Anthrax Lethal Toxin Rapidly Activates Caspase-1/ICE and Induces Extracellular Release of Interleukin (IL)-1β and IL-18

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Anthrax lethal toxin (LT), a critical virulence factor for Bacillus anthracis, has been demonstrated to cleave and to inactivate mitogen-activated protein kinase kinases (MAPKKs) that propagate prosurvival signals in macrophages (1–5). Whether this action of anthrax LT leads to the production of proinflammatory cytokines by macrophages has been more controversial (6, 7). We now report that anthrax LT treatment leads to the specific extracellular release of interleukin (IL)-1β and IL-18 by the murine macrophage cell lines, RAW264.7 and J774A.1. Studies of the processing of IL-1β reveal that the levels of activated/cleaved IL-1β in RAW264.7 and J774A1 cells are increased following treatment with anthrax LT. Enhanced processing of IL-1β directly correlates with increased levels in the activation of its upstream regulator, IL-1β-converting enzyme/Caspase-1 (ICE). The extracellular release of IL-1β and IL-18 in response to anthrax LT is ICE-dependent, as an ICE-specific inhibitor blocks this process. These data indicate that ICE, IL-1β, and IL-18 are downstream effectors of anthrax LT in macrophages, providing the basis for new bioassays for anthrax LT activity and representing potential therapeutic targets.

Patients with anthrax infection recognized at late stages have high mortality even with appropriate antibiotic therapy (8), which is likely due to the effects of bacterial toxins that persist following death of the pathogen. One of these toxins, anthrax LT,1 comprises anthrax protective antigen (PA) and anthrax lethal factor (LF). Anthrax PA binds target cells and allows entry of the enzymatically active anthrax LF (9). LF, in turn, inactivates mitogen-activated protein kinase kinases (MAPKKs) through cleavage at specific recognition sites (1–5). MAPKKs are critical intermediates in signal transduction cascades that ultimately lead to activation of the NF-κB family of transcription factors that promote macrophage survival (4). Although some of the elements underlying the mechanism of action of anthrax LT-induced apoptosis have now been elucidated, the etiology of species- and cell-specific differences in sensitivity to anthrax LT remains unclear. In addition, the role of other downstream effectors, such as cytokines, is disputed. In this regard, seemingly contradictory reports have been published that either support or reject roles for proinflammatory cytokines in responses to anthrax LT (6–7).

We now report that anthrax LT treatment induces rapid activation of ICE, a caspase family enzyme responsible for the processing of IL-1β and IL-18 into active forms. Anthrax LT treatment results in the extracellular release of IL-1β and IL-18 by murine macrophage cell lines in a manner dependent upon the activation of ICE. Studies of the action of anthrax lethal toxin on the regulation of IL-1β reveal that anthrax lethal toxin likely enhances both the cleavage of cytokine proforms and the release of intracellular stores of preprocessed cytokine. These data provide the basis for developing new anthrax LT bioassays and reveal new potential therapeutic targets for treating anthrax infection.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—RAW264.7 and J774A1 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% Pen-Strep (BIO-Source International, Camarillo, CA) on low attachment 24-well plates (Corning, Inc.) at a concentration of 1–2 million cells/ml. Cell viability was assessed by trypan blue staining followed by enumeration using a hemocytometer. Only cultures with >99% viability were used for experiments. As indicated, certain cell cultures also received varying doses of anthrax LT and/or Z-WEHD-FMK (ICE inhibitor).

Reagents—Recombinant anthrax PA and lethal factor LF were purchased from List Biological Laboratories, Inc. (Culver City, CA) and were stored as 1 mg/ml stock solutions in 1:1 glycerol:water. The endotoxin levels present in the PA and LF preparations from a representative lot using the company’s manufacturing process were reported to be 11.9 EU/mg and 12.4 EU/mg, respectively (List Biological Laboratories, Inc., lot testing data). Caspase-1 inhibitor, Z-WEHD-FMK (R & D Systems, Minneapolis, MN) was reconstituted in MeOH/SO to make a stock solution of 20 mM. Caspase-1 inhibitor was applied 30 min prior to LT application.

ELISA Assays—IL-1α, IL-1β, IL-6, IL-18, and TNF-α cytokine levels were determined by ELISA using commercial ELISA kits (R & D Systems). Cell-free supernatants were harvested after cell centrifugation and assayed neat or diluted according to the manufacturer’s protocol. Absorbance readings were performed using a microplate reader (Dynatech Laboratories, Chantilly, VA). Each assay was measured in duplicate and averaged. ELISA results were expressed in pg/ml, with exception of the cytokine screening results (Fig. 1, A and B), which were expressed as a ratio of cytokine levels in treated versus untreated cells.

