Interferons Increase Cell Resistance to Staphylococcal Alpha-Toxin

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Many bacterial pathogens, including Staphylococcus aureus, use a variety of pore-forming toxins as important virulence factors. Staphylococcal alpha-toxin, a prototype beta-barrel pore-forming toxin, triggers the release of proinflammatory mediators and induces primarily necrotic death in susceptible cells. However, whether host factors released in response to staphylococcal infections may increase cell resistance to alpha-toxin is not known. Here we show that prior exposure to interferons (IFNs) prevents alpha-toxin-induced membrane permeabilization, the depletion of ATP, and cell death. Moreover, pretreatment with IFN-alpha decreases alpha-toxin-induced secretion of interleukin 1beta (IL-1beta). IFN-alpha, IFN-beta, and IFN-gamma specifically protect cells from alpha-toxin, whereas tumor necrosis factor alpha (TNF-alpha), IL-6, and IL-4 have no effects. Furthermore, we show that IFN-alpha-induced protection from alpha-toxin is not dependent on caspase-1 or mitogen-activated protein kinases, but requires protein synthesis and fatty acid synthase activity. Our results demonstrate that IFNs may increase cell resistance to staphylococcal alpha-toxin via the regulation of lipid metabolism and suggest that interferons play a protective role during staphylococcal infections.

Staphylococcus aureus is a ubiquitous bacterial pathogen, causing a wide spectrum of severe infections, such as abscesses, pneumonia, endocarditis, and sepsis (18). Many clinical isolates of S. aureus produce a variety of pore-forming toxins (alpha-toxin, gamma-toxin, binary leukocidins LukF and LukS, and Panton-Valentine leukocidin) among other virulence factors (28). Staphylococcal pore-forming toxins contribute to the pathogenesis of staphylococcal infections by inducing death in susceptible cells and triggering the release of proinflammatory mediators (16). Staphylococcal alpha-toxin is a prototype beta-barrel pore-forming toxin and an important virulence factor of S. aureus (3, 28). Staphylococcal alpha-toxin induces primarily necrotic cell death via a mechanism that involves binding to the cell plasma membrane, oligomerization of the toxin, and formation of pores (3, 8). The efflux of intracellular potassium and the influx of sodium are followed by the loss of transmembrane potential, shutdown of ATP production, and predominantly necrotic cell death (3). These processes are accompanied by the release of proinflammatory and vasoactive mediators, contributing to the pathogenesis of staphylococcal infections (18).

Host cells have varying degrees of sensitivity to the cytolytic effects of alpha-toxin (3). Platelets and red blood cells are generally very sensitive to alpha-toxin-induced cytolysis (2). Among nucleated cells, epithelial and endothelial cells are more sensitive than are lymphocytes, mononuclear phagocytes, or fibroblasts, whereas neutrophils show a high degree of resistance to alpha-toxin-induced cytolysis (39). Recent studies demonstrated that epithelial cells cope with the subcytolytic concentrations of alpha-toxin and related pore-forming toxins by activating mitogen-activated protein kinases (MAPKs) or caspase-1-mediated mechanisms of plasma membrane repair (9, 11, 12). It has been reported that the production of interleukin-1beta (IL-1beta) requires protein synthesis but requires fatty acid synthesis activity. Our results demonstrate that IFNs may increase cell resistance to staphylococcal alpha-toxin via the regulation of lipid metabolism and suggest that interferons play a protective role during staphylococcal infections.

Materials and Methods

Reagents and cells. Staphylococcal alpha-toxin was purchased from Toxin Technology (Sarasota, FL) or purified as described previously (26). Streptolysin O was purchased from Sigma-Aldrich (St. Louis, MO). IFNs and other cytokines were purchased from PBL InterferonSource (Piscataway, NJ), R&D Systems (Minneapolis, MN), or PeproTech (Rocky Hill, NJ). Caspase inhibitors were purchased from BD Biosciences (San Jose, CA); other inhibitors were from EMD Biosciences (San Diego, CA). The human lung epithelial cell line AS49 and monocytic cell line THP-1 were purchased from ATCC (Manassas, VA). Mouse splenocytes were isolated from C57BL/6 mice (8- to 10-week-old females;
RESULTS

