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Neutrophil Elastase Mediates Pathogenic Effects of Anthrax Lethal Toxin in the Murine Intestinal Tract

Hui Fang,* Chen Sun,* Lixin Xu,* Robert J. Owen,* Roger D. Auth,* Philip J. Snyo, † and David M. Frucht* 

Neutrophils isolated from BALB/c or C57BL/6 mice and treated in vitro with anthrax lethal toxin release bioactive neutrophil elastase, a proinflammatory mediator of tissue destruction. Similarly, neutrophils isolated from mice treated with anthrax lethal toxin in vivo and cultured ex vivo release greater amounts of elastase than neutrophils from vehicle-treated controls. Direct measurements from murine intestinal tissue samples demonstrate an anthrax lethal toxin-dependent increase in neutrophil elastase activity in vivo as well. These findings correlate with marked lethal toxin-induced intestinal ulceration and bleeding in neutrophil elastase−/− animals, but not in neutrophil elastase+/− animals. Moreover, neutrophil elastase−/− mice have a significant survival advantage over neutrophil elastase+/+ animals following exposure to anthrax lethal toxin, thereby establishing a key role for neutrophil elastase in mediating the deleterious effects of anthrax lethal toxin. The Journal of Immunology, 2010, 185: 5463–5467.

Abbreviations used in this paper: EF, edema factor; LT, lethal toxin; NE, neutrophil elastase; PA, protective Ag.

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Materials and Methods

Animals

BALB/c, C57BL/6, and NE−/− mice (B6.129 × 1-Elane<sup>tm1Sds/J</sup> [backcrossed on C57BL/6 background for at least 10 generations]) (20) (The Jackson Laboratory, Bar Harbor, ME) were studied under a protocol approved by a Food and Drug Administration Animal Care and Use Committee.

Cell isolation and toxin experiments

Bone marrow-derived neutrophils were prepared and cultured, as previously described (15). In vitro assays involved the treatment of neutrophils in the presence or absence of LT (2.5 μg/ml PA and 1 μg/ml LF). For ex vivo experiments, lyophilized LT components were resuspended in water, diluted in PBS to a total volume of 1 ml, and injected i.p. into mice. Mice were sacrificed 24 h later, and neutrophils were cultured ex vivo. Experiments examining the role of NE in modulating short-term effects of LT on the intestinal tract involved the comparison of NE−/− mice versus age- and sex-matched background control NE+/+ mice treated for 24 h with i.p. LT. Fixed, paraffin-embedded tissues were stained with H&E (Histoserv, Germantown, MD) and evaluated in a blinded fashion by a board-certified veterinary pathologist. Whole slide images were acquired using an Aperio ScanScope at ×20 magnification (ScanScope CS, Aperio, CA). Relevant areas were converted into jpeg format at low magnification (×4) and high magnification (×10) for the generation of figures. For experiments examining the role of elastase in mediating gross pathological effects of LT, gastrointestinal (GI) tracts were dissected from study animals and photographed with a Nikon D40 camera. Stool hemoglobin assays involved the resuspension of 0.002 g stool in 1 ml PBS. This suspension was vortexed vigorously and centrifuged at 10,000 × g for 10 min to remove nonsoluble debris. The resulting supernatants were assayed for hemoglobin concentration using the mouse hemoglobin ELISA kit (Immunology Consultants Laboratory, Newberg, OR). Toxin lethality experiments used 9- to 12-wk-old female animals treated with i.p. LT. The JMP software package was used for survival analysis, using the log rank test.

