Macrophages Are Sensitive to Anthrax Lethal Toxin through an Acid-dependent Process*

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Anthrax lethal toxin, which consists of two proteins, protective antigen and lethal factor, is lethal for experimental animals. This study describes the first in vitro system demonstrating lethality of the toxin. Mouse peritoneal macrophages are killed within 1 h of exposure to the toxin. Neither protein component alone shows any toxic activity. The minimal effective concentration of protective antigen and lethal factor was \(10^{-2}\) and \(10^{-3}\) g/ml, respectively. None of the several established cell lines examined was killed. Cells could be totally protected from the toxin by pretreatment with agents, such as amines or monensin, which dissipate intracellular proton gradients and raise the pH of intracellular vesicles. This protection was reversible and could be overcome by lowering the intravesicular pH. Antitoxin added after preincubation with amines was unable to protect cells subsequently exposed to low pH treatment. These results suggest that anthrax lethal toxin requires passage through an acidic endocytic vesicle in order to exert its toxic effect within the cytosol.

The toxic nature of anthrax infection was suspected by Koch (1) in his earliest studies of its pathogenesis. Later studies by Bail and Weil (2) and others (3) using extracts of infected tissue suggested that aggressins or toxins were important factors in anthrax virulence. Subsequently, a toxin was demonstrated definitively by Smith and co-workers (4). Further research has established the existence of two toxin complexes (5, 6). The edema toxin consists of two proteins: protective antigen, or Factor II, together with edema factor, or Factor I. Each protein has an apparent molecular weight of approximately 85,000-90,000 (7). This toxin produces edema in experimental animals (8, 9) and edema factor has recently been shown to be a calmodulin-dependent adenylate cyclase (10). The lethal toxin consists of protective antigen plus a third protein, lethal factor, or Factor III, with an apparent molecular weight of 83,000 (7). It is this toxin that is lethal for several animal species (8, 9). The only biological system presently available for studying lethal toxin is lethality in experimental animals. The mechanism of action of the toxin remains unknown. In this communication, I report the first in vitro system demonstrating the toxicity of anthrax lethal toxin and show that the expression of toxin activity requires an acidic intracellular environment.

MATERIALS AND METHODS

Peritoneal exudate macrophages were obtained from male C3H/HeNHisd mice (Harlan/Sprague Dawley, Inc., Walkersville, MD) as previously described (12). They were plated at 7.5 X 10^6 cells in 1 ml in a 2-cm² 24-well tissue culture plate unless otherwise noted. After 1-2 h, the nonadherent cells were removed by washing, and complete medium, consisting of Dulbecco’s modified Eagle's medium containing 20% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% L cell conditioned medium (11) was added. Protective antigen, lethal factor, edema factor, and a goat antiserum to lethal factor were generously supplied by S. Leppla (this Institute). The protective antigen, lethal factor, and edema factor toxin components were purified by previously published methods to at least 90% homogeneity when analyzed by sodium dodecyl sulfate gels (13).

Cell toxicity was determined by the amount of cytoplasmic lactate dehydrogenase present in control or toxin-treated cell monolayers. Lactic dehydrogenase was measured in cells lysed in 100 µl of 0.05% digitonin, by oxidation of lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (14), using an automated analyzer (COBAS, Roche Analytical Instruments, Inc., Nutley, NJ). All the lactic dehydrogenase released from toxin-treated monolayers was recovered in the medium and was nondetectable at 500 X g. Thus, loss of lactic dehydrogenase from the monolayer represents cell lysis. Results are expressed as the per cent of control cellular lactic dehydrogenase \(\pm S.E\) in triplicate cultures unless otherwise indicated. Toxicity was also measured by exclusion of trypan blue (0.05 g/100 ml) from cells incubated in phosphate-buffered saline. The established cell lines, L929 and 3T3, were obtained from the American Type Culture Collection (Rockville, MD). MRC-5 and FRL-103 cells were from The Salk Institute (Swiftwater, PA).

RESULTS AND DISCUSSION

Initial experiments clearly indicated that the combination of protective antigen and lethal factor, each at 1 µg/ml, was cytotoxic for mouse peritoneal macrophages and caused destruction of the cell monolayer after a 24-h exposure. One hundred per cent of the toxin-treated cells were dead as determined by trypan blue exclusion while untreated control cells remained alive. Protective antigen or lethal factor alone caused no morphological change in the macrophages and the cells were all viable. Quantitation of the cytotoxicity by release of cytoplasmic lactic dehydrogenase (Fig. 1), revealed the minimal effective concentration of lethal factor to be \(10^{-3}\) µg/ml when protective antigen was present at 1 µg/ml. The minimal effective concentration of protective antigen was \(10^{-2}\) µg/ml when the lethal factor concentration was maximal (1 µg/ml). Protective antigen or lethal factor alone at 1 µg/ml was completely nontoxic. Identical results were obtained by using trypan blue exclusion as the measure of cytotoxicity. Five different preparations of protective antigen and lethal factor showed similar potencies. Cell death was first detectable after 1-h exposure to protective antigen + lethal factor (each at 1 µg/ml) and was complete by 4 h. Edema toxin (protective antigen and edema factor, each at 1 µg/ml) caused no toxicity after 4 h.

