Oxidized ATP Protection against Anthrax Lethal Toxin

Mahtab Moayeri, Katherine E. Wickliffe, Jason F. Wiggins, and Stephen H. Leppla*

*Microbial Pathogenesis Section, National Institute of Allergy and Infectious Diseases,
National Institutes of Health, Bethesda, Maryland 20892*

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***Bacillus anthracis* lethal toxin (LT) induces rapid lysis (<90 min) of murine macrophages from certain inbred strains. The mechanism for LT-induced cytotoxicity is currently unknown. We hypothesized that the ATP-activated macrophage P2X7 receptors implicated in nucleotide-mediated macrophage lysis could play a role in LT-mediated cytotoxicity and discovered that a potent P2X7 antagonist, oxidized ATP (o-ATP), protects macrophages against LT. Other P2X7 receptor antagonists, however, had no effect on LT function, while oxidized nucleotides, o-ADP, o-GTP, and o-ITP, which did not act as receptor ligands, provided protection. Cleavage of the LT substrates, the mitogen-activated protein kinases, was inhibited by o-ATP in RAW274.6 macrophages and CHO cells. We investigated the various steps in the intoxication pathway and found that binding of the protective-antigen (PA) component of LT to cells and the enzymatic proteolytic ability of the lethal factor (LF) component of LT were unaffected by o-ATP. Instead, the drug inhibited formation of the sodium dodecyl sulfate-resistant PA oligomer, which occurs in acidified endosomes, but did not prevent cell surface PA oligomerization, as evidenced by binding and translocation of LF to a protease-resistant intracellular location. We found that o-ATP also protected cells from anthrax edema toxin and diphtheria toxin, which also require an acidic environment for escape from endosomes. Confocal microscopy using pH-sensitive fluorescent dyes showed that o-ATP increased endosomal pH. Finally, BALB/cJ mice injected with o-ATP and LT were completely protected against lethality.**

Anthrax toxin consists of three polypeptides that form two binary toxins. The lethal toxin (LT) and edema toxin (ET) each contain the receptor binding protein, protective antigen (PA), combined with an enzymatic cargo protein. Lethal factor (LF), a protease which cleaves members of the mitogen-activated protein kinase family (MEKs), and edema factor (EF), a calmodulin-dependent adenylate cyclase, are transported into the cell cytoplasm by PA oligomers. These oligomers can form only after binding and cleavage of PA from an 83-kDa protein (PA83) to its 63-kDa form (PA63) by furin at the cell surface. The heptameric PA63 oligomers carrying LF and/or EF are then conformationally altered in an intracellular acidic compartment to allow passage of their cargo to the cytosol through a pore (for a review, see reference 9).

LT is present at high concentrations in the blood of infected animals and is sufficient to induce some symptoms of anthrax and lethality in animal models (4, 21, 41, 11, 29, 30, 56). Although many LT-mediated effects in cells and animals have been described over the years, the actual mechanism by which LT causes cell or animal death is unknown. Studying the toxin's unique induction of rapid lysis in murine macrophage lines (such as RAW264.7 and J774.A1 cells) or in primary macrophages from BALB/cJ mice may provide a better understanding of its function and cellular targets (23). The macrophage lysis assay also provides a rapid screening method for inhibitors of each step of the intoxication pathway (31). Therefore, while the role that macrophage sensitivity plays in anthrax pathogenesis and LT-mediated lethality has been questioned (11, 34, 37, 42), the study of macrophage lysis can provide important clues

about LT function. Currently, no link between MEK cleavage and LT cytotoxicity has been established and no mechanism for LT-mediated cytotoxicity has been proposed.

P2X7 (previously known as P2Z) receptors are members of the P2 purinergic receptor family responsible for cell responses to extracellular nucleotides. These receptors play many roles in immunomodulation and signaling through binding of extracellular ATP, which is often released from cells in a controlled manner via nonlytic events or more rapidly due to cell damage (for reviews, see references 15 and 16). This ubiquitous receptor is itself an ATP-gated ion channel, the prolonged activation of which leads to progressive increase in the size of a nonselective membrane pore, allowing passage of molecules of up to 900 Da (10, 16, 50). In macrophages, ATP activation of the P2X7 receptor and subsequent pore formation are associated with induction of rapid cytotoxicity (15, 18, 44), and ATP resistance of macrophage lines has been shown to correlate with lack of P2X7 receptor expression (8, 16).

