Biochemical and Physiological Changes Induced by Anthrax Lethal Toxin in J774 Macrophage-like Cells

P.C. Hanna, S. Kochi, and R.J. Collier

Department of Microbiology and Molecular Genetics and the Shipley Institute of Medicine,
Harvard Medical School, Boston, Massachusetts 02115

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Experiments were performed to probe the mechanism by which *Bacillus anthracis* Lethal Toxin (LeTx) causes lysis of J774 macrophage-like cells. After incubation of cells with saturating concentrations of the toxin, two categories of effects were found, which were distinguishable on the basis of chronology, Ca^{2+} -dependence, and sensitivity to osmolarity. The earliest events (category I), beginning 45 min postchallenge, were an increase in permeability to ^{22}Na and ^{86}Rb and a rapid conversion of ATP to ADP and AMP. Later events (category II) included alterations in membrane permeability to ^{45}Ca , ^{51}Cr , ^{36}Cl , $^{35}\text{SO}_4$, ^3H -amino acids, and ^3H -uridine, beginning at 60 min; inhibition of macromolecular synthesis, leakage of cellular lactate dehydrogenase and onset of gross morphological changes, at ~ 75 min; and cell lysis, beginning at 90 min. Category II events exhibited an absolute requirement for extracellular Ca^{2+} and were blocked by addition of 0.3 M sucrose to the medium, whereas category I events were attenuated, but not blocked, by either of these conditions. On the other hand, both ATP depletion and the category II events were blocked in osmotically stabilized medium that was also isoionic for Na^+ and K^+ . This suggests that permeabilization of the plasma membrane to monovalent cations and water may be the earliest of the physiological changes described here. The resulting influx of Na^+ and efflux of K^+ would be expected to cause depletion of ATP, via increased activity of the Na^+/K^+ pump. Subsequently the influx of Ca^{2+} , induced by depletion of ATP, imbalances in monovalent cations, and/or more dramatic changes in permeability due to influx of water, would be expected to trigger widespread changes leading ultimately to cytolysis.

INTRODUCTION

The Lethal Toxin (LeTx) of *Bacillus anthracis* plays a central role in the virulence of the disease anthrax (Stephen, 1986; Friedlander, 1990; Leppla, 1991), but its primary action on sensitive cells is unknown. LeTx is comprised of two noncovalently linked proteins: protective antigen (PA, 83 kDa) and lethal factor (LF, 89 kDa), both of which are required to form active toxin (Stephen, 1986). *B. anthracis* also produces another toxin, Edema Toxin (EdTx), which is composed of PA and EF (edema factor, 88 kDa), and is responsible for the edema seen in cutaneous anthrax infections (Stephen, 1986). PA, the protein in common between LeTx and EdTx, serves to bind EF or LF to cell-surface receptors and mediate their transfer to the cytoplasm (Leppla, 1982, 1991; Stephen, 1986; Friedlander, 1990). There EF functions as a Ca^{2+} -, calmodulin-dependent ade-

nylate cyclase (Leppla, 1982). No enzymatic activity has been reported for LF, however.

Evidence for the importance of LeTx in anthrax pathogenesis is compelling: 1) the lethality and symptoms of systemic anthrax infection are mimicked in test animals challenged with purified lethal toxin (Stephen, 1986; Friedlander, 1990; Leppla, 1991); 2) immunity to LeTx protects animals from *B. anthracis* challenge (Welkos and Friedlander, 1988; Iacono-Conners *et al.*, 1991); and 3) *B. anthracis* strains with insertional "knock-out" mutations in either the gene for PA or that of LF are attenuated (Cataldi *et al.*, 1990; Pezard *et al.*, 1991).

The recent discovery (Friedlander, 1986) that primary macrophages (M ϕ s) and the M ϕ cell line J774A.1 are uniquely sensitive to LeTx has fostered experiments to characterize the toxin's mode of action. Emerging from these and earlier studies is the following model for LeTx action. After PA binds to cellular receptors, the N-ter-

minal 20 kDa is cleaved by a cellular protease and removed, which permits LF binding to the residual receptor-bound PA₆₃ (Friedlander, 1990; Koehler and Collier, 1991; Leppla, 1991). The lethal toxin complex (PA₆₃-LF) is then internalized by receptor-mediated endocytosis and, after acidification of the endosome, LF is presumably translocated into the cytosol (Friedlander, 1986; Blaustein *et al.*, 1989; Escuyer and Collier, 1991; Koehler and Collier, 1991). In an unknown manner, LF then induces M ϕ lysis within 1–2 h postchallenge (Friedlander, 1986). A strict requirement for extracellular Ca²⁺ in LeTx-induced cytolysis has been demonstrated (Bhatnagar *et al.*, 1989). Although LeTx binds to and enters all cell types tested, only M ϕ s are killed (Friedlander, 1986, 1990; Leppla, 1991). The basis of this selectivity is not understood.

To elucidate underlying causes of LeTx-induced cytotoxicity, we examined physiologic and biochemical alterations occurring in M ϕ J774A.1 cells in response to LeTx challenge. Changes in cellular ATP levels, macromolecular synthesis and permeability of the plasma membrane to a variety of markers were characterized with respect to kinetics, LeTx concentration-dependence, and dependence on extracellular Ca²⁺ and osmolarity. From these studies we have defined two categories of LeTx-induced events. The earliest includes an increased membrane permeability to ²²Na and ⁸⁶Rb (a K⁺ analogue). These changes are concurrent with, and may be responsible for, loss of ATP levels. These events can occur at relatively low toxin concentrations and do not require extracellular Ca²⁺. The second, later, category involves changes in generalized membrane permeability, inhibition of macromolecular synthesis, visible morphological alterations, and cell lysis. These late events require net fluxes of Na⁺ and K⁺, entry of water into the cell, and the presence of extracellular Ca²⁺.

