Recognition of Host Immune Activation by Pseudomonas aeruginosa

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It is generally reasoned that lethal infections caused by opportunistic pathogens develop permissively by invading a host that is both physiologically stressed and immunologically compromised. However, an alternative hypothesis might be that opportunistic pathogens actively sense alterations in host immune function and respond by enhancing their virulence phenotype. We demonstrate that interferon-γ binds to an outer membrane protein in Pseudomonas aeruginosa, OprF, resulting in the expression of a quorum-sensing dependent virulence determinant, the PA-I lectin. These observations provide details of the mechanism by which prokaryotic organisms are directly signaled by immune activation in their eukaryotic host.

Although opportunistic infection has been traditionally viewed as a passive phenomenon in which exploitative pathogens invade a weakened host, recent advances in the understanding of bacterial virulence gene regulation would suggest that this process is much more complex than previously appreciated (1). Because bacteria are constantly assessing the cost versus benefit of expressing virulence (2), it is logical that they might develop a contingency-based system to recognize physiological and immunological disturbances in their hosts.

Although host cells are known to express receptors that bind bacteria for the purpose of activating the immune system, it must be considered that bacteria themselves might possess specialized receptors that in turn recognize and respond to host immune activation.

We studied this possibility using the human opportunistic pathogen, Pseudomonas aeruginosa, because its virulence gene regulation is well studied. We used the type I P. aeruginosa lectin (PA-I or lecA), an adhesin of P. aeruginosa, as a representative readout for virulence expression in this organism. Previously, we demonstrated that within the intestinal tract of a stressed host, the lethality of P. aeruginosa is dependent on the expression of the PA-I lectin, which causes increased permeability to its lethal cytotoxins across the intestinal epithelium (3). PA-I has also been shown to induce apoptosis in respiratory epithelial cells, which suggests that PA-I may be directly cytotoxic to epithelial cells (4). Finally, the expression of PA-I (lecA) is dependent on the quorum-sensing (QS) signaling system (5), a core system of virulence gene regulation that controls multiple virulence genes in P. aeruginosa.

We considered that immune elements might directly activate the virulence of P. aeruginosa. As a physiologically relevant in vitro source of such immune factors, supernatants from antigen-stimulated T cells, which express an array of cytokines (6), were evaluated for their ability to increase PA-I expression in P. aeruginosa strain PLL-EGFP27853, a PA-I-GFP reporter (7) that was readily available and verified in a previous report by our laboratory (8). PA-I expression was increased by supernatant from activated T cell cultures, as assessed by enhancement of fluorescence in the PA-I-GFP fusion reporter strain (Fig. 1A), but not in controls. To determine whether this effect was due to specific cytokines, the reporter strain was individually exposed to human IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α). Of these, only IFN-γ induced a significant increase in PA-I expression that started at early stationary phase of growth (Fig. 1B). None of the cytokines tested had any significant effect on bacteria growth (Fig. 1C).

Immunodepletion of IFN-γ resulted in the complete loss of its PA-I inducing capacity (Fig. 1A), which suggests that IFN-γ was the critical component in the activated T cell media that induced PA-I expression (Fig. 1A). Next, we examined PA-I expression in the completely genomically sequenced strain of P. aeruginosa, PAO1 (9), after exposure to human recombinant IFN-γ, TNF-α, IL-2, IL-4, IL-8, and IL-10 (7). Northern blot analyses revealed that only IFN-γ was capable of inducing lecA gene transcription (Fig. 1D).

Vilurine in P. aeruginosa is highly regulated by the QS signaling system, a hierarchical system of virulence gene regulation that is dependent on bacterial cell density and growth phase (10–12). To determine the effect of growth phase on the response of P. aeruginosa
to IFN-γ, bacteria were harvested at various growth phases after exposure to IFN-γ, and PA-I mRNA and protein were measured (7). Both transcription and translation of PA-I increased in response to IFN-γ, starting at early stationary phase of growth (Fig. 1E and F). PA-I protein expression was also dose dependent (Fig. 1G). Taken together, these results suggest that PA-I expression in P. aeruginosa is enhanced in the presence of IFN-γ in a growth-dependent manner.

To determine whether IFN-γ induced PA-I by activation of the QS signaling system, we measured rhl gene expression in response to IFN-γ (7). RhlII is the gene required for the synthesis of C2-HSL (C2-homoserine lactone), a core QS signaling molecule that plays a central role in the expression of PA-I (5). IFN-γ induced rhlII transcription in PAO1 (Fig. 2, A and B), and C2-HSL synthesis increased significantly after exposure to IFN-γ (Fig. 2C). Activation of the QS system by IFN-γ also led to the increase of pyocyanin (PCN), another QS-dependent virulence product (13) (Fig. 2D). In addition, rhlI and rhlII were required for the production of PCN and PA-I expression in response to IFN-γ, because the increase of these two virulence factors by IFN-γ was abolished in mutant strains (Fig. 2, E and F). Finally, supernatant from P. aeruginosa exposed to IFN-γ, but not controls, altered the barrier function of cultured epithelial cells (fig. S1). Taken together, these data suggest that the QS system plays a key role in the response of P. aeruginosa to IFN-γ and that IFN-γ can shift the virulence of P. aeruginosa against epithelial cells.