IFNs protect cells from alpha-toxin. In agreement with previous studies (17, 30), massive cell death was observed after the exposure of human lung epithelial cell line A549 to 0.5 μg/ml of alpha-toxin (Fig. 1A). Cell death was also noticeable for A549 cells treated with the high dose of IFN-α alone (1,000 U/ml), but was negligible at lower doses of IFN-α. Remarkably, pretreatment of A549 cells with 100 U/ml or 1,000 U/ml of IFN-α for 24 h significantly decreased alpha-toxin-induced cell death. In addition, IFN-α-pretreated A549 cells retained both live (green) and ethidium homodimer (red) and fluorescence microscopy. The data are means ± SD of five (B) or four (E) independent experiments, each performed at least in triplicate. The data in panels A and D are means ± SD (error bars) of triplicates and are representative of two independent experiments.

IFN-α protection was also evident after staining with calcine AM (for live cells) and ethidium homodimer (for dead cells) (Fig. 1C). In situ analyses of dead and live cells were performed by staining A549 cells with calcine AM and heat shock protein 90 and expressed as percent relative to the nontreated control group.

Statistical analyses. The difference between the experimental groups was evaluated using a one-way analysis of variance test and a post hoc Bonferroni test. A two-way analysis of variance test was applied to evaluate the effects of serum deprivation or inhibitors on IFN-α-induced protection. Since cycloheximide (CHEX) and cerulenin dramatically decreased baseline ATP levels, their effects on IFN-α-induced protection from alpha-toxin were analyzed by comparing alpha-toxin-induced decreases of ATP levels in the groups treated with CHEX or the carrier solution (0.1% vol/vol dimethyl sulfoxide [DMSO]) for CHEX or 0.1% vol/vol ethanol for cerulenin. All calculations were performed with GraphPad Prism software, version 4 (GraphPad Software, San Diego, CA).

FIG. 1. IFN-α protects A549 and THP-1 cells from alpha-toxin. A549 or THP-1 cells were pretreated with the indicated concentrations of IFN-α for 24 h prior to challenge with alpha-toxin for an additional 24 h. (A and D) Cell death was measured based on membrane permeability for PI in nucleated cells by using the Guava ViaCount reagent and flow cytometry. (B and E) Relative ATP levels were measured at 24 h after alpha-toxin exposure. (C) In situ analyses of dead and live cells were performed by staining A549 cells with calcine AM (green) and ethidium homodimer (red) and fluorescence microscopy. The data are means ± SD of five (B) or four (E) independent experiments, each performed at least in triplicate. The data in panels A and D are means ± SD (error bars) of triplicates and are representative of two independent experiments.
interferons protect against alpha-toxin

ATP induced by streptolysin O, a pore-forming toxin from Streptococcus pyogenes, or from valinomycin, a potassium ionophore (data not shown).

Human monocytic THP-1 cells are naturally more resistant to alpha-toxin than are A549 and many other cells (7); therefore, higher concentrations of alpha-toxin were required to trigger cell death in THP-1 cells (Fig. 1D). The pretreatment of THP-1 cells with 1,000 U/ml IFN-α for 24 h significantly decreased alpha-toxin-induced cell death (Fig. 1D) and loss of ATP (Fig. 1E). These data indicate that the resistance of mononcytic cells to alpha-toxin is further increased by pretreatment with IFN-α.

A number of other cells, such as monocytic cell line U937, nontransformed human fibroblast line HFL-1, and skin epithelial cell line HaCaT, could be protected from alpha-toxin by pretreatment with IFN-α (data not shown). Moreover, splenocytes isolated from mice that were pretreated with IFN-α in vivo were more resistant to alpha-toxin than were splenocytes from control mice (data not shown). Thus, pretreatment with IFNs increases the resistance of cells to the cytolytic action of alpha-toxin in various cell systems.

Previous studies demonstrated that alpha-toxin and other pore-forming toxins trigger the secretion of IL-1β, TNF-α, IL-8 and a number of other proinflammatory mediators (1, 7, 29, 30, 33–35). We found that IFN-α-pretreated THP-1 cells secreted less IL-1β than did control cells after exposure to 1 or 5 μg/ml of alpha-toxin (Fig. 2). Although the challenge with 5 μg/ml of alpha-toxin resulted in similar levels of production of TNF-α and IL-8 from control and IFN-α-pretreated cells, the latter cells secreted less TNF-α and IL-8 after stimulation with 1 μg/ml of alpha-toxin. These data suggest that treatment with IFN-α decreases proinflammatory responses to alpha-toxin by raising the threshold for cell activation.

