Interleukin-8 Production by Human Airway Epithelial Cells in Response to *Pseudomonas aeruginosa* Clinical Isolates Expressing Type a or Type b Flagellins

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*Pseudomonas aeruginosa* lung infection is a major cause of morbidity and mortality worldwide. *P. aeruginosa* flagellin, the main structural protein of the flagellar filament, is a virulence factor with proinflammatory activity on respiratory epithelial cells. *P. aeruginosa* bacteria express one of two isoforms of flagellin (type a or b) that differ in their primary amino acid sequences as well as in posttranslational glycosylation. In this study, the distribution of type a and b flagellins among 3 *P. aeruginosa* laboratory strains and 14 clinical isolates (1 ulcerative keratitis, 3 cystic fibrosis, and 10 acute pneumonia isolates) was determined, and their abilities to stimulate interleukin-8 (IL-8) production by human airway epithelial cells was compared. By comparison with the PAK (type a) and PAO1 (type b) prototype laboratory strains, 10/14 (71.4%) of clinical isolates expressed type a and 4/14 (28.6%) expressed type b flagellins. Among four cell lines surveyed, BEAS-2B cells were found to give the greatest difference between constitutive and flagellin-stimulated IL-8 production. All 17 flagellins stimulated IL-8 production by BEAS-2B cells (range, 700 to 4,000 pg/ml). However, no discernible differences in IL-8 production were evident when comparing type a versus type b flagellins or flagellins from laboratory versus clinical strains or among the clinical strains.

*Pseudomonas aeruginosa* is a Gram-negative, aerobic, rod-shaped bacterium with a unipolar flagellum. *P. aeruginosa* is a clinically important opportunistic human pathogen, and its respiratory tract infections are a leading cause of morbidity and mortality in patients with cystic fibrosis, ventilator-associated pneumonia, and compromised immune systems (6). Hospital-acquired pneumonia constitutes the second leading type of pneumonia, and compromised immune systems (6). Hospital-mortality in patients with cystic fibrosis, ventilator-associated spiratory tract infections are a leading cause of morbidity and clinically important opportunistic human pathogen, and its re-...
39, 43–45). The *P. aeruginosa* flagellar typing system was developed based upon the analysis of defined laboratory strains, and to our knowledge, clinical isolates, particularly from acute bacterial pneumonia patients, have not been extensively characterized in this manner. Therefore, the present study was undertaken to assess the distribution of type a and b flagellins among a panel of *P. aeruginosa* clinical isolates and to compare the abilities of the two protein isoforms to stimulate a proinflammatory response by respiratory epithelial cells.

**MATERIALS AND METHODS**

**Cells.** 16HBE14o– is a simian virus 40 (SV40) T-antigen-transformed human bronchial epithelial cell line (7) provided by Dieter Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA). BEAS-2B is an SV40 T-antigen-transformed human bronchial cell line (34) provided by Sekhar Reddy (Johns Hopkins University, Baltimore, MD). A549, an alveolar type II cell line derived from a lung adenocarcinoma (14), and NCI-H292, a human mucocutaneous dermoid pulmonary carcinoma (2), were from the American Type Culture Collection (Manassas, VA). Human embryonic kidney A549 (Invitrogen, Carlsbad, CA).

**Bacteria.** *P. aeruginosa* laboratory strains PAK, PAK/fliC, PAO1, and Richmond were provided by Alice Prince (Columbia University, New York, NY). PAK/fliC (12) is a nonmotile isogenic mutant of PAK in which the fliC gene encoding flagellin was replaced with a homologous gene interrupted by a gentamicin resistance cassette (38). *P. aeruginosa* clinical isolates from cystic fibrosis (CF) patients (PA149, PA383, and CF3) and an ulcerative keratitis patient (PA6294) were provided by Gerald Pier (Harvard University, Boston, MA) (40). Ten *P. aeruginosa* isolates (PA0241, PA0255, PA0296, PA0312, PA0327, PA0476, PA0542, PA0554, PA05043, and PA05482) from acute pneumonia patients at the University of Maryland Medical Center in Baltimore, MD, were provided by Richard Venezia. Bacteria were collected on cloth swabs and eluted into 1 ml of Trycyclothase solution (Becton Dickinson, Franklin Lakes, NJ), and 0.1 ml aliquots were streaked for isolation on MacConkey agar plates (Becton Dickinson). Isolated colonies were cultured in Luria-Bertani (LB) broth, confirmed as *P. aeruginosa* by Gram staining and growth on Cetrimide agar (Becton Dickinson), and stored at −80°C in 50% glycerol in LB broth.

