INFECTION AND IMMUNITY, Nov. 2007, p. 5175–5184 Vol. 75, No. 11
0019-9567/07/$08.00+0 doi:10.1128/IAI.00719-07

Anthrax Protective Antigen Cleavage and Clearance from the Blood of Mice and Rats[▽]

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Received 28 May 2007/Returned for modification 19 June 2007/Accepted 14 August 2007

Bacillus anthracis **protective antigen (PA) is an 83-kDa (PA83) protein that is cleaved to the 63-kDa protein (PA63) as an essential step in binding and internalizing lethal factor (LF). To assess in vivo receptor saturating PA concentrations, we injected mice with PA variants and measured the PA remaining in the blood at various times using PA83- and PA63-specific enzyme-linked immunosorbent assays. We found that both wild-type PA (WT-PA) and a receptor-binding-defective mutant (Ub-PA) were cleaved to PA63 independent of their ability to bind cells. This suggested a PA-acting protease activity in the blood. The protease cleaved PA at the furin cleavage sequence because furin site-modified PA mutants were not cleaved. Cleavage measured in vitro was leupeptin sensitive and dependent on calcium. Cell surface cleavage was important for toxin clearance, however, as Ub-PA and uncleavable PA mutants were cleared at slower rates than WT-PA. The cell binding-independent cleavage of PA was also verified by using Ub-PA (which is still cleaved) to rescue mice from toxin challenge by competitively binding circulating LF. This mutant was able to rescue mice even when given 12 h before toxin challenge. Its therapeutic ability was comparable to that of dominant-negative PA, which binds cells but does not allow LF translocation, and to the protection afforded through receptor clearance by WT-PA and uncleavable receptor binding-competent mutants. The PA cleavage and clearance observed in mice did not appear to have a role in the differential mouse susceptibility as it occurred similarly in lethal toxin (LT)-resistant DBA/2J and LT-sensitive BALB/cJ mice. Interestingly, PA63 was not found in LT-resistant or -sensitive rats and PA83 clearance was slower in rats than in mice. Finally, to determine the minimum amount of PA required in circulation for LT toxicity in mice, we administered time-separated injections of PA and LF and showed that lethality of LF for mice after PA was no longer measurable in circulation, suggesting active PA sequestration at tissue surfaces.**

Anthrax toxin, a major contributor to pathogenesis during infection by *Bacillus anthracis*, consists of two enzymatic polypeptides, lethal factor (LF) and edema factor and the receptor-recognition protein protective antigen (PA). Lethal toxin (LT) consists of PA and LF, which are individually nontoxic. PA (83 kDa) binds to the cellular receptors ANTXR1 (TEM8) and ANTXR2 (CMG2) (2, 24) and is cleaved to its 63-kDa form (PA63). Oligomerization of PA63 to a heptamer creates binding sites for LF that allow its translocation to the cell cytosol (for a review, see reference 10). LF is a metalloproteinase which cleaves and inactivates members of the mitogen-activated protein kinase kinase family (MEKs) (4, 22, 27). Previous studies indicated that PA83 (the 83-kDa form of PA) cleavage occurs primarily at the cell surface due to the action of cell surface proteases such as furin (6, 8, 18). However, an early study reported that PA in the blood of spore-infected guinea pigs exists primarily in the PA63 form and that blood from a variety of animal species contain PA protease activity (5). A recent follow-up to that study isolated complexes of PA63-bound LF from plasma of infected rabbits and guinea pigs with functional activity for cleavage of MEK proteins (20). Details regarding the cleavage and clearance of PA in vivo are relevant to understanding the toxin's role in pathogenesis and also because modified PA proteins are being developed as potential anticancer drugs (13–15).