MATERIALS AND METHODS

Anthrax Lethal Toxin Preparation

B. anthracis strain RP9 was the kind gift of M. Mock (Institute Pasteur) and was maintained as spore stocks. *B. anthracis* RP9 is nonencapsulated and has an insertion mutation in the gene encoding the edema factor, allowing for EF-free production of the PA and LF exoproteins (Pezard *et al.*, 1991). Six-liter cultures were grown in defined RM toxin-production medium, as reviewed in (Leppla, 1988). Culture supernatants were filter-sterilized by passage through a 0.22- μ m filter (Millipore, Bedford, MA) and concentrated to one liter with the Minitan Ultrafiltration System (Millipore). Ammonium sulfate was added to 75%, and the protein pellet was collected and suspended in 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl pH 8.0 and dialyzed extensively against the same buffer. An efficient one step purification was performed by FPLC MonoQ anion exchange (Pharmacia, Piscataway, NJ) with a 20 mM Tris-HCl pH 8.0 buffer and linear 0–400 mM NaCl gradient elution over 40 min. PA eluted at 130–140 mM NaCl, and LF eluted at 250–270 mM NaCl. Both PA and LF were determined to be 90–95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and neither PA alone nor LF alone was cytotoxic to J774A.1 cells. For cytotoxicity assays,

unless otherwise noted, final concentration of lethal toxin was PA = 0.1 μ g/ml; LF = 0.1 μ g/ml.

Cell Culture

The toxin-sensitive murine macrophage cell line J774A.1 was obtained from the American Type Culture Collection (ATCC TIB 67, Rockville, MD) and maintained in Minimal Essential Medium (MEM) supplemented with Earl's salts, 10% fetal calf serum, penicillin (200 μ g/ml), streptomycin (100 μ g/ml), and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0. Cells were grown in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C in 75-cm² plastic flasks (Corning, Corning, NY). Under these conditions, J774A.1 cells had a doubling time of 36–48 h. For the cytotoxicity experiments, cells were harvested by scraping with a rubber policeman, and plated at a density of 5×10^5 cells/ml in 2 cm², 24-well dishes (Corning), and grown 36–48 h to ~80–90% confluence ($1-2 \times 10^6$ cells/well).

Phase Contrast Microscopy

Cells (300 μ l of 10^6 cells/ml) were seeded into Lab-Tek chamber slides (Nunc, Naperville, IL) and grown to ~50–75% confluence (18 h). Cells were washed once with serum-free media and challenged with lethal toxin (PA + LF), PA alone, LF alone or blank in serum-free medium at 37°C. At the indicated times, the sides of the chamber slides were removed and phase contrast microscopy performed with an Olympus BH-2 microscope (Olympus Optical, Tokyo, Japan). LeTx-induced cell lysis was reported previously (Friedlander, 1986), and morphological alterations include loss of cytoplasmic density ("ghosting"), membrane fusion, and cell flattening. No nuclear segmentation is observed at any time. These changes also can be discerned by the practiced eye directly in the 24-well culture dishes with lower magnification ($\times 100$ – $\times 200$).

ATP Quantification

Cells were prepared as described under *Cell Culture*, washed once in serum-free medium, and challenged in serum-free medium at 37°C. At the indicated times, challenge medium was discarded, and the cells were scraped and lysed in 300 μ l of lysis buffer (0.2% Triton X-100, 4 mM EDTA, 0.1 mM Tris-HCl, pH 8.5). ATP levels were measured (5- μ l samples) with the high-sensitivity firefly luciferin/luciferase ATP bioluminescence HS system (Cat 567-701, Boehringer Mannheim Biochemicals, Indianapolis, IN) according to manufacturers specifications. Light emission at 562 nm was measured in a SLM-Aminco SPF-500C spectrofluorometer (SLM Instruments, Urbana, IL) (emission band pass = 20 nm, gain = 100, filter 3, 15°C). Light intensity was linearly proportional to standard ATP concentration ranges from 10^{-12} to 10^{-8} M. For each sample, seven data points (16 readings/point) were recorded at 3-s intervals beginning at 5 s after ATP addition to the reaction mix. The highest and lowest readings were discarded, and the mean of the remaining five points was determined and normalized to sample protein concentration.

To compare the amounts of ATP versus ADP versus AMP, the less sensitive reverse phase high-performance liquid chromatography system of DePetrillo *et al.* (1990) was used (Hewlett-Packard HPLC series 1050 equipped with a 18×4 cm Rainin C-18 column Hewlett-Packard, Dusseldorf, Germany). Cell supernatants and lysates were collected at the indicated times and run on a 30-min, 0–30% linear acetonitrile gradient in 30 mM potassium phosphate pH 7.0 containing 10 mM tetrabutylammonium phosphate. Peaks corresponding to ATP (24 min), ADP (19 min), and AMP (15 min) standards monitored at 254 nm were normalized to cellular concentrations with the assumptions described in Figure 1 legend.