We hypothesized that the rapid (<90 min) LT-mediated lysis of macrophages could involve activation of the P2X7 receptor through paracrine effects of ATP released from the cells. Because all the effects of P2X7 receptors, including the ATP-mediated cytotoxicity, can be abrogated by antagonists that include periodate-treated ATP (oxidized ATP [o-ATP]) (43), we tested the effects of this compound and other P2X7 antagonists on LT-mediated macrophage lysis. We found that o-ATP protects LT-sensitive macrophages and mice independently of P2X7 receptor function by inhibiting effective LF translocation to the cytosol through inhibition of sodium dodecyl sulfate (SDS)-resistant PA oligomer formation. We also present data on the effectiveness of this novel LT inhibitor in protecting against toxin-induced lethality in mice.

* Corresponding author. Mailing address: Building 30, Room 303, National Institutes of Health, Bethesda, MD 20892. Phone: (301) 594-2865. Fax: (301) 480-0326. E-mail: sleppla@niaid.nih.gov.

MATERIALS AND METHODS

Materials. PA, LF, EF, and diphtheria toxin (DT) were purified as previously described (7, 33, 55), yielding a single band by Coomassie-stained SDS-polyacrylamide gel electrophoresis (PAGE). For cytotoxicity assays, toxin was prepared in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) prior to addition to cells. Toxin for animal injections was prepared in sterile phosphate-buffered saline (PBS) with or without o-ATP. Concentrations and doses of LT refer to the amounts of each component (i.e., 1,000 ng LT/ml is 1,000 ng PA plus 1,000 ng LF/ml; 100 μ g LT is 100 μ g PA plus 100 μ g LF). Rabbit polyclonal antibodies to PA and LF were developed in our laboratory by standard immunization methods (<http://www.nal.usda.gov/awic/pubs/antibody/guidelin.htm>). MEK1 N-terminal (NT) antibody and purified six-His- and glutathione *S*-transferase (GST)-tagged MEK1 used for in vitro proteolysis assays were purchased from Upstate Biotechnologies (Waltham, MA). MEK2 and MEK3 N-terminal antibodies and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Infrared-dye-conjugated secondary antibody (IRDye800CW immunoglobulin G [IgG]) was purchased from Rockland Immunochemicals (Gilbertsville, PA). The 1 $\times$ trypsin-EDTA (0.05% trypsin in Hanks balanced salt solution) used in limited cell surface proteolysis assays was purchased from Invitrogen (Carlsbad, CA), and o-ATP, o-ADP, o-GTP, o-ITP, KN-62, and PMSF (phenylmethylsulfonyl fluoride) were purchased from Sigma (St. Louis, MO). o-ATP reduced with sodium borohydride, oxidized adenosine, the P2 receptor antagonists pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), iso-PPADS, and NF279, and the P2X7 antagonists MRS2326, MRS2361, and MRS2409 were the kind gifts of Kenneth Jacobson (National Institute of Diabetes and Digestive and Kidney Diseases, NIH) (47). LysoTracker Red DND-99 and acridine orange were purchased from Molecular Probes (Eugene, OR) and bafilomycin from Calbiochem (San Diego, CA).