We chose to analyze the rates of cleavage and clearance of PA in the circulation of healthy mice and rats after bolus injection of PA in order to avoid potential proteolytic artifacts that could arise from anthrax infection. By injecting a range of PA doses, we sought to identify amounts that saturate receptors in mice and then to determine rates of cleavage and clearance of the circulating PA. In addition to wild-type PA (WT-PA), we analyzed the cleavage, clearance, and therapeutic potential of a PA mutant altered in the receptor-binding domain so that it cannot bind cells (Ub-PA) and PA mutants modified at the furin cleavage sequence. We present data comparing these mutants and discuss the implications of their similar or contrasting cleavage, clearance, and therapeutic abilities.

MATERIALS AND METHODS

Toxins. LF and all PA variants were purified from *B. anthracis* as previously described (21, 26). PA variants PA-U7, PA-U2, PA-L1 (all uncleavable by furin) have been previously described (13–15). In two of these proteins, the furin cleavage site was altered to cleavage sites for matrix metalloproteinase (PA-L1) or urokinase plasminogen activator (PA-U2). The third (PA-U7) has the cleavage sequence deleted. Ub-PA contains three mutations (D683A, L685E, and Y688K) at the receptor binding site and cannot bind to cells (23). A dominant-negative PA mutant (PA-DN) contains two point mutations (K397D and D425K) that prevent proper channel function and LF translocation (25). Toxin for animal injections was prepared in sterile phosphate-buffered saline (PBS).

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▽ Published ahead of print on 27 August 2007.

For cytotoxicity assays, toxin was prepared in serum-free Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) prior to addition to cells.

Antibodies. Anti-PA rabbit polyclonal antibody 5308 was developed in our laboratory. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). Infrared dye-conjugated secondary antibodies were purchased from Rockland Immunochemical (Gilbertsville, PA). Anti-PA monoclonal antibodies 14B7 and 1G3 have been previously described (11, 12), and affinity-purified preparations were made by the National Institute of Allergy and Infectious Diseases core facility. The hybridoma cell line 1E9 was developed (using as antigen the N-terminal 20-kDa fragment partially purified from an in vitro PA cleavage) during the same studies which generated 1G3 (11). In addition to the affinity-purified 1E9 monoclonal antibody, a different preparation of 1E9 was prepared from hybridoma cells. These were adapted to serum-free CCM1 medium (HyClone Laboratories, Logan, UT) by first growing cells in one part DMEM and two parts CCM1 without additives. Cells were next passaged in complete CCM1 medium containing 25 mM HEPES and 50 μg/ml gentamicin. Cells were grown in roller bottles, and the culture supernatant was precipitated with 70% saturated ammonium sulfate, followed by dialysis against 10 mM HEPES. This product is referred to as 1E9-PPT. CCM1 medium alone was precipitated in parallel in a similar manner and is referred to as CCM1-PPT.

Animals. BALB/cJ and DBA/2J mice (8 to 12 week old; 20 to 22 g) were purchased from Jackson Laboratories (Bar Harbor, ME). Fischer F344 and Lewis rats (175 to 200 g) were purchased from Taconic Farms (Germantown, NY). Animals were injected intraperitoneally (i.p.) or intravenously (i.v.) with different doses of individual or combined toxin components, as described in each experiment. Animals were bled by cardiac puncture directly into syringes coated with heparin (10 to 20 USP units/ml) or EDTA (5 mM) for plasma collection or into serum separator tubes (Sarstedt, Newton, NC). Plasma used in these studies was platelet-poor plasma, as blood samples were centrifuged at 3,500 rpm for 10 to 15 min prior to removal of the upper plasma layer.

