Epithelial Cells Are Sensitive Detectors of Bacterial Pore-forming Toxins*

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Epithelial cells act as an interface between human mucosal surfaces and the surrounding environment. As a result, they are responsible for the initiation of local immune responses, which may be crucial for prevention of invasive infection. Here we show that epithelial cells detect the presence of bacterial pore-forming toxins (including pneumolysin from Streptococcus pneumoniae, α-hemolysin from Staphylococcus aureus, streptolysin O from Streptococcus pyogenes, and anthrolysin O from Bacillus anthracis) at nanomolar concentrations, far below those required to cause cytolsis. Phosphorylation of p38 MAPK appears to be a conserved response of epithelial cells to sublytic concentrations of bacterial pore-forming toxins, and this activity is inhibited by the addition of high molecular weight osmolytes to the extracellular medium. By sensing osmotic stress caused by the insertion of a sublethal number of pores into their membranes, epithelial cells may act as an early warning system to commence an immune response, while the local density of toxin-producing bacteria remains low. Osmosensing may thus represent a novel innate immune response to a common bacterial virulence strategy.

Elaboration of pore-forming toxins (PFT) is a common theme in bacterial pathogenesis. Many distantly related species produce protein toxins capable of puncturing eukaryotic membranes, suggesting that this strategy may be an example of convergent evolution. However, the PFT are a heterogeneous group made up of several distinct structural classes. Typically PFT are secreted as monomers, which insert into host cell membranes, form homooligomers, and finally lead to the disruption of membrane integrity. The consequences of pore formation for the host cell may be dire, leading to cell death by rupture or to the induction of apoptosis. This cytositis may, in turn, confer a survival advantage to the bacterium allowing it to better adhere to the damaged cells, to enter previously inaccessible sites, or to escape immune responses. In many cases, most notably the major Gram-positive pathogens Staphylococcus aureus, Streptococcus pneumoniae, and Streptococcus pyogenes, PFT are essential for full virulence of the organism, although their precise mechanisms of action in vivo are not well understood (3–5).

Here we show that respiratory epithelial cells, the initial point of contact between many common bacterial pathogens and their human hosts, respond to sublytic, nanomolar concentrations of distinct classes of bacterial PFT through phosphorylation of the cytoplasmic protein p38 mitogen-activated protein kinase (MAPK). Activation of p38 MAPK during bacterial infection has been shown to be crucial to local production of cytokines such as the chemokine interleukin (IL)-8 (6) and to the development of an effective immune response in vivo (7). In this regard, we have observed previously that S. pneumoniae strains that lack the PFT pneumolysin (Ply) induce lower chemokine responses associated with diminished neutrophil influx and delayed clearance in a murine model of mucosal colonization (8). Our findings are also consistent with the observation that survival of Caenorhabditis elegans challenged with PFT appears to be critically dependent on its ability to activate members of the MAPK family (9). Here we demonstrate that toxin-induced p38 MAPK activation requires pore formation and is inhibited by relief of osmotic stress. This finding demonstrates a role for the epithelium in detection of bacterial PFT at sublytic concentrations and has implications for understanding the early stages of immune responses to bacterial products during colonization or disease.

MATERIALS AND METHODS

Bacterial Strains and Products—S. pneumoniae strains D39 (10) and its Ply-deficient derivative D39ply (3), kindly provided by D. Briles, were grown as described (6). S. aureus RN6390 and its α-hemolysin-deficient derivative RN6390/bla (A. Cheung, Dartmouth University) were grown in tryptic soy broth. Purified Ply and toxoid PdB (Ply-W433F) were the gift of J. Paton. Purified recombinant anthrolysin O (ALO) (11) was the gift of Richard Rest. Other reagents were from Sigma unless otherwise noted.

Epithelial Cell Lines and Culture Conditions—A549 (CCL-185), Detroit 562 (CCL-138), and human embryonic kidney 293 (HEK, CRL-1573) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 1 mM sodium pyruvate, 10% fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 mg/ml streptomycin.

