Effect of *Bacillus anthracis* lethal toxin on human peripheral blood mononuclear cells

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Abstract Lethal toxin (LeTx) plays a central role in anthrax pathogenesis, however a cytotoxicity of LeTx has been difficult to demonstrate in vitro. No cytolytic effect has been reported for human cells, in contrast to murine cell lines, indicating that cell lysis can not be considered as a marker of LeTx activity. We have recently shown that murine macrophage-like RAW 264.7 cells treated with LeTx or infected with anthrax spores underwent changes typical of apoptotic death. Here we demonstrate that cells from human peripheral blood display a proapoptotic behavior similar to murine cells. TUNEL assay detected a nucleosomal degradation typical of apoptosis in peripheral blood mononuclear cells (PBMC) treated with LeTx. Membrane staining with apoptotic dyes was detected in macrophages derived from monocytes in presence of LeTx. The toxin inhibited production of proinflammatory cytokines in PBMC stimulated with a preparation of *Bacillus anthracis* cell wall. Infection of PBMC with anthrax spores led to the appearance of a large population of cells stained positively for apoptosis, with a reduced capacity to eliminate spores and vegetative bacteria. The aminopeptidase inhibitor, bestatin, capable of protecting cells from LeTx, restored a bactericidal activity of infected cells. These findings may be explained by LeTx expression within phagocytes and support an important role of LeTx as an early intracellular virulence factor contributing to bacterial dissemination and disease progression.

Key words: Anthrax; Apoptosis; Bactericidal activity; Lethal toxin; Peripheral blood; Mononuclear cell

1. Introduction

Macrophages have long been implicated in the development of inhalational anthrax. In the process of antimicrobial response the alveolar macrophages engulf the inhaled spores and carry them to mediastinal lymph nodes. A cascade of intracellular reactions becomes induced to eliminate pathogen. However, lethal doses of spores can overwhelm a bactericidal capacity of macrophages. As a result, some spores survive and germinate within the macrophages producing an antiphagocytic capsule and two toxins (lethal toxin, LeTx; and edema toxin, EdTx). Escape of bacilli from infected cells allows release and proliferation of the bacteria in the lymphatic system and consequent development of systemic disease [1]. LeTx plays a central role in anthrax pathogenesis, and macrophages were suggested as major mediators of its activity because it has been shown that mice depleted of macrophages are resistant to lethal doses of toxin [2–4]. However, the toxicity of LeTx in vitro was difficult to demonstrate. Cytolysis after LeTx treatment was reported only in a few cases, such as peritoneal exudate macrophages from C3H/HeNHsd mice [5], as well as murine macrophage cell lines, RAW 264.7 and J774.A1 [2,6]. No cytopathic effect was reported for human blood cells [5,7] indicating that cell lysis was not a biologically relevant effect of LeTx or perhaps that human cells of monocyte/macrophage origin were not susceptible to LeTx.

We have recently shown that RAW 264.7 cells undergo changes typical of apoptotic death at concentrations lower than required for lysis [8]. In agreement with this we detected cellular membrane apoptotic changes in the cells infected with anthrax spores. Here we demonstrate that the infected human peripheral blood mononuclear cells (PBMC) behave similar to the murine cells. In both systems a bactericidal capacity of cells decreases as a result of either LeTx treatment or intracellular infectious process. A correlation between LeTx-induced apoptosis and the impairment in monocyte/macrophage function is further supported by finding that a LeTx inhibitor, bestatin, displays a protection of the infected cells.