Enzyme-linked immunosorbent assays (ELISAs) and Western blot analyses. Immunolon-II high-binding 96-well plates (Dynatech, Chantilly, VA) were coated overnight with monoclonal antibodies (1 μg/ml in PBS) or 1E9-PPT or CCM1-PPT (1:1,000 dilution). Control wells contained PBS only (no capture antibody). Plates were washed two to three times with wash buffer (10 mM PBS, 0.05% Tween 20), followed by blocking with 1% gelatin (Bio-Rad, Hercules, CA) for 45 min. Plates were again washed three times with wash buffer, and diluted serum or plasma samples or PA83/PA63 standards prepared in PBS were added to each well. Following a 2-h incubation, plates were washed three times, and anti-PA polyclonal 5308 antibody (1:8,000 in PBS) was added to all wells for 2 h. Following five washes, HRP-conjugated anti-rabbit antibody (1:2,000) was added to all wells. After another five washes, plates were developed using HRP-tetramethyl benzidine developer and STOP solution (both from R&D Systems, Minneapolis, MN), and the optical density was read at 450 nm. Western blot analyses were performed utilizing polyclonal anti-PA 5308 (1:5,000) or monoclonal antibodies 14B7, 1G3, and 1E9-PPT (all at 1:500) as primary antibodies. Blotting was performed using the Odyssey LI-COR blocking solution (LI-COR Biosciences, Lincoln, NE), Rockland Immunochemical (Gilbertsville, PA) infrared dye (IRDye 800)-conjugated secondary antibodies (anti-rabbit, 1:5,000; anti-mouse, 1:2,000) and the Odyssey Infrared Imaging System (LI-COR Biosciences). For 1G3 antibody, 5% milk was used in some blots for blocking and primary antibody incubations.

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 μg/ml gentamicin (all from Invitrogen) at 37°C in 5% CO_2. Cells were seeded into 96-well plates 24 to 48 h prior to assays. Sera collected from PA-injected or control animals were serially diluted two- to fourfold in medium, followed by the addition of a set concentration of LF prior to treatment of macrophages with this mixture. Cell viability was assessed after 150 min by the addition of MTT [3-(4, 5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) at a final concentration of 0.5 mg/ml. 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 μl/well of 0.5% (wt/vol) sodium dodecyl sulfate (SDS)–25 mM HCl in 90% (vol/vol) isopropanol, and shaking the plates for 5 min prior to reading the A_{570}. PA standard dose response curves were performed on the same plates. The 50% effective concentration values for dilution curves of collected sera were calculated, and activity-based PA concentrations were back-calculated by comparison to standard PA toxicity curve 50% effective concentration values using GraphPad Prism (version 4.0) software.

Cleavage assays. Ninety-six-well plates containing PA (10 μg/ml) in 15 mM HEPES–2 mM NaCl or, alternatively, in DMEM–10 mM HEPES were treated with serial two- or fourfold dilutions of heparin-plasma, EDTA-plasma, or serum collected from BALB/cJ mice. For some experiments, reaction mixtures in 15 mM HEPES were supplemented with one of the following: $CaCl_2$ (2 mM), NaCl (100 mM), or EDTA (50 mM). All reactions were allowed to proceed for 2 h at room temperature. A 10-μl sample from each reaction mixture was then diluted 1:5 in a new 96-well plate, and 5-μl samples (total PA, 10 ng) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), followed by Western blotting using anti-PA polyclonal serum 5308. In experiments testing the ability of protease inhibitors to inhibit PA cleavage, 96-well plates containing PA (10 μg/ml) in DMEM with 10 mM HEPES and 10-fold dilutions of each inhibitor were treated with heparin-plasma (final concentration of 2.5%, 5%, or 20%). Alternatively, for quantification of cleavage inhibition, 96-well plates with PA (50 μg/ml) in 15 mM HEPES–2 mM $CaCl_2$ and 10-fold dilutions of the inhibitors were treated with heparin-plasma (5%) for 2 h prior to removing samples for PA63 measurements by ELISA. Inhibitors were purchased from Sigma, Calbiochem (San Diego, CA), or Roche Applied Science (Indianapolis, IN). The highest concentration for all inhibitors was 200 μg/ml, except pepstatin and bestatin (100 μg/ml) and calpain inhibitor I (500 μg/ml). For the Complete EDTA-free protease inhibitor (Roche) cocktail stock solution, one-eighth of a tablet was dissolved in 100 μl of reaction buffer prior to being diluted sixfold in the first well of each 96-well plate. Samples were processed as described above for Western blotting.