**Flagellin purification.** Flagellin was purified as described previously (47). Briefly, overnight liquid cultures in mineral salts medium (7.0 mg/ml K2HPO4, 3.0 mg/ml KH2PO4, 1.0 mg/ml [NH4]2SO4, 50 μg/ml MgSO4, 7H2O, 2.5 μg/ml FeCl3, 0.4 mg/ml NaCl, 0.4 mg/ml sodium succinate, pH 7.0) were centrifuged at 5,000 × g for 15 min at 4°C, the bacterial pellet was resuspended in 20 mM Tris-HCl, pH 8.0, and flagella were sheared in a commercial blender (Black and Decker, Towson, MD) for 90 s at room temperature. The suspension was centrifuged at 16,000 × g for 30 min at 4°C to remove bacteria, and saturated [NH4]2SO4 was added to the supernatant to a final concentration of 20% (vol/vol). The precipitate was collected by centrifugation at 16,000 × g for 30 min at 4°C, resuspended in 20 mM Tris-HCl, pH 8.0, and extensively dialyzed in resuspension buffer. Protein concentrations were determined by the method of Bradford (3) with crystalline bovine serum albumin (BSA) as the standard (Bio-Rad, Richmond, CA). As a negative control, a mock flagellin preparation was prepared in an identical manner using an extract of nonmotile PAK/fliC bacteria.

**Endotoxin removal.** Endotoxins were removed from flagellar preparations using polymyxin B-agarose (Sigma, St. Louis, MO). Flagellin proteins (10 μg) were resuspended in phosphate-buffered saline (PBS), 100 mg of polymyxin B-agarose was added, and the samples were incubated for 1 h at 4°C with constant agitation. The suspensions were centrifuged at 18,000 × g for 5 min, and the supernatant was aspirated and saved. The Limulus aemobocyte lysate assay (Sigma) was used according to the manufacturer’s directions to detect the presence of any remaining endotoxins in the samples. No endotoxin was detected (Sigma). Equal protein aliquots (20 μg) were resolved on 10% SDS-acrylamide gels, transferred to PVDF membrane, and stained with 0.25% Coomassie blue (Sigma). Protein concentrations were determined by the method of Bradford. Tissue culture plates were blocked with 5% nonfat dry milk in 10 mM Tris-HCl, pH 7.4, containing 1% Tween 20, and reacted with rabbit antiserum to *P. aeruginosa* flagellin (provided by Joseph S. Lam, University of Guelph, Guelph, Ontario, Canada) diluted 1/10,000 in blocking buffer and enhanced chemiluminescence substrate (GE Healthcare, Piscataway, NJ).

**ELISA.** Clinical isolates were washed with PBS and lysed at 4°C with PBS containing 0.1% SDS, 0.5% sodium deoxycholate, 1.0% Triton X-100, 50% glycerol, and 1.0% protease inhibitor cocktail (Sigma). Equal protein aliquots (20 μg) were transfected onto 100 mM 2-mecaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, reblocked, and probed with β-tubulin antibody. Immunoreactive bands were identified by comigration of prestained protein size markers (Bio-Rad).

**RESULTS**

**P. aeruginosa flagellin stimulates IL-8 production by human airway epithelial cells.** Initially, we compared four human airway epithelial cell lines for flagellin-stimulated IL-8 production. Flagellin purified from the PAK laboratory strain migrated as a single band on SDS-acrylamide gels that was immunoreactive with a flagellin polyclonal antiserum and devoid of detectable immunoreactivities with antisera against *P. aeruginosa* pilin and endotoxin (Fig. 1). A mock preparation purified in an identical manner from the PAK/fliC isogenic mutant lacking a functional fliC gene was free of detectable protein and nonreactive with the flagellin antiserum. Treat-
ment of human airway epithelial cell lines with the mock and purified flagellin preparations revealed a diverse pattern of proinflammatory responses, as assessed by IL-8 production (Fig. 2A). 16HBE14o cells produced relatively high IL-8 levels in response to treatment with 10 ng/ml of flagellin or an identical amount of the negative control preparation. Flagellin-treated BEAS-2B and A549 cells produced intermediate levels of IL-8, while NCI-H292 cells produced the lowest chemokine levels, all 3 of which were significantly greater than those of negative control-treated cells. The greatest difference between IL-8 production by flagellin and the control was manifested by BEAS-2B cells (10.1-fold). The heterogeneous IL-8 responses could not be explained by disparate expression of TLR5 protein, the cellular receptor for bacterial flagellin (Fig. 2B). Whole-cell lysates of all four cell lines contained TLR5 protein as an 100-kDa doublet, which is a result of differential glycosylation (35), although it is possible that different levels of TLR5 expression on the cell surface may have accounted for the varied IL-8 responses. Using a range of flagellin concentrations, a dose response in IL-8 (0 to 30 ng/ml flagellin) and TNF-α (0 to 50 ng/ml flagellin) production by BEAS-2B cells was observed (Fig. 3).

