Anthrax Lethal Toxin Induces Ketotifen-Sensitive Intradermal Vascular Leakage in Certain Inbred Mice

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*Bacillus anthracis* lethal toxin (LT) is a bipartite toxin composed of protective antigen (PA) and lethal factor (LF). Injection of LT produces clinical signs characteristic of anthrax infection, including pleural edema and vascular collapse in various animal models. We utilized the classic Miles leakage assay to quantify vascular leakage in mice. LT injected intradermally induced leakage as early as 15 to 25 min in some inbred mouse strains, but not in others, whereas PA or LF individually did not induce leakage. A third component of anthrax toxin, edema factor, did not induce leakage alone or with PA. Leakage was quantified in eight mouse strains, and no correlation was found between sensitivity to intradermal leakage and sensitivity to the lethality of systemically administered LT. The leakage could be inhibited by ketotifen, an inhibitor of mast cell degranulation, but not by azelastine, a histamine receptor 1 antagonist, or by ketanserin, a serotonin 5-HT2A receptor antagonist. LT was cytotoxic to MC/9 mast cells (in vitro) by 7 h after toxin treatment but did not induce histamine release from these cells. Mast cell-deficient mice exhibited the leakage event and had no increased resistance to systemic LT. Human umbilical vein endothelial cells were resistant to LT over 12 h, with only 20% of cells succumbing by 24 h, suggesting that endothelial cell killing is not the cause of the rapid LT-mediated leakage event. We describe here a ketotifen-sensitive vascular leakage event induced by LT which is the most rapid in vivo or in vitro LT-mediated effect reported to date.

Anthrax, the disease caused by *Bacillus anthracis*, is a worldwide bioterrorism concern. Anthrax toxin, a major virulence factor of this organism, consists of three polypeptides: protective antigen (PA), lethal factor (LF), and edema factor (EF). PA is required for binding and translocation of EF and LF into target cells (5). The injection of lethal toxin (LT is LF plus PA) into animals is sufficient to induce some symptoms of anthrax infection, including pleural effusions indicative of vascular leakage and lethality (3, 4, 6, 10, 23, 24, 28). Early studies suggested that LT kills animals by inducing nonspecific shock-like manifestations (3, 36), and recent studies with mice and rats have confirmed an LT-mediated cytokine-independent vascular collapse (6, 28). Humans and primates infected by aerosol exposure to spores present pleural effusions as the most common symptom of disease (8, 20, 40). Histopathological analyses of human subjects with inhalational anthrax infections as well as studies of nonhuman primates and other animals show hemorrhaging in various organs resulting from destruction of both large and small vessels (1, 7, 11, 15, 16, 38). However, while observations of pulmonary edema, inflammation, endothelial necrosis, vessel inflammation, increased vascular permeability, and hemorrhage have been associated with bacterial infection, they are not seen in systemic models of mouse and rat LT toxicity (6, 28). Clearly, LT is a single virulence factor in the arsenal of *B. anthracis* and contributes to some but not all the pathology observed with spore infection. However, a reductionist approach that dissects the LT-mediated effects leading to vascular collapse has great value for understanding the pathogenesis induced by this bacterium.

Recently, LT-mediated endothelial cell killing has been proposed to contribute to the vascular pathology observed during the course of anthrax (21). Since this LT-induced endothelial cytotoxicity occurs gradually (over 72 h) and death from LT-mediated vascular collapse can occur in as little as 45 min (9), we investigated mechanisms by which LT can alter vascular permeability. We used the classic Miles assay (27) to directly investigate and quantify LT as well as edema toxin (ET [PA plus EF])-mediated vascular leakage in the mouse model. We find that in this model, vascular leakage is caused only by LT and can be seen in some inbred strains but not in others. A correlation between susceptibility to a previously established lethal dose of systemic LT and induced leakage was not found. The leakage is inhibited by ketotifen, a histamine H1 receptor antagonist which also can act as an inhibitor of mast cell, basophil, and eosinophil degranulation (14). However, additional experiments show that it is unlikely that mast cells play a role in the observed rapid LT-mediated leakage. We present a novel assay for assessing LT-mediated effects on the vasculature. This study is the first direct demonstration of LT-induced leakage from vessels.