RESULTS

Characterization of monoclonal antibodies used for PA detection by ELISA. We first developed ELISAs measuring total PA (PA83 plus PA63) and specific for furin-cleaved PA (PA63) to determine the concentrations of PA proteins remaining in serum and plasma samples collected after bolus injections of mice and rats. The monoclonal antibodies used to capture PA were purified immunoglobulins of antibodies 14B7, 1G3, and 1E9 and an ammonium sulfate precipitate of a 1E9 hybridoma culture supernatant (1E9-PPT). The well-characterized 14B7 antibody recognizes an epitope in the receptor-binding domain of PA (11, 12). Not surprisingly, it recognizes PA83 and PA63 equally well in ELISAs and by Western blotting (Fig. 1A). Monoclonal antibody 1G3 is thought to recognize the PA63 oligomer and to compete with LF at its binding site, which spans two monomer units on the heptameric PA63 (7, 12). We show that it also captures PA83, but with roughly 100-fold weaker reactivity than for PA63 (Fig. 1B). This observation was also made in the original study characterizing this antibody (12). This antibody is unable to recognize the SDS-resistant PA63 oligomer by Western blotting (data not shown), indicating a possible conformational epitope reactivity. It recognizes denatured PA83 and PA63 equally well by SDS-PAGE with Western blotting when 5% skim milk is present in the blocking and primary antibody incubation steps but not when the Odyssey LI-COR blocking buffer is used (Fig. 1B, inset; also data not shown).

Although monoclonal antibody 1E9 was obtained from mice immunized with a sample of the PA 20-kDa N-terminal domain, we found that this antibody (only when prepared as the 1E9-PPT preparation) specifically captured PA63 in the ELISA, with no reactivity to PA83 (Fig. 1C). Surprisingly, this antibody preparation (again, only when prepared as the 1E9-PPT) reacted well with PA83 by Western blotting but had very poor reactivity with denatured PA63 in SDS-PAGE-Western blots (Fig. 1C, inset). However, 1E9 immunoglobulin G purified from ascites fluid using protein G columns was equally effective in capturing both PA83 and PA63 in the ELISA format (Fig. 1D), clearly indicating that this antibody was not a PA20 monoclonal. Therefore, 1E9-PPT was able to bind PA63 specifically and in a dose-dependent manner, while pu-