Bacterial Culture Conditions—Streptococcal Blot—A549, HEK, or Detroit 562 cells grown to confluence in 6-well plates were weaned from serum and antibiotics overnight. Bacteria were grown in liquid culture to mid-log phase (A620 = 0.5), collected by centrifugation, washed in phosphate-buffered saline (PBS), and resuspended in DMEM without serum or antibiotics. Serial dilutions were plated to confirm bacterial density. 1 × 10^7 colony-forming units/ml of S. pneumoniae or S. aureus was added to the epithelial monolayer (multiplicity of infection = ∼10). In some experiments, PBS-washed bacteria were sonicated prior to use. Also, where indicated, bacterial toxins or purified TNF-α, diluted to the indicated concentrations in DMEM, were added to the cells in lieu of bacteria. Plates were spun at 150 × g for 5 min to apply bacteria to epithelial cells and incubated at 37 °C, 5% CO₂.
for the indicated incubation periods. After washing with sterile PBS, cells were lysed in 1% Triton X-100 with protease and phosphatase inhibitors, and aliquots were separated on a 10% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes and probed using the PhosphoPlus p38 MAPK antibody kit (Cell Signaling). Duplicate samples were run on separate gels for detection of total and phosphorylated p38 MAPK. Blots were not stripped and reprobed, and each experiment was repeated at least three times. In some experiments, A549 cells were loaded with BAPTA/AM (10 μM, Invitrogen) for 45 min at 37 °C, washed twice with PBS, and incubated in fresh DMEM for 45 min prior to stimulation. In experiments using dextran or cellulose (20 μm average particle size) and purified PFT, these reagents were added simultaneously. Because HEK cells adhere poorly if weaned from serum, unweaned cells were used in experiments with this cell line. Because A549 cells detach if incubated at 37 °C in the presence of EGTA, experiments using EGTA were carried out at room temperature.

In all cases, epithelial cell viability exceeded 95%. As an independent marker for cell damage or death, release of lactate dehydrogenase into supernatants of cells exposed to toxins for 45 min at 37 °C was assessed using a commercial kit (Roche Applied Science). Positive control lysis was with 1% Triton X-100 in PBS.

Interleukin-8 Enzyme-linked Immunosorbent Assay—A549 cells were grown to confluence in 96-well plates and incubated in serum-free DMEM overnight. Quadruplicate wells were treated with Ply or PdB diluted to the indicated concentrations in serum-free DMEM. Where indicated, pretreatment of cells with 20 mM SB203580 (Calbiochem) was for 30 min at 37 °C, and SB203580 was present during incubation of A549 cells with toxin or toxoid preparations. After 18 h, supernatants were removed, and the concentration of IL-8 was determined using a commercially available enzyme-linked immunosorbent assay (Pharmingen OptEIA). Conditions were compared by one-way analysis of variance with Tukey test for multiple comparisons (Prism GraphPad software).

Hemolytic Assay—Purified Ply or PdB was diluted in PBS. 80 μl of diluted toxin was added to 160 μl of lysis buffer (PBS plus 0.1% bovine serum albumin, 10 mM dithiothreitol) and 80 μl of PBS-washed horse erythrocytes (2%) in a 96-well V-bottom plate. The plate was incubated at 37 °C for 30 min and centrifuged at 150 × g for 10 min; then supernatants were collected. Optical density at 415 nm was recorded in a plate reader (Bio-Rad). Triplicate wells at each concentration were assayed, and each experiment was repeated at least three times. In some experiments, 5 mM EGTA was added to the buffer prior to the addition of toxin or erythrocytes. MgCl₂ or CaCl₂ in PBS was added to the buffer along with EGTA to give indicated concentrations.

RESULTS AND DISCUSSION

PFT Are Necessary and Sufficient for Activation of p38 MAPK—Previous studies have demonstrated activation of p38 MAPK in the setting of pneumococcal pneumonia as an essential component of host control of bacterial replication in the lung (7). In addition, activation of p38 MAPK in human cells in vitro by S. pneumoniae has been described (12, 13). Stringaris et al. (14) reported activation of p38 MAPK and calcium-dependent cell death in neuroblastoma cells treated with purified Ply and speculated on its relevance to the pathogenesis of pneumococcal meningitis. Likewise, S. aureus activation of host p38 MAPK has been recognized but not attributed to the pore-forming nature of its α-hemolysin (15). S. pyogenes has been reported to activate osteoblasts and keratinocytes via p38 MAPK, but the importance of streptolysin O (SLO) in these systems is not addressed (16, 17).

