Microarray Analysis Reveals Induction of Lipoprotein Genes in Mucoid *Pseudomonas aeruginosa*: Implications for Inflammation in Cystic Fibrosis

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The main cause of the high morbidity and mortality of cystic fibrosis (CF) is the progressive lung inflammation associated with *Pseudomonas aeruginosa* colonization. During the course of chronic CF infections, *P. aeruginosa* undergoes a conversion to a mucoid phenotype. The emergence of mucoid *P. aeruginosa* in CF is associated with increased inflammation, respiratory decline, and a poor prognosis. Here we show, by the use of microarray analysis, that upon *P. aeruginosa* conversion to mucoidy there is an induction of genes encoding bacterial lipopolysaccharides. *P. aeruginosa* lipopolysaccharides are potent agonists of Toll-like receptor 2 (TLR2) signaling. The expression of TLR2 in human respiratory epithelial cells was ascertained by Western blot analysis. Human respiratory epithelial cells responded in a TLR2-dependent manner to bacterial lipopolysaccharides derived from *Pseudomonas* lipopolysaccharides induced in mucoid strains. The TLR2 proinflammatory response was further augmented in CF cells. Thus, the excessive inflammation in CF is the result of a global induction in mucoid *P. aeruginosa* lipopolysaccharides that act as proinflammatory toxins (here termed lipotixons) superimposed on the hyperexcitability of CF cells. Blocking the signaling cascade responding to bacterial lipotixons may provide therapeutic benefits for CF patients.

Cystic fibrosis (CF) is the most common lethal inheritable disease affecting Caucasians (19). CF is caused by mutations in the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR), resulting in multiorgan malfunctions, particularly within the respiratory, gastrointestinal, hepatobiliary, and reproductive tracts (29, 44). The lung complications in CF include chronic respiratory infections, which are the main cause of CF remaining an incurable lethal disease (15). The predominant CF pathogen is *Pseudomonas aeruginosa*: the lungs of >90% of all CF patients eventually become colonized with this bacterium (11). A classical feature of *P. aeruginosa* strains infecting CF patients is that they mutate into a mucoid, exopolysaccharide alginate-overproducing form in a process referred to as the conversion to mucoidy (15). The conversion to mucoidy is concomitant with the establishment of chronic bacterial colonization (20, 27). Infections with mucoid *P. aeruginosa* are associated with heightened inflammation, tissue destruction, and pulmonary function decline (3). It has been recognized that the establishment of mucoid *P. aeruginosa* biofilms correlates with a poor prognosis for CF patients (11, 20, 27). Conversion to mucoidy results from mutations that render the *P. aeruginosa* stress response sigma factor AlgU constitutively active (21, 22). This in turn activates genes of the alginate biosynthesis pathway and additional genes that still need to be fully characterized (9, 10).

However, the overproduction of alginate, an immunologically inert exopolysaccharide associated with mucoid conversion, cannot explain the increased inflammation in CF. This makes it likely that additional, less conspicuous, but potentially more damaging products of *P. aeruginosa* are produced by mucoid *P. aeruginosa* in the CF host. Here we describe the previously unappreciated induction of proinflammatory products in mucoid *P. aeruginosa* and how they affect signaling pathways in host respiratory cells. Using microarray analysis, we found that the most prominently induced genes in mucoid *P. aeruginosa* encode lipopolysaccharides. We show that these *P. aeruginosa* products cause the activation of NF-κB in human lung epithelial cells and that this occurs through Toll-like receptor 2 (TLR2).

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The mucoid *P. aeruginosa* strain PAO578II (*mucA22 sup-2*) and its isogenic nonmucoid PAO6665 (*algU::Tc*) derivative have been described previously (7). For RNA isolation, strains were cultured at 37°C overnight in Luria broth. One milliliter of the overnight culture was used to inoculate 100 ml of Luria broth containing 0.3 M NaCl, and the culture was grown for 4 h at 37°C to mid-log phase (optical density at 600 nm of 0.5). **Microarray analysis.** For microarray analysis and primer extension, RNAs were isolated with TRIzol (Invitrogen Life Technologies, Carlsbad, Calif.) and an RNeasy kit (Qiagen). cDNAs were purified with a Qiaquick PCR purification kit (Qiagen). The RNAs were removed by the addition of 1 N NaOH and incubation at 65°C for 30 min. The reaction was neutralized with 1 N HCl, and the cDNAs were fragmented with 0.6 U of DNase I (Amersham Pharmacia Biotech) per μg of cDNA for 10 min at 37°C, followed by heat inactivation. Chips were hybridized overnight at 50°C and then...
washed, stained, and scanned the next day according to the steps of the Affymetrix Microarray Suite software specified for the Pseudomonas chip. The results from three independent experiments were merged for each strain. The merged data were used for comparisons, and statistical significance was assessed with Student’s t-test.

