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Lethal toxin of *Bacillus anthracis* causes apoptosis of macrophages

Serguei G. Popov,* Rafael Villasmil, Jessica Bernardi, Edith Grene, Jennifer Cardwell, Aiguo Wu, Darya Alibek, Charles Bailey, and Ken Alibek

Hadron Advanced Biosystems, Inc., 10900 University Boulevard, MSN 1A8, Manassas, VA 20110, USA

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Abstract

Lethal toxin is a major anthrax virulence factor, causing the rapid death of experimental animals. Lethal toxin can enter most cell types, but only certain macrophages and cell lines are susceptible to toxin-mediated cytolysis. We have shown that in murine RAW 264.7 cells, sublytic amounts of lethal toxin trigger intracellular signaling events typical for apoptosis, including changes in membrane permeability, loss of mitochondrial membrane potential, and DNA fragmentation. The cells were protected from the toxin by specific inhibitors of caspase-1, -2, -3, -4, -6, and -8. Phagocytic activity of macrophages was inhibited by sublytic concentrations of lethal toxin. Infection of cells with anthrax (Sterne) spores impaired their bactericidal capacity, which could be reversed by a lethal toxin inhibitor, bestatin. We suggest that apoptosis rather than direct lysis is biologically relevant to lethal toxin intracellular activity.

Keywords: Anthrax; Apoptosis; Bactericidal activity; Lethal toxin; Macrophages

The pathogenesis of anthrax and methods of treating the disease have recently been the topics of great interest due to concerns over the use of *Bacillus anthracis* as a biological weapon and the recent anthrax attacks in the US. The most common view of inhalational anthrax holds that inhaled spores are engulfed by alveolar macrophages, which carry the bacteria to the mediastinal lymph nodes. The spores germinate within the migrating macrophages producing an antiphagocytic capsule and two toxins (lethal toxin or LeTx; and edema toxin or EdTx). Lysis of infected macrophages allows the release and proliferation of the bacteria in the lymphatic system [1]. The bacteria then enter the bloodstream and continue to proliferate. Death is attributed to severe respiratory distress and multi-system organ failure caused by sepsis and septic shock [2–5].

Lethal toxin is essential for *B. anthracis* virulence. The toxin consists of two protein subunits, lethal factor (LF) and protective antigen (PA). The current model suggests that LeTx attacks sensitive cells by the binding of heptamerized PA to a recently identified cell surface receptor [6] and subsequent endocytosis of LF into the cell cytosol [7,8]. Macrophages play a central role in LeTx activity, because mice depleted of macrophages are resistant to lethal doses of toxin [3–5]. It has been demonstrated that the toxin can enter most cell types, but only certain macrophages and macrophage-like cell lines are susceptible to cytolysis by the toxin [7]. Intracellularly, LF targets MAPKKs 1, 2, 3, 4, and 6 [9–12], though this activity does not appear to be related to cell susceptibility [10]. The mechanism of LeTx intracellular activity remains largely unknown. It has recently been shown that, in contrast to early reports, LeTx-mediated destruction of macrophages does not cause overproduction of proinflammatory cytokines and oxidative radicals [11,13]. It has also been suggested that LeTx may actually act to impair the proinflammatory response of host macrophages and thus enhance bacterial virulence [15]. However, no experimental data exist to prove this hypothesis.

We have undertaken studies on the mechanism of proinflammatory inhibition in macrophages exposed to lethal toxin. We conclude that methods to inhibit the activity of LeTx within macrophages could potentially be used as an early treatment of anthrax.

* Corresponding author. Fax: +1-703-993-8447.
E-mail address: serguei.popov@hadron.com (S.G. Popov).

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Experimental procedures

LF and PA, isolated as described by Park and Leppla [14], were provided by Dr. S. Leppla (NIH, USA). *B. anthracis* (Sterne) spores were prepared in LB agar broth. When the ratio of spores to vegetative bacteria reached 99:1, the spores were pelleted and washed five times with distilled water. Flow cytometry experiments were carried out in a FACSCalibur Becton Dickinson Immunocytometry System. Statistical analysis was performed by Student's t test. Error bars correspond to ±95% confidence.