Because it is required for synergistic enhancement of epithelial cytokine production during polymicrobial infection (6), we hypothesized that pneumolysin, the single protein toxin of S. pneumoniae, might be sufficient to induce p38 MAPK phosphorylation in respiratory epithelial cells. Treatment of A549 respiratory epithelial cells with S. pneumoniae D39, but not its isogenic Ply-deficient mutant D39Δp, led to the phosphorylation of p38 MAPK as determined by Western blotting (Fig. 1A). To assess whether this was a property specific to S. pneumoniae and its toxin, we performed a similar experiment using S. aureus, another
major Gram-positive pathogen. Importantly, although S. aureus does produce pore-forming toxins, of which α-hemolysin is the most abundant (7), these are structurally unrelated to Ply and form considerably smaller pores in eukaryotic cells. Despite these differences, we found that wild-type S. aureus RN6390 activated epithelial p38 MAPK in a manner similar to S. pneumoniae. Correspondingly, the isogenic α-hemolysin-deficient mutant RN6390 hla was attenuated in its ability to activate epithelial p38 MAPK (Fig. 1B).

To confirm that PFT were sufficient as well as necessary for activation of epithelial p38 MAPK in response to Gram-positive bacteria, we stimulated epithelial cells with purified S. aureus α-hemolysin, Ply, or other members of the cholesterol-dependent cytolysin family, SLO and ALO (Fig. 2). In each case, purified PFT were sufficient to cause p38 phosphorylation in epithelial cells and required either high ng/ml or low μg/ml quantities (corresponding to nanomolar concentrations). These in vitro results correlate with and may account for our previous observations that Ply-expressing but not non-expressing S. pneumoniae induce mucosal production of macrophage inflammatory protein-2 (a murine homolog of interleukin-8) and subsequent recruitment of neutrophils during murine nasopharyngeal colonization (8). At the toxin concentrations used, epithelial cell viability was unaffected and exceeded 95% as assessed by trypan blue exclusion. In addition, lactate dehydrogenase release from A549 cells treated with toxins at these concentrations was <5% of the positive control lysis in all cases (data not shown). At a 10-fold higher concentration of Ply (1 μg/ml), we noted release of >65% of total cellular lactate dehydrogenase. Thus, we found that subcytolytic concentrations of each toxin led to increased p38 MAPK phosphorylation.

Phosphorylation of p38 MAPK was detectable within 15 min after exposure of cells to toxin and reached its maximal level by 30 min (Fig. 2E). Likewise, we noted p38 MAPK phosphorylation in response to Ply in an unrelated cell line, Detroit 562 nasopharyngeal cells, suggesting that these effects were not specific to A549 cells (Fig. 2F). We attempted to confirm p38 MAPK phosphorylation in response to PFT in primary murine epithelial cells but were hampered by endogenous phosphorylation of p38 MAPK, making differences difficult to assess (data not shown).

Toxin-mediated Pore Formation Activates p38 MAPK—It has been proposed that some PFT have effects on eukaryotic cells that are independent of pore formation. Specifically, Ply and other members of the cholesterol-dependent cytolysins are putative ligands for the host pattern-recognition molecule toll-like receptor-4 (TLR4) independent of their pore-forming activities (18–20). Non-hemolytic Ply toxoids are equally efficient compared with native Ply as TLR4 agonists (18). Because S. aureus and S. pneumoniae produce PFT that are not structurally related, yet both induce epithelial p38 MAPK phosphorylation, we hypothesized that pore formation, rather than recognition of specific toxin motifs, might be an essential step in the p38 MAPK response to toxins. We used the Ply toxoid PdB, which has a point mutation in the region that is essential for pore formation, to distinguish the pore-forming properties of Ply from its potential action on receptors such as TLR4. PdB inserts into cholesterol-containing membranes and oligomerizes...
but does not form functional pores (21). To confirm the non-cytolytic quality of PdB, we used an assay of horse erythrocyte hemolysis (Fig. 3A). Measurement of hemoglobin release from horse erythrocytes is a convenient measure of pore formation, although it takes place at substantially lower doses than the ones relevant to epithelial cell stimulation. Treatment of A549 cells with purified Ply, but not with equivalent concentrations of PdB, led to the phosphorylation of p38 MAPK (Fig. 3B). Similar results were obtained using HEK cells, which lack TLR2 and TLR4 (22), suggesting that the mechanism of p38 activation is independent of these receptors (data not shown). This finding as well as the observation that structurally unrelated PFT activate p38 MAPK suggests that pore formation, rather than any effect of Ply or PdB on TLR4-mediated signaling, is the mechanism of toxin-induced activation of this host cell pathway.

