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Characterization of Macrophage Sensitivity and Resistance to Anthrax Lethal Toxin

ARTHUR M. FRIEDLANDER,* RAKESH BHATNAGAR, STEPHEN H. LEPPLA,† LARRY JOHNSON, AND YOGENDRA SINGH†

U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21702-5011

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Anthrax lethal toxin, which consists of two proteins, protective antigen and lethal factor, is cytolytic for macrophages. Macrophages from different mouse strains were found to vary in their sensitivities to toxin. C3H mouse macrophages lysed by lethal factor concentrations of 0.001 μg/ml were 100,000 times more sensitive than those from resistant A/J mice. We analyzed various stages of the intoxication process to determine the basis for this resistance. Direct binding studies with radioiodinated protective antigen revealed that the affinity (K_d, approximately 0.5 nM) and number of receptors per cell (25,000 to 33,000) were the same in sensitive and resistant cells. Proteolytic activation of protective antigen by a cell surface protease and subsequent binding of lethal factor were also the same in both sensitive and resistant macrophages. Resistant A/J macrophages were not cross-resistant to other toxins and a virus which, like lethal toxin, require vesicular acidification for activity, implying that resistance is not due to a defect in vesicular acidification. When introduced into the cytosol by osmotic lysis of pinosomes, lethal factor in the absence of protective antigen was cytolytic for the sensitive macrophages while resistant cells were unaffected. Thus, lethal factor by itself possesses the toxic activity of lethal toxin. These results suggest that macrophage resistance is due to a defect at a stage occurring after toxin internalization. A/J macrophages may lack the putative lethal factor target in the cytosol or be defective in the further processing or activation of lethal factor in the cytosol or in endocytic vesicles.

The two anthrax protein exotoxins, edema toxin and lethal toxin, are important virulence factors of *Bacillus anthracis*. The toxins are similar to many other bacterial and plant protein toxins in having a binding (B) component responsible for binding the toxin to receptors on target cells and an active (A) component responsible for toxic activity (17, 27). The anthrax toxins are unusual in that the B and A components exist as separate, noncovalently linked proteins. They are unique in that they have identical B components. The lethal toxin consists of the B protein, protective antigen (PA) (82.7 kDa), together with a second protein, lethal factor (LF) (approximately 83 kDa). The same B protein, PA, together with a third protein, edema factor (EF) (88.8 kDa) composes the edema toxin. The edema toxin produces edema when injected into the skin of experimental animals (1, 53), and EF has been identified as a calcium- and calmodulin-dependent adenylate cyclase (27). The lethal toxin is lethal for animals of several species (1, 53), but the nature of the toxic and presumed enzymatic activity of LF remains unknown. Our initial studies (29) with radioactive toxin components confirmed the B+A model and showed that cells bind PA to a single class of high-affinity receptors. More recent work has confirmed this observation (10). The PA is then cleaved at or near Arg-167 by a cell surface protease, generating a cell-bound, C-terminal, 63-kDa protein (PA63) which now possesses a second binding site to which LF or EF binds with high affinity. The N-terminal fragment produced, of approximately 20 kDa, is released from the cell surface. The cell-bound complex of the 63-kDa PA plus LF or EF is then transported inside the cell by receptor-mediated endocytosis, passing through an acidic compartment before translocation to the cytosol (12, 18). This model is supported by the recent finding that deletion of the protease-sensitive site of PA near Arg-167 creates a protein that is resistant to cleavage by the cell surface protease and nontoxic when given with LF (51). Further evidence is provided by the observation that the 63-kDa PA, under acidic conditions, can insert and form channels in membranes (24).

We previously reported that macrophages from C3H mice are lysed by anthrax lethal toxin while nonmacrophage cell lines are resistant, suggesting that macrophages are uniquely sensitive to the acute cytolytic effects of the toxin (12). In this report, we show that macrophages from some mouse strains are resistant to lethal toxin and examine the basis for this resistance, by using sensitive and resistant cells, in our attempts to understand the mechanism of action of anthrax lethal toxin.

MATERIALS AND METHODS

Cell culture and cytotoxicity. Peritoneal exudate macrophages induced by starch or thioglycolate were obtained from C3H/HeNHsd (C3H) (Harlan-Sprague-Dawley, Inc., Walkersville, Md.) and A/J (Jackson Laboratories, Bar Harbor, Maine) mice. Cells (5×10^5 to 7.5×10^5) were cultured in 2-cm^2 24-well tissue culture plates as previously described (12), unless otherwise indicated. Cytotoxicity was determined by the release of cytoplasmic lactic dehydrogenase (LDH) from the cell monolayer (12) and by inhibition of protein synthesis measured by incorporation of [^{35}S]methionine (2). Results from representative experiments are presented as the percentages of cellular LDH remaining in control cells incubated without toxin, based on the means of triplicate samples differing by less than 10%. The susceptibilities of C3H and A/J macrophages to lethal toxin were the same whether the cells were induced by starch or thioglyco-

* Corresponding author.
† Present address: Laboratory of Microbial Ecology, National Institute of Dental Research, Bethesda, MD 20892.

late. Unless indicated, all experiments used starch-induced cells.

Toxin binding and analysis. PA was radioiodinated with ^{125}I-labelled Bolton-Hunter reagent (2,000 Ci/mmol; Amersham, Chicago, Ill.) to a specific activity of 1×10^7 to 1.5×10^7 cpm/μg and LF was labelled with chloramine T to a specific activity of 1.5×10^7 to 2×10^7 cpm/μg, respectively, as described previously (2).