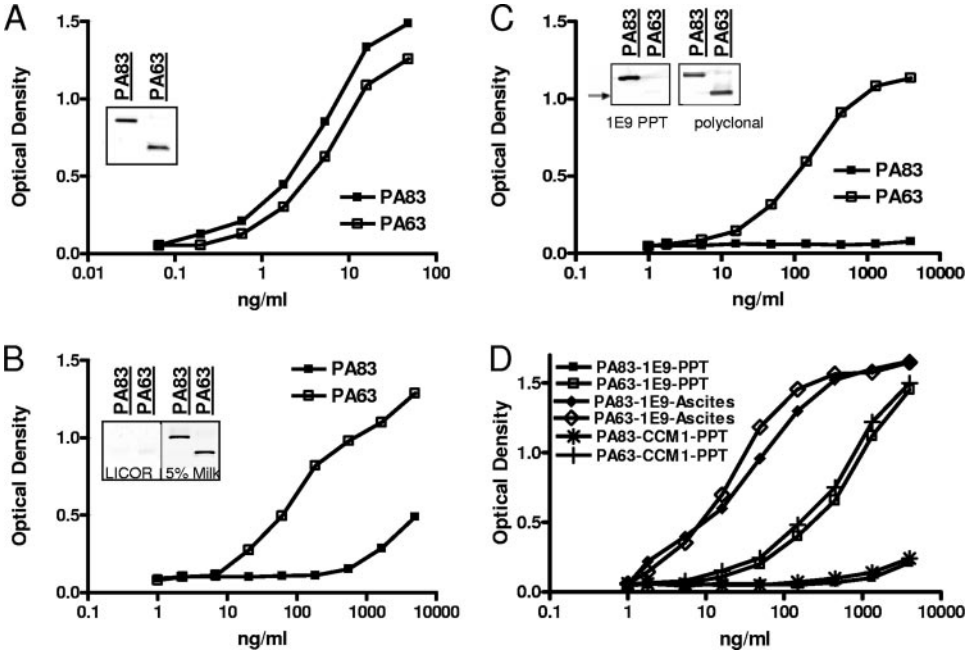


FIG. 1. Monoclonal antibody detection of PA83 and PA63. (A) Detection of PA83 and PA63 by sandwich ELISA using 14B7 antibody for PA capture. Inset gel shows Western blot detection of PA83 and PA63 (2 ng/well loaded for each protein) probed with 14B7. (B) Similar ELISA experiment as in panel A, using monoclonal antibody 1G3. Inset gels show Western blots of PA83 and PA63 (8 ng/well loaded for each protein) with 1G3 antibody using Odyssey (LI-COR) blocking buffer or 5% skim milk. (C) Similar ELISA experiment as in panel A, using 1E9 PPT for PA capture. Inset gel shows concentrations detected by Western blotting of PA83 and PA63 with 1E9-PPT compared to anti-PA polyclonal antibody (4 ng/well loaded for each protein). ELISA results for panels A to C are representative of >50 ELISAs. (D) Detection of PA83 and PA63 by ELISA using 1E9-PPT, purified 1E9 ascites, and CCM1-PPT for antigen capture.

rified 1E9 antibody bound both PA83 and PA63. To explain this discrepancy, we tested ammonium sulfate precipitates of the CCM1 medium in which the hybridoma was grown and found the same PA63 binding specificity, indicating that a component in the medium was sufficient for PA63-specific capture in ELISAs (Fig. 1D). Thus, we believe that the 1E9 hybridoma, when grown in ascites, may produce an antibody having unique reactivity that may merit further study. However, the PA63-specific binding activity in 1E9-PPT is not due to the 1E9 monoclonal antibody but is the result of a PA63-binding material present in CCM1 medium (Fig. 1C). Therefore, throughout this work, wherever 1E9-PPT was used for PA63-specific ELISAs, it is in fact the CCM1 medium component capturing PA63. While all the experiments reported in this work for PA63 measurements by ELISA were performed with 1E9-PPT, similar results were obtained in tests with CCM1 medium precipitates.

Clearance of PA from circulation. Our first goal was to determine the concentrations of PA required for receptor saturation. For the purposes of all our studies, "receptor" refers to all PA receptor/binding sites including the two currently identified (ATXR1 and ATXR2) and any potentially unidentified receptors. We first measured (by 14B7 ELISA) the amount of total PA (PA83 plus PA63) remaining in the circulation of BALB/cJ mice after i.v. injection of a range of PA doses (5 to 400 μg). The approximate 100% lethal doses (LD_{100}s) of PA for a BALB/cJ mouse, when coinjected with an equal amount of LF, are 100 μg (i.p.) (17) and 35 μg (i.v.) (data not shown). For these clearance studies PA alone was injected into the mice. After 2 h, approximately 15 to 20% of the injected PA was found in circulation with doses of 100 to 400 μg (assuming for the purposes of calculating the initial injected concentration at time zero that the mouse has an average blood volume of 1.8 ml). Lower doses of 10 to 50 μg, however, all resulted in the same low concentrations of remaining circulating PA (<1 μg/ml) (Fig. 2A). PA amounts fell to less than 0.5% of injected dose (for 100 and 200 μg/1.8 ml/mouse) by 6 h (0.5 to 1 μg/ml) and were hardly measurable at 24 h (Fig. 2B). The times required for PA to be reduced to half the initial injected concentrations ($t_{1/2}$), calculated for a one-compartment model, were 0.17 h for the 20 μg dose ($t_{1/2}$ of 10 min) and 0.88 h for the 100 and 200 μg doses ($t_{1/2}$ of 53 min).