Cytotoxicity assays. RAW264.7 cells (ATCC, Manassas, VA) were grown in DMEM with 10% fetal calf serum, 2 mM Glutamax, 2 mM HEPES, and 50 μ g/ml gentamicin (all from Invitrogen, Carlsbad, CA) at 37°C in 5% CO₂. Cells were seeded into 96-well plates 24 to 48 h prior to assay and grown to 80 to 90% confluence. In macrophage protection assays, in which the drug was introduced first, cells were treated with o-ATP or other drugs in duplicate at various concentrations for 10 min prior to the addition of a set concentration of LT per assay. Cell viability was assessed 150 min after LT addition by addition of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) at a final concentration of 0.5 mg/ml. The cells were then further incubated with MTT for 40 min, and the blue pigment produced by viable cells was dissolved by removing all medium; adding 50 μ l/well of 0.5% (wt/vol) SDS, 25 mM HCl in 90% (vol/vol) isopropanol; and shaking the plates for 5 min prior to reading the A_{570} using a microplate reader. In similar experiments, cells were first treated with twofold dilutions of LT at 4°C for 1 h, and unbound toxin was removed by washing the cells with DMEM. Following a shift to 37°C, a single fixed concentration of o-ATP (1 mM) was added at various time points (20, 40, and 65 min). Cell viability was then assessed as described above after 180 min. For DT toxicity assays, CHO WTP4 cells (35) were grown in alpha modified essential medium (AMEM) supplemented exactly as with DMEM, as described above. Cells were treated with DT in the presence of 200 μ M or 500 μ M o-ATP or no drug for 90 min prior to removing all media containing DT and drug and washing the cells once with AMEM, followed by adding 100 μ l/well of complete AMEM. The cells were then incubated for 46 h at 37°C prior to the addition of MTT and assessment of cell viability as described above. For ET assays, RAW264.7 cells in 96-well plates were treated with o-ATP at various concentrations for 10 min prior to the addition of ET (100 ng/ml). The cells were incubated with ET for 60 min, and cyclic AMP (cAMP) production was assessed using the 96-well BioTRAK cAMP enzyme immunoassay from Amersham Pharmacia Biotech (Piscataway, NJ) according to the manufacturer's protocol.

PA binding and MEK cleavage assays. RAW264.7 or CHO WTP4 cells were grown in 10-cm plates or six-well plates to 90% confluence. The cells were treated with 500 or 1,000 μ M o-ATP, o-ADP, or medium (control) for 10 min prior to the addition of LT at 1 μ g/ml and incubation at 37°C for various lengths of time. In some experiments, cells were treated with 0.5 μ M bafilomycin for 30 min prior to the addition of LT. The medium was removed, and the cells were washed five times in ice-cold PBS, followed by lysis in RIPA buffer (1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS in PBS) plus COMPLETE protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein concentrations in lysates were quantified using the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL) to ensure equal loading on gels. For oligomer visualization, 4 to 20% Novex gradient gels (Invitrogen, Carlsbad, CA) were used, and electrophoresis was performed for a long enough period to allow

FIG. 1. o-ATP protects against LT-mediated macrophage lysis. RAW264.7 cells were treated with o-ATP for 10 min prior to LT (500 ng/ml) addition, and cell viability was assessed after 150 min. Percent viability was calculated relative to medium-treated cells. The results shown are for a single experiment representative of multiple assays.

proteins of <40 kDa to run off the gels in order to ensure entry of the oligomer into the gel prior to transfer to nitrocellulose. Western blot analysis was performed using anti-PA (1:5,000), anti-MEK1 NT (1:2,000), anti-MEK2 NT (1:1,000), anti-MEK3 NT (1:1,000), or anti-LF (1:1,000) antibody. For detection, horseradish peroxidase-labeled secondary IgG (1:2,000) and chemiluminescence were used in most experiments. Alternatively, antibody was detected using infrared-dye-conjugated secondary IgG (1:5,000) and the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). For removal of cell surface-bound toxin, RAW264.7 cells or CHO WTP4 cells in six-well plates were first treated in duplicate with LT (1 μ g/ml) in the presence or absence of 1 mM o-ATP for 45 min at 37°C. The medium was removed, and the cells were washed three times with PBS. Half the wells received enough 1 $\times$ Trypsin-EDTA (Invitrogen, Carlsbad, CA) to cover the cells, followed by immediate removal of all the solution. The cells were then observed under the microscope, and upon rounding or lifting of 80 to 90% in trypsin-treated wells (<2 min), 3 ml of DMEM containing 30% fetal bovine serum-1 mM PMSF was added to all the wells. All cells were removed, washed once in a >10 $\times$ excess volume of PBS, and resuspended in RIPA buffer containing protease inhibitor cocktail and PMSF. After protein quantification with bicinchoninic acid, samples were treated with SDS loading buffer, boiled, and applied to SDS-PAGE for Western blotting with anti-PA and anti-LF antibodies.