For direct binding studies, cells in 2-cm^2 wells were cooled to 4°C, washed, and incubated for the indicated times with ^{125}I-PA in cold (4°C) minimum essential medium with Earle's salts, without bicarbonate but containing 1% bovine serum albumin and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4 (binding medium). The cell monolayers were then washed and dissolved in 0.1 N NaOH; bound radioactivity was determined, and cell protein was measured on duplicate or triplicate samples by an automated Lowry procedure (30). Specific binding was determined by the difference in the binding that occurred in the absence and presence of a 100-fold molar excess of unlabelled PA. For binding of ^{125}I-LF, cells in culture (2.5×10^6 in 4.5-cm^2 wells) were cooled, preincubated in binding medium with unlabelled PA, washed, and reincubated at 4°C with various concentrations of ^{125}I-LF, as indicated below. Specific binding was determined by using unlabelled LF as described for ^{125}I-PA. Binding data were analyzed by the method of Scatchard by using the LIGAND program (39).

For determination of proteolytic cleavage of cell-bound ^{125}I-PA to PA63, the cell monolayers were washed, solubilized in sodium dodecyl sulfate (SDS) sample buffer (0.0625 M Tris-HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue, pH 6.8), and analyzed on 10% gels by SDS-polyacrylamide gel electrophoresis and autoradiography.

Viral replication in macrophages. Vesicular stomatitis virus, Indiana strain, originally obtained from the American Type Culture Collection, was provided by P. Jahrling, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md. Viral stocks were grown in BHK cells. Macrophages grown in 2-cm^2 wells were infected with a multiplicity of infection of 1 for 1 h at 37°C as previously described (25). Viral growth at various times was measured by plating dilutions of the medium and counting plaques on Vero cells.

Introduction of LF into the cytosol. Gelonin and LF were introduced directly into the cytosol of cells by the method of osmotic lysis of pinosomes (44). Cells cultured in 2-cm^2 wells were washed once with hypertonic medium (0.5 M sucrose, 10% polyethylene glycol, 1% bovine serum albumin in L-15 medium) and incubated in 0.2 ml of hypertonic medium containing various amounts of gelonin or LF for 9 min at 37°C. The monolayer was then washed with 2 ml of hypotonic medium (six parts L-15 medium to four parts water) and incubated in 2 ml of hypotonic medium for 2.5 min. Cells were then washed and reincubated in normal medium for 16 h, at which time cytotoxicity was measured by inhibition of protein synthesis or release of cellular LDH as described above.

Reagents. PA and LF were purified as previously described (28), as was *Pseudomonas* exotoxin A (26). Modeccin was obtained from Pierce Chemicals (Rockford, Ill.), the transferrin-CRM 107 diphtheria toxin conjugate was generously supplied by V. Johnson and R. Youle (National Institutes of Health, Bethesda, Md.), gelonin was from Sigma Chemicals (St. Louis, Mo.), polyethylene glycol 1000 was from J. T. Baker Chemical Co. (Philipsburg, N.J.), and [^{35}S]methionine (1,100 Ci/mmol) was from New England Nuclear Corp. (Boston, Mass.).

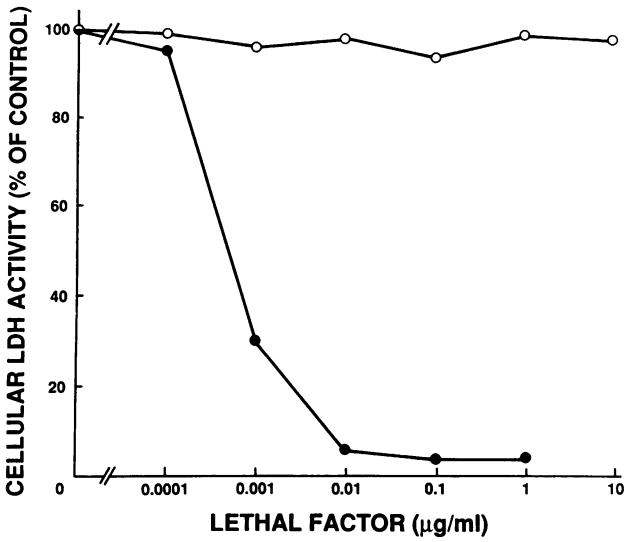

FIG. 1. Relative sensitivities of C3H (●) and A/J (○) macrophages to anthrax lethal toxin. Macrophages from each strain were exposed to PA (1 μg/ml) together with various concentrations of LF. After incubation at 37°C for 18 h, cytotoxicity was determined by measuring the amount of LDH released from the cell monolayer. Controls consisted of cells incubated without toxin. PA itself at 1 μg/ml caused no LDH release.

RESULTS

Our initial studies showed that peritoneal macrophages from C3H mice were lysed by anthrax lethal toxin, with the minimal effective concentration of LF being 0.001 μg/ml (12). Preliminary studies revealed that macrophages from different mouse strains were either sensitive, like C3H, or resistant (13). Macrophages from the A/J mouse strain were highly resistant to cytolysis by lethal toxin, and we used them in this study to determine the basis of resistance by comparing sensitive and resistant cells. Figure 1 shows the relative sensitivities of C3H and A/J macrophages to cytolysis by anthrax lethal toxin. In the presence of excess PA (1 μg/ml), C3H macrophages were lysed by LF concentrations of 0.001 μg/ml while A/J macrophages were resistant to 10 μg/ml. In other experiments, A/J macrophages showed less than 50% cytolysis even when exposed to LF and PA, each at 100 μg/ml (data not shown). Thus, the A/J macrophages were more than 100,000 times less sensitive to lysis by anthrax lethal toxin than were C3H cells. These results were confirmed by measuring cytotoxicity by trypan blue exclusion and protein synthesis inhibition (data not shown).