Cells and cell culture. The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC TIB-71; Manassas, VA). Cells were cultured in supplemented DMEM/F12 medium (Gibco, USA) with phenol red (2 mM glutamine, 10% heat-inactivated fetal bovine serum, 100 units/ml streptomycin–penicillin, 0.1 mM non-essential amino acids, and 0.5 mM 2-mercaptoethanol) at 37 °C in a 5% CO_2 atmosphere. Cells were harvested using a Cellstripper solution (Mediatech, USA).

Cell treatment with lethal toxin. Murine cells were treated with lethal factor at concentrations between 4 and 64 ng/ml. PA concentration was kept constant at 500 ng/ml. Toxin was prepared immediately before the experiment.

Apoptosis assay. For staining with YO-PRO-1/propidium iodide (Vybrant Apoptosis Assay Kit #4, Molecular Probes, USA), cells were stripped from plastic and suspended in 900 μl of PBS with 100 μl of stain (prepared according to manufacturer's instructions). Staining was carried out at 4 °C for 30 min. For JC-1 staining, cells were stripped and suspended in 1 ml of a 10 μg/ml JC-1 stain (Molecular Probes, USA) working solution in PBS. Staining was carried out at 4 °C for 10 min. At least 4000 cells were counted for each sample over a constant period of time. Staurosporine (Alexis Biochemicals, USA) was used as an inducer of apoptosis at 5 μM.

TUNEL assay. The In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, USA) was used to measure DNA fragmentation during apoptosis. The technique uses deoxynucleotidyl transferase to incorporate labeled nucleotides to apoptotic DNA strand breaks in situ.

MTT Assay. MTT (Sigma–Aldrich, USA) was used to analyze cell viability of RAW 264.7 cells after treatment with LeTx. MTT was diluted in PBS and then added to cells at 1 mg/ml. After 1 h of incubation the supernatant was removed and MTT reagent (91% (v/v) isopropanol, 4% (v/v) of 1 M HCl, and 5% (v/v) of 10% (w/v) sodium dodecyl sulfate) was added. Spectrophotometric readings were then taken on a μ-Quant spectrophotometer (Bio-Tek Instruments, USA).

Caspase inhibitors. The following inhibitors were used: Z-WEHD-FMK for caspase-1, Z-VDVAD-FMK for caspase-2, Z-DEVD-FMK for caspase-3, Z-YVAD for caspase-4, Z-VEID-FMK for caspase-6, Z-IETD-FMK for caspase-8, Z-LEHD-FMK for caspase-9, Z-AEVD-FMK for caspase-10, and Z-LEED-FMK for caspase-13 (R&D Systems, USA). The inhibitors were diluted in DMSO according to manufacturer's instructions. RAW 264.7 cells (1×10^6 cells/well) were preincubated for 15 h in media with 20 μM inhibitor. Then, LeTx (4 ng/ml of LF, 500 ng/ml of PA) was added to the culture. After 4 h of toxin treatment, cells were stripped and stained YO-PRO-1/propidium iodide stain.

Blocking of Fas-L with antibody. RAW 264.7 (1×10^6 cells/well) received 1 ml of media with anti-FasL neutralizing antibody (R&D Systems, USA) and LeTx (4 ng/ml of LF, 500 ng/ml PA) was added to the culture. After 15 h of toxin treatment cells were stripped and stained with YO-PRO-1/propidium iodide stain as above.