MATERIALS AND METHODS

Materials. Highly pure PA, LF, and mutant LF E687C were purified as previously described (37). Purified endotoxin-free EF was a kind gift of Wei-Jen Tang, University of Chicago, Chicago, IL. Doses of ET or LT refer to the amount of each component (i.e., 100 μg LT is 100 μg PA plus 100 μg of LF). All drugs except for azelastine were purchased from Sigma Aldrich (St. Louis, MO); azelastine was purchased from LKT Laboratories (St. Paul, MN).

Animals. BALB/cJ, DBA/2J, C3H/HeJ, C3H/HeOuJ, WBB6F1/J-Kitw, and colony-matched wild-type homozygous control mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c nude, C57BL/6J nude, and...
C3H hairless (C3.Cg/TifBomTac-ht) mice were purchased from Taconic Farms (Germantown, NY). C3H nude mice were purchased from The National Cancer Institute Animal Production Area (Frederick, MD). Mice were used when they were 8 to 12 weeks old. Except for C3H hairless and nude animals, all mice were shaved 24 h prior to intradermal (i.d.) injections. For assessing susceptibility to systemic LT, mice were injected intraperitoneally (i.p.) with 100 µg LT and observed over 5 days for signs of malaise or death. Extensive studies of LT toxicity in mice with this dose of LT were the basis for the selected dose (28, 29). Fischer 344 rats were purchased from Taconic Farms (Germantown, NY) and used at weights of 150 to 180 g. Rats were injected intravenously (i.v.) in the tail vein with 12 µg LT, which has been shown by our studies to result in death within 60 min (unpublished observations) with or without 250 µg of the mast cell stabilizer drug ketotifen (18, 19), and monitored for the exact time to death.

**Miles assay.** The Miles assay uses i.v. injection of Evans blue dye (which binds to endogenous serum albumin) as a tracer to assay macromolecular leakage from peripheral vessels after i.d. injection of test substances (27, 41). Nude mice and normal shaved mice were injected i.v. with 200 µl of 0.1% Evans blue dye (Sigma Chemical Co., St. Louis, MO). After 10 min, 30 µl of test toxin or control samples (PA only, LF only, EF only, or phosphate-buffered saline) were injected i.d. in both left and right flanks as well as at single or dual dorsal sites. To quantify the extents of leakage, equally sized (1.0 to 1.5-cm diameter) skin regions surrounding i.d. injection sites were always removed 60 min after injection and placed in formamide (1 ml) at 41°C for 48 h, allowing for dye extraction. The A600 of samples was read, and extents of leakage were calculated by comparison with phosphate-buffered saline-, PA-, or LF-treated controls. In experiments testing the effects of drugs on LT-mediated leakage, mice were injected i.v. with Evans blue as described above, and the drug was introduced systemically through i.p. injection 10 min after dye injection. LT was introduced by i.d. injection 30 min after the injection of Evans blue. Alternatively, drug was introduced locally by i.d. injection and LT was injected in the same site after 10 min.