Toxin binding and proteolytic activation by C3H and A/J cells. To understand the basis for the marked difference in susceptibilities to lethal toxin, we analyzed and compared various stages of the intoxication process in the sensitive C3H and resistant A/J macrophages. The first step in the interaction of lethal toxin with cells is binding of PA to a cell surface receptor. To determine whether resistance could be due to deficiencies in the receptor itself, we measured the binding of ^{125}I-PA to C3H and A/J macrophages. Scatchard analysis of the binding data (Fig. 2) revealed that both sensitive C3H and resistant A/J cells had a single class of

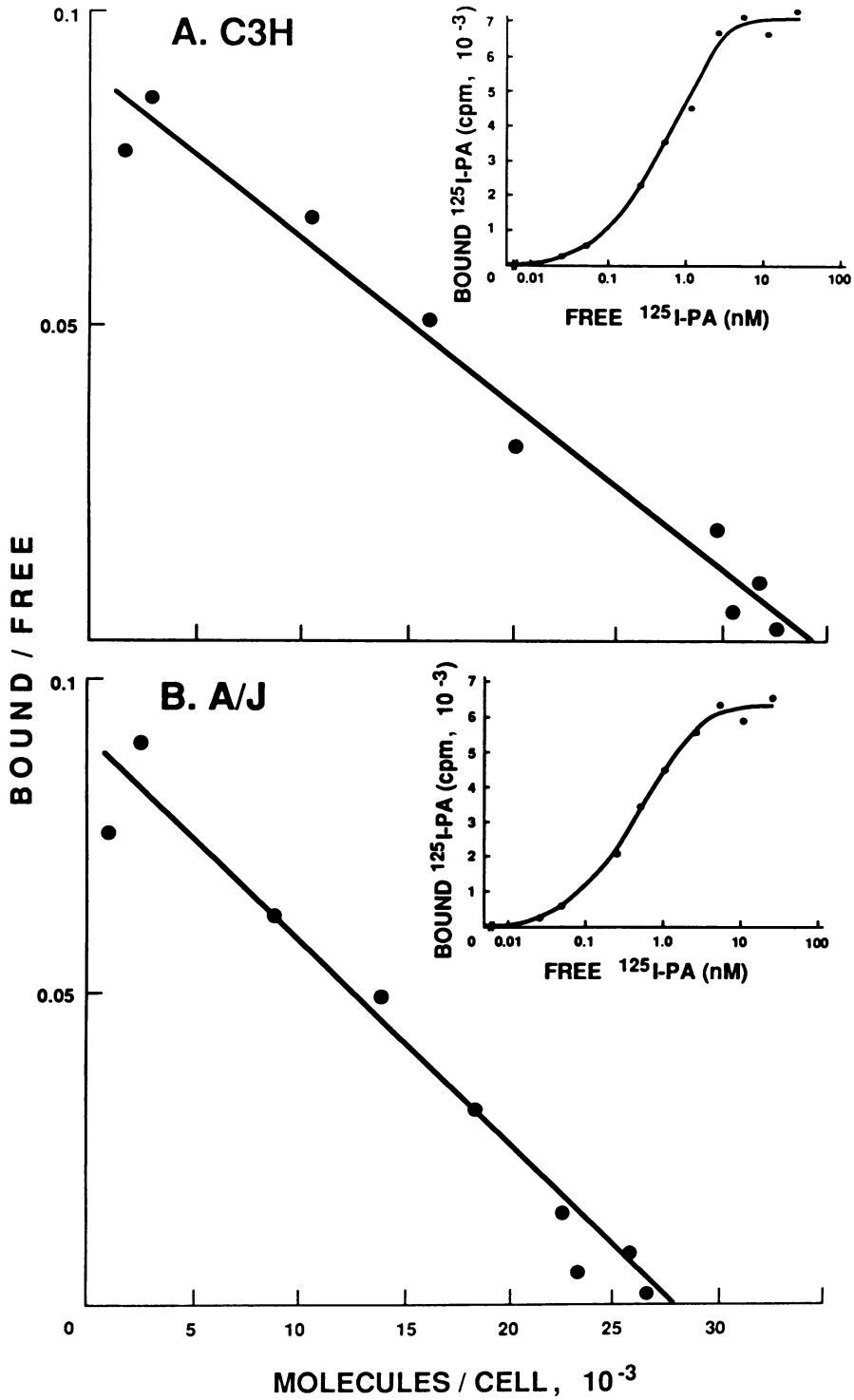

FIG. 2. Scatchard analysis of direct binding of ^{125}I-PA to sensitive C3H and resistant A/J macrophages. Cells were incubated with increasing concentrations of ^{125}I-PA (0.028 to 28 nM) for 16 h at 4°C in the absence or presence of a 100-fold molar excess of unlabelled PA. Specifically bound radioactivity was determined and the data were analyzed by the method of Scatchard by using the LIGAND program as described in Materials and Methods. (Insets) specific binding of ^{125}I-PA plotted against the concentration of free ^{125}I-PA at equilibrium.

high-affinity receptors for PA with essentially identical dissociation constants (K_ds, 0.59 and 0.50 nM, respectively) and similar numbers of binding sites per cell (32,900 and 25,600, respectively). Similar results were obtained in other experiments measuring the displacement of radioactive PA by increasing amounts of unlabelled ligand, suggesting that the radioactive PA behaved identically to the unlabelled PA (data not shown). Thus, the resistance of A/J macrophages

