Internalization and Processing of *Bacillus anthracis* Lethal Toxin by Toxin-sensitive and -resistant Cells* 

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Anthrax lethal toxin consists of two separate proteins, protective antigen and lethal factor (LF). Certain macrophages and a mouse macrophage-like cell line, J774A.1, are lysed by low concentrations of lethal toxin. In contrast, another macrophage cell line, IC-21, and all other cell types tested were resistant to this toxin. To discover the basis for this difference, each step in the intoxication process was examined. No differences between sensitive and resistant cells were found in receptor binding or proteolytic activation of protective antigen, steps that are required prior to LF binding. To determine whether resistance results from a defect in translocation to the cytosol, we introduced LF into J774A.1 and IC-21 cells and a nonmacrophage cell line (L6 myoblast) by osmotic lysis of pinocytic vesicles. Only J774A.1 cells were lysed; no effect was observed in IC-21 and L6 cells. These results suggest that resistant cells either lack the intracellular target of LF or fail to process LF to an active form. The relatively low potency of LF introduced into J774A.1 cells by osmotic lysis suggests that protective antigen may also be required at a stage subsequent to endocytosis.

*Bacillus anthracis* produces two toxins, which we designated edema toxin and lethal toxin (1–3). Both toxins fit the general model of many protein toxins in possessing a binding component (B domain), responsible for binding to receptor, and an active component (A domain) possessing toxic, and usually enzymatic activity. The anthrax toxins diverge from the structure of most AB-type toxins in two respects: first, the A and B domains exist as two separate, noncovalently linked proteins; and second, the edema and lethal toxins share a common B component. A total of three proteins are involved. Thus, the anthrax lethal toxin consists of protective antigen (PA, 82.7 kDa) and lethal factor (LF, approximately 83 kDa), while the edema toxin consists of PA and edema factor (EF, 88.8 kDa). Consistent with the AB model, each individual component lacks toxicity. Edema toxin produces edema in the skin of animals (1), a response which led to the demonstration that EF is a calmodulin-dependent adenylate cyclase (4). Lethal toxin kills a number of species of experimental animals (1, 5) and is assumed to be the major factor causing death in anthrax. No enzymatic activity has yet been demonstrated to be associated with LF, and the nature of the putative intracellular target of LF is unknown. The genes for all three of the anthrax toxin proteins have been cloned and sequenced (6–8).

Recent studies (9) have shown that PA binds to high affinity cell-surface receptors and is then cleaved by a cell-surface protease at residue Arg-167, releasing the NH2-terminal fragment (19.2 kDa). The receptor-bound, COOH-terminal, 63.5-kDa fragment (PA63) contains a site to which LF or EF binds with high affinity. Subsequently the complex is internalized by receptor-mediated endocytosis and translocated into the cytosol (3).

Edema toxin (PA + EF) has been shown to raise intracellular cAMP levels in many types of eukaryotic cells, suggesting that most cells possess PA receptors and internalize EF (4). In contrast, lethal toxin (PA + LF) has been shown to be highly cell type-specific. Macrophages from C3H mice are lysed by lethal toxin while nonmacrophage-like cells are resistant (3). In order to characterize the mechanism of toxin internalization and to identify the mechanism of action of LF, we compared the action of lethal toxin on sensitive and resistant cells.