Western Blotting—Cell pellets were lysed on ice for 45 min in a buffer containing 20 mM Tris-Cl, 0.5 mM NaCl, 5 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1% Triton-X-100, and a protease inhibitor mixture (Sigma). Protein extracts were generated from centrifuged lysates, and 50 μg was loaded on a 4–12% NuPage gradient gel (Invitrogen). These protein extracts were electrophoretically separated and then transferred to 0.2-μm nitrocellulose membranes (Bio-Rad). Western blotting was performed using standard techniques as de-
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RESULTS

Anthrax Lethal Toxin Specifically Induces Production of IL-1β and IL-18—We investigated cytokine production in two murine macrophage cell lines known to be sensitive to anthrax LT, RAW 264.7 (Fig. 1A) and J774A.1 (Fig. 1B). Cultures were treated with anthrax LT at a fixed concentration of 1 μg/ml LF and 2.5 μg/ml PA. This dose was toxic for both cell lines within 24 h (not shown). Supernatants were collected following 24 h of treatment and analyzed for production of a panel of proinflammatory cytokines produced by activated macrophages. Anthrax LT did not increase the extracellular levels of TNF-α, IL-1α, and IL-6 produced by either RAW264.7 or J774A.1 cells. However, levels of IL-1β and IL-18 were increased by anthrax LT treatment in both cell lines. In the experiment shown in Fig. 1A, extracellular levels of IL-1β and IL-18 in anthrax LT-treated RAW264.7 cells were 7-fold and 19-fold greater than base-line levels, respectively. Extracellular levels of IL-1β and IL-18 were also increased in J774A.1 cells in response to anthrax LT treatment (Fig. 1B). However, compared with RAW264.7 cells, the relative cytokine inductions were more variable and the absolute levels of induced IL-1β and IL-18 were somewhat lower in J774A.1 cells (Fig. 1, A–D). As a control, we confirmed that the induction of IL-1β and IL-18 required the presence of the complete anthrax LT, as neither LT nor PA alone induced extracellular release of IL-1β and IL-18 in RAW264.7 cells (Fig. 1C) or J774A.1 cells (Fig. 1D). Plateau levels of induced IL-1β and IL-18 were observed at anthrax LF doses starting at 0.1 μg/ml for both RAW264.7 cells (Fig. 2, A and B) and J774A.1 cells (not shown).

As both components of the anthrax LT used in our experiments were recombinant proteins generated in bacterial cell lines, it was critical to rule out that this effect was due to endotoxin contamination. This possibility was unlikely, since PA alone or LF alone did not induce IL-1β or IL-18. Moreover, even at the highest dose of anthrax LT used in our experiments (PA, 2.5 μg/ml and LF, 10 μg/ml), the levels of residual lipopolysaccharide (LPS) would be predicted to be ~0.15 EU/ml (Fig. 2, A and B). By comparison, LPS doses of ≥100 EU/ml were required for induction of IL-1β comparable with that resulting from anthrax LT treatment (Fig. 2A). IL-18 was not induced in RAW264.7 cells even at this dose (Fig. 2B). These findings indicated that the inductions of IL-1β and IL-18 by anthrax LT were not due to contaminating endotoxin in the toxin preparations but were instead an effect of anthrax LT.

Anthrax Lethal Toxin Enhances Processing of IL-1β into Its Active Form—We next investigated the effect of anthrax LT on IL-1β regulation. The production of extracellular IL-1β is regulated at several levels, including at the level of post-translational cleavage of its proform and at the level of extracellular release (11). As shown in Fig. 3, IL-1β was present in both its proform (33 kDa) and active form (17 kDa) at baseline in both RAW264.7 (Fig. 3A) and J774.A1 (Fig. 3B) cells. The presence of intracellular activated IL-1β in our culture conditions was not unexpected, as monocytes adhered up to 20 h had been described previously to contain activated IL-1β (12). Following treatment with anthrax LT, however, there was an increase in the ratio of the levels of processed IL-1β compared with unprocessed IL-1β in both RAW264.7 cells (1 h post-treatment, Fig. 3A) and J774.A1 cells (2–4 h post-treatment, Fig. 3B). These data indicated that while some of the extracellular IL-1β likely resulted from the release of preformed stores of the processed cytokine, anthrax LT also enhanced production of the active cytokine by increasing the processing of the IL-1β proform.