2. Materials and methods

2.1. Reagents

Chromatographically purified recombinant *Bacillus anthracis* lethal factor (LF) and protective antigen (PA) were kindly provided by Dr. S. Leppla (National Institutes of Health, USA) [9,10]. *B. anthracis* (Sterne) spores were prepared similar as described in [11]. Cells from the overnight starter culture grown in NB medium (Gibco, USA) without antibiotic at 37°C were plated onto NB agar and further incubated at 37°C. When the ratio of spores to vegetative bacteria detected by phase-contrast microscopy reached 99:1, the spores were removed from the agar surface with water, pelleted at $5000 \times g$ for 30 min, washed five times with distilled water, then the concentration was adjusted to 1×10^9 spores/ml. Flow cytometry experiments were carried out in a FACSCalibur™ Becton Dickinson Immunocytometry System (BD Biosciences, USA). Statistical analysis was performed by Student's *t*-test. Error bars correspond to 95% confidence.

2.2. Cells and cell culture

PBMC and monocytes were isolated from whole blood obtained

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Abbreviations: LF, lethal factor; PA, protective antigen; LeTx, lethal toxin; CW, *Bacillus anthracis* cell wall preparation; MAPKK, mitogen-activated protein kinase kinase; PBMC, human peripheral blood mononuclear cells; AM, activated monocytes

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from 41 American Red Cross volunteers (Rockville, MD, USA) using Fico/Lite LymphoH (Atlanta Biologicals, USA) according to established procedures [12]. Monocytes were further isolated from PBMC by cell attachment onto a tissue culture dish overnight. The non-adherent cells were washed away, and the adherent fraction containing isolated monocytes was removed using a Cellstripper solution (Mediatech, USA). Alternatively, monocytes were negatively selected using a magnetically activated cell sorting (MACS) cell isolator (Milteny Biotec Inc., Germany). PBMC and monocytes were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, USA) at 37°C in 5% CO_2. Phenol red and antibiotics were omitted when indicated.

2.3. Cell treatment with LeTx

Human cells were treated with 0.05–0.5 μg/ml of LF. PA concentration was kept constant at 0.1 or 0.5 μg/ml. When indicated, cells were preactivated with recombinant INF-γ (100 U/ml; R&D Systems, USA) for at least 24–48 h.

After incubation at 37°C (5% CO_2) for 48 h, the supernatants were collected and stored at −80°C.

2.4. Apoptosis assay

For staining with YO-PRO®-1/propidium iodide (Vibrant Apoptosis Assay Kit #4, Molecular Probes, USA), cells were stripped from plastic and suspended in 900 μl of phosphate-buffered saline (PBS) with 100 μl of stain (prepared according to manufacturer's instructions). Staining was carried out at 4°C for 30 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 50 μM.

TUNEL assay was carried out with in situ cell death detection kit (Roche Molecular Biochemicals, USA). Cells were stripped from plastic and suspended in 100 μl of PBS.

2.5. Preparation of cell wall

The *B. anthracis* cell wall has been obtained essentially as described by Lemaire et al. [13] with modifications introduced by Chauvaux et al. for *B. anthracis* [14]. The cell wall components isolated by this method are mainly peptidoglycan and teichoic acid [13]. *B. anthracis* (Sterne strain) was grown in LB medium (Gibco, USA). Bacterial cultures were incubated until they reached late exponential phase and were then harvested by centrifugation (5000×*g*, 37°C, 10 min), washed in distilled endotoxin-free water, and boiled in 8% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) for 30 min. Cells were incubated at room temperature overnight and centrifuged at 25,000 *g* for 20 min. Supernatant was discarded and the cell pellet was resuspended in 4% SDS, boiled for 15 min, and centrifuged at 25,000 *g* for 20 min. The remaining cell pellet was resuspended in distilled water and centrifuged at 25 000×*g* for 20 min. This procedure was repeated three times. Finally, the pellet was resuspended in 2 M NaCl and centrifuged at 25 000×*g* for 20 min; the pellet was then resuspended in distilled water and centrifuged at 25 000×*g* for 20 min. Supernatant was discarded and the remaining pellet dried. The concentration of cell walls was determined by measuring turbidity of the suspension at 600 nm. Cell walls were checked for absence of aerobic and anaerobic bacterial contamination using various bacterial growth media, and for endotoxin (*Escherichia coli*) using the *Limulus* amebocyte lysate test (Sigma-Aldrich). The peptide content determined by Bio-Rad protein assay (USA) is 0.9% (w/w), teichoic acid content is 4.0% (w/w).