**Cell culture.** Primary normal human bronchial epithelial cells (NHBEs) (Cambrex Bio Science, Baltimore, Md.) were cultured in bronchial epithelial medium (BEGM; Cambrex Bio Science). IB3-1 (47) is a CF-affected human airway epithelial cell line. Genotypically, IB3-1 is a compound heterozygote containing the ΔF508 mutation and W1282X. The C38 and S9 cell lines, created by correcting IB3-1 cells for chloride conductance by the introduction of functional CFTR (8), were grown in LHC-8 medium (Bio-fluids, Rockville, Md.) supplemented with 10% fetal bovine serum and antibiotics. S9 and C38 cells are both functionally complemented for the major known CFTR effects. They differ in that the CFTR cDNA used to complement IB3-1 cells encodes a complete CFTR molecule in the case of S9 cells, while it lacks the first CFTR extracellular loop in the case of C38 cells (47). NuLi-1 cells, derived from normal human airway epithelial cells by J. Zabner (46), were cultured in collagen-coated plastic dishes (Sigma, St. Louis, Mo.) in serum-free bronchial epithelial cell growth medium with supplements (Clonetics/BioWhittaker, San Diego, Calif.). The 9/HTeO (Sigma, St. Louis, Mo.) in serum-free bronchial epithelial cell growth medium

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**RESULTS**

The most highly induced genes in mucoid *P. aeruginosa* encode lipoproteins. It has been shown that the conversion to mucoidy in *P. aeruginosa* CF isolates occurs via mutations in the mucA gene (22) that activate the alternative sigma factor AlgU (21, 22). We tested how the activation of AlgU in a mucoid *P. aeruginosa* strain carrying the most common mucA mutation found in CF isolates affected global gene expression. PAO578II carries the mucA22 mutation and an additional sup-2 mutation, also common among CF isolates, that renders it responsive to growth conditions for the maximal production of alginate (35). The strain PAO6865 is an algU knockout (algU::Tc) derivative of PAO578II (4). When the microarray expression data (Fig. 1) for PAO578II and PAO6865 were compared, the analysis revealed massive and selective lipoprotein induction in mucoid *P. aeruginosa*, with high expression ratios (Fig. 1, crosses, and Table 1). We found that 70% of the genes showing induction above 30-fold encoded uncharacterized lipoproteins (Table 1). The highest levels of AlgU-dependent expression were observed with the lipoprotein genes lptE and lptG. Two of the lipoprotein-encoding genes, lptA and lptG, have known AlgU-dependent promoters (10). The majority of the other highly induced genes encoding products with lipoprotein leader sequences contained an AlgU promoter consensus motif (Table 2). Since lipoproteins have been implicated in inflammatory processes and the pathogenesis of several important bacterial infections, including *Mycobacterium tuberculosis* (5), *Treponema pallidum* (40), *Listeria monocytogenes* (12), and *Borrelia burgdorferi* (32), this observation warranted further analysis.

**P. aeruginosa** lipopeptides stimulate NF-κB activation in human lung epithelial cells. Considering the preponderance of lipoprotein genes induced in mucoid *P. aeruginosa*, we next investigated whether lipopeptides and/or lipoproteins could induce inflammation in clinically relevant host cells. *P. aeruginosa* colonization is largely limited to the lower airways, and the bronchioles of the CF lung are where respiratory epithelial cells are exposed to *Pseudomonas* products. To determine...
TABLE 1. Activation of lipoprotein gene expression in mucoid P. aeruginosaa