The data shown in Fig. 2A suggested that toxin receptors saturate at doses above 50 μg and might have suggested that doses below this amount would be entirely removed from circulation. However, even the 5-μg dose produced a measurable concentration (125 ng/ml) of circulating PA at 2 h (Fig. 2A). Thus, it was necessary to consider whether the material detected might be due to PA breakdown, PA dissociation from receptor, or PA modification occurring in unrecognized ways in the bloodstream. As one way to characterize the PA remaining in the collected blood samples, we tested its ability to kill LT-sensitive RAW264.7 macrophages in the presence of a fixed concentration of LF (200 ng/ml) (Fig. 2C). These activity-based PA values correlated strongly with the values obtained by ELISA for all doses of toxin of >20 μg (Fig. 2D). Sera from mice given the 5- and 10-μg doses (Fig. 2D and data not

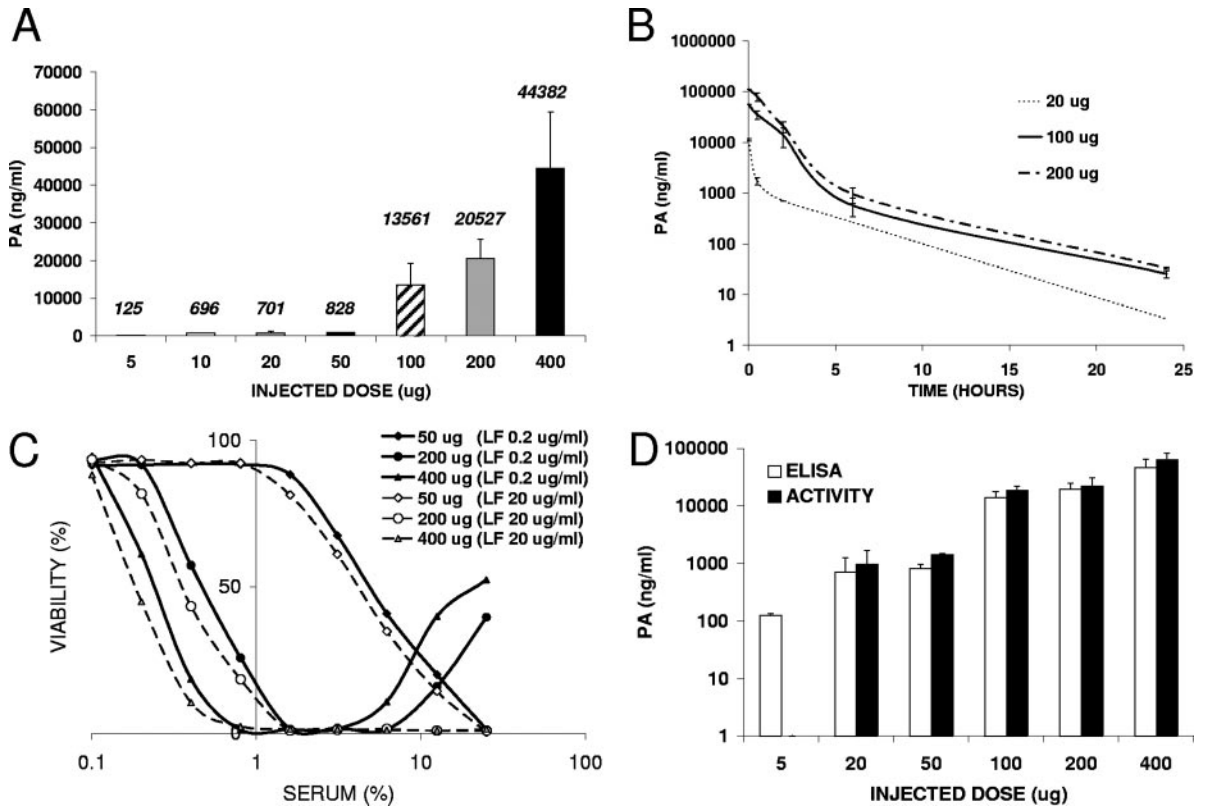


FIG. 2. Clearance of PA from circulation. (A) BALB/cJ mice were injected i.v. with 5 to 400 μg of PA, and serum or plasma was collected 2 h later and analyzed for PA by ELISA using 14B7 monoclonal antibody as the capture antibody. The average values calculated from all experiments (serum and plasma collections) are shown numerically above each column. Standard deviation was calculated based on following *n* values with the indicated doses: 5 μg ($n = 2$ mice; six ELISAs), 10 μg ($n = 4$ mice; eight ELISAs), 20 μg ($n = 6$ mice; eight ELISAs), 50 μg ($n = 4$ mice; six ELISAs), 100 μg ($n = 16$ mice; eight ELISAs), 200 μg ($n = 6$ mice; six ELISAs), 400 μg ($n = 10$ mice; eight ELISAs). (B) PA concentrations in serum of BALB/cJ mice injected i.v. with 20, 100, or 200 μg of PA bled at 0.5, 2, 6, and 24 h were measured by 14B7 ELISA as follows: 0.5 h, $n = 2$ for all doses; 2 h, $n = 6$ for 20- and 200-μg doses and $n = 16$ for 100-μg dose; 6 h, $n = 4$ for 20- and 200-μg doses and $n = 10$ for 100-μg dose; and 24 h, $n = 4$ for all doses. All values are actual measured PA concentrations except zero time concentrations of PA, which were calculated based on an approximate 1.8-ml blood volume for the BALB/cJ mouse. All sigmoidal dose response (variable slope) analyses for calculations of concentrations based on PA ELISA standard curves and one-compartment half-life calculations were performed using GraphPad Prism, version 4.0, software. (C) Macrophage toxicity of serum collected at 2 h after injection of PA (50 μg, 200 μg, or 400 μg) in assays