2.6. Cytokine release assay

PBMC (1×10^6/ml) were plated in 24-well tissue plates (Nalge Nunc, USA) and varying amounts of cell wall and LeTx were added for 48 h. Cell culture supernatants were harvested and analyzed for released cytokines (TNF-α, IL-6, IL-1β, and IFN-γ; IL-10; BD Pharmingen) by enzyme-linked immunosorbent assay (ELISA) according manufacturer's recommendations.

2.7. Alamar blue assay

Alamar blue is a water-soluble, non-toxic, fluorometric/colorimetric growth indicator that measures cell metabolism. Cellular growth and metabolism reduce Alamar blue dye and cause a color change from blue to red. Alamar blue has been extensively used in experiments on antibacterial effects of different substances [15]. *B. anthracis* cells grow as chains of different length. It makes the assay based on plaque forming unit counts unreliable. In this case, the Alamar blue assay is especially suitable for quantitative measurement of bacterial viability. The Alamar blue assay has been used by us in a microplate format [16]. Each plate included control wells with known amount of *B. anthracis* spores for determination of the dose–response curve.

3. Results

3.1. LeTx causes apoptotic changes in activated monocytes (AM)

High level of systemic bacteremia is a typical feature of anthrax. To study pathogenicity of *B. anthracis* on human blood cells, we tested the effect of LeTx on freshly isolated PBMC and monocytes. The cells were resistant to lysis in presence of LeTx, in contrast to mouse RAW 264.7 cells. At the highest concentration of LeTx tested (0.5 μg/ml LF and 0.5 μg/ml PA), no lysis or changes in fluorescence staining patterns (see below) were detected after 15–24 h incubation