Phagocytic activity of LeTx-treated cells. To measure spore phagocytosis by macrophages, RAW 264.7 were grown in media without phenol red or antibiotics (2×10^5/well), spores were added, and phagocytosis was allowed to occur for 30 min. The supernatant was removed and cells were washed six times with an equal volume of PBS. This washing procedure has been shown to remove more than 85% of uningested spores from control wells. Cells were lysed with 1% aqueous saponin (Sigma, USA) for 5 min. After lysis, Alamar Blue (Biosource, USA) in media without phenol red was added to each well and fluorescence was measured according to manufacturer's instructions. Fluorescence intensity was shown to be linearly proportional to the concentration of spores at a range of 1×10^4–1×10^6 spores/well.

Bactericidal activity of LeTx-treated cells. To measure the bactericidal activity of RAW 264.7 macrophages to LeTx, a procedure similar to the one described above was carried out except that cells were pretreated with bestatin (Sigma–Aldrich, USA) for 1 h, then incubated with spores for 3 h. The plate was spun by low speed centrifugation, supernatant was removed, and Alamar Blue measurements were taken.

Results

Sublytic concentrations of LeTx cause apoptosis-like changes in cellular cytoplasmic and mitochondrial membranes

The mouse macrophage cell line RAW 264.7 is sensitive to LeTx and is widely used in anthrax studies [4]. At concentrations close to 100 ng/ml of LF in the presence of PA (usually 100–500 ng/ml), RAW 264.7 cells undergo rapid lytic death [3]. However, much lower concentrations of the toxin have been reported to inhibit cellular cytokine production [3,13,15]. We therefore studied the means by which sublytic concentrations of LeTx interfere with cell signaling. To detect changes in plasma membrane permeability, monolayers of RAW 264.7 cells were treated with a range of LeTx concentrations and screened by flow cytometry using YO-PRO-1, a green fluorescent dye capable of detecting early apoptosis-specific changes in membrane permeability and composition, and propidium iodide (PI), a late apoptosis/necrosis-specific red fluorescent dye. Incubation of cells with PA alone (500 ng/ml) or in combination with sublytic concentrations of LF causes a shift in the green fluorescence intensity, characteristic of apoptosis (Fig. 1A). At an LF concentration of 4 ng/ml, a shift in green fluorescence indicative of early apoptotic changes was observed. As the concentration of LF was increased to 8 ng/ml, both green and red fluorescence greatly increased without substantial lysis, indicating the onset of late apoptotic events. DNA fragmentation is a typical feature of apoptosis [16]. To confirm the occurrence of DNA fragmentation in cells exposed to LeTx, we used the TUNEL assay. Increased fluorescence of LeTx-treated cells was detected, compared to untreated control, indicative of DNA fragmentation (Fig. 1B).

Mitochondria are known mediators of the 'intrinsic' apoptotic pathways [17]. We therefore studied mitochondrial involvement in LeTx-induced cell death using a specific dye, JC-1, capable of changing color depending on mitochondrial transmembrane potential. RAW 264.7 cells were treated with LeTx and stained with JC-1. The transmembrane potential of intact mitochondria de-