Membrane Permeability Alterations

For studies measuring the efflux of radiolabeled markers, nearly confluent J774A.1 cell monolayers were prepared as described above and

incubated overnight with the cytoplasmic markers $^{86}\text{RbCl}$ (4 $\mu\text{Ci}/\text{ml}$, $\text{Na}_2^{51}\text{CrO}_4$ [25 $\mu\text{Ci}/\text{ml}$], or for 2 h with $\text{Na}_2^{35}\text{SO}_4$ (50 $\mu\text{Ci}/\text{ml}$), ^3H -AIB (a nonmetabolizable amino acid), or ^3H -uridine (2 $\mu\text{Ci}/\text{ml}$) (McClane *et al.*, 1988b). Radioactivity was purchased from Dupont/NEN (Boston, MA). Cultures were then washed twice with serum-free medium and challenged at 37°C with LeTx or controls. At the indicated times, culture supernatant was gently removed and counted. The amount of radioactivity released from cells was calculated with the formula of Thelestam and Mollby (1975) where percent maximal release = $100\% \times (\text{sample induced release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$.

Spontaneous release represents radiolabel released into the medium without toxin challenge. The maximal release of cytoplasmic label represents the total available cytoplasmic radioactivity at the end of radiolabeling (i.e., before challenge) and was determined after cell membrane rupture by the addition of 1 ml of 1% Triton X-100 to each well. For ^{51}Cr studies, maximal release was $\sim 100\,000$ cpm/well and spontaneous release from cells was linear with time from 100 cpm/well at $t = 0$ h to 10 000 cpm/well at $t = 5$ h. ^{86}Rb maximal release was $\sim 45\,000$ cpm/well with a linear spontaneous release ranging from 4000 cpm/well at $t = 0$ h to 40 000 cpm/well at $t = 5$ h. ^3H -AIB and ^3H -uridine maximal release was $\sim 20\,000$ cpm/well with a linear spontaneous release ranging from 200 cpm/well at $t = 0$ h to 1500 cpm/well at $t = 5$ h. At $t = 0$ h, 15–25% of ^3H -uridine was precipitable with trichloroacetic acid (e.g., RNA); the remaining cpm was in TCA-soluble pools.

Release of lethal toxin-induced cellular lactate dehydrogenase (LDH) into the media was measured with the methods of Friedlander (1986) and a kit supplied by Sigma Diagnostics (Product 500C; St. Louis, MO).

For studies measuring influx of $^{22}\text{NaCl}$ (0.01 $\mu\text{Ci}/\text{ml}$, NEN/Dupont), $^{45}\text{CaCl}_2$ (10 $\mu\text{Ci}/\text{ml}$, NEN/Dupont), and Na^{36}Cl (0.1 $\mu\text{Ci}/\text{ml}$, Amersham, Arlington Heights, IL), cultures were prepared as described above and challenged with LeTx or controls in the presence of the radiolabel in serum-free medium and incubated at 37°C. At the indicated times, medium was removed, and the cells were quickly washed twice with ice-cold medium containing 0.3 M sucrose. The cells were then lysed with 1% Triton X-100 and counted. Data are presented as percent of control influx where 100% of control is the amount of label taken into unchallenged cells for the given timepoint. Control incorporation of ^{22}Na and ^{36}Cl was linear with time from 4000 cpm/well at $t = 0$ h to 39 000 cpm/well at $t = 3$ h, and control incorporation of ^{45}Ca was linear with time from 500 cpm/well at $t = 0$ h to 3500 cpm/well at $t = 4$ h.

Macromolecular Synthesis Measurement

The rate of incorporation of ^3H -thymidine (1 $\mu\text{Ci}/\text{ml}$), or ^3H -leucine (0.5 $\mu\text{Ci}/\text{ml}$) precursors (2–10 Ci/mmol, Dupont/NEN) into trichloroacetic acid-precipitable DNA, RNA or protein was performed by the methods of McClane and McDonel (1979). Briefly, cells were prepared as above and washed once with serum-free medium. Lethal toxin or control was added to cells in serum-free medium, simultaneously with a ^3H -precursor (^3H -leucine was added in leu⁻ medium). At the indicated times, culture supernatants were discarded and the cells were washed twice with serum-free medium and collected into lysis buffer. TCA-precipitable counts were determined and normalized to protein concentration.

Kinetics of LeTx-Induced Cellular Pathologies

The kinetics of onset of various LeTx-induced alterations in J774A.1 cultures was first examined under the presumption that early toxin events are central for cytolysis. In all instances, three parameters were assayed from the same dishes of cells for each time point: visible alterations in morphology, ATP levels, and either ion fluxes, or macromolecular synthesis. In this manner it was possible to obtain a relative chronology of LeTx pathologies under identical experimental conditions.

Toxin Challenge in Ca^{2+} -Free or Osmotically Stabilized Media

The above LeTx assays were repeated under Ca^{2+} -free conditions (Bhatnagar *et al.*, 1989) by washing J774A.1 cultures twice with Ca^{2+} -free medium before challenge in the same medium. For these studies, Ca^{2+} -free MEM was purchased from Gibco/BRL (Paisley, Scotland) and was supplemented with Earl's salts, penicillin (200 $\mu\text{g}/\text{ml}$), streptomycin (100 $\mu\text{g}/\text{ml}$), and 10 mM HEPES, pH 7.0. Ca^{2+} -free medium was then treated with CHELEX 100 (Bio-Rad, Richmond, CA) for 1 h, and EDTA was added to a final concentration of 2 mM just before use.