**P. aeruginosa** flagellin stimulates NF-κB and IL-8 production by TLR5-transfected HEK293T cells. Flagellin binding to TLR5 activates the NF-κB transcription factor, leading to induction of IL-8 and TNF-α by epithelial cells (13). To directly demonstrate that TLR5 was responsible for flagellin-stimulated IL-8 production, TLR5 was overexpressed in HEK293T cells in conjunction with an NF-κB-luciferase gene reporter plasmid. HEK293T cells were shown previously to be minimally responsive to non-pseudomonad flagellins, while TLR5-transfected HEK293T cells exhibited robust IL-8 production following flagellin stimulation (15). HEK293T cells transfected with a TLR5 expression plasmid, but not the pcDNA empty vector, had significantly greater NF-κB activation, as measured by increased luciferase activity, following treatment with 1, 10, or 100 ng/ml of flagellin, compared with cells treated with the negative control (Fig. 4A). Treatment of TLR5-HEK293T cells with 10 ng/ml of flagellin also was correlated with increased IL-8 levels in culture supernatants compared with negative control-treated cells or compared with flagellin-treated pcDNA-transfected cells (Fig. 4B). Thus, **P. aeruginosa** flagellin stimulated NF-κB and IL-8 production through TLR5.

**Purification of flagellins from** **P. aeruginosa** clinical isolates and IL-8 production. Flagellins isolated from laboratory reference strains of **P. aeruginosa** have been classified as type a (e.g., the PAK strain) or type b (e.g., the PAO1 strain) based upon their molecular mass and reactivity with type-specific antisera (28). However, no published studies have compared the distributions of type a and b flagellins among **P. aeruginosa** clinical isolates. Therefore, we purified flagellin proteins from a panel of bacterial strains isolated from ulcerative keratitis (n = 1), cystic fibrosis (n = 3), or acute pneumonia (n = 10).
patients and classified them as type a or b based upon their mobilities on SDS-acrylamide gels (Fig. 5) and reactivities with flagellin typing antiserum (Table 1). We first confirmed that all isolates were motile on 0.3% LB agar (data not shown). Next, the purified flagellins were used to stimulate IL-8 production by BEAS-2B cells. As shown in Fig. 6A, a broad distribution in IL-8 levels in cell culture supernatants was observed, ranging from 700 to 4,000 ng/ml. However, no significant differences in IL-8 levels were observed when grouped according to clinical status or flagellin type (Fig. 6B). In summary, the distribution of type a and b flagellins had no specified pattern among the P. aeruginosa clinical strains, and no differences in IL-8 production were seen between the type a and b flagellins.

**FIG. 4.** Flagellin stimulates NF-κB and IL-8 production by TLR5-transfected HEK293T cells. (A) HEK293T cells were cotransfected with a TLR5 expression plasmid or pcDNA empty vector plus the pGL4.32 plasmid containing NF-κB response elements linked to a firefly luciferase reporter gene and the pRL-SV40 plasmid encoding *Renilla* luciferase, the cells were incubated for 24 h and treated for 6 h with the indicated concentrations of PAK flagellin or the negative control, and relative luciferase activity in cell lysates was determined. (B) HEK293T cells were transfected with the TLR5 plasmid or pcDNA vector, incubated for 24 h, and treated for 6 h with 10 ng/ml of flagellin, and IL-8 levels in culture supernatants were measured by ELISA. Each bar represents the mean ± SD (n = 3), *, significantly increased IL-8 levels comparing flagellin- and negative control-treated cells (P < 0.05).

**FIG. 5.** SDS-PAGE of flagellins from *P. aeruginosa* laboratory and clinical strains. Purified flagellins were resolved by SDS-PAGE and stained with Coomassie blue. The migration of prestained molecular mass marker proteins (M) is indicated on the left. The assignment as type a or b flagellins based upon molecular mass and reaction with typing antiserum is indicated above each flagellin band. Lab, laboratory strains; CF, cystic fibrosis isolates; UK, ulcerative keratitis isolate.