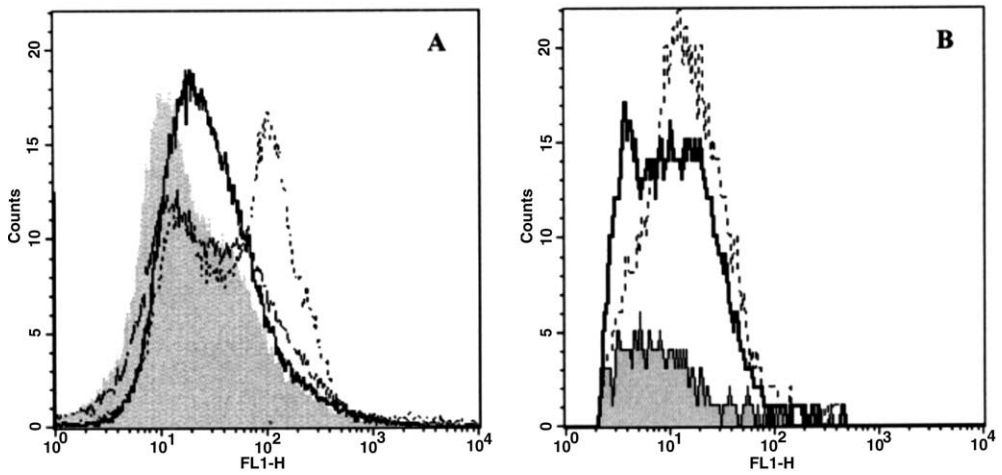

Fig. 1. Staining of LeTx-treated Raw 264.7 cells with YO-PRO-1 (A) and TUNEL assay of nucleosomal degradation of RAW 264.7 cells in the presence of LeTx (B). Cells were treated with different concentrations of toxin for 4 h in culture media. Staining was measured by flow cytometry. In (A) thick solid line, PA (500 ng/ml); thin solid line, PA (500 ng/ml) + LF (4 ng/ml); dashed line, PA (500 ng/ml) + LF (8 ng/ml). Gray area, untreated control cells. In (B) thick solid line, 16 ng/ml LF; 500 ng/ml PA; gray area, 500 ng/ml PA alone; dashed line, staurosporine (5 μM) as positive control. Fluorescence in a green channel was recorded and the population of apoptotic cells was gated using a positive control as reference.

creased upon LeTx treatment, consistent with the onset of apoptosis (Fig. 2). Staurosporine, a drug known to cause mitochondrial toxicitiy [17], caused JC-1 staining changes similar to LeTx.

Inhibition of caspases decreases LeTx pathogenicity

Apoptosis is carried out via the action of a number of initiator and effector caspases [18]. Inhibition of caspases using specific inhibitors of caspase-1, -2, -3, -4, -6, and -8 leads to different degrees of cellular protection against LeTx, with inhibition of caspase-4, -6, and -8 being the most effective (Fig. 3). This finding is consistent with a model in which LeTx-assisted apoptosis is initiated at the level of death receptors and often involves the activation of caspase-8. This latter event is usually followed by the activation of effector caspases, such as caspase-3 and -6 [24]. We were unable to demonstrate activation of caspase-9, in spite of established LeTx-induced mitochondrial damage.

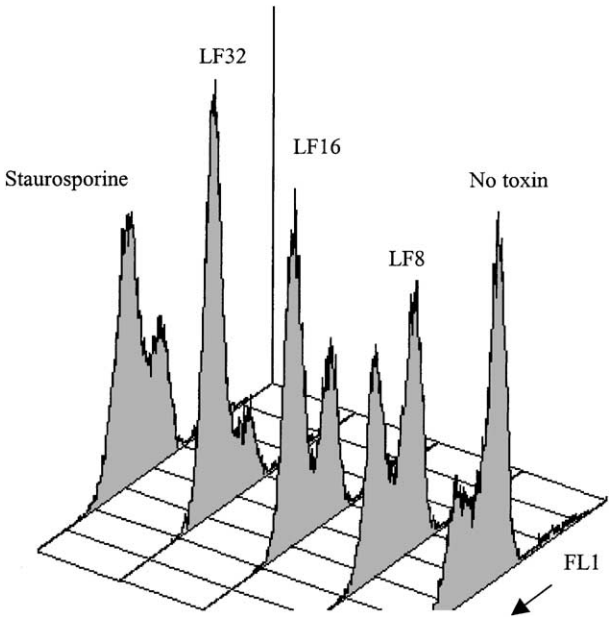

Fig. 2. Flow cytometry analysis of RAW 264.7 cells stained with a mitochondrial transmembrane potential-sensitive dye, JC-1. Histograms of fluorescence in green channel correspond to (from right to left): control cells without toxin, cells treated for 4 h with LeTx (8, 16, and 32 μg/ml LF, 500 ng/ml PA), and cells treated with staurosporine (5 μM; positive control).