**EXPERIMENTAL PROCEDURES**

Cells obtained from the American Type Culture Collection (Rockville, MD) included J774A.1 (10) and IC-21 (11) and a rat myoblast line, L6 (12). For most experiments, cells (2–3 × 10⁶ in 1 ml) were grown in 2-cm² wells of 24-well tissue culture plates in Dulbecco's modified Eagle's medium (for J774A.1 and L6) or RPMI medium (for IC-21) for 48 h before use. All media contained 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). PA and LF were purified as described earlier (13). Gelonin was obtained from Sigma. For measuring cytotoxicity, lethal toxin (PA + LF) was added directly into media and cultures were incubated 3 h. Lactate dehydrogenase released in the cells was assayed as described previously (5). For assay of toxin binding and nicking, PA was labeled with [125I]-Bolton-Hunter reagent (2000 Ci/mmol, Amersham Corp.) to approximately 7.5 × 10⁶ cpm/µg. Labeled PA (3 × 10⁶ cpm/well) was incubated with cells for 8 h at 4°C in wells containing 0.6 ml of minimum essential medium with Earle's salts (EMEM) without bicarbonate and containing 1% bovine serum albumin (BSA) and 20 mM Hepes, pH 7.4. Cells were washed 4 times with cold EMEM and dissolved in sodium dodecyl sulfate (SDS) sample buffer (final concentrations: 5% glycerol, 1% SDS, 100 mM diethiothreitol, and 0.01% bromphenol blue). After counting to determine cell-associated radioactivity, a portion of the sample was analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography. Bands were excised and counted to determine the conversion of native PA (83 kDa) to PA63.

The procedure for osmotic lysis of pinosomes was essentially that previously described (14, 15). Briefly, 2–5 × 10⁶ cells were suspended by scraping (J774A.1 and IC-21) or by trypsin treatment (L6), washed with Ca- and Mg-free phosphate-buffered saline, and incubated 10 min at 37°C in 0.5 ml of osmotic lysis medium (1 M sucrose in L-15 medium containing 1% BSA) containing various amounts of LF.

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1 The abbreviations used are: PA, protective antigen; LF, lethal factor; EF, edema factor; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PA63, COOH-terminal 63.5-kDa fragment of PA; EMEM, minimum essential medium with Earle's salts; SDS, sodium dodecyl sulfate.

2 D. Robertson, personal communication.
Sensitivity to Anthrax Lethal Toxin

After this time, 10 ml of hypotonic medium (six parts of L-15 to four parts of water) was added, and the cells incubated for 2 min at 37 °C. The cells were centrifuged, washed three times with normal culture medium, and plated in the recommended medium in 24-well dishes. After incubation for 12 h at 37 °C, inhibition of protein synthesis was determined by incorporation of [35S]methionine (800 Ci/mmol, Amer sham Corp.) as described previously (16), and cytotoxicity was measured by lactate dehydrogenase release.

RESULTS AND DISCUSSION

To study the basis of sensitivity and resistance to anthrax lethal toxin, we selected two mouse macrophage-like cell lines, J774A.1 and IC-21, and a rat myoblast line, L6. The latter was found in preliminary experiments to have a large number of receptors. Cells were exposed to PA (0.1 µg/ml) and increasing concentrations of LF for 3 h (Fig. 1). LF at 0.1 µg/ml caused 82% cell death in J774A.1 cells, while L6 and IC-21 did not show any cytotoxicity at any concentration of LF tested. Even at 50 µg/ml, LF was not toxic to L6 and IC-21 cells in the presence of PA. We tested several other cell lines and primary cells and found that all were resistant to lethal toxin (data not shown). Earlier studies showed that the growth rates of certain cell lines were decreased when cells were plated at very low densities and exposed to lethal toxin for several days. However, no acute effects or cell death were noted in those cells.

The first step in the expression of toxicity of lethal toxin is binding of PA to the cell-surface receptor. Therefore, we attempted to measure any differences in binding of PA to these cell lines. The data presented in Table I show that all three types of cells bound PA. Because a near-saturating concentration of PA was used, and nonspecific binding was <20% of the total, the amount of PA bound was an accurate estimate of the number of binding sites. For the macrophage lines, we found approximately 8000 sites/cell. The amount of PA bound to the cell lines showed no correlation with sensitivity to lethal toxin.

The next step in the process of toxicity is proteolytic cleavage of PA bound to cell-surface receptors. To determine whether both sensitive and resistant cell types could cleave PA, cells were incubated with 125I-PA at 4 °C for 8 h, and cell-associated PA was analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 2 shows that sensitive and resistant cells cleaved PA to PA63. The L6 and J774A.1 cells cleaved about 60% of the PA, while the IC-21 cells cleaved 30%. In longer incubations, the PA was completely nicked by all three cell lines (data not shown). These results show that the resistance of L6 and IC-21 was not due to inability to cleave PA.