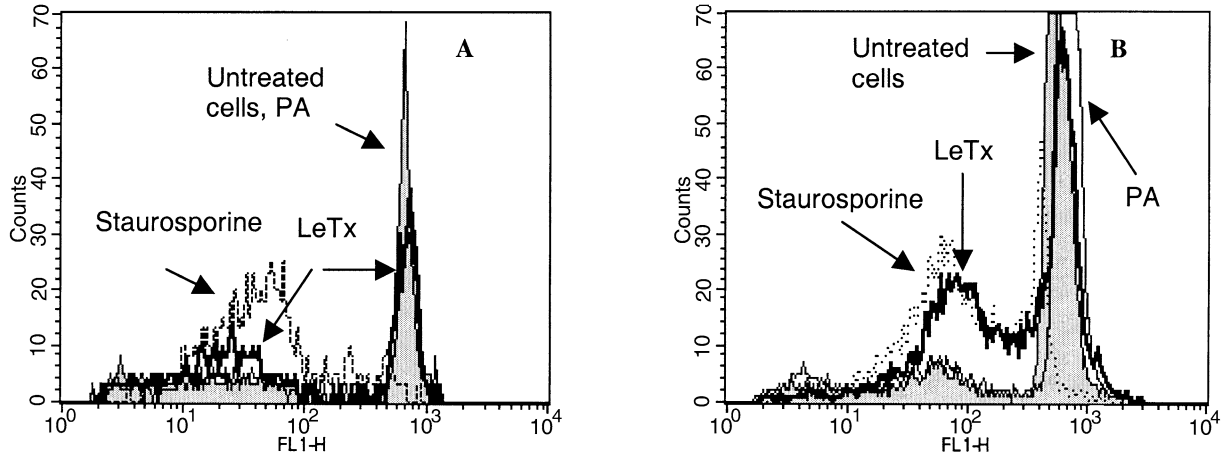

Fig. 1. Staining of LeTx-treated human peripheral blood monocytes preincubated with IFN-γ (100 U/ml) for 48 h. Cells were treated with different concentrations of toxin for 15 h in culture media with (A) or without (B) 10% FCS. Cells were analyzed by flow cytometry after staining with YO-PRO-1 (apoptosis-specific, green fluorescent) and propidium iodide (PI; late apoptosis and necrosis-specific, red fluorescent) using a gate region corresponding to necrotic and apoptotic cells. Thick line, PA (0.5 μg/ml)+LF (0.5 μg/ml); dotted line, control with staurosporine (50 μM). Gray area, PA (0.5 μg/ml) and untreated cells.

using cells isolated from different donors (data not shown). This is consistent with the results of earlier studies unable to demonstrate cytolysis in cells of human origin upon treatment with anthrax culture filtrates or purified anthrax toxins [5,7]. Human blood monocytes, however, are capable to respond to LeTx treatment by the induction of bystander lymphocyte proliferation [17]. We therefore isolated peripheral blood monocytes and studied the behavior in presence of LeTx. Human monocytes undergo rapid spontaneous apoptosis upon culture in vitro unless provided with specific survival signals such as IFN-γ, or microbial products such as lipopolysaccharide (LPS) [18,19]. In our experiments monocytes were activated with IFN-γ for 48 h and then treated with LeTx for 15–24 h. The activated monocytes (AM) were used for flow cytometry experiments after staining with 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. Signals from the cells that acquired additional green and/or red fluorescences during the course of treatment were gated and plotted as histograms in Fig. 1. A spontaneous cell death in control experiments with untreated cells resulted in the appearance of the high intensity peak in Fig. 1A (about 50% of total cells). Treatment with PA only did not lead to the additional change of the histogram. Cells treated with LeTx, compared to untreated cells, showed a higher intensity of fluorescence in the region typical of early apoptotic cells (first peak in Fig. 1A) similar to that of staurosporine as a positive control for apoptosis [20]. Removal of serum from the culture media substantially increased the rate of spontaneous apoptosis (Fig. 1B) in agreement with previous data [21]. In these stress conditions the AM became considerably more susceptible to LeTx (Fig. 1B). The number of green fluorescent cells in the early apoptotic region relative to untreated control increased, while the number of double-stained

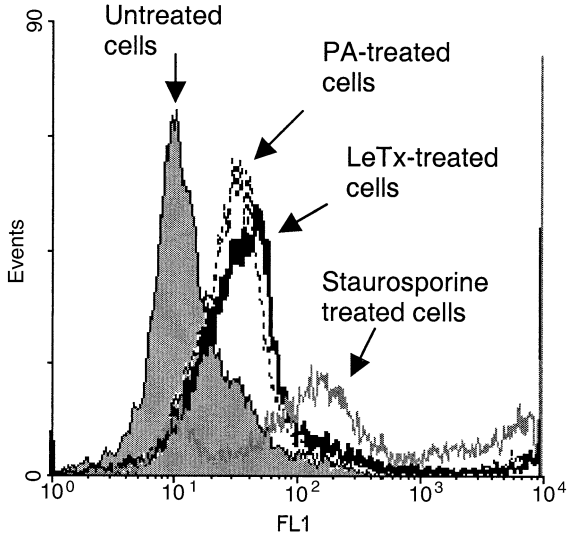

Fig. 2. TUNEL assay of nucleosomal degradation of PBMC in the presence of LeTx. Cells were preincubated with IFN-γ (100 U/ml) for 48 h and treated with toxin for 15 h in culture media. Staining was measured by flow cytometry. Dotted line, PA (0.5 μg/ml); thick solid line, PA (0.5 μg/ml)+LF (0.5 μg/ml); thin solid line, staurosporine (50 μM) as positive control. Gray area, LeTx-untreated control cells incubated in the conditions of the assay. Fluorescence in a green channel was recorded.