<table>
<thead>
<tr>
<th>Gene no.</th>
<th>Gene name</th>
<th>Description</th>
<th>Fold activation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA1323</td>
<td>lptF</td>
<td>Lipoprotein LptF</td>
<td>53</td>
<td>0.004</td>
</tr>
<tr>
<td>PA3691</td>
<td>osmE</td>
<td>Lipoprotein OsmE</td>
<td>49</td>
<td>6.0 x 10^-5</td>
</tr>
<tr>
<td>PA3819</td>
<td>lptB</td>
<td>Lipoprotein LptB</td>
<td>49</td>
<td>0.0002</td>
</tr>
<tr>
<td>PA5526</td>
<td>lptG</td>
<td>Lipoprotein LptG</td>
<td>49</td>
<td>0.0002</td>
</tr>
<tr>
<td>PA3692</td>
<td>lptA</td>
<td>Lipoprotein LptA</td>
<td>35</td>
<td>4.8 x 10^-6</td>
</tr>
<tr>
<td>PA0737</td>
<td>lptD</td>
<td>Lipoprotein LptD</td>
<td>34</td>
<td>8.8 x 10^-6</td>
</tr>
</tbody>
</table>

a The data shown are for all P. aeruginosa genes with activation exceeding 30-fold in mucoid cells and with P values of <0.001.

b Genes are ordered by decreasing levels of induction.
c A Lpt designation indicates the presence of a typical lipoprotein signal sequence.
d PA03692 lptF was included based on a genetic linkage to PAO3691 lptE and a statistically significant induction (P < 0.005).

whether P. aeruginosa lipoproteins activate NF-κB in human respiratory epithelial cells, we first measured NF-κB-dependent promoter activity by use of a luciferase reporter plasmid in primary human bronchial epithelial cells (NHBEs). We exposed NHBEs to the lipopeptides LPTA(6) and LPTB(6) (9), derived from Pseudomonas lipoproteins induced in mucoid cells. Responses to LPTA(6) and LPTB(6) were detected (Fig. 2A) that were comparable to the response to the standard bacterial lipoprotein (sBLP) (2, 17). NHBEs did not respond to P. aeruginosa LPS. As a negative control, we used a palmitoylated cysteine residue (Pam,Cys) that does not stimulate TLR2 (2, 17). Pam,Cys did not cause NF-κB activation, consistent with a requirement for both the acyl groups and a peptide moiety for lipopeptide recognition by TLRs (2). These results show that lipopeptides derived from lipoproteins induced in mucoid P. aeruginosa activate NF-κB in human respiratory epithelial cells.

TLR2 is expressed in human lung epithelial cells and responds to P. aeruginosa lipoproteins. In contrast to its relatively high expression in lymphoid tissues, TLR2 is believed to be expressed only at a low level in epithelial cells, although TLR2 has been detected in human epithelial HeLa cells (36). For this study, we extended these investigations to verify whether TLR2 is expressed in human respiratory epithelial cells. TLR2 protein expression was detected by Western blots in the bronchial epithelial cell line IB3-1, derived from a CF patient, by using a monoclonal antibody against human TLR2 (Fig. 2B). Equal amounts of TLR2 were observed in IB3-1 cells and in their CFTR-corrected, genetically matched derivatives C38 and S9 cells (Fig. 2B). Similar results were obtained with primary NHBEs. Since TLR2 is known to be the receptor for bacterial lipoproteins, we next tested whether TLR2 is involved in P. aeruginosa lipopeptide-stimulated NF-κB activation in NHBEs (37, 41, 45). In cotransfection experiments with hTLR2 and DN-TLR2 cDNAs, a dependence on TLR2 for LPTA(6) stimulation was observed, as detected by an NF-κB-dependent luciferase reporter assay (Fig. 2C). In addition to NHBEs, two human respiratory epithelial cell lines, 9HTEo (28) and NuLi-1 (46), were tested. Both the dependence on TLR for stimulation with LPTA(6) (Fig. 2A and B) and the presence of TLR2 (Fig. 3C) were demonstrated in experiments with 9HTEo- and NuLi-1 cells.

TLR2 is involved in Pseudomonas lipoprotein-induced NF-κB activation in CF cells. We next assessed the role of Pseudomonas lipoproteins and TLR2 in the stimulation of NF-κB in IB3-1 cells, derived from a compound heterozygote CF patient carrying the ∆F508 CFTR and W1282X CFTR alleles. In addition to the CFTR mutant cell line IB3-1, its CFTR-corrected derivatives C38 and S9 were included in the study. IB3-1, C38, and S9 cells were transfected with TLR2 or DN-TLR2, exposed to the LPTA lipopeptide, and tested for the ability to activate the NF-κB reporter gene. The CF

TABLE 2. P. aeruginosaa AlgU (a5) promoter consensus sequence in front of the lpt genes