Controls recovering LeTx activities that are inhibited by Ca^{2+} -free conditions are performed by removing the challenge medium at the indicated times, twice washing the cells and incubating them in Ca^{2+} -containing medium (no toxin) and scoring as described above.

Osmotic stabilizers are agents such as sucrose and dextran that do not penetrate the plasma membrane of cells but inhibit the action of some of the cytolytic toxins by temporarily decreasing the colloid-osmotic swelling due to influxes of water into the cell following toxin-induced permeability of the membrane to certain ions (Donnan effect) (Bernheimer, 1947; McClane, 1984; Harris *et al.*, 1991). For osmotic studies, sucrose was added to serum-free MEM to a final concentration of 0.3 M. Cells were washed twice in sucrose-containing media, and the various LeTx challenges were performed in the same media as described above.

Controls recovering LeTx activities that are inhibited by osmotic stabilizers in the challenge media are performed by removing the sucrose-containing media, twice washing the cells and incubating them in sucrose-free media (no toxin), and scoring as described above.

In indicated studies, isoionic challenge medium (10 mM NaCl, 150 mM KCl, 20 mM HEPES pH 7.1) was used in the presence or absence of the osmotic stabilizer sucrose. Controls recovering LeTx activities that are inhibited by the combination of isoionic and isosmotic conditions are performed by removing the protective solution, twice washing the cells, incubating them in normal medium (no toxin), and scoring as above.

Other Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemicals (St. Louis, MO). Protein determinations were performed with Coomassie Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard. TCA-precipitations were performed as described (McClane *et al.*, 1988b).

RESULTS

To investigate the molecular basis of LeTx-induced cytolysis of J774A.1 macrophage cells, we assayed changes in ATP levels, membrane permeability to ions and other markers, macromolecular synthesis and cell morphology, upon challenge with a saturating level of the toxin (0.1 $\mu\text{g}/\text{ml}$ each of PA and LF). ATP levels in LeTx-treated or control cells were measured with a firefly luciferin/luciferase assay. M ϕ s challenged with LeTx showed dramatic decreases in ATP, beginning ~ 45 min (Figure 1, ●). The ATP concentration, initially at 1–2 mM, showed a $>50\%$ decrease by 60 min and $>90\%$ decrease by 75 min. Mock challenged cells (Figure 1, ○) or cells challenged with LF or PA alone maintained initial concentrations of ATP throughout the experiment. At no time could ATP be detected in the extracellular medium; and no endogenous ATPase activity was found for purified LeTx. As shown in Table 1, by

Figure 1. Effect of lethal toxin on macrophage ATP levels. J774A.1 cells were incubated in LeTx (●—●), LeTx in Ca^{2+} -free medium (■—■), LeTx in medium containing 0.3 M sucrose osmotic-stabilizer (▲—▲), or control medium (○—○). LeTx (0.1 $\mu\text{g}/\text{ml}$ each of PA and LF) was added at $t = 0$ h, and cells were incubated at 37°C . At the indicated times, samples were collected and lysed. ATP was determined by bioluminescence and normalized to protein concentration. Additional controls not shown include Ca^{2+} -free medium alone and medium containing 0.3 M sucrose alone, neither of which had any effect on ATP levels. Samples were assayed in triplicate. SEM for each data point was $<8\%$ of the indicated value ($n = 5$). Control M ϕ s contained $\sim 1.6 \times 10^{-11}$ mol of ATP/ μg cellular protein. From the calculated value of $\sim 1 \times 10^{-4}$ μg protein/cell and an assumed cellular volume of 4×10^{-9} cm^3 , the ATP levels in control cells was estimated to be 1–2 mM.

60 min after addition of toxin to cells, over one-half of the ATP had been hydrolyzed, mainly to ADP, and the total intracellular pool of adenosine nucleotides remained constant. By 120 min after LeTx addition, ATP had been almost completely degraded, and significant release of ADP and AMP into the extracellular medium had occurred.

Next we examined the effect of LeTx on permeability of the plasma membrane to a variety of radiolabeled markers. Efflux of ^{86}Rb (a K^+ analogue) from prelabeled cells was observed beginning at 45 min and was essentially complete by 75 min (Figure 2a). This effect coincided with the depletion of ATP (Figure 1, ●). In contrast, efflux of ^{51}Cr (Figure 2b), ^3H -uridine (Figure 2c), ^3H -AIB (Figure 2d), $\text{Na}^{35}\text{SO}_4$ (Figure 2e), or cellular LDH (Figure 2f) was not observed before 60 min and was not complete until 2–3 h. Experiments (Figure 3) mea-

suring influx of ions into M ϕ s showed that ^{22}Na entered the cell beginning at 45 min (Fig. 3a, ●), whereas influx of ^{45}Ca and ^{36}Cl was not observed until 60 min (Fig. 3, b and c, ●). Alterations in cellular morphology were not discernible before 75–90 min, with extensive cell lysis becoming apparent at 90–120 min. No membrane permeability alterations or morphologic damage were apparent in control cells (Fig. 3, a–c, ○) or in cells challenged with PA alone or LF alone. These results are generally consistent with an earlier study (Bhatnagar *et al.*, 1989), which showed that ^{45}Ca influxes began at 75 min, preceding the release of cellular lactate dehydrogenase from the cell at ~ 120 min.