LF and EF bind avidly to the receptor-bound PA63 and are believed to enter cells by endocytosis, with subsequent delivery to the cytosol where LF is assumed to inactivate an essential target cell process. No biochemical methods are known for measuring productive internalization of the PA63-LF complex. If the defect in toxin-resistant cells is at this stage, it would follow that such cells could be killed if LF were introduced into the cytoplasm by artificial means. Therefore, we tested indirectly for the integrity of the internalization process by putting LF into the cytosol through osmotic lysis of pinosomes (15). Conditions for osmotic lysis in macrophages and L6 cells were standardized by using the single chain, ribosome-inactivating protein, gelonin, which has no cell-binding capacity and is not toxic when added externally. Gelonin introduced into the cytosol of J774A.1 and L6 cells by osmotic lysis inhibited protein synthesis in a dose-dependent manner with a 50% effective concentration of about 100 ng/ml in both cell types (data not shown). This positive control was run in parallel with each experiment.

Different concentrations of LF were internalized into sensitive and resistant cells by osmotic lysis of pinosomes. Fig. 3 shows the toxicity (lactate dehydrogenase remaining in the cells, panel A) and the [35S]methionine incorporation (panel B) 12 h after internalization of LF. LF induced 85% leakage of cellular lactate dehydrogenase and 95% inhibition of protein synthesis in J774A.1 cells. There was no significant change in lactate dehydrogenase level or capacity to synthesize proteins in IC-21 or L6 cells after internalization of LF. LF alone added externally had no effect on lactate dehydrogenase release or protein synthesis in any of the cell lines, even when incubated for 12 h at concentrations of 500 µg/ml (data not shown). The ability of LF internalized by osmotic lysis to kill J774A.1 is the first direct evidence that LF has an effector activity, as is required if lethal toxin is to conform to the AB model of toxins. This suggests that LF, like EF, may have an enzymatic activity that can function in the absence of PA.

The resistance of IC-21 and L6 to LF even after osmotic

Table I

| Cell type | PA bound (ng/mg cell protein) |
|-----------|-----------------------------|
| J774A.1   | 4.11 ± 0.16                 |
| L6        | 11.60 ± 0.36                |
| IC-21     | 5.08 ± 0.30                 |

3 S. Leppla, unpublished studies cited in Ref. 2.
bating in cold 

The medium was replaced with cold 

with cold 

cultured in 12-well tissue culture plates. Cells were cooled by incu-

J774A.1; 

surface proteases in sensitive and resistant cells. Cells were 

containing 1% BSA and 25 mM Hepes and 125I-PA (1 pg/ml, 1 pg 

3000 cpm was loaded in each lane. Lane A, 125I-PA stock; lane B, 

Cy-21; lane C, IC-21; lane D, L6.

lysis shows that these cells lack the ability to perform some 

step subsequent to, or distinct from, the translocation of toxin 

to the cytosol. A defect in internalization could not explain 

the differences in sensitivity seen in Fig. 3. The suggestion 

that cells resistant to lethal toxin are able to internalize LF 

is consistent with earlier evidence that most cell types are 

sensitive to anthrax edema toxin (4, 17). Since LF and EF 

compete for binding to PA63 and for internalization (4), it is 

probable that most cells do internalize LF. The conclusion 

drawn here differs from that reached after analysis of the 

resistance of mouse and rat cells to diphtheria toxin, where 

resistance was attributed to a defect or lack of the transport 

process (18, 19).

We also used the osmotic lysis technique to test whether 

LF is active in resistant cells only when complexed with PA63. 

In a single preliminary experiment, a mixture of LF and PA63 

(200 µg/ml each) had no effect on IC-21 cells treated with the 

osmotic lysis procedure. This result lends some support to the 

idea that resistant cells lack the intracellular target of LF.