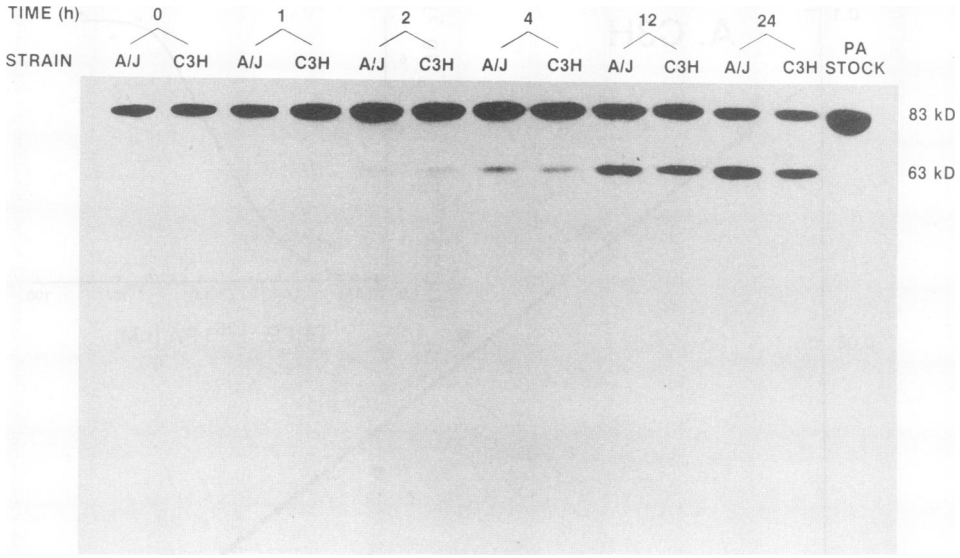

FIG. 3. Time course of proteolytic cleavage of cell surface PA by sensitive C3H and resistant A/J macrophages. Cells cultured in 2-cm^2 wells were cooled by incubation on ice in cold Hank's balanced salt solution for 10 min. ^{125}I-PA (1 μg/ml) in cold binding medium was then added and cells were incubated on ice for 1 h. The medium was then removed, and cells were washed with cold medium four times and reincubated in binding medium on ice for various times. After the 1-h incubation with ^{125}I-PA (0 time) and at the indicated times after reincubation, cells were washed and solubilized in SDS sample buffer and equal counts for both cell types at each time point were analyzed on 10% gels by SDS-polyacrylamide gel electrophoresis and autoradiography. PA, stock of ^{125}I-PA applied to cells. The numbers on the right indicate the positions and apparent molecular masses of the PA species.

was not due to a lack or deficiency in the receptor itself, as determined by its binding characteristics.

We next examined whether resistance could be due to an inability to proteolytically activate the cell-bound, intact 82.7-kDa PA to PA63, thereby failing to generate the binding site for LF. Sensitive and resistant cells were incubated with ^{125}I-PA for 1 h at 4°C. The cells were then washed and reincubated at 4°C for various periods of time. As seen in Fig. 3, the preparation of ^{125}I-PA consisted essentially entirely of the intact 82.7-kDa species. The ^{125}I-PA bound to cells after a 1-h exposure similarly has a molecular mass of 82.7 kDa, as expected. However, there was progressive conversion, over time, of cell surface bound PA to PA63 in both sensitive C3H and resistant A/J macrophages. After 24 h at 4°C, approximately 50% of the cell surface PA was converted to PA63 in both sensitive C3H and resistant A/J macrophages. There was no difference in proteolytic cleavage by the cell surface protease of sensitive and resistant cells. Thus, cellular resistance was not due to a deficiency in the cell surface protease which cleaves PA to expose a binding site for LF.

The final process in the interaction of toxin with the cell surface is the binding of LF to proteolytically activated PA63. To determine whether the LF binding sites are the same in sensitive and resistant cells and whether decreased LF binding could be responsible for resistance, cells were first incubated with nonradioactive PA for 16 h at 4°C to allow PA to be converted to PA63. The cells were then washed and direct binding experiments were performed with increasing concentrations of ^{125}I-LF. The sensitive C3H and resistant A/J macrophages bound LF to similar degrees (data not shown). Scatchard analysis of the data revealed that LF bound to a single class of high-affinity receptors which appear to be identical in the sensitive and resistant cells (data not shown). The affinity and number of receptors in the sensitive C3H cells (K_d, 0.084 nM; 18,000 binding sites per cell) were the same as in the resistant A/J macrophages (K_d, 0.086 nM; 20,900 binding sites per cell). This implies that the LF binding site is identical in the sensitive and resistant cells and that resistance to lethal toxin is not due to a deficiency in LF binding but, rather, to a postbinding event.

Relative sensitivities of C3H and A/J macrophages to other toxins and to viral infection. We previously reported that, after binding, lethal toxin is internalized by receptor-mediated endocytosis and passes through an acidic intracellular compartment in order to express its cytotoxicity (12). Acidification of intracellular vesicles is similarly required by many other toxins (41) and viruses (20, 32) for penetration into the cytosol. We therefore determined whether A/J macrophages were also more resistant to other toxins and a virus that require acidification for cytotoxicity or replication. This would suggest that the macrophages are defective in vesicular acidification. The resistant A/J and sensitive C3H macrophages were equally sensitive to inhibition of protein synthesis by *Pseudomonas* exotoxin A, modeccin, and a transferrin-CRM 107 diphtheria toxin conjugate which introduces diphtheria toxin into cells by means of the transferrin receptor (23). The 50% inhibitory doses of each of the respective toxins for both A/J and C3H cells were approximately 10^{-11}, 4×10^{-13}, and 5×10^{-10} M (data not shown). Similarly, we found that vesicular stomatitis virus replicated to an equivalent peak titer at 18 h after infection, 10^7 PFU/ml in both resistant A/J and sensitive C3H macrophages (data not shown).