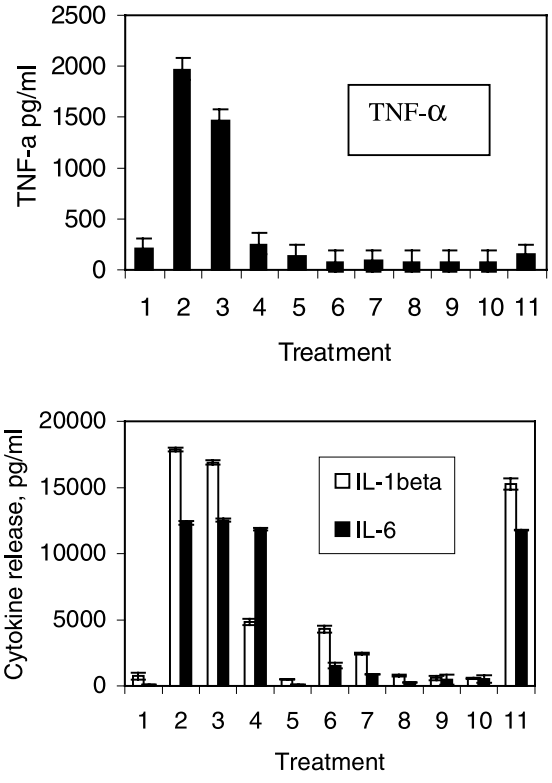

Fig. 3. Production of proinflammatory cytokines by human PBMC activated by CW in presence of LeTx. Cytokine release was detected by ELISA after 48 h stimulation: cells only control (lane 1); CW, 1 μg/ml (lane 2); CW, 0.5 μg/ml (lane 3); CW, 0.1 μg/ml (lane 4); LF, 0.5 μg/ml+PA, 0.1 μg/ml (lane 5); CW, 1 μg/ml+LF, 0.5 μg/ml+PA, 0.1 μg/ml (lane 6); CW, 0.5 μg/ml+LF, 0.5 μg/ml+PA, 0.1 μg/ml (lane 7); CW, 0.1 μg/ml+LF, 0.5 μg/ml+PA, 0.1 μg/ml (lane 8); LF, 0.5 μg/ml (lane 9); PA, 0.1 μg/ml (lane 10); LPS, 0.01 μg/ml (lane 11).

cells decreased, showing a change in a cellular death status. We also detected LeTx-induced nucleosomal fragmentation typical for apoptosis [22] using TUNEL assay (Fig. 2). The in situ modification of the assay we used employs terminal deoxynucleotidyl transferase to incorporate fluorescent nucleotide derivatives into the DNA strand breaks generated in the apoptotic process. The results in Fig. 2 show that both treatments with LeTx and PA alone induced DNA fragmentation detected by flow cytometry as an increase in cell fluorescence.

3.2. *LeTx suppresses the innate immune response and inhibits bactericidal activity of PBMC*

Bacterial infection typically induces a proinflammatory response of PBMC and other immune cells as a result of the innate signaling through receptors capable of recognizing a variety of antigens of both Gram-negative and Gram-positive bacteria [23]. We suggested that one of the possible pathogenic functions of LeTx could consist in the suppression of the innate response of monocytes/macrophages and, perhaps, other cell types present in PBMC. Induction of proinflammatory cytokines in LeTx-treated PBMC by surface antigens of anthrax bacillus, such as cell wall components (CW), could be used as a sensitive indicator of this process. Indeed, PBMC stimulated with anthrax CW responded by strong increase in the production of IL-1β, TNF-α and IL-6, typical for the innate immune response (Fig. 3), whereas LeTx treatment