**Cytotoxicity experiments.** MC9 mast cells were obtained from ATCC (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium supplemented with t-glutamine (2 mM), 2-mercaptoethanol (0.05 mM), Rat T-STIM (BD Biosciences-Discovery Labware, Bedford, MA) (10%), and fetal bovine serum (FBS, 10% final concentration; Invitrogen-GIBCO BRL, Gaithersburg, MD). Cells were seeded at a density of 10^6 cell/well in 96-well plates prior to treatment with various LT concentrations or PA-only controls. After 6, 12, and 24 h, viability was assessed byPromega’s CellTiter 96 AQu这么久 One Solution cell proliferation assay (Promega, Madison, WI) per the manufacturer’s protocol. Alternatively, toxicity assays were performed in medium provided with all supplements except FBS (serum-free medium). In other experiments, pooled human umbilical vein endothelial cells (HUV/ECs) at third to fifth passage were obtained from Cambrex Corp. (Cambrex, Walkersville, MD) and grown in an EGM-MV Bulletkit (Cambrex, Walkersville, MD) in flasks pretreated with endothelial cell attachment factor (Sigma, St. Louis, MO). For cytotoxicity experiments, cells were seeded in 96-well plates in an EGM-MV Bulletkit. On the day of assays, this medium was replaced with M199 medium (Sigma, St. Louis, MO) supplemented with 10% FBS or human serum (Sigma, St. Louis, MO), and cells were resuspended in 96-well plates at a density of 2 × 10^3/ml wash and treated with various concentrations of LT in triplicate. Cell viability was assessed as described for MC9 cells, except that 24, 48, and 72 h time points were used.

**Histamine assay.** Histamine release from MC9 cells was measured in 96-well plates following 2, 6, 12, and 24 h of LT exposure at various doses by use of a histamine enzyme-linked immunosorbent assay kit from IBL (Hamburg, Germany) per the manufacturer’s protocol. This kit detects histamine concentrations of as low as 0.3 ng/ml.

**HUV/EC permeability assay.** HUV/EC monolayers were cultured on Transwell-Clear cell culture inserts (6.5-mm diameter, 0.4-µm pore size; Corning-Costar, Acton, MA) in 24-well plates, creating a two-chamber culturing system consisting of a luminal compartment (inside the insert) and a subluminal compartment (the tissue culture plate well). Prior to seeding cells, inserts were coated with endothelial cell attachment factor (Sigma, St. Louis, MO). Prewarmed CS-C medium (Sigma, St. Louis, MO) containing 10% iron-supplemented calf serum and 1% endothelial cell growth factor (Sigma, St. Louis, MO) was added to wells prior to insert placement. A HUV/EC cell suspension (200 µl of 5 × 10^6 cells/ml) was added to each insert. Cells were cultured at 37°C in 5% CO2 for up to 21 days to ensure proper formation of a monolayer. For testing barrier function, medium was changed to RPMI supplemented with 10% FBS or to RPMI without serum. To assess barrier function, horseradish peroxidase enzyme (Sigma, St. Louis, MO) was added to the inserts (10 µg/well). LT (1 µg/ml) or control treatments of PA alone (1 µg/ml) or LF alone (1 µg/ml) were added to duplicate wells, and every hour (for 12 h), a sample of 10 µl was taken from the subluminal compartment and tested for the enzymatic activity of horseradish peroxidase by adding 100 µl substrate [2,2’-azino-bis(3-ethylbenzthiazolin 6-sulfonic acid)] (A-3129; Sigma, St. Louis, MO) and reading at 405 nm.

**RESULTS**

**In vivo measurement of vascular leakage.** The Miles assay was used to test LT- and ET-mediated leakage in mice. Evans blue was injected i.v. into BALB/c nude mice, followed by i.d. injection of ET (EF plus PA), LT (LF plus PA), and PA and LF alone at various doses (1 µg, 10 µg, 25 µg, 50 µg). Only LT (PA plus LF) caused a leakage effect, which was seen as a blue zone surrounding the site of injection (Fig. 1). The extent of leakage correlated with the LT dose injected and was observed as early as 15 to 25 min, reaching a maximum by 60 min (data not shown). We selected a dose of 25 µg for all future experiments.