In vitro MEK cleavage assays. Purified N'-GST-MEK1-HIS-C' fusion protein (125 ng) obtained from Upstate Biotechnologies was cleaved by the addition of 25 ng of LF or LF pretreated with 1, 2, or 4 mM o-ATP. Cleavage was in a reaction buffer of 50 mM Tris-HCl, pH 7.4, 1 mM NaCl and a total volume of 10 μ l. The reactions were stopped after 60 min by adding SDS loading buffer and boiling them. One-tenth of each reaction mixture (12.5 ng MEK1) was subjected to SDS-PAGE, followed by Western blotting using MEK1 NT antibody (1:1,000). The cleavage of the N terminus of MEK1 fused to GST resulted in a significant size change detected by Western blotting.

Microscopy. CHO WTP4 cells were grown in eight-chamber Lab-Tek Chambered no. 1 Borosilicate Coverglass slides (no. 1554411; Nalgene Nunc International, Rochester, NY) to 50 to 60% confluence and treated with 0.5 μ M bafilomycin (30 min) or 1 mM o-ATP (for 15 min) prior to the addition of dye. Acridine orange (16 μ M) or LysoTracker Red (60 nM) was added and incubated for 20 min or 1 h, respectively. The cells were then washed three times with DMEM without phenol red (Invitrogen, Carlsbad, CA) prior to image collection on a Leica SP2-UV405 confocal microscope (Leica Microsystems, Exton, PA) using a 63 $\times$ oil immersion objective. LysoTracker Red (excitation, 561 nm; emission, 568 to 641 nm) and acridine orange (excitation, 514 nm; emission, 618 to 666 nm) images were collected simultaneously with differential interference contrast images. The images were processed, and fluorescence was quantified using Leica TCS-SP software (version 2.1537). The relative fluorescence associated with each treatment was determined by averaging the total fluorescence minus background ($n = 10$ /slide). Relative fluorescence values for the o-ATP

FIG. 2. Oxidized nucleotides protect against LT. RAW264.7 cells were treated with each compound shown for 10 min prior to LT (1,000 ng/ml) addition, and cell viability was assessed after 150 min. Percent viability was calculated relative to medium-treated cells. The results shown are for a single experiment representative of multiple assays.

and bafilomycin treatments were then calculated as percentages of untreated controls.

Animals. BALB/cJ mice (10 to 16 weeks old; 20 to 24 g) were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were injected intraperitoneally (i.p.) with 1.0 ml of 100 μ g LT alone (100 μ g PA plus 100 μ g LF/ml in PBS) or 100 μ g LT in an o-ATP-PBS solution of 9 mM or 4.5 mM (equivalent to 4.5 mg and 2.25 mg/mouse). The animals were monitored every 12 h for 144 h for signs of malaise or mortality. For experiments involving pre- or posttreatment of mice with drug, o-ATP was injected (4.5 mg/mouse; i.p.) 3 h prior to or after LT injection (100 μ g; i.p.), and the animals were monitored similarly.

RESULTS

Oxidized-ATP protects against LT-mediated macrophage lysis. LT-sensitive RAW264.7 macrophages treated with o-ATP for 10 min prior to the addition of LT were protected from lysis in a dose-dependent manner at concentrations of 200 μ M

(Fig. 1). This protective range correlates with the high concentrations necessary for o-ATP P2X7 receptor antagonism (16). Concentrations higher than 1,000 μ M caused 5 to 25% toxicity over the course of the assay.