<table>
<thead>
<tr>
<th>Gene category and no.</th>
<th>Gene name</th>
<th>AlgU consensusa</th>
<th>Distance (bp) from initiation codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lpt-encoding genes with AlgU promoter consensus sequence</td>
<td>lptD</td>
<td>TGAACCTTGATGTTGACGATGCGCTACACCTGCT</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>CTGAGAATTCTCCCTTGGTTCGAGCGGGGCACCAAGGCAA</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>lptG</td>
<td>AAGAAATTTCCCTGTGGCGCTGAGCGGGCACCAAACGTGGTCGAC</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>lptA</td>
<td>TGAAGATCTGCACGCGCGCGCGCGCGCGGCTAAGGCGCTA</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>lptB</td>
<td>TGAAGATCTGCACGCGCGCGCGCGCGCGGCTAAGGCGCTA</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>osmE</td>
<td>GCGAGAATCTCCGCGGGGCGCCCGGGCGGTTCGACCAAGGCAA</td>
<td>37</td>
</tr>
</tbody>
</table>

a The AlgU consensus sequence was GAACTT (16 or 17 bp)-TCCAA (5 or 6 bp). The consensus matches are shown in bold; underscored residues map mRNA 5' ends.
(IB3-1) cells showed a higher reactivity to P. aeruginosa lipopeptides than the CFTR-corrected, genetically matched C38 and S9 cells, in a TLR2-dependent manner (Fig. 4A). Transfection with TLR2 further enhanced LPTA-induced NF-κB activation in the mutant IB3-1 cells. Taken together, these data indicate that the TLR2-dependent activation of NF-κB in response to Pseudomonas lipopeptides is augmented in CFTR mutant epithelial cells.

Mucoin P. aeruginosa lipopeptides induce IL-8 production. IL-8 is a potent chemoattractant for neutrophils that has been implicated in a neutrophil infiltration and inflammatory cascade in CF (26). Elevated levels of the chemokine IL-8 represent one of the hallmarks of excessive inflammation in CF (3, 15, 19). Thus, we tested whether lipopeptides corresponding to the highly induced lipoproteins in mucoid P. aeruginosa could induce IL-8 production in human lung epithelial cells. To measure IL-8 production, we stimulated confluent NHBE monolayers for 24 h with P. aeruginosa LPS, with 1 or 10 μg of lipopeptide/ml, or a palmitylated cysteine control or left the cells unstimulated. Both lipopeptides, LPTA(6) and LPTB(6), induced detectable IL-8 levels in culture supernatants (Fig. 4B). Furthermore, the induction of IL-8 production was at least partially suppressed with TLR2-blocking antibodies (Fig. 4B). IL-8 was not induced in response to either LPS or palmitylated cysteine. Thus, primary human respiratory epithelial cells have the machinery to recognize and respond to bacterial lipoproteins and produce IL-8 upon stimulation with Pseudomonas lipopeptides.

**DISCUSSION**

The results of this study show that P. aeruginosa causes excessive inflammation in CF due to a massive and selective induction of lipoprotein genes upon conversion to mucoidy. The lipopeptides derived from such proteins show a strong proinflammatory potential in primary lung epithelial cells. In our view, these products are toxic to the CF host and thus should be considered bacterial toxins. Due to their significance in CF and their implications in pathogenesis in other infections (1, 24, 33), lipoproteins should be viewed as a double-edged sword in host-pathogen interactions: they can serve both as agents causing excessive host damage by the pathogen in some situations, such as in CF. We have consequently designated the highest expressing lipoprotein genes in mucoid P. aeruginosa as genes encoding lipotolins (LPTs): lptA, lptC, lptD, lptE, lptF, and lptG (Table 1).

Increased levels of IL-8 have been noted in the sputum and bronchoalveolar lavage of patients with CF (3, 34). IL-8 is known to play a major role in inflammatory pathogenesis in the airways of CF patients (3, 23, 34, 39). Synthetic lipopeptides corresponding to the N termini of the mature, processed LPTs caused IL-8 production in primary human epithelial cells, as shown here, and in human macrophages derived from peripheral blood monocytes (9). Previous studies have shown IL-8 secretion in response to P. aeruginosa in human bronchial epithelial cells (6, 7, 42), although the factors responsible have not been identified. Our results show that the primary human respiratory epithelial cells have the machinery to recognize and respond to bacterial lipoproteins and that the production of IL-8 in CF may be due to stimulation with Pseudomonas lipopeptides.