Direct introduction of LF into the cytosol, bypassing receptor-mediated endocytosis. To determine whether the resistance of A/J cells is due to an inability to internalize and translocate toxin to the cytosol, we attempted to bypass the normal receptor-mediated endocytic process and introduce LF directly into the cytosol. Our initial attempts with direct microinjection were unsuccessful because of the high degree of cytotoxicity caused by the injection procedure in these

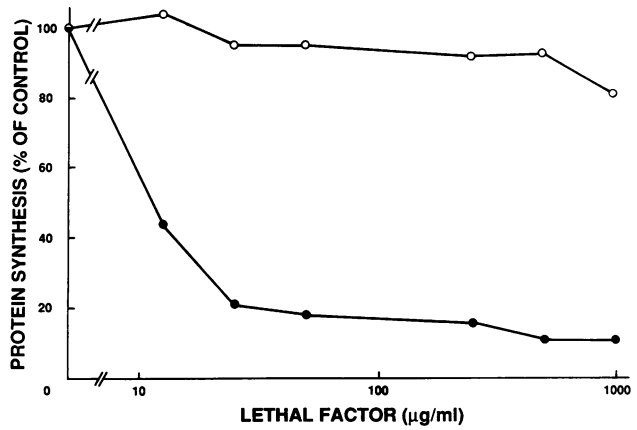

FIG. 4. Toxicity of LF introduced into the cytosol of C3H (●) and A/J (○) macrophages by osmotic lysis of pinosomes. LF was introduced into macrophages by osmotic lysis of pinosomes and protein synthesis was measured as described in Materials and Methods.

cells. We also tried to translocate LF from the plasma membrane by lowering the pH, as had been reported for diphtheria toxin (9, 48) and for anthrax edema toxin with Chinese hamster ovary cells (18). No toxicity could be induced in C3H or A/J cells after binding of PA63 and LF in the cold, each at concentrations up to 50 μg/ml, followed by treatment at pH 4.5 and reincubation in normal medium with NH_4Cl and antibody to LF to prevent entry by receptor-mediated endocytosis (data not shown).

We next used the method of osmotic lysis of pinocytic vesicles to introduce LF into the cytosol. This procedure has been previously used to deliver ricin and its A chain directly into the cell cytosol of ricin-resistant cells (15). First, we established that sensitive C3H and resistant A/J macrophages were equally susceptible to the introduction of proteins by the osmotic lysis procedure. For this purpose, we used the ribosome-inactivating protein gelonin, which lacks a B component and is relatively nontoxic when given to cells in normal medium (54). When gelonin was introduced by the osmotic lysis procedure, the lethal toxin-resistant A/J and -sensitive C3H macrophages were equally sensitive, with 50% inhibition of protein synthesis occurring with approximately 0.2 μg/ml in both cell types (data not shown). Gelonin, when added in normal isotonic medium, caused 50% inhibition of protein synthesis only at concentrations higher than 20 μg/ml in both cell types (data not shown).

We then introduced LF, by itself, into the cytosol of sensitive and resistant cells by osmotic lysis of pinosomes. LF internalized by osmotic lysis was cytotoxic, in a dose-dependent manner, for sensitive C3H macrophages as measured by either inhibition of protein synthesis (Fig. 4) or release of cellular LDH (data not shown). The concentration causing 50% inhibition of protein synthesis varied from 10 to 50 μg/ml in four different experiments. In contrast, LF, at concentrations of up to 1,000 μg/ml, when introduced by osmotic lysis was not cytotoxic for the resistant A/J cells. LF when added by itself in normal isotonic medium for 10 min was not toxic for sensitive C3H macrophages. The concentration giving 50% inhibition of protein synthesis was higher than 1,000 μg/ml (data not shown). When LF was given with PA in normal medium for 10 min, 50% inhibition of protein synthesis occurred at a concentration of approximately 1 μg/ml each for LF and PA (data not shown). Thus, LF by itself in the absence of PA possesses the cytotoxic activity of lethal toxin, and resistant A/J macrophages remain resistant to LF when it is introduced directly into the cytosol.

DISCUSSION

In the experiments described here, we sought to characterize the basis for the marked difference in sensitivities to anthrax lethal toxin between sensitive C3H and resistant A/J macrophages. A/J mice, whose macrophages are relatively resistant, have been reported to be relatively resistant to intravenous injection of lethal toxin; this resistance is manifested by a fourfold increase in time to death compared with other mouse strains (59). Thus, these results suggest that the macrophage may be an important target of the lethal toxin in vivo and may determine, in part, the susceptibility of the whole animal to the toxin. A/J mice, in contrast to C3H mice, are deficient in C5 and have an impaired leukocyte inflammatory response (58). Mouse strains, including A/J, with C5 deficiency have increased susceptibilities to infection with an attenuated *B. anthracis* strain (58), *Listeria monocytogenes* (14), *Streptococcus pneumoniae* (56), and *Haemophilus influenzae* (57). However, C5 deficiency is unlikely to be related to the resistance of A/J macrophages to anthrax lethal toxin because macrophages from other C5-deficient mouse strains were not resistant to lethal toxin (13). Macrophages from A/J but not C3H/HeN mice also have defective tumoricidal capacity (3) and are permissive for growth of *Legionella pneumophila* (60). However, in preliminary studies with macrophages from different mouse strains described in references 3 and 60, we found no correlation between tumoricidal capacity or growth in *L. pneumophila* and resistance to anthrax lethal toxin (13). Further study will be needed to determine the genetic basis of resistance to lethal toxin.

The resistance to lethal toxin could be due to defects occurring at any step in the intoxication process, from binding to cell surface receptors, internalization into endocytic vesicles, translocation of the LF from vesicles to the cytosol, and interaction of LF with its putative target molecule within the cytosol. Our results show that the resistance of A/J macrophages is not due to the lack or deficiency of the cell surface receptor for PA (Fig. 2) or the cell surface protease activity (Fig. 3). The PA63 species generated by both resistant A/J and sensitive C3H cells were the same size on SDS-polyacrylamide gel electrophoresis and appeared to function identically as receptors for LF in that the affinities and receptor numbers were the same in both cell types.