We next hypothesized that IFN-γ may directly bind to a protein on the surface of P. aeruginosa, leading to virulence up-regulation. Consistent with this, we observed that IFN-γ avidly bound to whole fixed cells of P. aeruginosa in a dose-dependent manner (Fig. 3A). The vast majority of bacterial cells (73% ± 3.2% versus 8.5% ± 2.5%) bound IFN-γ (Fig. 3B and fig. S2). The binding capacity of the IFN-γ to P. aeruginosa was not affected significantly by the growth phase of bacteria (fig. S3A). To determine whether IFN-γ bound to membrane or cytosolic fractions of P. aeruginosa, equal protein concentrations of each fraction were prepared (7), and results showed that IFN-γ preferentially bound to membrane fractions by enzyme-linked immunosorbent assay (ELISA) (fig. S3B). Furthermore, IFN-γ binding to P. aeruginosa membranes was diminished upon proteinase K treatment (fig. S3C), which suggests that IFN-γ binds to a protein on the bacterial cell membrane. Binding was specific to IFN-γ, because no binding was observed with any other cytokines tested (fig. 3D). Taken together, these data indicate that IFN-γ binds specifically to a membrane protein (s) on P. aeruginosa.

P. aeruginosa membrane proteins solubilized with mild detergents (7) retained their binding capacity to IFN-γ (Fig. 3C), thus making it possible to isolate the putative binding protein by immunoprecipitation. Membrane proteins were next separated by non-denaturing gel electrophoresis, transferred to polyvinylidene difluoride membranes, and hybridized with IFN-γ followed by biotin-labeled antibody to IFN-γ; results revealed a single immunoreactive band at 35 kD that was dependent on the dose of IFN-γ (Fig. 3D). Immunoprecipitation against the P. aeruginosa fractionated membrane protein isolated a 35-kD protein that was IFN-γ dependent (Fig. 3E). Use of ESI-TRAP LC-MS-MS ion trap (electrospray ionization–tandem mass spectrometry) identified the 35-kD protein to be the P. aeruginosa outer membrane porin OprF (Fig. 3F) (14). We next verified that OprF was a major binding site for IFN-γ by showing that solubilized membrane proteins from OprF mutant strain (15) displayed reduced binding to IFN-γ (Fig. 4A). Immunoprecipitation of solubilized membrane protein confirmed the role of OprF by showing complete loss of the ~35-kD band in the OprF mutant strain (Fig. 4B). Further evidence supporting the role of OprF in the IFN-γ response was found when mutant strains failed to increase PA-I protein expression after exposure to an effective stimulating dose of IFN-γ as compared with the wild-type strain (Fig. 4, C and D). When OprF was reconstituted in the mutant strain S1899 using the plasmid pUCP24/OprF,
responsiveness to IFN-γ was reestablished, with an increase in PA-I protein expression (Fig. 4E). Finally, ELISA binding assays between purified OprF and IFN-γ demonstrated that OprF binds directly to human IFN-γ (Fig. 4F) in a dose-dependent manner.

Although it was previously recognized that various cytokines, including IL-1β (16) and TNF-α (17), can affect the growth and virulence properties of bacteria, little progress had been made on the mechanistic details of these initial observations. For example, although TNF-α has been shown to display high-affinity binding to Shigella flexneri, the specific receptor that mediates this response has not yet been identified. In addition, although TNF-α has been shown to induce Shigella flexneri to become more invasive against cultured epithelial cells, the specific signaling pathways and virulence factors that mediate this response remain unknown. The observation here that P. aeruginosa binds INF-γ through OprF, resulting in activation of the QS system, provides specific information on the mechanisms by which certain bacteria sense and respond to the host immune system. That INF-γ binding to OprF induces P. aeruginosa to express both PA-I and pyocyanin, both of which are capable of disrupting epithelial cell function, is intriguing given that, in contrast to binding to TNF-α, the main function of INF-γ is bacterial clearance (18).

Data from the present study provide molecular evidence that certain opportunistic pathogens such as P. aeruginosa may have evolved a contingency-based mechanism to mount an effective countermeasure to immune activation by their host. It remains to be clarified whether other virulence regulators that activate the QS system, such as the quinolone signaling system (19, 20) and the lasRI system (5), may be involved in the transduction of membrane signaling in P. aeruginosa to IFN-γ. Finally, the design of appropriate animal models using INF-γ knockout mice and OprF mutant strains would be...
A Phenyllalanine Clamp Catalyzes Protein Translocation Through the Anthrax Toxin Pore

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The protective antigen component of anthrax toxin forms a homoeptameronic pore in the endosomal membrane, generating a narrow passageway for the enzymatic components of the toxin to enter the cytosol. We found that, during conversion of the heptamer to the pore, the seven phenylalanine-427 residues converged within the lumen, generating a radially symmetric heptad of solvent-exposed aromatic rings. This $\omega$-clamp structure was required for protein translocation and comprised the major conductance-blocking site for hydrophobic drugs and model cations. We conclude that the $\omega$-clamp serves a chaperone-like function, interacting with hydrophobic sequences presented by the protein substrate as it unfolds during translocation.

Anthrax toxin is composed of three nontoxic proteins, which combine on eukaryotic cell surfaces to form toxic, noncovalent complexes. [See (1) for a review.] Protective antigen (PA), the protein translocase component, binds to a cellular receptor and is activated by a furin-family protease. The resulting 63-kD receptor-bound fragment, PA$_{63}$, self-assembles into the prepore, which is a ring-shaped homoeptamer (Fig. 1A). The prepore then forms complexes with the two ~90-kD enzymatic components, lethal factor (LF) and edema factor (EF). These complexes are endocyotosed and delivered to an acidic compartment (2). There, the prepore undergoes an acidic pH-dependent conformational rearrangement (3) to form an ion-