In vitro, ex vivo, and in vivo elastase activity assays

In vitro and ex vivo elastase activities were assessed using a real-time fluorimetric assay (18, 21) adapted to measure elastase activity in cell-free tissue culture supernatants. During in vitro experiments, cells were first cultured with or without LT for 24 h, whereas during ex vivo experiments, neutrophils were obtained from C57BL/6 mice treated with LT or PBS for 24 h. For each experimental condition, 2 × 10<sup>6</sup> washed cells were resuspended in 250 μl reaction buffer (5 mM KCl, 147 mM NaCl, 1.9 mM KH<sub>2</sub>PO<sub>4</sub>, 1.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.5 mM glucose, 1.5 mM CaCl<sub>2</sub>, 0.3 mM MgSO<sub>4</sub>, and 1 mM MgCl<sub>2</sub>) and incubated at 37°C for 4–5 h. Cell-free supernatants were analyzed for elastase activity after addition of 50 μl elastase substrate (20 μM N-methylsuccinyl-Ala-Ala-
Pro-Val-7-amido-4-methylcoumarin, Sigma-Aldrich, St. Louis, MO). Sample fluorescence was assessed at 5-min intervals for 100 min using a Victor 1420 Multilabel Counter (PerkinElmer, Waltham, MA; 355 nm excitation/460 nm emission; 7,220–10,000 lamp units). In vivo elastase activity was measured directly from isolated tissues, which were minced in PBS, filtered through a 70 μM nylon cell strainer, and microcentrifuged to remove nonsoluble debris. Protein levels in the supernatants were assessed, sample volumes were normalized, and elastase activity was measured as above, with the exception that the Victor counter lamp energy was adjusted to 1,920 U for in vivo experiments.

Results

Anthrax LT-treated neutrophils release NE in vitro and ex vivo

To account for potentially confounding strain-specific effects (22), neutrophils were studied from both C57BL/6 and BALB/c strains of mice. Neutrophils were treated in vitro with or without LT for 24 h and then assessed for elastase release. Increased elastase activity was detected in LT-treated C57BL/6 and BALB/c neutrophil cultures, compared with control cultures (Fig. 1A, 1B, respectively). The specificity of this assay for NE was confirmed by the absence of detectable elastase activity in the supernatants of LT-treated neutrophils derived from NE−/− mice (data not shown). Next, C57BL/6 and BALB/c mice were treated with or without lethal doses of LT. The mice were sacrificed following 24 h of treatment (prior to development of severe signs of intoxication). Cell culture supernatants from neutrophils were then assessed for elastase activity ex vivo. Enhanced elastase activity was observed in neutrophils cultures from LT-treated C57BL/6 and BALB/c mice (Fig. 1C, 1D, respectively).

NE promotes intestinal ulceration following LT administration

Administration of LT results in the appearance of blood-stained feces 24 h following administration to mice (6). To address the role of NE following LT administration directly, we compared the pathogenic effects of LT administration on NE−/− versus NE+/+ mice. First, intestinal elastase activity was determined in NE+/+ (C57BL/6 background control) versus NE−/− mice following LT treatment. Analysis of small intestine samples revealed a >5-fold increase in elastase activity in LT-treated NE+/+ animals compared with PBS-treated animals, whereas a slightly greater than 2-fold increase in elastase activity was noted in NE−/− mice treated with LT (Fig. 2A). These data were consistent with the conclusion that the increase in elastase activity observed in wild-type C57BL/6 mice was predominantly due to NE, whereas other endogenous enzymes with elastase activity that are expressed in the GI tract (e.g., pancreatic elastase) (23) played a smaller role. In addition, LT administration also significantly increased intestinal elastase activity in BALB/c mice in vivo (data not shown), indicating that this observation was not strain dependent.

As shown in Fig. 2B, LT caused accumulation of hemoglobin in fecal samples from the small intestine (left panel) and colon (right panel) in NE+/+ animals, whereas evidence of intestinal bleeding was not observed in LT-treated NE−/− mice. Intestinal bleeding observed in NE+/+ directly correlated with histological findings, which revealed multifocal ulcerations in the intestinal mucosa of all LT-treated NE+/+ animals (Fig. 2C, middle panel). These ulcers were large, but the vast majority of the intestinal mucosa was normal. In contrast, LT-treated NE−/− mice showed relatively normal intestinal histology following LT exposure, similar to PBS-treated controls (Fig. 2C, left panel). Only one of three of the NE+/+ animals treated with LT for 24 h showed evidence of ulceration (Fig. 2C, right panel). This particular animal had a relatively small, isolated ulcer. We used a lower dose of LT to compare gross GI pathology between NE+/+ and NE−/− mice, in support of this observation was not strain dependent.