To probe the effects of LeTx on the cells' synthetic capabilities, we examined the kinetics of LeTx-induced inhibition of protein, RNA and DNA synthesis. The observation that LeTx inhibits M ϕ protein synthesis was previously used as an assay method for LeTx cytotoxicity (Bhatnagar *et al.*, 1989). Results presented in Figure 4 indicate a simultaneous inhibition in the rates of incorporation of radiolabeled precursors into RNA, protein, and DNA, beginning ~ 75 min after toxin challenge. Because macromolecular synthesis was unaffected until relatively late in the intoxication process, it is likely that the global inhibition results from earlier toxin-induced events such as ATP depletion or the loss of the small molecules required for macromolecular synthesis (e.g., ions, cofactors, amino acids, and nucleotides).

Data in Figure 5 compare the LeTx concentration-dependence of ATP depletion with that of ^{51}Cr release, at 90 min. A significant ($>20\%$) loss of ATP was observed at the relatively low concentration of 0.1 ng/ml of LF ([PA] = 0.1 $\mu\text{g}/\text{ml}$), whereas release of ^{51}Cr from the cell did not occur at concentrations below 5.0 ng/ml LF ([PA] = 0.1 $\mu\text{g}/\text{ml}$). Membrane fluxes of ^{22}Na and ^{86}Rb gave concentration-dependence curves virtually identical to that of the ATP-depletion curve, whereas curves for ^{45}Ca , ^{36}Cl , $^{35}\text{SO}_4$, ^3H -AIB, ^3H -uridine, and LDH fluxes, as well as those for inhibition of macromolecular synthesis, were virtually identical to that

Table 1. Fate of cellular ATP^a after lethal toxin challenge

	$t = 0$ min		$t = 60$ min		$t = 120$ min	
	Intracellular (mM)	Extracellular (mM)	Intracellular (mM)	Extracellular (mM)	Intracellular (mM)	Extracellular (mM)
ATP	2.0 ^b	<0.1	0.8	<0.1	0.2	<0.1
ADP	<0.1	<0.1	1.1	<0.1	0.7	0.3
AMP	<0.1	<0.1	0.2	<0.1	0.3	0.1

^a Intracellular and extracellular ATP, ADP, and AMP measurements were performed at the indicated times after LeTx challenge by HPLC as described in MATERIALS AND METHODS. Nucleotide amounts were normalized to cellular concentrations with the assumptions described in Figure 1 legend.

^b Figures represent the mean of three trials performed in duplicate. SEM for each point was 12% of the indicated figure.

Figure 2. Effect of lethal toxin on membrane permeability: efflux of radiolabeled cytoplasmic markers. J774A.1 cells were prelabeled with ^{86}Rb (a), ^{51}Cr (b), ^3H -uridine (c), ^3H -AIB (d), or $^{35}\text{SO}_4$ (e) as described and incubated in LeTx (●—●), LeTx in Ca^{2+} -free medium (■—■), LeTx in medium containing 0.3 M sucrose (▲—▲), or control medium (○—○). LeTx (0.1 $\mu\text{g}/\text{ml}$ each of PA and LF) was added at $t = 0$ h. At the indicated times, the toxin-induced radioactivity released into the culture medium was determined, and after subtraction of background release, the data were plotted as the percentage of total cellular radiolabel, as defined under MATERIALS AND METHODS. Lactate dehydrogenase determinations (f) were performed as described by Friedlander (1986). Controls not shown included mock challenges of cells in Ca^{2+} -free medium and medium containing 0.3 M sucrose. Neither of these media induced release of cellular radioactivity above background. In activity-rescue experiments (Δ — Δ), the 0.3 M sucrose challenge medium was removed after 2 h (arrow), and cells were then washed twice and incubated with control medium (sucrose-free and toxin-free) at 37°C and assayed for release of radiolabel, as described above. Experiments were performed in triplicate. SEM for each data point was $<6\%$ of the indicated value ($n > 4$).

of ^{51}Cr release. Alterations in cell morphology and cytolysis were not seen at LF concentrations below 10 ng/ml ([PA] = 0.1 $\mu\text{g}/\text{ml}$). At very high toxin doses (10 $\mu\text{g}/\text{ml}$ LF, 10 $\mu\text{g}/\text{ml}$ PA), the kinetics of onset of M ϕ pathologies was identical with that seen at the 100-fold lower toxin concentration used in earlier experiments (Figures 1–4), indicating that a defined lag period is required before onset of toxin activities. This lag period may represent, at least in part, time required for LeTx binding and internalization (Friedlander, 1990).