**IFN-α protects cells from alpha-toxin without interfering with alpha-toxin binding and oligomerization.** Next, we examined whether treatment with IFN-α affects alpha-toxin binding and oligomerization by incubating control and IFN-α-pretreated THP-1 cells with 35S-labeled alpha-toxin and subjecting the lysates of washed cells to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiography revealed that alpha-toxin was present predominantly in the monomeric form (34 kDa) at the start of the incubation (Fig. 3A). In agreement with results of previous studies (40, 41), amounts of alpha-toxin were essentially equal in monomeric and heptameric forms at 2 h of incubation, although the overall amounts of membrane-bound alpha-toxin decreased, probably due to shedding. Control and THP-1 cells showed similar amounts of monomeric and heptameric forms of alpha-toxin, indicating that pretreatment with IFN-α has no effect on alpha-toxin binding or oligomerization.

In the next set of experiments, we evaluated the effects of IFN-α on alpha-toxin-induced plasma membrane permeabilization in THP-1 cells. Although the initial β-barrel pores formed by heptamerized alpha-toxin are permeable to monovalent cations only (38, 40, 41), the exposure of the cells to microgram concentrations of alpha-toxin results in the formation of pores permeable to larger molecules, such as propidium iodide (PI) (8). Therefore, to evaluate alpha-toxin-induced membrane permeabilization, we stained control and IFN-α-pretreated THP-1 cells with PI prior to, and at early time points after, exposure to alpha-toxin (Fig. 3B). IFN-α alone had no effect on the number of PI-positive cells. A considerable fraction of control cells became permeable to PI as early as 2 h after alpha-toxin exposure, whereas very few IFN-α-pretreated cells became permeable to PI (18.0% ± 2.2% cells were PI positive in the control group versus 3.8% ± 0.5% PI-positive cells in the IFN-α-pretreated group; P < 0.01; data are means ± standard deviations [SD] of triplicate samples from a representative experiment). By 4 h of alpha-toxin exposure, 36.1% ± 3.4% cells in the control group became permeable to PI, whereas only 6.7% ± 0.3% PI-positive cells were found in the IFN-α-pretreated group (P < 0.01). Remarkably, a fraction of IFN-α-pretreated cells showed a decrease in forward scatter without membrane permeabilization, which may indicate transient ion fluxes and cell volume decrease.

Since the detection of alpha-toxin-induced permeabilization using flow cytometry was problematic in A549 cells, which are cultured under adherent conditions, we replicated our experiments using primary splenocytes from control and IFN-α-pretreated mice. Similar numbers of PI-positive cells were found in splenocytes isolated from control (13.5% ± 1.3%, n =
3) and IFN-α-pretreated mice (13.7% ± 1.2%) following ex vivo culture for 4 h without additional stimulation. The number of PI-positive splenocytes in the control group almost doubled to 27.5% ± 1.5% after treatment with alpha-toxin for 4 h, whereas little increase was found for the splenocytes from IFN-α-pretreated mice (18.3% ± 0.2%, significantly different from control; P < 0.01). Thus, our data suggest that IFN-α protects cells from alpha-toxin by interfering with the formation of alpha-toxin pores.

It is known that alpha-toxin triggers rapid depletion of ATP and that alpha-toxin-resistant cells quickly repair membrane lesions and recover their pools of ATP (38, 41). To determine the effects of IFN-α on the early alpha-toxin-triggered loss of ATP, we measured changes in ATP levels for up to 6 h after exposure to alpha-toxin. The levels of ATP in control and IFN-α-pretreated groups increased without alpha-toxin treatment, which may reflect ongoing cell proliferation (Fig. 3C). Treatment of control THP-1 cells with 5 μg/ml of alpha-toxin resulted in dramatic depletion of ATP within the first 2 to 3 h. However, IFN-α-pretreated cells retained high levels of ATP within 6 h after exposure to alpha-toxin. Thus, our data suggest that pretreatment with IFN-α prevents early loss of ATP following exposure to alpha-toxin.