CF cells showed increased responses to lipopeptide stimulation relative to genetically matched CFTR-corrected cell lines. This indicates that CF cells can be primed for TLR2 signaling to levels that are higher than normal. Thus, both the
FIG. 3. TLR-dependent induction of NF-κB-mediated transcription in bronchial epithelial cells stimulated with *P. aeruginosa* lipopeptide. 9/HTEo- (A) and NuLi-1 (B) cells (both derived from normal human lung cells) were transiently cotransfected with an NF-κB-responsive luciferase reporter plasmid and TLR2 or DN-TLR2 and then incubated for 6 h in the presence of 10 μg of lipopeptide (LptA)/ml. The data were normalized to a cotransfected β-galactosidase construct and are means ± standard deviations. (C) Western blot analysis of TLR2 protein expression in NuLi and 9/HTEo- cells with an anti-hTLR2 monoclonal antibody (IMG-319). Equal amounts of proteins were loaded. *, *P* < 0.05.

FIG. 4. Proinflammatory action of LptA lipopeptide in the CF bronchial epithelial cell line IB3-1 and its CFTR-corrected derivatives C38 and S9 and TLR2 dependence of the response. (A) Induction of NF-κB-mediated transcription in vitro. For a luciferase assay, IB3-1, C38, and S9 cells were transiently transfected with an NF-κB-responsive luciferase reporter plasmid and TLR2 or DN-TLR2 and then incubated for 6 h in the presence of 5 μg of lipopeptide/ml. The data were normalized to a cotransfected β-galactosidase construct. (B) *P. aeruginosa*-based lipopeptides induce inflammatory IL-8 chemokine production by NHBEs. Confluent monolayers of NHBEs were left unstimulated (in medium) or were incubated for 24 h with *P. aeruginosa* LPS, 1 or 10 μg of lipopeptide/ml (sBLP, LPTA(6), or LPTB(6)), or a palmitylated cysteine control. (C) NHBEs were stimulated with LPTA(6) and LPTB(6) in the absence or presence of 20 μg of TLR2-blocking antibody (TL2.1)/ml. *, *P* < 0.05; **, *P* < 0.01.
pathogen and the host conspire to bring about excessive inflammation in CF. The differences in TLR2 responsiveness between CF and CFTR-corrected cells cannot be explained, however, by differences in the expression levels of TLR2 in CF and CFTR-corrected cells, as we did not observe any dissimilarity between IB3-1, C38, and S9 cells (Fig. 2B). Thus, it is likely that other parts of the signaling pathway downstream of or parallel to the TLR2 function differ between CF and CFTR-corrected cells. For example, recent data that were published while this study was under review suggest that TLR2 may be distributed slightly differently within plasma membrane domains in CF and normal cells (25). Furthermore, TLR2 activity may be additionally amplified in CF by asialoliganglioside gangliotetraosylceramide (aGM1) (38), as aGM1 is increased in CF respiratory epithelial cells (18) due to hyperacidiﬁcation of the trans-Golgi network in CF cells (29, 30). Also, note that in our experiments, transfection with DN-TLR2 did not eliminate the endogenous TLR2 response (Fig. 2C). One explanation for this observation is that the endogenous, preassembled signaling complexes responding to lipopeptides [Fig. 2C, control and LPTA(6)] may be resistant [Fig. 2C, LPTA(6) versus DN-TLR2+LPTA(6)] in respiratory epithelial cells to the superimposed expression of DN-TLR2 (defined as dominant negative) in myeloid cells.

While in most challenges the TLR response may serve to limit infections, in other situations it can result in tissue destruction, as in CF and other diseases (2). The pattern recognition receptors of the host play a beneﬁcial role in inducing innate clearance mechanisms. However, when preexisting conditions preclude smooth clearance of the invading pathogens, the same receptors may become targets for the destructive action of bacterial toxins such as LPTs, as in the case of P. aeruginosa in CF.

The microarray data and follow-up experiments presented here help to explain critical aspects of the runaway inﬂammatory processes in the CF lung. Other factors linked to the genetic lesion in CFTR and other modifier genes in CF patients certainly contribute to the colonization and establishment of P. aeruginosa in the lung (16). Superimposed on such preconditioned milieu, the previously unrecognized massive induction of genes encoding proinﬂammatory lipoproteins is likely responsible for the tissue destruction that causes high morbidity and mortality of CF patients. We envision that a simultaneous induction of a large number of lipoprotein genes may result in an additive action, although additional more complex synergisms cannot be excluded.

The knowledge of the molecular basis for the excessive inﬂammation in CF via bacterial lipoproteins and TLR2 may provide targets for blocking or dampening the signaling cascade, including potential applications of neutralizing or blocking antibodies, thus eliminating the factors that are ultimately responsible for respiratory failure in CF patients.

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REFERENCES


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