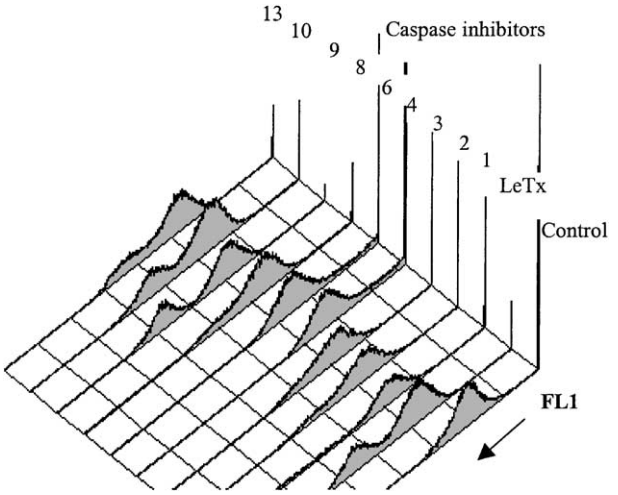

Fig. 3. Effect of caspase inhibitors on staining pattern of RAW 264.7 cells as measured by flow cytometry. Cells were incubated with one of the caspase inhibitors (20 μM) for 15 h, then LeTx (4 ng/ml of LF + 500 ng/ml PA) for 4 h, and stained as described above (Fig. 1A). Numbers above the histograms correspond to caspase numbers. Histograms of LeTx-treated and untreated cells are marked as LeTx and control, respectively.

Fas/Fas ligand interaction is involved in lethal toxin activity

The experiments described above on caspase inhibition in LeTx-treated cells suggested the possibility that cellular death receptors may be involved. To decipher the signaling effects of LeTx, it is important to determine which of the major apoptotic pathways [19], TNF-α, FasL, TRAIL/Apo2L, or Apo3L-induced, is targeted by LeTx. The TNF-α pathway can be ruled out based on published data showing a decrease in LPS-stimulated TNF-α production in the presence of LeTx [11]. TRAIL/APO-2L is a newly identified member of the TNF family that induces apoptosis in cancer cells without affecting most non-neoplastic cells, both in vitro and in vivo [19]. The Fas/FasL system is the best characterized of the known apoptotic pathways [20]. We therefore decided to explore the possibility that Fas mediates LeTx-induced apoptosis. Cell surface staining for Fas using FITC-labeled specific antibodies showed that RAW 264.7 cells express a detectable amount of surface Fas, which was greatly increased after stimulation with murine IFN-γ, TNF-α, and GM-CSF. However, treatment of the cells with LeTx did not induce Fas expression on the cell surface (data not shown).

To show that Fas/FasL interaction is involved in the LeTx-induced cell death, we used specific anti-FasL antibody (TNFSF6, R&D Systems, USA), raised against the extracellular domain of recombinant murine FasL. When added to cells, this antibody neutralizes the activity of recombinant FasL. LeTx cytotoxicity in RAW 264.7 cells was effectively abrogated by treatment with anti-FasL antibody (Fig. 4). In the absence of the antibody, cells were almost completely killed by an overnight incubation with LeTx. Incubation with the antibody greatly reduced the number of dead cells, whereas the number of apoptotic cells increased, indicating a substantial retardation in apoptotic processes. Higher antibody concentrations showed some increase in cell death; however, we did not study this effect further, since it could be explained by FasL crosslinking.

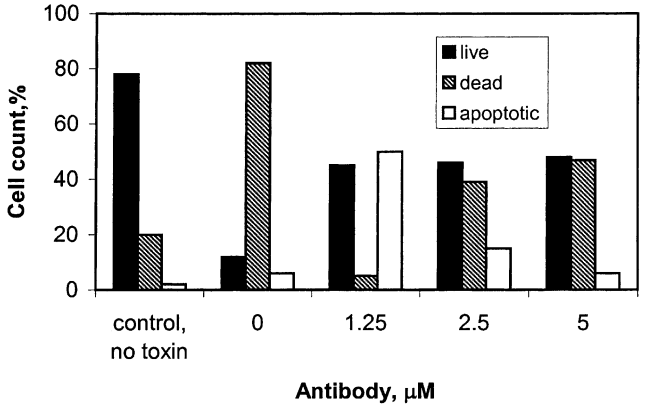

Fig. 4. Anti-FasL neutralizing antibody treatment protects RAW 264.7 cells from killing by LeTx. Cells were incubated with the indicated concentration of antibody and LeTx (4 ng/ml LF, 500 ng/ml PA) for 15 h. Cell numbers were determined by flow cytometry as in Fig. 1A.