IFN-α-induced protection from alpha-toxin is dependent on protein synthesis and activity of fatty acid synthase. Recent studies implicated MAPKs in the protection of cells from subcytolytic concentrations of alpha-toxin and other structurally related pore-forming toxins (11, 12). Extracellular signal-regulated kinase and p38 MAPKs are activated by type I IFNs and participate in the regulation of cell survival (15). However, we found that well-characterized inhibitors of p38 (SB203580) and extracellular signal-regulated kinase (UO126) had no significant effect on IFN-α-induced protection of THP-1 or A549 cells from alpha-toxin (data not shown).

Most of the biological effects of IFNs are realized via expression of IFN-stimulated genes, many of which are involved in the regulation of cell death and survival (6). During our initial screening experiments, we found that IFN-α showed maximal protection if used 16 to 24 h prior to alpha-toxin exposure, was less efficient if used 4 h prior to alpha-toxin, and showed no protection if used simultaneously with alpha-toxin (data not shown). These kinetics suggest that IFN-stimulated genes mediate protection from alpha-toxin. Therefore, we examined the effects of CHEX on the responses of control and IFN-α-pretreated cells to alpha-toxin. CHEX alone decreased ATP levels by 30 to 40% relative to ATP levels in the cells exposed to 0.1% DMSO, which was used as a carrier. However, both groups responded to alpha-toxin by similar reductions of the ATP levels (Fig. 4A and B) and the presence of CHEX had little effect on cell viability within the frame of the experiments (data not shown). When CHEX was added 24 h prior to alpha-toxin, ATP levels decreased to levels similar to those in cells treated with CHEX alone or IFN-α plus CHEX (data not shown). CHEX alone decreased ATP levels by 30 to 40% relative to ATP levels in the cells exposed to 0.1% DMSO, which was used as a carrier. However, both groups responded to alpha-toxin by similar reductions of the ATP levels (Fig. 4A and B) and the presence of CHEX had little effect on cell viability within the frame of the experiments (data not shown). When CHEX was added 24 h prior to alpha-toxin, ATP levels decreased to levels similar to those in cells treated with CHEX alone or IFN-α plus CHEX (Fig. 4A). There was no significant difference between the CHEX and IFN-α plus CHEX groups in ATP levels after treatment with 1 or 5 μg/ml alpha-toxin. These data indicate that CHEX abolishes the protective effects of IFN-α if added simultaneously with IFN-α. However, CHEX did not abolish the protective effects of IFN-α if added 23 h later, since cells treated with IFN-α plus CHEX retained significantly higher ATP levels.

**FIG. 3.** IFN-α protects cells without interfering with alpha-toxin binding and oligomerization. (A) Binding to and oligomerization on control and IFN-α-pretreated THP-1 cells of labeled alpha-toxin were assessed as described in Materials and Methods. The data are representative of two independent experiments. (B) Cell permeability for PI was measured by flow cytometry following short-term exposure of control or IFN-α-pretreated THP-1 cells. Representative dot plots are shown. (C) ATP levels in control and IFN-α-pretreated THP cells were measured following exposure to 5 μg/ml of alpha-toxin. Means ± standard deviations (error bars) of triplicate samples from a representative experiment are shown.
than did cells treated with CHEX alone after alpha-toxin exposure (Fig. 4B). In composite, these data suggest that the protective effects of IFN-α are dependent on protein synthesis prior to alpha-toxin exposure.

A recent study showed that the activation of caspase-1 and the induction of lipogenic genes facilitate epithelial cell recovery from the attack by alpha-toxin and a structurally related pore-forming toxin, aerolysin (9). It is well known that type I IFNs modify lipid metabolism and increase the activity of fatty acid synthase, a key enzyme involved in the biosynthesis of membrane phospholipids (22). We found that the treatment of THP-1 cells with IFN-α increased the abundance of fatty acid synthase, but slightly decreased the abundance of ATP citrate lyase, which is involved in the regulation of lipid biosynthesis by generating cytosolic acetyl-coenzymeA (Fig. 4C). This result coincided with the induction of pro-caspase-1 and STAT1.