Other P2 and P2X7 receptor antagonists do not protect against LT. Surprisingly, other P2X7 receptor antagonists were incapable of protecting RAW264.7 macrophages from LT function. Figure 2 shows the results for KN-62, but because KN-62 may have specificity for human over mouse P2X7 receptors (16), we also tested a panel of other antagonists. The general P2 receptor antagonists PPADS, iso-PPADS, and NF279 and the potent P2X7 antagonists MRS2326, MRS2361, and MRS2409 (2, 47) all had no effect on LT-mediated lysis (data not shown), while compounds normally inactive against the receptors, such as the oxidized forms of ADP, GTP, and ITP, were effective in protecting against LT (Fig. 2 and data not shown). Additionally, both sodium borohydride-reduced o-ATP and oxidized adenosine were unable to protect macrophages from LT (data not shown).

o-ATP inhibits LT-mediated MEK cleavage in cells, but not LT proteolytic function. To determine which step in LT toxicity was altered by o-ATP, we began by testing whether LF was delivered to its substrates in the cytosol. MEK1, MEK2, and MEK3 cleavage was followed in RAW264.7 cells treated with LT for 35, 60, or 85 min in the presence or absence of o-ATP. Cell lysates were monitored for MEK cleavage by Western blotting using N-terminal antibodies specific to epitopes spanning the cleavage sites for MEK1 and MEK2 and capable of monitoring a size shift in the case of MEK3. LT cleavage of MEKs was rapid and complete by 60 to 85 min in the absence of o-ATP but totally inhibited in its presence (Fig. 3A). To test if o-ATP was directly inhibiting LF proteolytic function, LF was pre-treated with various doses of o-ATP prior to the performance of in vitro MEK cleavage assays using purified N-terminally GST-tagged MEK1. As shown in Fig. 3B, o-ATP did not inhibit LF proteolytic function.

o-ATP does not inhibit PA binding and processing but prevents formation of SDS-resistant PA oligomers. To test whether

FIG. 3. o-ATP effects on LT-mediated MEK cleavage in cells and in vitro. (A) RAW264.7 cells were treated with LT (1 μ g/ml) in the presence or absence of o-ATP (1 mM) for various times prior to cell lysis, SDS-PAGE, and Western blotting using MEK1 NT antibody (1:2,000), MEK2 NT antibody, and anti-MEK3 NT antibody (both at 1:1,000). (B) Purified N'-GST-MEK1-HIS-C' fusion protein (125 ng) was cleaved for 1 h by addition of 25 ng of LF or LF pretreated with 1, 2, or 4 mM o-ATP (1 h). One-tenth of each reaction mixture was subjected to SDS-PAGE and Western blotting using MEK1 NT antibody (1:1,000). NT refers to no-treatment control cells, which had not received toxin.

FIG. 4. o-ATP effects on PA binding, processing, and oligomerization and LF uptake, (A) CHO WTP4 cells were treated with LT in the presence or absence of o-ATP, and Western blot analysis was performed with anti-PA polyclonal antibody (1:5,000). The cellular cross-reactive band above PA83 is an internal control verifying equal loading in all lanes. (B) CHO WTP4 lysates were prepared similarly to those in panel A, but with LT treatment in the presence of either 1 mM o-ATP or 1 mM o-ADP, and electrophoresed longer to ensure movement of SDS-resistant oligomers into the gels. Formation of the SDS-resistant PA oligomer was monitored by Western blotting with anti-PA polyclonal antibody (1:5,000). The image is a single gel showing the oligomeric species, PA83, PA63, and the cellular cross-reactive band. (C) CHO WTP4 and RAW264.7 lysates were prepared as described for panel A and analyzed for LF content by Western blotting using polyclonal anti-LF (1:1,000). (D) Limited cell surface proteolysis on cells treated with LT in the presence or absence of o-ATP (1 μ M) was performed as described in Materials and Methods prior to Western blot analyses (of the same samples on two separate gels, divided by the black bar) with anti-PA and anti-LF sera. The leftmost lane is purified PA83 control, and the next lane is a molecular weight marker (overloaded). In all gels, NT refers to no-treatment control cells, which had not received toxin or drug.