Membrane translocation of several protein toxins, including diphtheria toxin (9, 41, 48) and *Pseudomonas* exotoxin A (11, 36), and of many viruses (20, 32) from intracellular endocytic vesicles to the cytosol appears to require acidification of the vesicles. Several classes of cell mutants that are cross-resistant to different toxins or to toxins and viruses have been described as having defects in their abilities to acidify distinct populations of intracellular vesicles of the endosome-lysosome pathway (6, 34, 45–47, 50, 55). A/J macrophages were not more resistant to the cytotoxic effect of *Pseudomonas* exotoxin A, modeccin, or a transferrin-CRM 107 diphtheria toxin conjugate or to the replication of vesicular stomatitis virus, all of which require vesicular acidification. Furthermore, A/J macrophages did not show an increased sensitivity to ricin (data not shown), which has been observed in cells defective in endosomal acidification (45–47). These results all imply that resistance of A/J cells to

lethal toxin is not due to a defect in acidification or some other common vesicular function. Preliminary experiments with postnuclear supernatants (31) revealed no difference in the acidification abilities of A/J and C3H macrophages (data not shown). However, acidification defects present in selected vesicular compartments might not be detected with postnuclear supernatants, and assays of endosome and lysosome subpopulations may be required. It is also possible that lethal toxin may require a lower pH for translocation than the other toxins tested.

The resistance of A/J cells was not due to a lack of PA receptors and was unlikely to be caused by a generalized defect in vesicle acidification. However, it is possible that receptor abnormalities could impair membrane translocation of LF in A/J cells. Such a receptor defect has been postulated to explain the resistance of mouse cells to diphtheria toxin (40). Abnormalities in the receptor could also alter the internalization and proper targeting of the toxin within the cell, as has been described for other ligands (4, 5, 7, 21, 22, 35, 38, 43).

We attempted to introduce LF directly into the cytosol by various procedures to bypass the receptor-mediated route. We were not able to kill C3H or A/J macrophages by acid treatment of surface-bound PA63 plus LF. It is unclear why acid pH treatment was ineffective for lethal toxin while being reported to be effective for edema toxin (18). It is possible the different cell types used vary in their sensitivities to the procedure or that LF requires additional intracellular processing for translocation. However, in the experiments with edema toxin in which amines were used, it is likely that edema toxin was present in intracellular vesicles rather than on the plasma membrane at the time of low pH treatment so that toxin entered by the receptor-mediated pathway (18). The inability to translocate toxins from the cell surface by low pH treatment has also been noted with *Pseudomonas* exotoxin A and modeccin (41).

LF, when introduced directly into the cytosol by osmotic lysis of pinosomes, bypassing the normal receptor-mediated entry mechanism, killed sensitive C3H cells. In contrast, A/J macrophages remained resistant. The first finding clearly demonstrates that LF by itself and in the absence of PA possesses the cytotoxic activity of the two protein components of lethal toxin and that the normal receptor-mediated uptake mechanism is not required to produce toxicity. Secondly, the resistance of A/J cells is unlikely to be due to an impairment in internalization and translocation of LF to the cytosol, but rather is more likely due to a defect occurring at a later stage after LF reaches the cytosol. We have recently observed similar resistances to LF, introduced by the osmotic lysis procedure, in some macrophage-like and nonmacrophage established cell lines (52).

Perhaps the simplest explanation for the resistance of A/J macrophages is that they lack the putative cytosolic target molecule of LF responsible for cell death. Alternatively, the defect in resistant A/J cells may involve a processing of LF that is necessary for the full expression of its activity. LF may need to be modified enzymatically by proteolysis or in other ways to become activated. In this regard, it is thought that reduction of diphtheria toxin, necessary for its full activity, occurs within the cytosol (37). LF might also require an additional cofactor for activation or targeting to specific organelles for expression of its lethal effect. Additionally, resistant A/J cells may inactivate the LF within the cytosol or possess a mechanism for repairing damage due to LF which is absent in sensitive cells. Other workers have reported degradation of the adenylate cyclase of *Bordetella pertussis* and *B. anthracis* by lymphocyte lysates (16). Using lysates prepared as described by these workers, we found that C3H and A/J macrophages degraded ^{125}I-LF to equivalent degrees (data not shown), suggesting that resistance is not due to differential degradation in the cytosol. While the osmotic lysis experiments suggest that any defects in resistant cells occur within the cytosol, it is also possible that they may exist within the pinosomes. The postulated activation of LF by sensitive cells and its defect in resistant cells may occur, under the conditions of osmotic lysis, within the pinosomes and, under normal conditions, within endocytic vesicles. It is also possible that LF may be retained in the pinosome of A/J macrophages in contrast to C3H macrophages, and this differential retention may reflect the disposition in endocytic vesicles in the receptor-mediated pathway. Further study of the intracellular processing of ^{125}I-LF in A/J and C3H cells will be of interest. Processing of several other protein ligands has been reported to occur within endosomes (8, 19, 33, 42, 49). Finally, it is also possible that resistant A/J macrophages may be deficient at more than one stage of the intoxication process and possess multiple defects.