Previous studies of the effect of anthrax toxins on cells in vitro performed as early as 1911 showed that extracts of tissue from anthrax-infected animals inhibited leukocyte killing of anthrax bacilli (2). Subsequent workers reported that anthrax culture filtrates inhibited neutrophil chemotaxis (15) and the combination of all three toxin components together (protective...
tive antigen + edema factor + lethal factor) inhibited neutrophil phagocytosis (16). No cytopathic effect was noted and individual components were not tested. These previously observed effects may reflect the ability of the edema toxin (protective antigen + edema factor) present to raise the cyclic AMP level of cells (10) and inhibit their function (17). Anthrax culture filtrates produced no cytopathology in KB cells, primary mouse embryo cells, or a guinea pig spleen cell line (18). Similarly, Fedotova (19) noted no cytopathology of anthrax toxin for F1 or HeLa cells. However, he noted some growth inhibition in these cells and degenerative changes in guinea pig macrophages which were evident at 4 h but not at later times. Based on the present results, it is likely that these morphological effects were caused by the protective antigen + lethal factor in the crude preparation. However, no quantitation was reported and purified components were not used. The difference between the lack of cytotoxicity reported for various cell lines and the present results with macrophages may be due to differences in sensitivity to lethal toxin, although individual toxin components were not tested in the previous studies. Using purified protective antigen + lethal factor, each at 1 µg/ml, we found no significant cytotoxicity after 24-h exposure (<20% lethality) for four cell lines (MRC-5, L929, FRL-103, and 3T3) (data not shown). The basis for this marked difference in sensitivity between macrophages and other cells awaits further study.

In view of the large amount of evidence showing that some toxins (20, 21), other protein ligands (22), and viruses (23, 24) enter and exert effects on cells by an acid-dependent process, we next examined the effects on lethal toxin activity of agents which dissipate intracellular proton gradients. Preincubation of macrophages with the lysomotropic amines, NH4Cl and chloroquine, or with the ionophore monensin, for 20 min before exposure to protective antigen + lethal factor, completely protected them from killing by lethal toxin (Fig. 2). Other experiments demonstrated that the drugs themselves were nontoxic. The protection by NH4Cl was totally reversible by removing the NH4Cl along with the toxin 1 h after exposure to protective antigen + lethal factor (data not shown). These results strongly suggest that anthrax lethal toxin exerts its effect within the cytosol after passage through an acidic intracellular vesicle or compartment.

If the protection by amines is due to their ability to raise the pH of intracellular vesicles, then it might be possible to overcome such protection by lowering the pH as has been previously demonstrated with diphtheria toxin (25, 26), epidermal growth factor (22), and some viruses (23). Macrophages protected from lethal toxin by preincubation with NH4Cl were exposed for 10 min to media of varying pH in the presence of NH4Cl to reduce the pH of intracellular vesicles (27). They were then placed in toxin-free medium (pH 7.4) with NH4Cl and assayed for toxicity 24 h later. NH4Cl was present throughout the experiment. Exposure to media of increasingly lower pH overcame the inhibition of toxicity by NH4Cl (Fig. 3). Thus, at pH ≤4.75, complete

**FIG. 1.** Dose response of mouse peritoneal macrophages to the lethal effect of protective antigen + lethal factor. Macrophages were cultured, exposed to varying concentrations of protective antigen and lethal factor, and assayed for toxicity 24 h later by the amount of lactic dehydrogenase remaining in the cell monolayer as described in the text. The per cent of control cellular lactic dehydrogenase is plotted versus the concentration of lethal factor (log scale). Protective antigen was used at varying concentrations as indicated. PA, protective antigen; LDH, lactic dehydrogenase.