Inhibition of LeTx increases bactericidal activity of macrophages

The proapoptotic function of LeTx described above, combined with the observation that the LF, EF, and PA genes are expressed early in the infection process [1], has prompted us to study the possibility of detecting apoptotic changes in spore-infected RAW 264.7 cells. In cells stained with apoptotic dyes at different time points after the addition of spores, gating of FACS scatter plots allows almost complete separation of signals from spores/vegetative bacilli and infected cells on fluorescence plots (not shown). Within 4 h of infection the cell population corresponding to uninfected macrophages disappeared (Fig. 5A and B), while the intensity of cell stained positively for apoptosis-like membrane permeability changes considerably increased. These apoptotic changes in macrophages explain the ability of LeTx to reduce production of proinflammatory cytokines by stimulated cells and implicate LeTx as an early intracellular virulence factor secreted by vegetative bacilli within macrophages. We suggest that LeTx can therefore cause a reduction in phagocytic and/or bactericidal activity of macrophages against spores and germinating bacilli. RAW 264.7 cells treated with sublytic concentration of LeTx have decreased phagocytosis of *B. anthracis* (Sterne) spores (Fig. 6). We have also demonstrated that LeTx function within infected macrophages could be related to their reduced antimicrobial activity. We studied the survival of anthrax spores in RAW 264.7 cells treated with different amounts of bestatin, a known inhibitor of LeTx-induced intracellular signaling [21]. In the presence of bestatin, the antimicrobial activity of RAW 264.7 cells increased in a concentration dependent manner (Fig. 7). Under the conditions of the experiment the intrinsic antibiotic activity of bestatin was undetectable (data not shown).

Discussion

Our findings show that intoxicated mouse RAW 264.7 cells undergo changes in membrane permeability, DNA structure, and mitochondrial membrane potential that are typical of apoptosis [18,22,23]. We have also demonstrated that LeTx initiates activation of a number of caspases, including the initiator caspase-3 and the effector caspase-8, both of which are implicated in the current model of apoptosis [22–24]. The apoptotic events in LeTx-treated cells described above seem to be initiated by Fas/FasL interaction on the cell surface.