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FIGURE 1. Anthrax LT induces release of bioactive NE in vitro and ex vivo. Neutrophils from three C57BL/6 (A) or three BALB/c (B) mice were pooled and treated in the presence (●) or absence (○) of LT for 24 h. Cells were then assayed for the release of bioactive elastase in triplicate, as described. Relative elastase levels (change in fluorescence intensity/min) calculated from the average of triplicate neutrophil cultures are shown. Error shown represents SD; p values for LT versus none are <0.05 and <0.01 for A and B, respectively (two-tailed Student t test). Similar results were observed in a total of three independent experiments. C57BL/6 (C) or BALB/c (D) mice were treated with (●) or without (○) LT (C57BL/6, 250 μg PA and 100 μg LF; BALB/c, 100 μg PA and 40 μg LF) for 24 h. Neutrophils from these mice were then assayed for elastase release. Relative elastase levels calculated from three cultures derived from LT-treated mice and three cultures derived from mice treated with vehicle alone are shown. Error shown represents SD. C and D, p <0.04 and <0.01 LT versus PBS, respectively (two-tailed Student t test). Each BALB/c neutrophil culture was derived from a single animal (three animals/cohort), whereas C57BL/6 cultures were derived from pairs of animals to achieve adequate numbers of cells (six animals/cohort). Similar results were observed in a total of three independent experiments for each strain of mice tested (BALB/c and C57BL/6).
an effort to extend survival following LT administration. As is shown in Fig. 2D, NE<sup>−/−</sup> and NE<sup>+/+</sup> mice were treated with LT or PBS for a 3-d period. During this time period, two of four NE<sup>+/+</sup> animals died with marked evidence of intestinal bleeding (animals 2 [40 h] and 3 [56 h]), confirmed by stool hemoglobin assay (data not shown). In contrast, one of four NE<sup>−/−</sup> animals died (animal 7 [64 h]), and none had signs of more than mild/scant GI bleeding. The acute, lethal GI bleeding observed in a fraction of wild-type mice has been a sporadic event observed in numerous experiments, but has not been observed in LT-treated NE<sup>−/−</sup> mice.

**NE mediates lethal effects of anthrax LT**

Having demonstrated that NE is one of the factors responsible for mediating intestinal ulceration observed in LT-treated mice, we subsequently investigated whether NE affects mortality following administration of LT. To address this question directly, we compared survival of LT-treated NE<sup>−/−</sup> mice with that of NE<sup>+/+</sup> mice. In the first survival experiment, we employed ∼90% lethal dose of toxin (PA, 200 μg; LF, 80 μg). As shown in Fig. 3A, this dose led to the death of 10 of 20 of the NE<sup>+/+</sup> within 65 h, and 18 of 20 within 80 h of exposure. In contrast, deaths in the NE<sup>−/−</sup> animals...
NE-/- and monitored over a 7-d period (2 mice (6 of 10 NE-/- mice) appeared healthy 4 d following LT exposure, whereas none of the NE-/- mice died). As shown in Fig. 3, the survival advantage for NE-/- mice compared with NE+/+ mice (p < 0.03, log rank test). A, Twenty NE-/- and 20 NE+/+ mice were treated with anthrax LT (200 μg PA, 80 μg LF) and monitored over a 7-d period (p < 0.03, NE-/- versus NE+/+ mice, log rank test). B, Ten NE-/- and 10 NE+/+ mice were treated with anthrax LT (100 μg PA, 40 μg LF) and monitored over a 14-d period (p < 0.05, NE-/- versus NE+/+ mice, log rank test).