Anthrax Lethal Toxin Leads to Proteolytic Activation of ICE in Murine Macrophage Cell Lines—The proforms of IL-1β and IL-18 are both processed by ICE, an enzyme from the caspase family that cleaves the proforms of both of these molecules. The resulting cleavage products of IL-1β and IL-18 are then available for secretion into the extracellular environment in their bioactive form (13). As this upstream processing pathway is a common feature shared between IL-1β and IL-18, but not the
cytokine induction was dose-dependent. At a dose of 100 μg/ml Anthrax LT treatment alone led to induction of IL-1β and IL-18 in RAW264.7 cells treated with anthrax LF as shown. Anthrax PA was administered at a constant, non-limiting dose of 2.5 μg/ml. Extracellular levels of IL-1β (A) and IL-18 (B) were measured by ELISA in duplicate 24 h following treatment. As a control, selected cultures received varying doses of LPS as indicated. Each bar represents the average concentrations from duplicate ELISA assays. Shown is one representative experiment of four separate experiments. Bars indicate intra assay standard deviation.

FIG. 3. Processing of IL-1β in anthrax LT-stimulated RAW264.7 and J774A.1 cells. Cultures of RAW264.7 (A) and J774A.1 cells (B) were treated with a fixed dose of anthrax LT (1 μg/ml LF and 2.5 μg/ml PA) for increasing periods of times as indicated. Protein lysates generated from these cultures were analyzed by Western blotting for the presence of the cleaved ICE. Upon activation, the proform of ICE (p45) is ultimately cleaved into the two bioactive forms p20 and p10. As shown in Fig. 3A, the active p20 ICE product was detected at low levels at baseline in RAW264.7 cells, but its levels increased very rapidly following treatment with anthrax LT (15 min). The induction of bioactive p20 ICE could be detected in J774A.1 cells within 2 h of anthrax LT treatment (Fig. 3B). It is important to note, however, that we could not exclude the possibility that the apparent delay in the kinetics of ICE induction in J774A.1 cells compared with that in RAW264.7 cells was a consequence of the detection limit of the Western blotting assay and the higher basal levels of activated ICE in RAW264.7 cells compared with J774A.1 cells. In both RAW264.7 and J774A.1 cells, ICE activation was observed immediately prior to the onset of cell toxicity, which occurred 2–4 h following treatment (not shown).

ICE-specific Inhibitor Blocks Production of IL-1β and IL-18 in Anthrax LT-treated Murine and Human Macrophage Cell Lines—Subsequently, we confirmed that ICE was required for production of IL-1β and IL-18 in anthrax LT-treated macrophages using a specific inhibitor of activated ICE, W-WEHD-FMK. W-WEHD-FMK had no effect on cell viability. As shown in Fig. 5, anthrax LT treatment alone led to induction of IL-1β and IL-18 in RAW264.7 cells following 24 h of treatment. Administration of Z-WEHD-FMK prior to anthrax LT treatment blocked induction of IL-1β and IL-18. The inhibition of cytokine induction was dose-dependent. At a dose of 100 μM, W-WEHD-FMK blocked nearly 70% of the production of IL-1β (Fig. 5A) and IL-18 (Fig. 5B) induced by 1 μg/ml anthrax LF, a dose that is 10-fold greater than the minimum anthrax LT dose required to induce plateau levels of IL-1β and IL-18 production.

DISCUSSION

Anthrax LT is known to be a critical factor in the pathogenicity of Bacillus anthracis (9). Although the proximal targets of its action are known, the MAPKKs (1–5), the role of downstream effectors has been controversial (6–7). Our finding that anthrax LT activates ICE provides a possible explanation for the variability present in published reports. ICE plays roles in two potentially competing pathways, participating in both proapoptotic and proinflammatory pathways. Thus, species-specific, cell-specific, and activation state-specific variability to anthrax LT might be predicted based on the relative strength of these signaling pathways. It is important to note that ICE−/− mice have defects in the production of IL-1β and IL-18 but only subtle defects in apoptotic pathways (16–18). In many settings, the production of ICE-dependent proinflammatory cytokines could represent a more suitable biomarker for anthrax LT activity than its action to cause cell death.

The role of the activation of ICE and ICE-dependent cytokines in the pathogenesis of anthrax infection is poorly understood. Recently, however, it has been reported that anthrax LT rapidly increases serum levels of IL-1β in mice within 2 h of anthrax LT treatment, indicating that this anthrax toxin directly modulates IL-1β levels in vivo (19). Whether anthrax LT treatment in animal models augments serum levels of IL-18 in vivo has not yet been reported. As the ICE-dependent cytokines, especially IL-1β, are key host modulators of septic shock, the regulation of ICE activation by anthrax LT is a potential target for therapeutic intervention. These predictions will require confirmation in vivo, through examination of host responses to anthrax LT and to live anthrax infection in vivo. 

other cytokines tested in our panel, we hypothesized that anthrax LT treatment leads to activation of ICE. This hypothesis was supported by the finding of increased processing of IL-1β in response to anthrax LT (Fig. 3A and B).