o-ATP interfered with PA binding and processing or oligomer formation, lysates of CHO WTP4 and RAW264.7 cells that had been treated with LT or LT in the presence of 500 or 1,000 μ M o-ATP were analyzed by Western blotting using anti-PA antibody. PA binding and cleavage of PA83 to PA63 was not altered in the presence of o-ATP (Fig. 4A shows the results for CHO WTP4 cells). However, formation of the SDS-resistant PA oligomer, which requires the acidic endosome environment (9, 40), was inhibited by o-ATP (Fig. 4B). Formation of this oligomer is indicative of the PA pore transition that is required for LF translocation into the cytosol. The SDS-sensitive oligomer formed at the surfaces of cells following PA binding and cleavage was unaffected, as indicated by the cells' binding of LF equally in the presence or absence of o-ATP (Fig. 4C shows the results for both RAW264.7 and CHO WTP4 cells), since LF binding requires the formation of this oligomer (12). To test if the cell surface oligomer-bound LF was internalized, we performed limited cell surface proteolysis on CHO WTP4 cells treated with LT in the presence or absence of o-ATP, remov-

ing surface-bound PA83 with no effect on PA63 and LF, indicating that these proteins were internalized and protected from trypsin treatment (Fig. 4D).

o-ATP inhibits effective translocation of other toxins. The data mentioned above suggested that o-ATP was acting on translocation from endosomes, possibly through effects on acidification. We therefore examined the effects of o-ATP on ET-mediated cAMP production, which requires formation of the SDS-resistant PA63 oligomer and translocation from late endosomes. We also analyzed effects on DT killing of CHO WTP4 cells, which requires toxin release from acidic early endosomes (32, 45). As seen in Fig. 5A and B, o-ATP inhibited both toxins. In the case of ET, the fact that o-ATP has been previously shown to modify a mammalian adenylate cyclase at the ATP-binding site (57) suggested that the toxin could also be directly inhibited by o-ATP. We tested the direct effects of o-ATP on the enzymatic function of EF and found that 1 mM o-ATP caused a fourfold reduction in cAMP production in assays containing 0.5 mM ATP and 1 pg/ml EF (data

FIG. 5. o-ATP effects on ET, DT, and LT. (A) RAW264.7 cells were treated with serial dilutions of o-ATP prior to the addition of ET and cAMP measurement. (B) CHO WTP4 cells were treated with DT in the presence of 200 μ M or 500 μ M o-ATP, and cell viability was assessed following assay protocols described in Materials and Methods. Percent survival was calculated relative to o-ATP-treated controls. (C) RAW264.7 cells were first treated with LT at 4°C for 1 h, and unbound toxin was removed. o-ATP (1 mM) was added at various time points after shift to 37°C, and cell viability was assessed by MTT assay. The results are for a single assay representative of three similar experiments.

not shown). The inhibition of DT function by o-ATP, however, argues for a general mechanism, potentially affecting toxin translocation. Furthermore, in experiments where o-ATP was added 20, 40, or 65 min after LT was allowed to enter cells, addition by 20 min resulted in full protection of cells, while addition at later times was less effective. These results are consistent with the hypothesis that once LT is translocated to the cytosol (15 to 20 min), o-ATP does not function as a protectant, while earlier treatment of cells allowed for full protection (Fig. 5C).

o-ATP alters endosomal pH. Because o-ATP prevented formation of the SDS-resistant PA oligomer, we hypothesized that o-ATP may alter endosomal pH, possibly through effects on the vacuolar ATPase. It has been shown that o-ATP modifies and inhibits many ATPases (13, 25, 26, 39, 46, 52). We tested the effects of o-ATP on endosomal pH directly by using two pH-sensitive dyes, acridine orange and LysoTracker Red. As a control, bafilomycin, an inhibitor of the vacuolar ATPase (6, 17, 54), which prevents PA oligomer formation (38 and data not shown), was used. The acridine orange dye is membrane permeant at neutral pH but becomes trapped and concentrated upon protonation in acidic compartments. A green fluorescence is associated with the diffuse cytoplasmic or nuclear staining with this dye, while a strong granular orange-red fluorescence is seen in normal cells upon accumulation of the dye in acidic endosomes (Fig. 6). This orange-red fluorescence has been shown to be almost totally abolished upon pretreatment of cells with bafilomycin (3, 54, 59), and we found the same result in our control experiments with CHO cells treated with this drug (Fig. 6). Endosomal acridine orange staining was also significantly reduced in o-ATP-treated cells, by almost 50% (Fig. 6). Similarly, LysoTracker Red is also retained in acidic compartments after protonation, and the red fluorescence of this dye in endosomes has also been shown to greatly diminish upon inhibition of the vacuolar ATPase (27, 28, 53, 58). We found almost complete loss of fluorescent staining in bafilomycin-treated CHO cells and a reduction to 30% staining in o-ATP-treated cells (Fig. 6).