**TABLE 1.** *Pseudomonas aeruginosa* flagellin purification and type classification

| PA isolate | Source          | Flagellin yield (μg/liter) | Flagellin mol mass (kDa) | Flagellin type |
|------------|-----------------|----------------------------|--------------------------|---------------|
| PAKΔfliC   | Laboratory      | NA*                        | NA                       | NA            |
| PAK        | Laboratory      | 65.6                       | 47.0                     | a             |
| PAO1       | Laboratory      | 50.5                       | 54.0                     | b             |
| Nottingham | Laboratory      | 76.8                       | 52.0                     | a             |
| PA149      | Cystic fibrosis | 42.7                       | 46.0                     | a             |
| PA383      | Cystic fibrosis | 96.8                       | 46.0                     | a             |
| CF3        | Cystic fibrosis | 40.6                       | 50.0                     | a             |
| PA6294     | Ulcerative keratitis | 22.5           | 52.0                     | a             |
| PA50241    | Pneumonia       | 61.9                       | 54.0                     | b             |
| PA50255    | Pneumonia       | 48.4                       | 50.0                     | a             |
| PA50296    | Pneumonia       | 31.1                       | 54.0                     | b             |
| PA50312    | Pneumonia       | 29.6                       | 52.0                     | a             |
| PA50327    | Pneumonia       | 51.3                       | 52.0                     | a             |
| PA50403    | Pneumonia       | 24.2                       | 52.0                     | a             |
| PA50459    | Pneumonia       | 36.4                       | 54.0                     | b             |
| PA50476    | Pneumonia       | 28.8                       | 52.0                     | a             |
| PA50482    | Pneumonia       | 21.8                       | 54.0                     | b             |
| PA50542    | Pneumonia       | 49.7                       | 50.0                     | a             |

*Micrograms of purified flagellin per liter of bacterial culture.

Flagellin molecular mass determined by SDS-PAGE with reference to prestained protein markers: type a flagellin, 54.0 kDa; type b flagellin, 45.0 to 52.0 kDa.

Flagellin type based upon molecular mass and reaction with flagellin typing antiserum.

NA, not applicable.
A short segment of 10 amino acids (LQRIRDLALO) in the NH₂-terminal region of P. aeruginosa flagellin has been shown to be important for binding to TLR5 and downstream NF-κB activation and proinflammatory cytokine/chemokine production (18, 45). A homologous region is present in the flagellins of most Gram-negative bacteria that have been examined (Salmonella enterica, Escherichia coli, Vibrio cholerae, and Listeria monocytogenes) (15, 18). Therefore, it is unlikely that sequence differences in the TLR5-binding region of the conserved NH₂-terminal domain of P. aeruginosa flagellin account for variation in IL-8 production from the different bacterial strains. More plausible, however, is a potential role for alteration in post-translational modifications, particularly O-linked glycosylation (1, 4) and/or tyrosine phosphorylation (19, 20). For example, IL-8 release from A549 cells stimulated with non-glycosylated flagellins was significantly reduced compared with that in wild-type flagellar preparations, indicating a role for glycosylation in the proinflammatory action of P. aeruginosa flagellin (44). Mapping of these glycosylation sites has revealed not only strain-specific variation in the sites of glycosylation (Thr-189, Ser-191, Ser-195, and Ser-260) but also heterogeneity in the particular glycan moieties attached to these sites (39, 45). Differences in flagellin glycosylation can be explained, in part, through strain-dependent genetic divergence of the respective flagellar glycosylation islands in the P. aeruginosa genome.

Given that the P. aeruginosa flagellum contributes to bacterial virulence and stimulates host innate immunity through a TLR5 \( \rightarrow \) NF-κB \( \rightarrow \) IL-8 pathway, recent interest has been generated in the development of a flagellar vaccine and vaccination efficacy has been demonstrated in several animal models of active and passive immunization. For example, Holder et al. (16) observed that immunization with flagella protected against subsequent P. aeruginosa challenge infection in the burned-mouse model. Saha et al. (37) developed a DNA vaccine encoding a mutant flagellin protein that was highly immunogenic, but its ability to interact with TLR5 was reduced by more than 100-fold. DNA vaccination of mice induced flagellum-reactive antibodies and provided protection against lethal P. aeruginosa infection in the lungs without blocking TLR5 activation. In mouse and rat models of P. aeruginosa-induced pneumonia, administration of human monoclonal antibodies to flagella provided protection against infection and decreased bacterially induced lung injury (22, 32). Active vaccination of mice with intact flagella induced protection against infection by PAK and PA01, although it was much less effective against P. aeruginosa clinical isolates (5). In contrast, immunization with flagellin monomers was suggested to induce antibodies capable of neutralizing innate immunity due to blockade of TLR5 activation. In human studies, P. aeruginosa lung infections were reduced in patients passively immunized with chicken IgY antiflagellin antibodies (31). Additionally, a double-blind randomized placebo-controlled phase III study of a P. aeruginosa flagellar vaccine in CF patients has been published, albeit these patients nonetheless became colonized with P. aeruginosa in spite of being vaccinated (10). Part of the problem in the latter study relates to the ability of the bacteria to downregulate flagellin biosynthesis in colonized CF patients who harbor biofilms of P. aeruginosa in the lungs (26, 27). In summary, while promising experimental results have been ob-
tain following flagellar vaccination in animal models of *P. aeruginosa* infection, the limited human clinical trials that have been performed are less encouraging. Future studies to identify optimal flagellar/flagellin vaccine candidates that elicit broadly protective immunity against *P. aeruginosa* infection may be applicable to patients with acute infections as well as to high-risk patients before the onset of chronic infection.

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