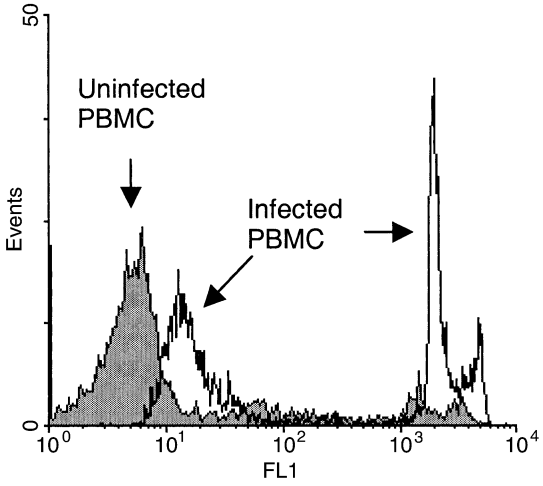

Fig. 4. Anthrax infection results in apoptosis of PBMC. Histogram of green fluorescence of PBMC in flow cytometry experiment 24 h after infection with anthrax (Sterne) spores. Gray area, uninfected cells. Staining was as in Fig. 1. The spore:cell ratio was 10:1. Signals from growing bacterial cells were excluded by gating using a scatter plot.

effectively abrogated the induction of the cytokine release. Similar effect was found in experiments with isolated AM (data no shown).

3.3. *Anthrax infection in AM results in apoptosis-positive cells*

The apoptotic morphology of human AM treated with LeTx, 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 apoptosis in spore-infected blood cells (Fig. 4). Flow cytometry offers a convenient way of monitoring the infectious process in cells stained with apoptotic dyes at different time points after the addition of spores. Gating of scatter plots allows almost complete separation of signals from spores/vegetative bacilli and infected cells on fluorescence plots. It has been found that the development of infection resulted in the gradual increase of the cell green fluorescence as well as the appearance of the additional peak with high both green and red fluorescences. Within 24 h of infection the cell population corresponding to uninfected cells disappeared, while the intensity of cell stained positively for apoptosis-like membrane permeability changes considerably increased.

We also studied survival of anthrax spores in the infected human PBMC. In order to evaluate the role of the expressed LeTx in the intracellular infection, the infected cells were treated with different amounts of bestatin, a known inhibitor of LeTx [24]. In the presence of bestatin, the antimicrobial activity of PBMC increased in a concentration-dependent manner (Fig. 5). Under the conditions of the experiment the intrinsic antibiotic activity of bestatin was undetectable (data not shown).

4. Discussion

LeTx is considered to be a major anthrax virulence factor. Paradoxically, experiments with animals demonstrated that the strains of mice sensitive to toxin were relatively resistant to anthrax infection [25,26]. Both humans and rodents are susceptible species, however, no correlates were found between LeTx activity detectable in vitro and the infectious process. There are only a limited number of available in vitro systems where biological activity of LeTx has been demonstrated. The murine macrophage-like cell lines, RAW 264.7 and J774.A1 are among the most susceptible to the cytolysis by LeTx, while a majority of other cells, including human white blood cells, are resistant [5,7]. The intracellular enzymatic activity of LeTx toward mitogen-activated protein kinase kinases (MAPKKs) [27–30] does not correlate with the resistance.

This study aimed to investigate the cytopathological effect of LeTx in human PBMC and its relevance to human disease.