**Comparison of LT-mediated vascular permeabilities in different mouse strains.** We repeated the Miles assay using 25 µg LT in BALB/c nude, BALB/cj nude, C57BL/6j nude, C3H/HeJ normal, C3H/HeJ nude, C3H/OuJ, C3H hairless, and DBA/2J mice. We observed leakage in the BALB/cj mice as well as in all tested nude strains of mice, in the lipopolysaccharide (LPS) responder C3H/HeJ, and in its nonresponder matched strain, C3H/HeOuJ (Fig. 2). Leakage was seen only with wild-type LF and not when a proteolytically inactive LF was used in combination with PA (Fig. 2, insert). Statistically significant differences in the extents of leakage were not observed between the various strains that showed leakage. Surprisingly, the genetically related (but mutant) C3H hairless...
strain did not show any leakage. DBA/2J mice also did not show leakage. Previous assessment of the susceptibilities of these mouse strains to systemic LT treatment showed that DBA/2J mice are the most resistant strain, while C3H/HeJ and C3H/OuJ mice exhibit relatively high resistance compared with BALB/cJ mice (29). The C3H hairless mouse, however, shows susceptibility to systemic LT similar to that of the BALB/cJ mouse (data not shown). The leakage observed in the C3H/HeJ and C3H/OuJ mice does not correlate with the higher degree of resistance to LT seen in those animals, and the lack of leakage in C3H hairless mice does not correlate with the systemic sensitivity of that strain.

**Ketotifen inhibits LT-mediated intradermal vascular leakage.** Because LT induces a cytokine-independent, shock-like death in mice and rats, we hypothesized that anaphylactic shock-like events due to mast cell degranulation or release of histamine from other cells could be the cause of LT-mediated vascular leakage and shock (26, 39). To test this hypothesis, we utilized the drug ketotifen, which inhibits mast cell, basophil, and eosinophil degranulation and interferes with histamine function (14), protecting against shock (2, 35) and inhibiting LPS-mediated leakage by preventing mast cell degranulation, in a Miles assay (18, 19). Ketotifen or saline (control) was introduced to mice systemically by i.p. injection 30 min prior to LT injections or locally at the site of i.d. injection. We found that both i.p. and i.d. introductions of ketotifen were very effective in reducing LT-mediated leakage of Evans blue at the site of i.d. toxin injection (Fig. 3). This inhibition was not due to direct inhibition of LT activity by the drug, as ketotifen premixed with LT did not result in any effect in macrophage toxicity assays (data not shown). We proceeded to test the ability of ketotifen to prevent LT-mediated toxicity in Fischer F344 rats. This rat strain is very susceptible to LT, with 12 μg LT (PA plus LF) resulting in death in an average of 64 ± 3 min (n = 3). Premixing 12 μg LT with 250 μg ketotifen prior to injection did not fully protect the animals but did extend the time to death significantly (P = 0.014) to an average of 84 ± 8 min (n = 3) (data not shown). Rats pretreated with ketotifen 45 min prior to toxin administration also showed a similarly extended time to death.

**LT is cytotoxic to MC/9 mast cells but does not induce histamine release.** We tested the direct effect of LT on mast cells in vitro by performing toxicity assays using the MC/9 mast cell line and various LT doses over 6- to 24-h periods. LT (at 250 ng/ml) killed 25 to 30% of MC/9 cells over the first 6 h of treatment (Fig. 4A). Approximately 50% killing was observed after 24 h when assays were performed in medium containing 10% serum. We had previously noted that the killing of various cells by LT is amplified in the absence of serum (data not shown); therefore, we also tested LT-mediated toxicity for MC/9 cells using serum-free medium (Fig. 4B). Killing was increased to 80 to 90% when assays were performed in serum-free medium at the 1 g/ml dose, compared to only 60% maximal killing in complete medium. At a dose of 100 ng/ml, the difference was also significant, with only 20 to 25% killing in complete medium, compared to 60% killing in serum-free medium. However, when the medium from LT-treated MC/9 cells was assessed for histamine release (2 to 24 h) due to degranulation, free histamine was not detected in any LT-treated wells at a level beyond that seen for untreated controls (data not shown). Because MC/9 cells spontaneously release histamine, it may be difficult to assess small changes in histamine release induced by LT in this system.
Mast cell-deficient mice exhibit LT-mediated leakage and are susceptible to LT killing. To further test the potential role of mast cells in the observed LT-mediated leakage, we tested the mast cell-deficient Kit mouse (WBB6F1/J-Kit<sup>W</sup>/Kit<sup>W-v</sup>) (12, 22) and colony-matched wild-type control mice for LT-mediated leakage. Both groups of mice showed leakage levels similar to those for BALB/cJ control mice (data not shown), indicating that mast cells alone were not involved in LT-mediated leakage induction. We compared the sensitivity to LT of these mast cell-deficient mutant mice (which harbor LT-resistant macrophages) to that of their wild-type colony-matched controls and found that, if anything, they were more susceptible to LT (Fig. 5), likely due to the anemia in this strain (33), which exacerbates hypoxia-mediated damage during LT shock (28). Thus, it appeared that the absence of mast cells did not result in any increased resistance to LT treatment.