The work described here was initiated to increase our 

understanding of anthrax lethal toxin cell entry, to account 

for the toxin's specificity for macrophages, and to help define 

the molecular or enzymatic mechanism of action of LF. The 
evidence that resistant cells bind and process toxin in the 
same manner as sensitive ones eliminates these steps as 
explanations for differences in sensitivity. The fact that LF 
alone or together with PA63 was unable to kill resistant cells, 
even when internalized by osmotic lysis, suggests that resist-

ant cells may entirely lack the putative intracellular target of 

LF action. Alternatively, resistant cells may be unable to 

activate LF. Possible activating events include binding of an 
esential enzymatic cofactor (analogous to the requirement of 
EF for calmodulin binding), transport of LF to a particular 
intracellular compartment, or proteolytic processing of LF. 

We consider that proteolytic activation is the least probable 
explanation, because preliminary experiments showed radio-
labeled LF was not cleaved in a specific manner after inter-

nalization, and partial proteolysis with several different en-

zymes failed to increase the potency of LF used for osmotic 

lysis.

While the data of Fig. 3 show that LF does have effector 
activity, the results are not those predicted if LF is a simple 
enzyme, like gelonin, that needs only to be delivered to the 
cytosol to achieve toxicity. Fig. 3 shows that the concentration 
of LF required to achieve killing of J774A.1 when internalized 
by osmotic lysis (50 µg/ml) was more than 1000-fold that 
needed when uptake occurred by receptor-mediated endocy-
tosis with PA. This ratio of potencies is very different from 
the analogous ratios that can be calculated for other toxins, 
such as the many plant proteins that inactive ribosomes. 
Proteins of this type include both those that fit the AB model 
(e.g. ricin) and those having a single (A) chain (e.g. gelonin). 
The single chain, ribosome-inactivating proteins are effective

4 S. Leppla, unpublished data.

**Fig. 2.** Proteolytic cleavage of protective antigen by cell 
surface proteases in sensitive and resistant cells. Cells were 
cultured in 12-well tissue culture plates. Cells were cooled by incu-
bating in cold EMEM for 10 min and then placing the plates on ice. 
The medium was replaced with cold EMEM without bicarbonate 
containing 1% BSA and 25 mM Hepes and 125I-PA (1 µg/ml, 1 µg = 
7.5 × 10⁶ cpm) and kept at 4 °C for 8 h. Cells were washed four times 
with cold EMEM, solubilized in SDS sample buffer, and analyzed on 
10% SDS-polyacrylamide gel electrophoresis. A volume containing 
3000 cpm was loaded in each lane. Lane A, 125I-PA stock; lane B, 
J774A.1; lane C, IC-21; lane D, L6.

**Fig. 3.** Toxicity of LF for sensitive and resistant cells after 
internalization by osmotic lysis of pinocytic vesicles. Cells were 
suspended in hypertonic medium (1 M sucrose in L-15 medium 
containing 1% BSA) containing different amounts of LF for 10 min 
at 37 °C, then exposed for 2 min to hypotonic medium (six parts of 
L-15 and four parts of water), washed, and plated. After 12 h incubation, lactate dehydrogenase (LDH) leakage (panel A) and protein 
synthesis (panel B) were determined.
at concentrations of 100–500 ng/ml when applied to cells either by osmotic lysis (20) or by attachment to receptor ligands (21). The AB molecules, such as ricin, when added externally, or the isolated ricin A chain introduced by osmotic lysis, also have generally similar effective concentrations (20). It follows that the osmotic lysis method is approximately as efficient in delivering proteins to the cytosol as are most receptor-mediated events. If one assumes that the osmotic lysis experiments that we performed also achieved cytosolic levels of LF approaching those occurring when PA is present, then it must be concluded that LF introduced alone into the cytosol has surprisingly low activity. The low potency of LF internalized by osmotic lysis suggests that introduction of LF alone into the cytosol is not sufficient to kill macrophages. This suggests that the PA63 protein may be required in some way to promote the toxic activity of LF. PA63 binding might be required to enzymatically activate LF; alternatively, interaction with PA63 and receptor may be needed to present LF correctly to an intracellular enzyme that activates LF.

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