A strict requirement for extracellular Ca^{2+} in LeTx-induced cytolysis and influx of Ca^{2+} into challenged

M ϕ s was demonstrated by Bhatnagar *et al.* (1989), who showed that elimination of Ca^{2+} from the medium or addition of the Ca^{2+} channel blocker verapamil, protected M ϕ s from LeTx. Their studies indicated that the Ca^{2+} -dependent step occurred after LeTx binding and internalization. To confirm and extend these findings, experiments were performed to determine the Ca^{2+} -dependence for LeTx-induced alterations in M ϕ function. Consistent with the earlier study (Bhatnagar *et al.*, 1989), we found an absolute requirement for Ca^{2+} for LeTx-

Figure 3. Effect of lethal toxin on membrane permeability: influx of radiolabeled ions. Influx of ^{22}Na (a), ^{45}Ca (b), or ^{36}Cl (c) into the cytoplasm of J774A.1 cells upon coincubation with LeTx (●—●), LeTx in Ca^{2+} -free medium (■—■), LeTx in medium containing 0.3 M sucrose (▲—▲), or control medium (○—○). LeTx (0.1 $\mu\text{g}/\text{ml}$ each of PA and LF) was added at $t = 0$ h. Data are plotted as percentage of control influx, where 100% represents radiolabel associated with nonchallenged cells at each time point after removing extracellular radioactivity by gently washing cells twice with 0.3 M sucrose in MEM at 37°C . Cells were lysed with 1% Triton X-100 before counting. Influx of ^{22}Na into control cells was not linear after 3 h. In the absence of toxin, neither Ca^{2+} -free medium (^{22}Na experiment only) nor medium containing 0.3 M sucrose induced influx of radiolabel above background. Rescue of LeTx-induced ^{45}Ca influxes from the protection afforded by osmotic stabilizers (Δ — Δ) was performed at 2 h (arrow) by removing the 0.3 M sucrose medium, washing cells twice and then incubating in control medium (toxin-free and sucrose-free) and assaying for uptake of radiolabel as described above. Experiments were performed in triplicate. SEM for each data point was $<11\%$ of the indicated value ($n = 3$).

Figure 4. Lethal toxin effect on *M*φ macromolecular synthesis. Measurements of the rate of incorporation of ^3H -uridine, ^3H -leucine, or ^3H -thymidine precursors into TCA-precipitable RNA (a), protein (b) or DNA (c), respectively, were performed as described in the text. J774A.1 cells were incubated in LeTx (● — ●), LeTx in Ca^{2+} -free medium (■ — ■), LeTx in medium containing 0.3 M (▲ — ▲), or control medium (○ — ○), at 37°C . LeTx ($0.1\ \mu\text{g}/\text{ml}$ each of PA and LF) was added at $t = 0$ h. Controls (no toxin) not shown include incubation of cells with Ca^{2+} -free medium and medium containing 0.3 M sucrose. Neither medium affected macromolecular synthesis. Rescue of LeTx-induced inhibition of macromolecular synthesis from the protection afforded by osmotic stabilizers (Δ — — Δ) was performed at 2 h (arrow) by removing 0.3 M sucrose medium, washing cells twice and then incubating in control medium (toxin-free and sucrose-free), and assaying for TCA-precipitable incorporation of radioactivity as described above. Experiments were performed in duplicate. SEM for each data point was $<8\%$ of the indicated value ($n = 3$).

induced morphologic alterations, lysis, and macromolecular synthesis. Additional experiments showed that extracellular Ca^{2+} was required for membrane fluxes of $^{35}\text{SO}_4$, ^{36}Cl , ^{51}Cr , ^3H -AIB, ^3H -uridine, and LDH. In contrast, the earliest LeTx effects, ATP depletion and increased membrane permeability to ^{22}Na and ^{86}Rb were not absolutely dependent on extracellular Ca^{2+} . The absence of extracellular Ca^{2+} did not delay the onset of ATP depletion or ^{86}Rb and ^{22}Na fluxes (Figures 1, 2a, and 3a, ■) but did attenuate the magnitude of the LeTx effect by $\sim 50\%$. Merely omitting Ca^{2+} from the medium had no effect on ATP levels or ion fluxes. As additional controls, toxin-activity rescue experiments were performed after 2 h. In control studies, replacing the Ca^{2+} -

free challenge medium with toxin-free medium containing Ca^{2+} rescued $>85\%$ of full LeTx activity within 30 min, supporting the idea (Bhatnagar *et al.*, 1989) that extracellular Ca^{2+} is not absolutely required for LeTx binding and internalization into cells.

Addition of sucrose or other osmotic stabilizers to isomolar levels has been shown to protect cells from certain cytolytic toxins (Bernheimer, 1947; McClane, 1984; Harris *et al.*, 1991), presumably by inhibiting the influx of water after damage to the plasma membrane. We found that cells challenged with LeTx in medium containing 0.3 M sucrose were protected from morphological damage, changes in macromolecular synthesis (Fig. 4, ▲), and release of ^{51}Cr , ^3H -uridine, $^{35}\text{SO}_4$, ^3H -AIB, or LDH release (Fig. 2, b–f, ▲). Additionally, ^{45}Ca uptake was inhibited by sucrose in the challenge medium (Fig. 3b, ▲), suggesting that water influx is important for LeTx-induced calcium imbalances. When the osmotically stabilized medium was removed after 2 h and replaced with control medium lacking both sucrose and toxin, full LeTx activities were seen after 30 min additional incubation (Figs. 2, b–f, 3, b and c, and 4, a–c Δ). On the other hand, LeTx-induced ATP depletion (Fig. 1, ▲), ^{22}Na influx (Fig. 3a, ▲), and ^{86}Rb efflux (Fig. 2a, ▲) occurred in the presence of 0.3 M sucrose, albeit to $\sim 50\%$ of the full LeTx levels. Although the magnitude of LeTx-induced effects was diminished by the presence of sucrose, there was no alteration in the kinetics of their onset. The presence of 0.3 M sucrose alone did not affect cells in the absence of toxin. Collectively, these data suggest that disruption of membrane colloid-osmotic equilibrium alone is not absolutely essential for LeTx-induced ATP depletion or for membrane permeability to ^{22}Na and ^{86}Rb but is important for induction of some of the later LeTx effects resulting in cytotoxicity.