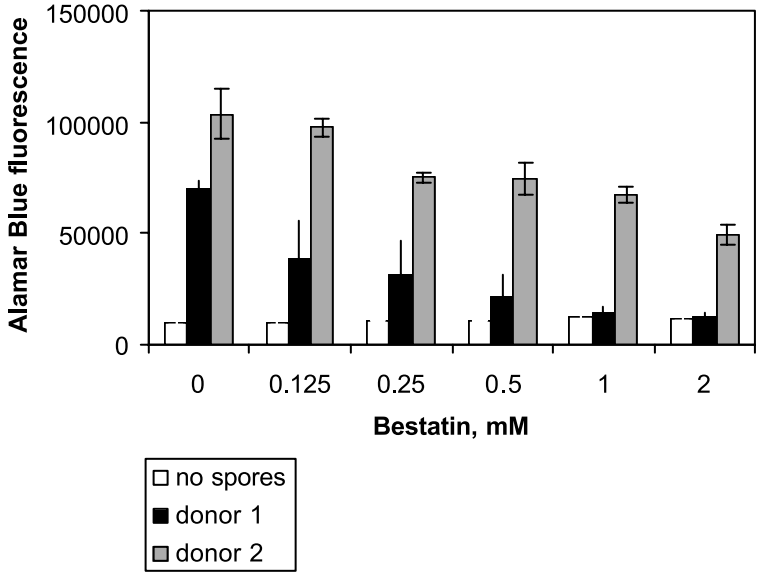

Fig. 5. Bestatin increases bactericidal activity of human PBMC infected with anthrax (Sterne) spores. Cells (2×10^5/well) were incubated with bestatin for 1 h, then spores were added to cells for 3 h. After incubation the cells were lysed, and the viability of remaining spores and vegetative bacteria was determined using the Alamar blue technique. The spore:cell ratio was 10:1. PBMC from two donors were used. Before addition of spores the PBMC were prestimulated with IFN-γ (100 U/ml) for 24 h.

Our findings show that anthrax infection in both mouse [8] and human phagocytes leads to the reduction in their bactericidal capacity against spores and germinating bacilli, and the appearance of apoptotic cells. Apoptosis changes in cell membrane and nucleosomal fragmentation can also be found in LeTx-treated human PBMC and monocytes. PA alone was also able to cause DNA fragmentation, perhaps as a result of its membrane permeabilizing activity. Similar effect was previously described for staphylococcal α-toxin capable of forming transmembrane pores [31].

Apoptosis and proliferation may be viewed in terms of a 'growth equation', where the fate of the cell depends on the relative intensity of growth and death stimuli [32]. LeTx is known to inactivate a MAPKK [27–30] but this inactivation per se does not provide a direct apoptotic stimulus. It is interesting to speculate that proliferation of susceptible cell lines like RAW 264.7 depends on the continuous signaling through MAPK pathways [33], while in human monocytes, rapidly undergoing spontaneous apoptosis in culture, a contribution of the MAPK survival stimulus is low. As a result, RAW 264.7 cells rapidly become apoptotic in presence of LeTx. In contrast, the effect of LeTx on human monocyte apoptosis can be detected only after an additional stress signal. We have demonstrated that conditions of serum withdrawal in presence of IFN-γ substantially increase the susceptibility of monocytes to LeTx (Fig. 1). Serum starvation is known to increase Fas-mediated apoptosis in human diploid fibroblasts [34], and to activate stress-responsive JNK pathway in neuronal cells [35]. We previously presented evidence that Fas – FasL interaction could be involved in the mechanism of LeTx activity in mouse RAW 264.7 cells [8]. Consistent with this, Fas activates the p38 and JNK pathways in Jurkat cells [36], the latter of which is known to be required for induction of FasL promoter activity in response to various stress stimuli [37].

The apoptotic changes in AM explain the ability of LeTx to reduce production of proinflammatory cytokines by cells stimulated through innate immune receptors [23]. Macrophage inactivation but not direct killing may contribute to bacterial dissemination and disease progression. The fact that bestatin, the inhibitor of cell signaling pathways induced by LeTx [17], reduces bacterial burden in infected AM implicates LeTx as an early intracellular virulence factor secreted by vegetating bacilli within macrophages. This conclusion is supported by the data on the reduced survival of the isogenic Sterne strain mutant with deletion of LeTx and PA genes, compared to the Sterne strain, in murine RAW 264.7 macrophages [11]. Our results suggest that a proinflammatory status of macrophages rather than cytolysis can be considered as a marker of cell susceptibility to LeTx.

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