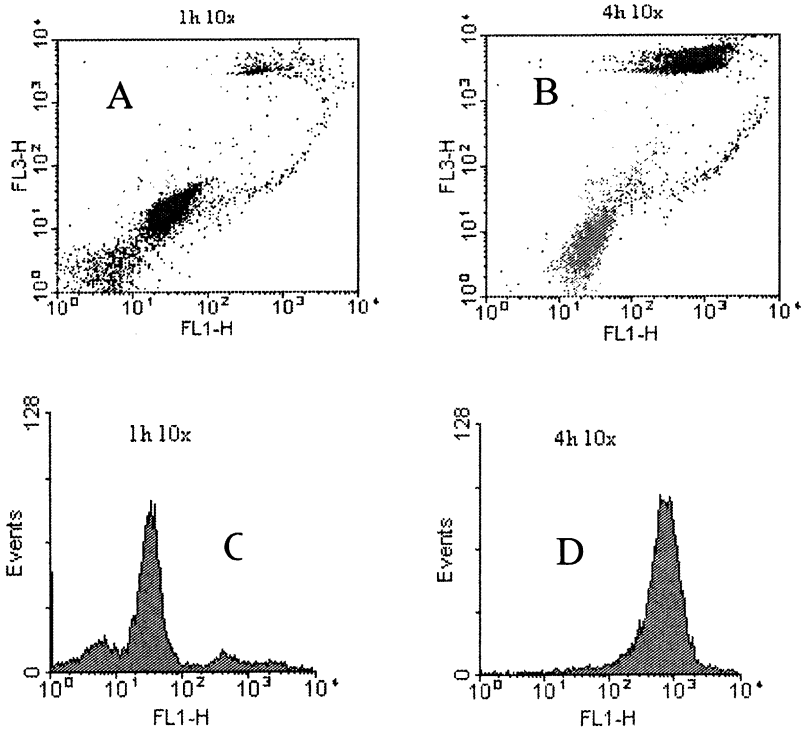

Fig. 5. Flow cytometry of RAW 264.7 cells (1×10^6/ml) after infection with anthrax (Sterne) spores (10×10^6/ml) at different times after staining as in Fig. 1A. At the beginning of infection, spores are undetectable in scatter channels (A). Signals from growing bacterial cells (B) do not appear to overlap with signals from apoptotic RAW 264.7 cells. Histograms (C, D) were gated to exclude signals from bacterial cells (B, gray dots).

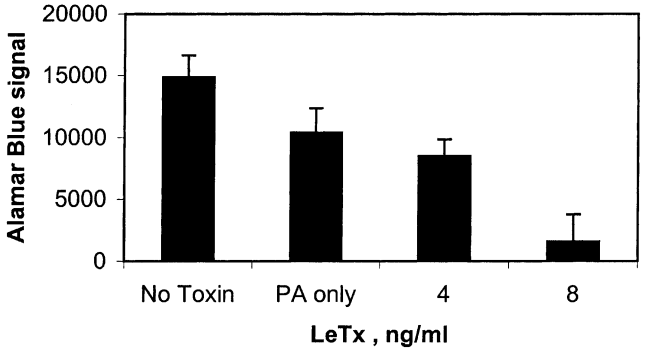

Fig. 6. LeTx decreases phagocytic capacity of RAW 264.7 cells. Spores (2×10^6/well) were added to cells (2×10^5/well) and incubated for 30 min. Cells were lysed and the viability of remaining spores and vegetative bacteria was determined using the Alamar Blue technique.

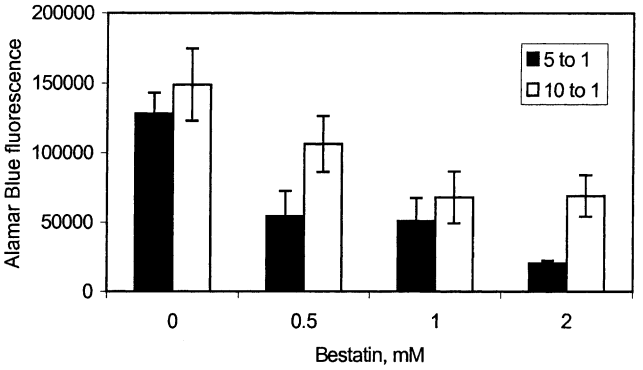

Fig. 7. Bestatin increases bactericidal activity of murine RAW 264.7 cells infected with anthrax (Sterne) spores. Cells (2×10^5/well) were incubated with bestatin for 1 h, then spores were added to cells for 3 h. After incubation the cells were lysed and the viability of remaining spores and vegetative bacteria was determined using the Alamar Blue technique. The spore:cell ratio was 5:1 (filled bars) and 10:1 (open bars).

