particularly valuable to design potential therapeutics that may decrease the overexpression of cytokines.

In this study, we determined that *B. cepacia* at an MOI >4:1 significantly induced HLE cells to secrete IL-8; in contrast, *P. aeruginosa* required an MOI >64:1 for significant induction of

Figure 7. Inhibition of NF- κ B down-regulates IL-8 secretion by *B. cepacia*-induced HLE cells. HLE cells were exposed to 1–1000 nM of dexamethasone 1 h before infecting with *B. cepacia*. The culture medium was then collected, and secreted IL-8 was quantified by ELISA. A total of 10 nM of dexamethasone significantly inhibited *B. cepacia*-induced IL-8 synthesis by HLE cells by $\geq 60\%$ (▨) in comparison with untreated *B. cepacia* (▩). This inhibition was dose dependent, and maximal inhibition was observed at 1 μ M of dexamethasone. HLE cells exposed to dexamethasone alone failed to secrete IL-8. The results obtained for the test samples were compared with that of cells exposed to *B. cepacia* in the absence of dexamethasone by the *t* test with a probability value of $p \leq 0.05$ considered to be significant. *** $p < 0.001$.

IL-8 secretion to occur. This finding is consistent to a recent study by Zughaier *et al.* (24), who demonstrated that the LPS from *B. cepacia* (strain J2315) is 10-fold more active in the induction of TNF- α by human monocytes compared with the LPS from *P. aeruginosa* (strain PAO1). However, as we did not achieve a maximal dose for *P. aeruginosa*, we cannot draw any conclusions regarding the efficacy of *P. aeruginosa* compared with *B. cepacia*.

Of the species of *Burkholderia*, those from genomovar III account for most of the clinical isolates and most of the epidemic strains. We compared the ability of two isolates from genomovar III (ET12, strain J2315 and ATCC 700070) to induce HLE cells to secrete IL-8. Both strains of *B. cepacia* were equally potent in the induction of IL-8 by HLE cells. Furthermore, this is the first report that confirms that *B. cepacia* ATCC strain 700070 belongs to genomovar III of the *B. cepacia* complex (J. Lipuma, personal communication).

In this study, we found that in the presence of actinomycin D (gene transcription inhibitor), *B. cepacia*-induced IL-8 secretion by HLE cells was completely inhibited. Similarly, there was $\geq 80\%$ inhibition of IL-8 secretion by HLE cells exposed

to *B. cepacia* in the presence of cycloheximide (protein translation inhibitor), suggesting that *B. cepacia*-induced IL-8 secretion in HLE cells involves *de novo* IL-8 protein synthesis and gene transcription.

PK-treated *B. cepacia* was able to induce IL-8 synthesis by HLE cells in a comparable manner to untreated *B. cepacia*, thus suggesting that the IL-8-inducing component of *B. cepacia* is not a protein but may be due to LPS. Further evidence to support this was obtained by the use of α CD14 receptor antibody. *B. cepacia*-induced IL-8 release by HLE cells was significantly abrogated by the use of α CD14 receptor antibody, indicating that IL-8 secretion is CD14 dependent. CD14 receptors are specific for LPS, their main role being in capturing LPS and then presenting the LPS to other receptors such as Toll-like receptors. The capturing of LPS by CD14 receptors is facilitated by a serum protein, LBP. In our studies, we found that in the presence of serum there was a dose-dependent increase in the IL-8 induction in *B. cepacia*-infected HLE cells (data not shown). The IL-8-inducing capability of *B. cepacia* was dependent on the percentage of serum present, suggesting 1) that as the serum concentration was increased, there may be more LBP present to facilitate LPS binding to CD14 receptors, and 2) that there also may be soluble CD14 (sCD14) receptors present in the serum. Therefore, as the percentage of serum was increased, the concentration of sCD14 receptors may also increase, allowing more LPS to bind. Our finding is consistent with a recent study by Zughaier *et al.* (24), who demonstrated that the LPS from other clinical isolates of *B. cepacia* induced TNF- α in human monocytes in a CD14-dependent manner.

In contrast, a study by Palfreyman *et al.* (14) reported that a non-LPS extracellular factor from *B. cepacia* induced IL-8 secretion in alveolar epithelial cells. The differences between our data and their data may be due to differences in the *B. cepacia* strains used between the two studies and also to the use of whole organisms (in this study) and only the culture medium (in their study).