**Effects of ketanserin and azelastine on LT-mediated leakage.** Because ketotifen not only is an H1 receptor antagonist and inhibitor of histamine release from many cells (14) but also has been shown to inhibit vascular leakage by other means, such as acting as a leukotriene inhibitor (17), we tested the similar histamine receptor 1 antagonist, azelastine, which also inhibits leukotrienes (17), as well as ketanserin, a serotonin 5-HT2A receptor antagonist (13, 25), for their abilities to inhibit LT-mediated leakage by use of both systemic (i.p.) and local (i.d.) introduction of the drugs. Ketanserin did not result in a significant decrease in LT-mediated leakage. Azelastine, however, did result in a 40% reduction in leakage levels but was not as effective as ketotifen (data not shown).
We investigated the effects of LT (1 μg/ml) on HUVECs treated with three doses of LT over 72 h in the presence of 10% FBS or human serum (HuSerum) compared to that of cells treated with LT in the absence of serum.

**Effects of LT on HUVEC viability and monolayer permeability.** We investigated the effects of LT (1 μg/ml) on HUVEC cell viability over 72 h and found 20 to 25% mortality by 24 h and 75% mortality by 72 h (Fig. 6A). The removal of serum during the first 24 h of the assay enhanced LT-mediated killing of cells (Fig. 6B). When testing the permeability of LT-treated HUVEC monolayers, we did not find any horseradish peroxidase enzyme traveling across these monolayers through 12 h after toxin treatment (data not shown).

**DISCUSSION**

We utilized the classic Miles assay of vascular leakage to quantitatively assess LT effects on vascular permeability. We report here that LF causes a very rapid increase in local vessel leakage. The doses of toxin that are required to produce leakage are relatively high compared to the systemically administered doses that lead to lethality. PA or LF alone did not induce leakage, and proteolytically active LF was required in combination with PA. Interestingly, although no leakage was seen in the LT-resistant DBA/2J mouse, no correlation between mouse strain sensitivities (29) and leakage levels was found. For example, the C3H/HeJ, C3H/HeOuJ, and C57BL/6J strains are all more resistant to systemically administered LT than BALB/c mice but show higher vascular leakage in the Miles assay system. However, all these mice are somewhat sensitive to LT, i.e., are killed by doses of toxin greater than 100 μg (data not shown), it is possible that no direct correlation between the extent of the rapidly induced leakage in the Miles assay and mortality would be discerned. Additionally, it is possible that it is not only the leakage event that is important to LT-mediated lethality but also the animal’s ability to respond to that leakage event, an ability that must be controlled by numerous genetic factors. For example, the C3H hairless mouse had a leakage pattern totally different from those of the other C3H strains, which had no leakage, and yet is more sensitive than other C3H strains to systemic LT lethality (data not shown). This mouse is mutant in the hairless (hr) gene, which encodes a nuclear receptor corepressor with histone deacetylase binding activity (32). The mutation results in a wide range of immune defects affecting many types of cells, including macrophages and T cells (34). It is fascinating that this mutation is sufficient to completely inhibit LT-mediated leakage in the C3H background. Analysis of the molecular basis by which the hr gene confers protection from LT-mediated leakage through the study of other hairless strains harboring this mutation is an area for future study. Additionally, it will be interesting to see if the C3H hairless and DBA/2J mice can be made sensitive to LT-mediated leakage by steroid consumption or adrenalectomy, treatments that sensitize DBA/2J animals to systemic LT (30).