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REFERENCES

1. **Beall, F. A., M. J. Taylor, and C. B. Thorne.** 1962. Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. J. Bacteriol. **83:**1274–1280.
2. **Bhatnagar, R., Y. Singh, S. H. Leppla, and A. M. Friedlander.** 1989. Calcium is required for the expression of anthrax lethal toxin activity in the macrophagelike cell line J774A.1. Infect. Immun. **57:**2107–2114.
3. **Boraschi, D., and M. S. Meltzer.** 1979. Macrophage activation for tumor cytotoxicity: genetic variation in macrophage tumoricidal capacity among mouse strains. Cell. Immunol. **45:**188–194.
4. **Canfield, W. M., K. F. Johnson, R. D. Ye, W. Gregory, and S. Kornfield.** 1991. Localization of the signal for rapid internalization of the bovine cation-independent mannose 6-phosphate/insulin-like growth factor-II receptor to amino acids 24–29 of the cytoplasmic tail. J. Biol. Chem. **266:**5682–5688.
5. **Chen, W. J., J. L. Goldstein, and M. S. Brown.** 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J. Biol. Chem. **265:**3116–3123.
6. **Colbaugh, P. A., M. Stookey, and R. K. Draper.** 1989. Impaired lysosomes in a temperature-sensitive mutant of Chinese hamster ovary cells. J. Cell Biol. **108:**2211–2219.
7. **Davis, C. G., I. R. van Driel, D. W. Russell, M. S. Brown, and J. L. Goldstein.** 1987. The low density lipoprotein receptor. J. Biol. Chem. **262:**4075–4082.
8. **Diment, S., K. J. Martin, and P. D. Stahl.** 1989. Cleavage of parathyroid hormone in macrophage endosomes illustrates a novel pathway for intracellular processing of proteins. J. Biol. Chem. **264:**13403–13406.
9. **Draper, R. K., and M. I. Simon.** 1980. The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. J. Cell Biol. **87:**849–854.
10. **Escuyer, V., and R. J. Collier.** 1991. Anthrax protective antigen interacts with a specific receptor on the surface of CHO-K1 cells. Infect. Immun. **59:**3381–3386.
11. **Farahbakhsh, Z. T., R. L. Baldwin, and B. J. Wisnieski.** 1986. Pseudomonas exotoxin A. Membrane binding, insertion, and traversal. J. Biol. Chem. **261:**11404–11408.

12. **Friedlander, A. M.** 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. **261**:7123–7126.
13. **Friedlander, A. M.** Unpublished data.
14. **Gervais, F., M. Stevenson, and E. Skamene.** 1984. Genetic control of resistance of *Listeria monocytogenes*: regulation of leukocyte inflammatory responses by the *Hc* locus. J. Immunol. **132**:2078–2083.
15. **Ghosh, P. C., R. B. Wellner, and H. C. Wu.** 1984. Osmotically induced microinjection of ricin bypasses a ricin internalization defect in a Chinese hamster ovary mutant cell line. Mol. Cell. Biol. **4**:1320–1325.
16. **Gilboa-Ron, A., A. Rogel, and E. Hanski.** 1989. *Bordetella pertussis* adenylate cyclase inactivation by the host cell. Biochem. J. **262**:25–31.
17. **Gill, D. M.** 1978. Seven toxic peptides that cross cell membranes, p. 291–314. *In* J. Jeljaszewicz and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, Inc., New York.
18. **Gordon, V. M., S. H. Leppla, and E. L. Hewlett.** 1988. Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. Infect. Immun. **56**:1066–1069.
19. **Hamel, F. G., B. I. Posner, J. J. M. Bergeron, B. H. Frank, and W. C. Duckworth.** 1988. Isolation of insulin degradation products from endosomes derived from intact rat liver. J. Biol. Chem. **263**:6703–6708.
20. **Helenius, A., M. Kielian, I. Mellman, and S. Schmid.** 1989. Entry of enveloped viruses into their host cells, p. 145–161. *In* R. W. Compans, A. Helenius, and M. B. A. Oldstone (ed.), Cell biology of virus entry, replication, and pathogenesis. Alan R. Liss, Inc., New York.
21. **Iacopetta, B. J., S. Rothenberger, and L. C. Kuhn.** 1988. A role for the cytoplasmic domain in transferrin receptor sorting and coated pit formation during endocytosis. Cell **54**:485–489.
22. **Jing, S., T. Spencer, K. Miller, C. Hopkins, and I. S. Trowbridge.** 1990. Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. J. Cell Biol. **110**:283–294.
23. **Johnson, V. G., D. Wilson, L. Greenfield, and R. J. Youle.** 1988. The role of the diphtheria toxin receptor in cytosol translocation. J. Biol. Chem. **263**:1295–1300.
24. **Koehler, T. M., and R. J. Collier.** 1991. Anthrax toxin protective antigen: low-pH-induced hydrophobicity and channel formation in liposomes. Mol. Microbiol. **5**:1501–1506.
25. **Lee, M.-T., and M. K. Warren.** 1987. CSF-1-induced resistance to viral infection in murine macrophages. J. Immunol. **138**:3019–3022.
26. **Leppla, S. H.** 1976. Large-scale purification and characterization of the exotoxin of *Pseudomonas aeruginosa*. Infect. Immun. **14**:1077–1086.
27. **Leppla, S. H.** 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentration in eukaryotic cells. Proc. Natl. Acad. Sci. USA **79**:3162–3166.
28. **Leppla, S. H.** 1988. Production and purification of anthrax toxin. Methods Enzymol. **165**:103–116.
29. **Leppla, S. H., A. M. Friedlander, and E. Cora.** 1988. Proteolytic activation of anthrax toxin bound to cellular receptors, p. 111–112. *In* F. Fehrenbach, J. E. Alouf, P. Falmagne, W. Goebel, J. Jeljaszewicz, D. Jurgens, and R. Rappuoli (ed.), Bacterial protein toxins. Gustav Fischer Verlag, Stuttgart, Germany.
30. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
31. **Marnell, M. H., L. S. Mathis, M. Stookey, S.-P. Shia, D. K. Stone, and R. K. Draper.** 1984. A Chinese hamster ovary cell mutant with a heat-sensitive, conditional-lethal defect in vacuolar function. J. Cell Biol. **99**:1907–1916.
32. **Marsh, M.** 1984. The entry of enveloped viruses into cells by endocytosis. Biochem. J. **218**:1–10.
33. **Matrisian, L. M., S. R. Planck, and B. E. Magun.** 1984. Intracellular processing of epidermal growth factor. I. Acidification of ^{125}I-epidermal growth factor in intracellular organelles. J. Biol. Chem. **259**:3047–3052.
34. **Merion, M., P. Schlesinger, R. M. Brooks, J. M. Moehring, T. J. Moehring, and W. S. Sly.** 1983. Defective acidification of endosomes in Chinese hamster ovary cell mutants "cross-resistant" to toxins and viruses. Proc. Natl. Acad. Sci. USA **80**:5315–5319.
35. **Miettinen, H. M., J. K. Rose, and I. Mellman.** 1989. Fc receptor isoforms exhibit distinct abilities for coated pit localization as a result of cytoplasmic domain heterogeneity. Cell **58**:317–327.
36. **Moehring, J. M., and T. J. Moehring.** 1983. Strains of CHO-K1 cells resistant to *Pseudomonas* exotoxin A and cross-resistant to diphtheria toxin and viruses. Infect. Immun. **41**:998–1009.
37. **Moskaug, J. O., K. Sandvig, and S. Olsnes.** 1987. Cell-mediated reduction of the interfragment disulfide in nicked diphtheria toxin. J. Biol. Chem. **262**:10339–10345.
38. **Mostov, K. E., A. de Bruyn Kops, and D. L. Deitcher.** 1986. Deletion of the cytoplasmic domain of the polymeric immunoglobulin receptor prevents basolateral localization and endocytosis. Cell **47**:359–364.
39. **Munson, P. J., and D. Rodbard.** 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. **107**:220–239.
40. **O'Keefe, D. O., and R. K. Draper.** 1985. Characterization of a transferrin-diphtheria toxin conjugate. J. Biol. Chem. **260**:932–937.
41. **Olsnes, S., and K. Sandvig.** 1988. How protein toxins enter and kill cells, p. 39–73. *In* A. E. Frankel (ed.), Immunotoxins. Kluwer Academic Publishers, Boston.
42. **Pease, R. J., G. D. Smith, and T. J. Peters.** 1985. Degradation of endocytosed insulin in rat liver is mediated by low-density vesicles. Biochem. J. **228**:137–146.
43. **Prywes, R., E. Livneh, A. Ullrich, and J. Schlessinger.** 1986. Mutations in the cytoplasmic domain of EGF receptor affect EGF binding and receptor internalization. EMBO J. **5**:2179–2190.
44. **Rechsteiner, M.** 1987. Osmotic lysis of pinosomes. Methods Enzymol. **149**:42–48.
45. **Robbins, A. R., C. Oliver, J. L. Bateman, S. S. Krag, C. J. Galloway, and I. Mellman.** 1984. A single mutation in Chinese hamster ovary cells impairs both Golgi and endosomal functions. J. Cell Biol. **99**:1296–1308.
46. **Roff, C. F., R. Fuchs, I. Mellman, and A. R. Robbins.** 1986. Chinese hamster ovary cell mutants with temperature-sensitive defects in endocytosis. I. Loss of function on shifting to the nonpermissive temperature. J. Cell Biol. **103**:2283–2297.
47. **Ryser, H. J.-P., R. Mandel, A. Hacobian, and W.-C. Shen.** 1988. Methotrexate-poly(lysine) as a selective agent for mutants of Chinese hamster ovary cells defective in endocytosis. J. Cell. Physiol. **135**:277–284.
48. **Sandvig, K., and S. Olsnes.** 1980. Diphtheria toxin entry into cells is facilitated by low pH. J. Cell Biol. **87**:828–832.
49. **Schaudies, R. P., R. M. Gorman, C. R. Savage, Jr., and R. D. Poretz.** 1987. Proteolytic processing of epidermal growth factor within endosomes. Biochem. Biophys. Res. Commun. **143**:710–715.
50. **Schmid, S., R. Fuchs, M. Kielian, A. Helenius, and I. Mellman.** 1989. Acidification of endosome subpopulations in wild-type Chinese hamster ovary cells and temperature-sensitive acidification-defective mutants. J. Cell Biol. **108**:1291–1300.
51. **Singh, Y., V. K. Chaudhary, and S. H. Leppla.** 1989. A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin *in vivo*. J. Biol. Chem. **264**:19103–19107.
52. **Singh, Y., S. H. Leppla, R. Bhatnagar, and A. M. Friedlander.** 1989. Internalization and processing of *Bacillus anthracis* lethal toxin by toxin-sensitive and -resistant cells. J. Biol. Chem. **264**:11099–11102.
53. **Stanley, J. L., and H. Smith.** 1961. Purification of factor I and recognition of a third factor of anthrax toxin. J. Gen. Microbiol. **26**:49–66.
54. **Stirpe, F., S. Olsnes, and A. Pihl.** 1980. Gelonin, a new inhibitor of protein synthesis, nontoxic to intact cells. J. Biol. Chem. **255**:6947–6953.