**FIG. 2.** Inhibition of the lethal effect of protective antigen + lethal factor by amines and monensin. Macrophages were preincubated for 20 min with NH4Cl (A), chloroquine (B), or monensin (C). Protective antigen + lethal factor, each at 0.1 µg/ml, was then added and toxicity was measured 4 h later as described in the text. Inhibitor was present throughout the experiment. The per cent control cellular lactic dehydrogenase is plotted versus the concentration of inhibitor. A, cells exposed to inhibitor plus protective antigen + lethal factor; B, cells exposed to inhibitor alone. LDH, lactic dehydrogenase.
totoxicity of anthrax lethal toxin was restored. Similarly, it was also possible to overcome the inhibition of toxicity afforded by monensin by treating cells for 10 min at pH 4.5 (data not shown). These results suggest that the protection by amines and monensin is due to the elevated vesicular pH and support the conclusion that a low pH is required for anthrax lethal toxin activity.

To determine the cellular location of the toxin after pretreatment with NH4Cl at the time when low pH exposure can reverse the inhibition of toxicity, we studied whether antitoxin, added in the cold, could block the effect of low pH treatment. We first showed that antibody to lethal factor, when added at 4°C, was able to block the effect of protective antigen + lethal factor, located on the cell surface after adsorption at 4°C (Table I). Additional controls showed that this protection was not affected by including 10 mM NH4Cl during the antibody incubation or by treating cells at pH 4.5 for 10 min after the antibody exposure. The next experiment showed that antitoxin, added at 4°C, after the NH4Cl block and immediately before the acid pH treatment, was unable to prevent the acid-induced toxicity (Table II). A further control showed that the amount of antitoxin used in the experiment was more than sufficient to neutralize all of the lethal factor initially added (data not shown). This result demonstrates that after pretreatment with NH4Cl, the toxin is not accessible to antitoxin and supports the idea that it is present in an intracellular location before expressing its toxic effect on the cell. This interpretation is consistent with previous reports suggesting that amines and monensin cause accumulation of diphtheria toxin (25, 26, 28) and several protein ligands (29) within acidic intracellular vesicles.

The protection against toxicity afforded by lysomotropic amines and monensin, the reversal of this protection by low pH treatment, and the inability of antitoxin to neutralize toxicity after amine treatment all suggest that anthrax lethal toxin must pass through an acidic endocytic vesicle to exert its toxic effect within the cytosol. The identification of the macrophage as a cell sensitive to anthrax lethal toxin provides an in vitro system which is necessary to study further the mechanism of toxin action.

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REFERENCES

1. Koch, R. (1877) Beiträge zur Biologie der Pflanzen 2, 277–310 in (1938) Medical Classics 2, 787–820
2. Bail, O., and Weil, E. (1911) Arch. Hg. Bakteriol. 73, 218–264
3. Cromartie, W. J., Watson, D. W., Bloom, W. L., and Heckly, R. J. (1947) J. Infect. Dis. 80, 14–27
4. Smith, H., and Keppie, J. (1954) Nature 173, 869–870
5. Lincoln, R. E., and Fish, D. C. (1970) in Microbial Toxins (Montie, T. C., Kadi, S., and Ajl, S. J. eds) Vol. III, pp. 362–414, Academic Press, New York

**TABLE II**

| Addition | Lethal toxin | Antitoxin | Cellular lactic dehydrogenase (milliunits/monolayer) |
|----------|--------------|-----------|-----------------------------------------------------|
|          |              |           | −         | +          | 11 f 1   |
|          |              |           | +         | +          | 12 ± 1   |
|          |              |           | −         | +          | 60 ± 4   |

**TABLE I**

Effect of antitoxin on the toxicity of lethal toxin bound to the cell surface

Macrophages were washed with cold medium and incubated at 4°C with protective antigen (10 μg/ml) plus lethal factor (10 μg/ml) for 4 h. They were then washed three times with cold medium and incubated with or without antitoxin (1:50 dilution of antiserum) for 4°C for 1 h. The cells were then washed three times with cold medium and toxicity was measured after a further 24-h incubation at 37°C in complete medium. Results represent the mean ± S.E. of triplicate wells.

| Addition | Lethal toxin | Antitoxin | Cellular lactic dehydrogenase (milliunits/monolayer) |
|----------|--------------|-----------|-----------------------------------------------------|
|          |              |           | −         | +          | 82 ± 3   |
|          |              |           | +         | +          | 10 ± 1   |
|          |              |           | −         | +          | 80 ± 3   |
|          |              |           | +         | +          | 80 ± 3   |
|          |              |           | −         | +          | 78 ± 4   |