Because of the rapidity of the vascular leakage event described here, which can be observed as early as 15 min, and the rapid 40-min death of Fischer F344 rats injected with LT (10), we considered whether LT may be inducing an anaphylactic shock-like event mediated by mast cell products, such as histamine (26, 39). We showed that MC/9 mast cells were in fact killed by LT in vitro. While the effect was not rapid, occurring over 6 to 24 h, it still was more rapid than that seen in another candidate cell target we tested, HUVECs. The relatively slow deaths of these two types of cells did not correlate with the very rapid LT-mediated leakage event, which occurred in 15 to 60 min. We therefore considered whether degranulation of mast cells (rather than their killing) could play a role in the observed leakage, much in the manner that injections of histamine as a control substance resulted in a similar leakage event in mouse skin (data not shown). Although the drug ketotifen, an inhibitor of histamine release from mast cells (14), impressively inhibited the LT-mediated vascular leakage event, we could not show LT-mediated induction of histamine from cultured MC/9 cells. Of course, the MC/9 liver mast cell line may not accurately represent mast cells in mouse skin. However, we also found that the mast cell-deficient knockout mouse used in our studies displayed strong LT-mediated leakage and no increased resistance to systemic LT. Therefore, it is possible that the additional effects of ketotifen on basophils and eosinophils (14) may explain its ability to inhibit leakage. Alternatively, ketotifen also functions as a potent histamine H1 receptor antagonist (14) and therefore could manifest its effects by interfering with histamine released from other sources. Additionally, ketotifen may play an inhibitory role against responses to other vascular mediators, such as bradykinin (31) as well as other rapidly released vasomodulators originating from the endothelium. However, it is clear that this very rapid LT-mediated event involves some form of membrane destabilization or transcription-translation-independent mediator, be-
cause the time in which it occurs does not allow for protein synthesis events which would lead to the production of proteins responsible for the leakage. Additionally, because LF alone did not induce leakage and the proteolytic function of LF was required for leakage, it is clear that cell entry mediated by PA is essential. This raises questions as to the cellular target of LF that would lead to this rapid leakage event. Mitogen-activated protein kinase kinases are the only currently known targets of this toxin, and the initiation of their cleavage occurs at about 15 min after the binding of LT to cells. However, it is difficult to imagine how the cleavage of mitogen-activated protein kinase kinases would lead to this vascular leakage. Interestingly, i.d. injection of LPS in rat skin has been shown to induce a similar dye leakage event in a similarly rapid fashion (18, 19). The mechanisms described for this leakage, however, vary from NO-mediated effects to mast cell degranulation and histamine release (18, 19).

It is important to note that the rapidity of LT-mediated leakage in these mice contrasts with the relatively long times before mice succumb to systemically administered LT (28, 29). If leakage were occurring systemically with the rapidity seen in skin, mice might be expected to die in minutes or hours, much like Fischer F344 rats. However, it is possible that the high doses of locally administered LT produce a rapid event that otherwise occurs slowly in systemically injected mice, due to lower receptor occupancy and toxin uptake in the latter situation. Alternatively, the specific cell types needed to increase vascular permeability may be most abundant in dermis. It is also possible that the LT-mediated leakage observed in this model is not mechanically related to the widespread vascular leakage, which takes several days to appear in systemically intoxicated mice. Finally, we recognize that the characterization of LT-mediated events like those described here may only indirectly contribute to understanding the pathology resulting from infection with a virulent B. anthracis. However, it is clear that LT is able to cause a very rapid chain of events resulting in localized vascular leakage. A complete understanding of how B. anthracis infections will progress will be aided by characterization of the intradermal vascular leakage event we describe here through identifying the key cell types and proteins targeted by LT that lead to leakage, explaining the mechanism by which ketotifen inhibits the leakage, and identifying the genetic differences that control susceptibility to leakage.

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