Figure 5. Lethal toxin concentration-dependence curves. J774A.1 cells were challenged with LeTx for 2 h at 37°C and assayed for cellular ATP (Figure 1) or efflux of ^{51}Cr (Figure 2). Data are plotted as percentage of total cellular ATP (● — ●) remaining after toxin challenge, where 100% represents ATP concentrations in unchallenged cells; or as percentage of maximal release of ^{51}Cr (□ — — □) into the medium. Toxin concentrations used were $0.1\ \mu\text{g}/\text{ml}$ PA, with varying amounts of LF (0.00001 – $10\ \mu\text{g}/\text{ml}$), as indicated. Toxin was added at $t = 0$ h. Results from concentration-dependence assays measuring other LeTx parameters are described in the text. Experiments were performed in triplicate. SEM for each data point was $<9\%$ of the indicated value ($n = 3$).

Finally, experiments were performed to determine causal relationships between the two early responses, namely ATP depletion versus membrane fluxes of Na^+ and K^+ . In a preliminary study, cells incubated for 12 h in the presence of 25 mM 2-deoxyglucose or 10 mM sodium azide showed a marked decrease in ATP levels (to 35% and 12% of control ATP levels, respectively) but did not lyse. This indicated that energy deprivation alone (at least to the levels achieved) is not sufficient to induce toxin-like cytolysis and suggested that, rather, the imbalance in Na^+ and K^+ might induce both ATP depletion and later effects. To determine whether inhibition of the Na^+ and K^+ fluxes across the plasma membrane induced energy depletion, we challenged cells in medium that was isoionic (10 mM NaCl, 150 mM KCl) and contained various concentrations of sucrose. As shown in Table 2, prevention of both monovalent cation fluxes and water movement, by balancing the intra- and extracellular concentrations of Na^+ , K^+ , and total solutes, virtually completely blocked ATP depletion, later events, and cytolysis. In additional studies, replacing the protective challenge solution with control medium restored LeTx-induced ATP depletion within 15 min, indicating that the conditions employed did not inhibit toxin binding or internalization.

DISCUSSION

LeTx triggers a cascade of events in macrophages that results in cell lysis within 2 h. Data presented in this paper define early physiological and biochemical alterations that precede lysis and may be key elements in

the lethal cascade. ATP depletion and increased permeability to ^{86}Rb and ^{22}Na , beginning at ~ 45 min, are now the earliest reported cellular responses attributable to LeTx. Although our studies did not distinguish ATP depletion from changes in ^{22}Na and ^{86}Rb fluxes chronologically, experiments with isoionic and isosmotic medium suggested that membrane fluxes of Na^+ , K^+ , and water may be responsible for ATP hydrolysis, and ultimately all other pathological effects of LeTx. Cellular energy levels and regulation of ions are intimately coupled, and it seems likely that the cell depletes its ATP pool in an attempt to compensate for altered ion imbalances.

To test if fluxes of Na^+ and K^+ also played a role in later nonspecific insults, perhaps via gross water influx, toxin challenges were performed in the presence of sucrose. The use of osmotic stabilizers in the challenge medium has been instrumental in delineating steps involved in the pathology of many cytolytic toxins (Bernheimer, 1947; McClane, 1984; Harris *et al.*, 1991). Osmotic stabilizers neutralize osmotic swelling resulting from ion imbalances and influx of water into the cell. It is clear from the current study that LeTx late events, including the influx of ^{45}Ca , are prevented by the addition of sucrose to the challenge medium. This protection is not merely due to inhibition of toxin binding or internalization because replacement of sucrose-containing media with sucrose-free medium rapidly restores LeTx activities. Also, early LeTx events occur (albeit to a lesser degree) in osmotically stabilized challenge medium with the same kinetics as in sucrose-free media. These findings suggest that while later events and cy-

Table 2. Protection of J774A.1 cells from LeTx using a combination of osmotic and ionic challenge conditions

Challenge solution	% Maximal ATP loss after addition of LeTx ^a	% Maximal cytolysis after addition of LeTx ^b
a. Normal medium	100%	100%
b. 10 mM Na^+ , 150 mM K^+	86%	82%
c. 0.30 M sucrose	50%	2%
d. 0.30 M sucrose + b	5%	1%
e. 0.125 M sucrose + b	4%	5%
f. 0.20 M sucrose + b	7%	3%
g. 0.15 M sucrose + b	11%	7%
h. 0.10 M sucrose + b	59%	65%
i. 0.05 M sucrose + b	91%	77%

^a Cellular ATP concentrations were measured 90 min after addition of toxin to cells by the luciferin/luciferase bioluminescence assay described in Figure 1. Nontoxin-challenged cells (0% loss of ATP) contained $\sim 1.5 \times 10^{-11}$ mol ATP/ μg protein. At 100% ATP loss, cellular concentrations were below 0.05×10^{-11} mol ATP/ μg protein. Figures represent the mean of three trials performed in duplicate and the SEM for each point was $<9\%$ of the indicated value.