To determine the role of fatty acid synthase in IFN-α-induced protection from alpha-toxin, we used cerulenin, a well-characterized inhibitor of this enzyme (23). We found that cerulenin decreased baseline ATP levels, sensitized cells to alpha-toxin, and reversed the protective effects of pretreatment with IFN-α (Fig. 5A). Similar effects of cerulenin were observed in A549 cells (data not shown). To determine the role of caspase-1, we exposed control and IFN-α-pretreated THP-1 cells to alpha-toxin in the presence of the caspase-1-specific, cell-permeable peptide inhibitor Ac-YVAD-CMK. We observed that neither the loss of ATP in control cells after alpha-toxin exposure nor the IFN-α-induced protection from alpha-toxin was altered in the presence of Ac-YVAD-CMK (Fig. 5B). Similarly, a cell-permeable pan-caspase inhibitor zVAD-FMK had no effect on alpha-toxin-induced loss of ATP or IFN-α-induced protection from alpha-toxin (Fig. 5C). However, IL-1β secretion in control cells exposed to alpha-toxin decreased in the presence of Ac-YVAD-CMK (Fig. 5D) or zVAD-FMK (Fig. 5E), which confirmed the efficiency of the inhibitors, since the secretion of IL-1β is dependent on caspase-1-mediated cleavage of pro-IL-1β. Thus, our data show that IFN-induced resistance to alpha-toxin is not dependent on caspases, but requires the activity of fatty acid synthase.

DISCUSSION

Our findings demonstrate that IFNs protect a broad spectrum of cells from staphylococcal alpha-toxin, which is a prototypical microbial toxin forming β-barrel pores. The protec-
The protective effects are shared by type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ), but not by other cytokines tested so far. Moreover, the protective effects are specific for alpha-toxin, as we found no protection from streptolysin O, and a previous study showed that type I IFNs actually sensitize lymphocytes to listeriolysin O, a pore-forming toxin from Listeria monocytogenes (4). The variations in the effects of IFNs are likely due to differences in specificity of binding at the molecular level and the mechanisms of pore formation between the pore-forming toxins (37).

The fact that IFNs are protective was surprising to some degree, given that the net effects of IFNs are usually associated with sensitization to apoptotic cell death via the induction of IFN-stimulated genes (6, 32). However, the mechanism of alpha-toxin-induced cell death is primarily necrotic (8) and there are several examples in the literature showing that IFNs may promote cell survival (14, 19, 27).

With respect to the mechanisms of IFN-induced protection from alpha-toxin, we found that IFN-α does not have any significant effect on alpha-toxin binding or oligomerization. However, we observed that pretreatment with IFN-α prevents membrane permeabilization and protects cells from the early depletion of ATP. We also found that IFN-α-mediated protection from alpha-toxin was dependent on timely protein synthesis, suggesting a role for IFN-stimulated genes. Furthermore, we found that IFN-α-mediated protection required the activity of fatty acid synthase, but was not dependent on the activity of MAPKs, caspase-1, or other caspases. Our findings are in line with those of other studies emphasizing the role of membrane lipid turnover in protection from pore-forming toxins (9, 21, 38). However, previous studies focused on cell responses to the efflux of potassium through the pores, which triggers the induction of lipogenic genes to repair the pores, whereas our study shows that IFN-α minimizes cell injury by preparing the cells to attack with alpha-toxin in advance.

Fatty acid synthase has previously been implicated in the regulation of membrane lipid turnover by IFNs (5). This enzyme is regulated at the level of gene transcription via sterol regulatory element binding proteins and at the level of enzyme activity via substrate availability (5). Further studies will be necessary to determine whether IFNs act primarily by increasing expression levels or activity of fatty acid synthase and whether other IFN-stimulated genes contribute to protection from alpha-toxin.

A number of studies showed that the ablation of alpha-toxin secretion or passive immunization against alpha-toxin decreases virulence of S. aureus (13, 20, 24, 25, 43). The recognition of S. aureus protein A by mononuclear phagocytes and/or plasmacytoid dendritic cells triggers the release of IFN-α, albeit at relatively low levels (31, 36). Our findings prompt further investigation of the role of type I IFNs in the pathogenesis of staphylococcal infections. It will be important to determine whether IFN-induced protection of the host cells from alpha-toxin may improve the outcome of staphylococcal infections and whether IFNs regulate cell resistance to other staphylococcal pore-forming toxins, such as leukocidins.
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