To examine this possibility directly, lysates from anthrax LT-treated RAW264.7 and J774A.1 cells were collected and analyzed by Western blotting for the presence of the cleaved ICE. Upon activation, the proform of ICE (p45) is ultimately cleaved into the two bioactive forms p20 and p10. As shown in Fig. 4A, the active p20 ICE product was detected at low levels at baseline in RAW264.7 cells, but its levels increased very rapidly following treatment with anthrax LT (15 min). The induction of bioactive p20 ICE could be detected in J774A.1 cells within 2 h of anthrax LT treatment (Fig. 4B). It is important to note, however, that we could not exclude the possibility that the apparent delay in the kinetics of ICE induction in J774A.1 cells compared with that in RAW264.7 cells was a consequence of the detection limit of the Western blotting assay and the higher basal levels of activated ICE in RAW264.7 cells compared with J774A.1 cells. In both RAW264.7 and J774A.1 cells, ICE activation was observed immediately prior to the onset of cell toxicity, which occurred 2–4 h following treatment (not shown).

ICE-specific Inhibitor Blocks Production of IL-1β and IL-18 in Anthrax LT-treated Murine and Human Macrophage Cell Lines—Subsequently, we confirmed that ICE was required for production of IL-1β and IL-18 in anthrax LT-treated macrophages using a specific inhibitor of activated ICE, Z-WEHD-FMK. Z-WEHD-FMK had no effect on cell viability. As shown in Fig. 5, anthrax LT treatment alone led to induction of IL-1β and IL-18 in RAW264.7 cells following 24 h of treatment. Administration of Z-WEHD-FMK prior to anthrax LT treatment blocked induction of IL-1β and IL-18. The inhibition of cytokine induction was dose-dependent. At a dose of 100 μM, W-WEHD-FMK blocked nearly 70% of the production of IL-1β (Fig. 5A) and IL-18 (Fig. 5B) induced by 1 μg/ml anthrax LF, a dose that is 10-fold greater than the minimum anthrax LT dose required to induce plateau levels of IL-1β and IL-18 production.

FIG. 4. Rapid activation of ICE in response to anthrax LT. Cultures of RAW264.7 (A) and J774A.1 cells (B) were treated with a fixed dose of anthrax LT (1 μg/ml LF and 2.5 μg/ml PA) for increasing periods of time as indicated. Protein lysates generated from these cultures were analyzed by Western blotting for the presence of the activated cleavage products of ICE, p20 (upper panels). Membranes were then stripped and re-blotted for β-actin to demonstrate equal protein loading (lower panels). Shown is one representative experiment of three separate experiments.
ICE−/− compared with wild type mice.

The finding that anthrax LT induces secretion of ICE-dependent cytokines has immediate implications for the development of bioassays for anthrax LT activity. Anthrax LT bioassays that are commonly used assess the action of anthrax LT to induce cell death in the murine cell lines, an action that might be different from its physiological action in vivo. Indeed, macrophages from some susceptible mouse strains are resistant to anthrax LT-induced toxicity (20). For this reason, other biomarkers should be considered, including both proximal (e.g. cleavage of MAPKK substrates (21, 22)) and new distal biomarkers for anthrax LT activity. Our results clearly indicate that ICE and ICE-dependent cytokines represent previously unrecognized distal biomarkers for anthrax LT activity that can be exploited for the development of bioassays. A role for caspase-1 inhibitors in the treatment of anthrax infection has been proposed by others as well (23).

The activation of ICE by anthrax LT has important implications beyond its utility as a basis for bioassays, introducing new scientific questions. For example, it will be important to determine whether the activation of ICE-dependent cytokine pathways by anthrax LT might have important functions in cells other than murine macrophages, including human cells. The role of IL-18 in the pathogenesis of late-stage anthrax infection warrants further study as well, as it is a recognized mediator of systemic inflammatory responses (13). Most importantly, it will be important to determine whether anthrax LT increases release of IL-1β and IL-18 at the level of ICE activation and/or cytokine release in vivo. If so, this would suggest the intriguing possibility that therapeutic modulation of ICE activity and ICE-dependent pathways could attenuate deleterious host responses to anthrax LT during infection.

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FIG. 5. Dose-dependent inhibition of IL-1β and IL-18 induction by an ICE inhibitor. Cultures of RAW264.7 cells were pretreated with or without increasing concentrations of Z-WEHD-FMK. Following 30 min of pretreatment, cultures were treated with or without a fixed concentration of anthrax LT as indicated. Values represent the average extracellular cytokine concentrations of IL-1β (A) and IL-18 (B) measured by ELISA in duplicate. Shown is one representative experiment of two separate experiments. Bars indicate intraassay standard deviation.
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