FIGURE 3. NE mediates the lethal effects of anthrax LT. A, Twenty NE-/- and 20 NE+/+ mice were treated with anthrax LT (200 μg PA, 80 μg LF) and monitored over a 7-d period (p < 0.03, NE-/- versus NE+/+ mice, log rank test). B, Ten NE-/- and 10 NE+/+ mice were treated with anthrax LT (100 μg PA, 40 μg LF) and monitored over a 14-d period (p < 0.05, NE-/- versus NE+/+ mice, log rank test).

Discussion

These studies demonstrate that anthrax LT treatment increases NE release in vitro, ex vivo, and in vivo. NE, in turn, is a critical host factor underlying toxin-induced intestinal ulceration, bleeding, and mortality. The recent observation that elevated circulating levels of NE are present in mice infected with toxigenic anthrax (17) supports the relevance of our findings during in vivo infection. Our data support a model in which LT-dependent NE release causes breakdown of the GI barrier. These findings could not only explain a mechanism underlying pathogenicity during GI infection in natural animal hosts (i.e., allowing systemic spread of B. anthracis from the intestinal lumen), but they support a novel mechanism underlying lethality in a murine model of intoxication. Understanding the pathogenic mechanisms of action of anthrax LT is critical, as high mortality rates are observed even in patients receiving an appropriate and sterilizing antibiotic treatment regimen. These high mortality rates are thought to be due to circulating anthrax toxin that persists in the absence of live infection (2).

Nevertheless, our findings highlight important questions that remain. For example, the mechanism(s) underlying NE-dependent GI bleeding in LT-treated mice warrants further investigation. Although a role for NE in mediating coagulopathy during anthrax infection has been proposed (17), other mechanisms involving direct effects on the integrity of intestinal and vascular barrier structures most likely play a role as well. LT’s well-described antiproliferative and immunosuppressive effects, along with the destructive action of NE, most likely combine to promote intestinal ulceration and bleeding. Increased fecal hemoglobin concentrations were noted in nearly all of the intestinal samples from wild-type mice treated with anthrax LT. Moreover, two LT-treated NE+/+ mice developed gross GI hemorrhage events that were most likely fatal (Fig. 2, D, animals 4 and 5). It remains to be determined whether the observed variability in bleeding levels represents the random nature of the location of the LT-induced intestinal ulcers (e.g., proximity to arteries) or whether other contributing factors are involved (e.g., differences in gut flora). It can be concluded, however, that NE-dependent intestinal bleeding is a leading or contributing factor to mortality in LT-treated mice. In addition, to address the relative role of NE in modulating host responses to anthrax LT, it is important to consider these results in the context of previous studies reporting a role for neutrophils and/or bleeding in the pathology caused by anthrax LT. For example, in an earlier study, we reported that anthrax LT enhances the release of superoxide by neutrophils (15). As elastase has been shown to enhance epithelial cell damage in an animal model of oxidative damage (24), it is possible that superoxide augments elastase-dependent intestinal pathology following LT administration. It is also likely that NE targets other host tissues in response to LT; vascular tissues surrounded by elastin-containing connective tissue would be likely targets as well. In this regard, microscopic evidence of anthrax LT-induced hemorrhage and/or vasculopathy outside of the intestinal tract has been reported by other investigators (6, 19).

Anthrax LT has been reported to induce neutrophilia in both C57BL/6 and BALB/c mice (6), but the role of neutrophils during intoxication has remained enigmatic. Through multiple experimental approaches, we have shown that anthrax LT exposure results in the release of NE, a major proinflammatory mediator. NE, in turn, is a critical factor underlying the breakdown of the intestinal barrier and the rapid death associated with exposure to LT. These findings highlight a central role for NE in mediating the deleterious effects of anthrax LT, thereby identifying NE as a potential target for therapeutic intervention.

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Disclosures

The authors have no financial conflicts of interest.

References