**Epithelial Cells Sense Bacterial Toxins**

**Epithelial IL-8 Response to PFT Requires p38 MAPK Activity**—Consistent with these findings, treatment of epithelial cells with Ply but not PdB led to the release of interleukin-8, a chemokine that functions as a potent neutrophil attractant. The chemical inhibitor of p38 MAPK activity, SB203580, was sufficient to reduce or abolish this response (Fig. 3C). Activation of ERK1/2 and c-Jun N-terminal kinase (JNK) were also detected in response to PFT (data not shown). However, we focused our investigations on the mechanism of the p38 MAPK response because SB203580 inhibited epithelial IL-8 release in response to microbial products (Fig. 3C and Ref. 6).

**Divalent Cations Enhance Toxin-mediated Pore Formation**—Studies of the effects of purified Ply on neutrophils have suggested a role for extracellular calcium in the initiation of host cell responses. Typically, chelation of divalent cations using EGTA has been associated with the inhibition of Ply-mediated effects. The mechanism of this effect has been hypothesized to involve entry of Ca$$^{2+}$$ ions through Ply pores (23–26). This is consistent with studies of PFT from *Escherichia coli*, showing that the insertion of pores into host cell membranes can induce calcium oscillations and transient currents that may activate host cell signaling pathways (27). We further investigated the role of divalent cations in Ply-induced host cell signaling.

Chelation of extracellular divalent cations with EGTA eliminated the ability of Ply to induce p38 MAPK phosphorylation in A549 cells (Fig. 4A). In contrast, the cell-permeant chelator BAPTA/AM (10 $\mu$M) has no effect on toxin-mediated p38 MAPK phosphorylation (B). Horse erythrocyte lysis by Ply at the concentrations indicated is inhibited in the presence of EGTA and is restored by the addition of MgCl$_2$ or CaCl$_2$ to the extracellular medium (C).
important for the activation of p38 MAPK by bacterial PFT, chelation of intracellular divalent cations with BAPTA/AM had no effect on Ply-mediated p38 MAPK activation (Fig. 4B). Concentrations of BAPTA/AM used in these experiments have been shown previously to efficiently inhibit Ca^{2+}-dependent pathways in epithelial cells (15). In our system, BAPTA/AM did enhance phosphorylation of ERK in A549 cells treated with thapsigargin (1 μM, 15 min) (data not shown) as described previously in hepatic epithelial cells (28).

EGTA chelation of extracellular divalent cations also increased the concentration of Ply needed to achieve the lysis of horse erythrocytes relative to conditions in which divalent cations were present (Fig. 4C), suggesting that these cations may be a previously unrecognized requirement for efficient formation of its pores. To confirm the dependence of this effect on the chelation capacity of EGTA, we added Mg^{2+} or Ca^{2+} salts in the presence of EGTA. Addition of either MgCl_{2} or CaCl_{2} increased the efficiency of Ply-mediated hemolysis. This is consistent with findings related to another unrelated PFT, α-latrotoxin, which requires divalent cations for efficient pore formation (29). Previous studies of Ply pores demonstrated that small- and medium-sized Ply channels are gated by divalent cations, whereas large ones are insensitive to the effects of these cations (21, 30). Our results suggest that it may be the larger, cation-insensitive pores that are responsible for the majority of cellular effects, including erythrocyte lysis and epithelial p38 MAPK activation.

Epithelial Cells Sense PFT via Osmotic Stress—Because p38 MAPK phosphorylation is involved in osmotic stress sensation (31), we hypothesized that activation of p38 MAPK by PFT might be a consequence of cellular processes normally used to sense membrane integrity. If that were the case, relief of osmotic stress caused by the sublethal disruption of membrane integrity by PFT ought to ameliorate p38 activation. We used high molecular weight dextran (M_{w} ~167,000) larger than the cut-off size shown previously to enter through pores created by a related toxin, SLO (32). Dextran of this size are sufficient to inhibit toxin-induced hemolysis through osmotic protection (21). Addition of high molecular weight dextran to the extracellular medium efficiently inhibited the p38 response of cells to Ply (Fig. 5A) or to S. aureus α-hemolysin (Fig. 5B) but not TNF-α treatment (Fig. 5B), consistent with the hypothesis that osmosensing plays an important role in this epithelial response to PFT. The absence of an effect on TNF-mediated p38 MAPK phosphorylation suggests that the effect of dextran is specific to signaling initiated by PFT. Likewise, a suspension of cellulose particles of molecular weight dextran to the extracellular medium efficiently inhibited p38 phosphorylation suggests that the effect of dextran is specific to signal-

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