Prevention of LeTx-induced cytotoxicity by anti-FasL neutralizing antibodies (Fig. 4) indicates a significant role for Fas/FasL interaction in this process. This finding is consistent with the observation that proteases of the caspase family, especially caspase-1 (ICE), -3, -4, and -8, are implicated in Fas-mediated apoptosis [25]. Caspase cleavage of intracellular proteins downstream of caspase-8 activation finally results in the disturbance of mitochondrial function and the release of cytochrome *c* from mitochondria. This in turn can induce the activation of an alternative branch of the caspase cascade through the activation of caspase-9. Although both Fas-mediated caspase activation pathways are functional in most cell types, in some mitochondrial events they are not required for efficient apoptosis [26,27]. Similarly, in our experiments, LeTx-treated cells displayed a strong loss of mitochondrial potential (Fig. 2). However, an inhibitor of caspase-9 was unable to protect cells from changes in the membrane permeability (Fig. 3).

Apoptosis has been earlier detected in several bacterial infections [22,23,28]. Certain pathogens have developed elegant mechanisms to modulate the fate of the host cell. However, in the case of anthrax, the action of LeTx has been previously considered to be cytolytic [3]. Some evidence, such as involvement of the proteasome in LeTx activity [29], consistent with the hypothesis of apoptosis, was however rejected by the authors. An indication of the importance of leukocyte apoptosis in anthrax was presented by Grinberg et al. [30] who found morphologically typical apoptotic lymphocytolysis after pathological analysis of the documented cases of anthrax from the Sverdlovsk epidemic.

We cannot rule out the possibility that under the conditions of our experiments both types of cell death, necrosis and apoptosis, may occur. Many bacterial pore-forming toxins can induce both necrosis and apoptosis [28]. Several reports [31–33] have indicated that these two processes can take place simultaneously in tissues or cell cultures exposed to the same stimulus. The choice of death pathway is thought to depend largely on the severity of the insult [34,35]. In cells exposed to staphylococcal α-toxin, the induction of either apoptosis or necrosis appears to be concentration dependent [36]. This may explain why necrotic-like death dominates over apoptotic death in RAW 264.7 cells in experiments described in the literature using high cytolytic concentrations of the toxin. In our opinion, sublytic concentrations of LeTx better reflect the initial death process in infected macrophages, as LeTx can be detected in blood only late in infection [2]. In our experiments, the infection of macrophages with anthrax (Sterne) spores leads to the appearance of a large population of cells with a membrane staining pattern typical of apoptosis (Fig. 1A). We found it difficult to detect DNA oligomerization in intoxicated RAW 264.7 cells using an agarose gel technique (data not shown), however, the more sensitive TUNEL assay confirmed the presence of DNA fragmentation (Fig. 1B). Several laboratories have shown that the early morphological changes in nuclear chromatin coincide with the appearance of high molecular weight fragments, while the appearance of DNA laddering is a rather late event, occurring during or after apoptotic body formation has taken place [16,37,38].

It has been previously shown that the *Yersinia* protein YopJ/P is able to decrease the production of TNF-α and thus promote apoptosis of infected cells (54). In both cases, cell death is induced indirectly by inhibition of survival pathways, rather than by direct triggering of a proapototic signal. It is therefore reasonable to conclude that in the case of both *Yersinia* and anthrax infections the ability to eliminate phagocytic cells through apoptosis and downregulate inflammatory cytokines contributes to bacterial dissemination and disease progression. We have directly demonstrated for the first time that LeTx-treated RAW 264.7 cells show a decrease in their phagocytic capacity, indicating a functional impairment of infected macrophages (Fig. 6). In agreement with this, we have further shown that bestatin, a known inhibitor of LeTx activity [21], is able to restore the bactericidal activity of mouse RAW 264.7 cells infected with anthrax spores (Fig. 7). Finally, we have demonstrated that the process of intracellular macrophage infection leads to the appearance of apoptotic cells (Fig. 5), as could be anticipated based on our finding of proapoptotic LeTx activity and data from other groups on the early expression of the toxin genes by intraphagocytic anthrax bacilli [1].

In summary, the present study suggests a novel mechanism of LeTx-induced cell death which may contribute significantly to the pathology of anthrax. The elucidation of the bacterial factors and mechanisms by which *B. anthracis* is able to trigger apoptosis of host cells may provide valuable information for new approaches to anthrax treatment.

Acknowledgments

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