^b Cytolysis was assayed 90 min after the addition of toxin to cells with the ^{51}Cr release assay of Figure 2b. In these experiments 100% cytolysis represents release of $\sim 55\%$ of total available cellular ^{51}Cr . Data was normalized for spontaneous release of radiolabel ($\sim 5\%$ total radiolabel) as described in MATERIALS AND METHODS. Figures represent the mean of three trials performed in duplicate and the SEM for each point was $<10\%$ of the indicated value.

tolysis depend on perturbation of the colloid-osmotic balance, early LeTx events can occur in the absence of gross water influxes.

Experiments were also performed to define, more precisely, where in the cytolytic cascade Ca^{2+} influxes fit. A strict dependence on influx of Ca^{2+} for LeTx-induced cytolysis has been clearly demonstrated (Bhatnagar *et al.*, 1989). It is conceivable that the early events described in this report and the consequent water influx serve primarily to disrupt Ca^{2+} homeostasis, and that increased cellular Ca^{2+} is then responsible for much of the subsequent damage. Results from this study showing that prevention of LeTx-induced Na^+ , K^+ , and water fluxes, with protective challenge media, prevent both Ca^{2+} influx, and cytolysis support this hypothesis. The early toxin events may directly affect cellular $[\text{Ca}^{2+}]$, 1) the plasma membrane-bound Ca^{2+} pumps responsible for maintaining low intracellular Ca^{2+} concentrations require ATP (Carfoli, 1987), 2) Ca^{2+} -channels can be regulated (e.g., opened) by changes in the levels of other ions, including Na^+ (Carafoli, 1987), and 3) Na^+ -induced entry of water can disrupt plasma membrane integrity, which permits gross Ca^{2+} influx (Bernheimer, 1947; Bhakdi and Trantum-Jensen, 1988; McClane *et al.*, 1988a). It is well recognized that increased cytoplasmic Ca^{2+} affects many metabolic systems and, at high concentrations, is toxic to cells, disrupting cytoskeletal structure, activating nucleases, proteases, phospholipases etc., and contributing to cell rupture (Orrenius *et al.*, 1989). There is also evidence accumulating of a synergistic relationship between disrupted Ca^{2+} homeostasis and oxygen free radicals as common mediators of cell death (Boobis *et al.*, 1989, Orrenius *et al.*, 1989), but this relationship has not yet been established in the LeTx-challenged M ϕ .

Multistep cascade patterns similar to those proposed for LeTx have been reported for other cytolytic toxins. For example, *Clostridium perfringens* enterotoxin induces early, Ca^{2+} -independent, events (including perturbations in membrane permeability to Na^+ and K^+) in the absence of lysis, and late, Ca^{2+} -dependent, events including generalized membrane damage, macromolecular synthesis inhibition, and lysis (McClane *et al.*, 1988b). Additionally, staphylococcal alpha toxin, a pore-forming toxin, allows passage of monovalent cations through the membrane, initiating a physiologic cascade with cytolytic consequences (Bhakdi and Trantum-Jensen, 1988). Evidence is also accumulating that an *Escherichia coli* haemolysin family of cytolytic toxins acts in a multistep manner. For instance, experiments utilizing low "sub-lytic" doses of *E. coli* haemolysin showed a twofold decrease in cellular ATP levels in both human monocytes and rat renal tubular cells in the absence of lysis (Welch, 1991). This concentration-dependence pattern, at a superficial level, resembles the LeTx dose-response data presented in Fig. 5.

Dramatic increases in membrane permeability to ions, ATP depletion, inhibition of synthetic processes, and cytolysis are diagnostic of a broad category of cell death commonly called necrosis (Wyllie *et al.*, 1980, Bobbis *et al.*, 1989). It is interesting to note that most well-characterized cytolytic toxins that elicit these necrotic cellular symptoms are membrane-active toxins, either forming physical pores in the plasma membrane (e.g., staphylococcal α -toxin, *E. coli* haemolysin) or else directly altering membrane functions (e.g., *C. perfringens* enterotoxin) (Bhakdi and Trantum-Jensen, 1988; McClane *et al.*, 1988a; Welch, 1991). In these instances, toxin activity is directed to the plasma membrane with no requirement for endocytosis or translocation into the cytoplasm. LeTx activity, in contrast, requires internalization, passage through an acidic vesicle, and (presumably) translocation into the cytoplasm (Friedlander, 1990). Unlike other membrane-active toxins, LeTx is specific for M ϕ s although it can bind to and enter most cell types (Friedlander, 1990; Leppla, 1991). In general, the membrane-active toxins tend to affect all cell types that permit binding. LeTx did not induce any of the characteristics of "apoptosis" (programmed cell death) (Wyllie *et al.*, 1980), including fragmentation of chromosomal DNA into nucleosome-sized ladders that are seen with certain other toxins acting in the cytoplasm (e.g., diphtheria toxin) (Chang *et al.*, 1989, Kochi and Collier, unpublished data).

Collectively, data presented here suggest a lethal scenario in which LeTx increases macrophage permeability to Na^+ and K^+ , which results in hydrolysis of ATP, and also contributes to misregulation of cellular colloid-osmotic equilibrium, Ca^{2+} influxes (and the serious Ca^{2+} -induced pathologies), global inhibition of macromolecular synthesis, changes in cell morphology, and generalized membrane rupture. Whether an Anthrax lethal toxin activity directly affects membrane permeability to selective ions or whether there are additional intermediate steps in the intoxication cascade remains to be determined.

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