We found no significant change in the expression of IL-1 β mRNA or in the secretion of IL-1 β in HLE cells infected with *B. cepacia*, indicating that perhaps IL-8 induction by HLE cells exposed to *B. cepacia* occurs *via* an IL-1 β -independent manner. This is at present under investigation in our laboratory. A recent report to support this observation demonstrated that the LPS from an environmental strain of *B. cepacia* (ATCC 25416) stimulated murine macrophages to secrete IL-6 and TNF with relative lack of IL-1 β -inducing ability (25). A similar trend has been observed with a periodontal pathogen, *Actinobacillus actinomycetemcomitans*, implicated in juvenile periodontitis, where synthesis of IL-6 secretion in gingival fibroblasts occurs independent of IL-1 β synthesis (26).

B. cepacia-induced IL-8 secretion in HLE cells may occur *via* the activation of the p38 MAPK pathway as significant inhibition of IL-8 was found after co-incubating *B. cepacia* with the p38 MAPK inhibitor SB203580. There was a marked increase in the protein for phosphorylated p38 MAPK after exposing HLE cells with *B. cepacia*. This enhancement for phosphorylated p38 MAPK protein was significantly diminished in the presence of the p38 MAPK inhibitor SB203580. In fact, SB203580 at a concentration as low as 3 μ M was able to diminish IL-8 secretion by

$\geq 80\%$. The ERK MAPK pathway may also play a role in *B. cepacia*-induced IL-8 secretion in HLE cells, as a specific inhibitor of ERK MAPK PD98059 (10 μ M) inhibited approximately 30% of IL-8 synthesis induced by *B. cepacia*. The role of the JNKs was not pursued in this study as there are no specific inhibitors for these kinases. Furthermore, as an SB203580 concentration as low as 3 μ M was able to reduce IL-8 secretion by $>80\%$ in HLE cells exposed to *B. cepacia*, we speculate that the JNK pathway by itself would be insufficient for expression of IL-8 genes postinfection with *B. cepacia* in HLE cells. Our observation correlates to a recent report that demonstrates that both the p38 and ERK MAPKs are activated after exposure to pyocyanin from *P. aeruginosa* by human airway epithelial cells to induce IL-8 secretion (27). In addition, they show that this IL-8 secretion is independent of PKC. In our study, we also found that *B. cepacia*-induced IL-8 secretion by HLE cells was not abrogated in the presence of inhibitors for PKC. Ratner *et al.* (28) also demonstrated that *P. aeruginosa* and *Staphylococcus aureus* activated both p38 and ERK MAPK pathways in 1HAEO⁻ tracheal cells. This activation occurred within 30 min of exposure to *P. aeruginosa* and 60 min of exposure to *S. aureus*. In this study, phosphorylation of p38 occurred within 10 min of exposure to *B. cepacia*.

It has long been known that dexamethasone induces transcription of the gene for the inhibitory protein I κ B α , an inhibitor of the translocation of NF- κ B, one of the transcriptional control elements for the IL-8 gene (20, 21). NF- κ B is normally retained in the cytoplasm through its association with the inhibitory molecule I κ B. Phosphorylation, ubiquitination, and proteolysis of I κ B allows NF- κ B to translocate to the nucleus and induces transcription of cytokine gene expression (29). In the presence of dexamethasone (10–1000 nM), IL-8 secretion by *B. cepacia*-infected HLE cells was significantly abrogated. The finding that *B. cepacia*-induced IL-8 synthesis by HLE cells is blocked by dexamethasone strongly suggests that NF- κ B is a key transcriptional element involved in IL-8 synthesis by this stimulus. The involvement of NF- κ B in our study strongly corresponds to a recent study that showed that there were elevated levels of NF- κ B in *B. cepacia*-infected Cfr $-/-$ mice (30). Clinically, the down-regulation of NF- κ B may be therapeutically beneficial as a recent report by Kazachkov *et al.* (31) described that a patient who had CF and was infected by *B. cepacia* and had extreme respiratory failure survived her illness after treatment with corticosteroids. They speculated that treatment with the corticosteroids might have decreased the *B. cepacia*-induced influx of proinflammatory cytokines and neutrophil-induced inflammation.

We conclude that a CF clinical isolate of *B. cepacia* (ATCC 700070) potentially induces IL-8 synthesis in HLE cells in a CD14- and MAPK-dependent manner. Colonization of *B. cepacia* in patients with CF may lead to extensive activation of the MAPKs, thus inducing overexpression of IL-8, consequently leading to tissue damage as a result of an increase in neutrophil activity as observed in patients with CF. The data from this study underscore the importance of defining the signaling pathways activated after exposure to *B. cepacia* to facilitate the design of future therapies to minimize acute inflammation in CF.

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