o-ATP protection of mice against lethal doses of LT. Based on previous studies showing a reduction of LT toxicity in mice using the basic lysosomotrope, chloroquine, which interferes with endosome acidification and LT translocation to the cell cytosol (1), we decided to test o-ATP for protection against LT lethality in mice. Based on an approximation of a 1:20 dilution in a whole mouse, we tested 9 mM and 4.5 mM o-ATP concentrations injected in a 1-ml volume i.p. (4.5 mg and 2.25 mg/mouse, respectively) for the ability to protect BALB/cJ mice against a lethal dose of LT (100 μ g; i.p.) (41). As shown in Fig. 7, administration of either dose of o-ATP with LT resulted in complete protection of the mice, with no signs of malaise at any point throughout the experiment, while all control mice treated with 100 μ g LT succumbed in 2 to 4 days. We then tested whether o-ATP was effective when administered before or after toxin injection. Administration of 4.5 mg o-ATP 3 h after LT injection did not result in any protection or delay in LT lethality (data not shown), as expected, since >90% of this dose of toxin is expected to be taken up by cells in mice by 2 h. However, administration of 4.5 mg o-ATP 3 h prior to LT attenuated toxin effects so that lethality was delayed by at least

FIG. 6. o-ATP effects on endosomes. CHO WTP4 cells treated with 0.5 μ M bafilomycin (BAF) or 1 mM o-ATP and untreated controls were stained with acridine orange (left) or LysoTracker Red (right). Fluorescence levels were quantified using Leica TCS-SP software, and relative fluorescence for the o-ATP and bafilomycin treatments were calculated as percentages of no-drug (untreated) controls (NT) and plotted as shown in the graphs. Paired *t* tests (99% confidence interval) were performed on the data using Graph Pad Prism 4.0 software and indicate the following *P* values for comparisons of the different data groups (for each dye, quantifications of the differences between all three groups are statistically significant): for acridine orange, NT and BAF ($P < 0.0001$), NT and o-ATP ($P = 0.0013$), BAF and o-ATP ($P = 0.0002$); for LysoTracker, NT and BAF ($P < 0.0001$), NT and o-ATP ($P = 0.0003$), BAF and o-ATP ($P = 0.0133$). The error bars indicate standard deviations.

32 to 48 h for all treated mice (data not shown). All o-ATP-pretreated mice did eventually succumb to LT.

DISCUSSION

Although the physiological functions of P2X7 receptors are not well known, the potent cytolytic effect caused by their sustained activation with ATP, especially as seen in macrophages, led us to investigate the possibility that they could play a role in the rapid LT-mediated lysis of macrophages. Initial experiments showing protection of macrophages from LT toxicity using o-ATP, a potent irreversible antagonist of the P2X7 receptor, led us to test other P2X7 antagonists, but none were protective. We also performed LT toxicity studies in the presence of ATPases, such as apyrase, with no effect on LT function (data not shown), arguing against a contribution by extracellular ATP to LT-mediated macrophage lysis. Although the concentrations of o-ATP needed for protection correlated well with those needed for P2X7 receptor

antagonism, the rapidity with which o-ATP was able to protect macrophages (10 min) did not correlate with the 1-h treatment usually needed to observe P2X7 function (16). Subsequently, the discovery that o-ATP inhibited LF-mediated MEK cleavage in cells led us to investigate the step at which this compound interfered with LF function.