55. **Timchak, L. M., F. Kruse, M. H. Marnell, and R. K. Draper.** 1986. A thermosensitive lesion in a Chinese hamster cell mutant causing differential effects on the acidification of endosomes and lysosomes. J. Biol. Chem. **261:**14154–14159.
56. **Toews, G. B., and W. C. Vial.** 1984. The role of C5 in polymorphonuclear leukocyte recruitment in response to *Streptococcus pneumoniae*. Am. Rev. Respir. Dis. **129:**82–86.
57. **Toews, G. B., W. C. Vial, and E. J. Hansen.** 1985. Role of C5 and recruited neutrophils in early clearance of nontypable *Haemophilus influenzae* from murine lungs. Infect. Immun. **50:**207–212.
58. **Welkos, S. L., and A. M. Friedlander.** 1988. Pathogenesis and genetic control of resistance to the Sterne strain of *Bacillus anthracis*. Microb. Pathog. **4:**53–69.
59. **Welkos, S. L., T. J. Keener, and P. H. Gibbs.** 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. Infect. Immun. **51:**795–800.
60. **Yamamoto, Y., T. W. Klein, C. A. Newton, R. Widen, and H. Friedman.** 1988. Growth of *Legionella pneumophila* in thioglycolate-elicited peritoneal macrophages from A/J mice. Infect. Immun. **56:**370–375.