**FIG. 3.** Reversal of the NH4Cl inhibition of protective antigen + lethal factor toxicity by low pH. Macrophages were preincubated with medium containing 10 mM NH4Cl for 30 min. Protective antigen (1 μg/ml) and lethal factor (0.1 μg/ml) were then added in the presence of 10 mM NH4Cl and cells were incubated for another 90 min. Cells were then washed and reincubated with medium containing 10 mM NH4Cl and no toxin for an additional 60 min. At that time they were exposed for 10 min to medium with 10 mM NH4Cl and 20 mM HEPES adjusted to different pH with HCl. The cells were then washed again and reincubated in medium with 10 mM NH4Cl (pH 7.4). Toxicity was measured 24 h later and expressed as the cellular lactic dehydrogenase activity (milliunits/monolayer) of triplicate wells ± S.E. versus pH of the medium during the 10-min exposure. A, toxin-treated cells; O, control cells. NH4Cl was present throughout the entire experiment. Cells not exposed to NH4Cl that were given protective antigen + lethal factor and treated at pH 7.2 had <5 milliunits/monolayer. LDH, lactic dehydrogenase.

**REFERENCES**

1. Koch, R. (1877) Beiträge zur Biologie der Pflanzen 2, 277–310 in (1938) Medical Classics 2, 787–820
2. Bail, O., and Weil, E. (1911) Arch. Hg. Bakteriol. 73, 218–264
3. Cromartie, W. J., Watson, D. W., Bloom, W. L., and Heckly, R. J. (1947) J. Infect. Dis. 80, 14–27
4. Smith, H., and Keppie, J. (1954) Nature 173, 869–870
5. Lincoln, R. E., and Fish, D. C. (1970) in Microbial Toxins (Montie, T. C., Kadi, S., and Ajl, S. J. eds) Vol. III, pp. 362–414, Academic Press, New York

1. The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
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6. Stephen, J. (1981) *Pharmacol. Ther.* 12, 501–513
7. Leplla, S. H., Ivins, B. E., and Ezzell, J. W., Jr. (1985) in *Microbiology—1985* (Leive, L., ed) pp. 63–66, American Society for Microbiology, Washington, D. C.
8. Stanley, J. L., and Smith, H. (1961) *J. Gen. Microbiol.* 26, 49–66
9. Beall, F. A., Taylor, M. J., and Thorne, C. B. (1962) *J. Bacteriol.* 83, 1274–1280
10. Leplla, S. (1982) *Proc. Natl. Acad. Sci. U. S. A.* 79, 3162–3166
11. Mauel, J., and Defendi, V. (1971) *Exp. Cell Res.* 65, 33–42
12. Friedlander, A. M., Jahrling, P. B., Merrill, P., and Tobery, S. (1984) *Infect. Immun.* 43, 283–288
13. Leplla, S. H. (1984) in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Greengard, P., and Robison, B. A., eds) Vol. 17, pp. 189–198, Raven Press, New York
14. Gay, R. J., McComb, R. B., and Bowers, G. H., Jr. (1968) *Clin. Chem.* 14, 740–753
15. Kashiba, S., Morishima, T., Kato, K., Shima, M., and Amano, T. (1959) *Biken J.* 2, 97–104
16. Keppie, J., Harris-Smith, P. W., and Smith, H. (1963) *Br. J. Exp. Pathol.* 44, 446–453
17. O’Brien, J., Friedlander, A., Dreier, T., Ezzell, J., and Leplla, S. (1985) *Infect. Immun.* 47, 306–310
18. Bonventre, P. F. (1965) *J. Bacteriol.* 90, 284–285
19. Fedotova, I. M. (1970) *Arthr. Patol.* 32, 30–33
20. Olsnes, S., and Sandvig, K. (1985) in *Endocytosis* (Pastan, I., and Willingham, M. C., eds) pp. 195–234, Plenum Press, New York
21. Middlebrook, J. L., and Dorland, R. (1984) *Microbiol. Rev.* 48, 199–221
22. King, A. C., and Cuatrecasas, P. (1982) *Biochem. Biophys. Res. Commun.* 106, 479–485
23. Helenius, A., Kartenbeck, J., Simons, K., and Fries, E. (1980) *J. Cell Biol.* 84, 404–420
24. Madahus, I. H., Olsnes, S., and Sandvig, K. (1984) *J. Cell Biol.* 98, 1194–1200
25. Sandvig, K., and Olsnes, S. (1980) *J. Cell Biol.* 87, 828–832
26. Draper, R. K., and Simon, M. I. (1980) *J. Cell Biol.* 87, 849–854
27. Poole, B., and Ohkuma, S. (1981) *J. Cell Biol.* 90, 665–669
28. Marnell, M. H., Stookey, M., and Draper, R. H. (1982) *J. Cell Biol.* 93, 57–62
29. Merion, M., and Sly, W. S. (1983) *J. Cell Biol.* 96, 644–650