Although early work associated almost all the effects of o-ATP in cell systems with its inhibition of P2X7 receptor function, more recent data indicate that many of the effects of o-ATP, such as anti-inflammatory effects and those on NF- κ B activation, occur in cell types lacking P2X7 receptors, as well as in macrophages from P2X7 knockout mice (5, 14, 49). These results have raised new questions about when o-ATP can reliably be used as a P2X7 receptor blocker and how it exerts its P2X7-independent effects (14). Beigi et al. (5) suggested that o-ATP may directly interact with and inhibit other receptors, such as Toll-like receptors, or gain access to the cell cytoplasm by fluid phase pinocytosis and modify target proteins having

FIG. 7. o-ATP protects BALB/cJ mice from LT-mediated lethality. BALB/cJ mice were injected (1 ml; i.p.) with 100 μ g LT alone or 100 μ g LT in an o-ATP-PBS solution of 9 mM or 4.5 mM (equivalent to 4.5 mg and 2.25 mg/mouse). The animals were monitored every 12 h for 7 days for signs of malaise or mortality. The data shown are from an experiment representative of three independent experiments in which slightly higher or lower concentrations of o-ATP were used with similar results. The graphed data are based on six mice per treatment.

affinity for ATP, such as kinases. In light of these recent discoveries, results obtained with this drug must be interpreted carefully and with the recognition that it may not be a highly selective P2X7 antagonist as previously thought. Additional complications arise when it is noted that o-ATP is a Schiff base-forming drug, and this category of compounds has been shown to have immunomodulatory effects on cells (48).

Historically, o-ATP was used as an affinity reagent for nucleotide binding proteins (36), where long incubations allowed covalent modification of ATP-binding sites in purified proteins (36, 22, 57), including many ATPases, such as the Na^+K^+ ATPase (46), mitochondrial F1 ATPase (13), Ca^{2+} ATPase from the sarcoplasmic reticulum (26, 39), $\text{Na}^+\text{Mg}^{2+}$ ATPase of *Acholeplasm laidlawii* (25), and *Escherichia coli* GroEL (52). Modification was often accompanied by loss of function. o-ADP and other oxidized nucleotides are also capable of the same effect on ATPases (13, 51). These examples, as well as our data showing interference with formation of the acid-dependent SDS-resistant oligomer due to increase in endosomal pH, suggest that o-ATP could be acting on the endosome vacuolar H^+ ATPase. However, o-ATP is cell impermeable, and it is unclear whether sufficient amounts could enter cells by fluid phase pinocytosis during the short exposure times of our experiments. The possibility that the endosomal ATPase is first modified at the cell surface is also problematic, as the ATP-binding domain resides in the cytosolic side of the complex and is not accessible to cell-impermeable agents. Our results have not definitively proven that o-ATP targets the vacuolar ATPase, but it is clear from microscopy analyses that endosomal pH is affected by this drug. We must also consider alternative o-ATP effects, which could include interference with various trafficking components, such as chaperones, which are subsequently unable to partake in or aid the oligomer conformational change. Many chaperones have ATP-binding sites and would be ideal targets. In light of the inhibitory effect on DT toxicity, it is likely that o-ATP affects a part of the general

cell endocytosis machinery involved in translocation from endosomes (such as the vacuolar ATPase), rather than any PA-specific components.

The ability of o-ATP to completely prevent LT-mediated effects in mice is a novel application of this drug. The only previous *in vivo* uses of o-ATP were numerous studies by a single laboratory on the inhibition of inflammatory responses in a rat model (24). However, because o-ATP is a regulator of interleukin-1 β in macrophages, as well as an inhibitor of NF- κ B activation (19, 20), it is possible that some of the *in vivo* effects of the drug are due to its immunomodulatory effects, in combination with its LT-inhibitory function. Interestingly, the survival shift seen with o-ATP pretreatment was similar to that noted in similar studies with chloroquine (1), which also results in delayed death of all treated mice from LT. However, these parallel survival shifts are not necessarily indicative of similar functions for the two drugs.

In summary, we have demonstrated that o-ATP protects cultured cells and mice from LT and provided evidence that the protection is due to prevention of endosome acidification. Although o-ATP acts on P2X7 receptors, this action is not involved in the process of LT-mediated macrophage lysis. Thus, protection against anthrax LT represents another example of P2X7-independent o-ATP effects. The investigation of the detailed cellular processes by which o-ATP prevents formation of the SDS-resistant PA63 oligomer required for PA pore formation and LF translocation will be pursued in future studies.

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