performed in vitro in the presence of 200 ng/ml or 20,000 ng/ml LF. Results are representative of four similar experiments performed with samples from two mice for each dose. (D) Comparison of PA concentrations in circulation at 2 h measured directly by ELISA compared to concentrations calculated from PA activity in the macrophage toxicity assay in the presence of LF in vitro. Standard deviations are based on averages from four experiments performed with duplicate samples from two individual mice per PA dose.

shown) had no measurable PA activity in the macrophage toxicity assay. We were surprised to find that sera from mice given the 200-μg and 400-μg doses of toxin showed a biphasic effect in the macrophage assay, causing toxicity at low concentrations but survival at higher concentrations (Fig. 2C). The survival was reversed when the concentration of LF in the assay was increased 100-fold to 20 μg/ml, suggesting that the inhibitory factor competes with LF. We hypothesized that the inhibitory component could be the N-terminal 20-kDa fragment generated from the cleavage of PA83 either at cell membranes followed by release to the circulation or through direct cleavage by a protease active in the blood of healthy animals, much in the manner reported for spore-infected animals (5, 20). PA20 generated by cleavage would be expected to be present in concentrations sufficient to compete only in sera of mice given the highest injected PA doses (200 and 400 μg), as was observed.

Cleavage and clearance of WT-PA and Ub-PA. To further characterize the PA remaining in the blood after i.v. injection, PA83 and PA63 concentrations were measured by ELISA as well as by Western blot analysis. Because PA has been reported to be cleaved in blood, we collected plasma as well as serum in parallel experiments. Approximately one-third of the PA in circulation at 2 h was PA63 (Fig. 3A), and cleavage was only slightly greater in serum than in plasma samples (data not shown), indicating that cleavage was not due to activation of clotting cascade proteases. Cleavage of PA to PA63 could be detected as early as 5 min after PA injection when Western blotting was used for detection. The PA63 was apparently cleared rapidly, so that its levels were not in a detectable range in blood collected at 6 h or later (Fig. 3A).

We next asked whether PA63 was generated by cell surface proteases and then released back into circulation or was it directly cleaved in the bloodstream. To answer this question,

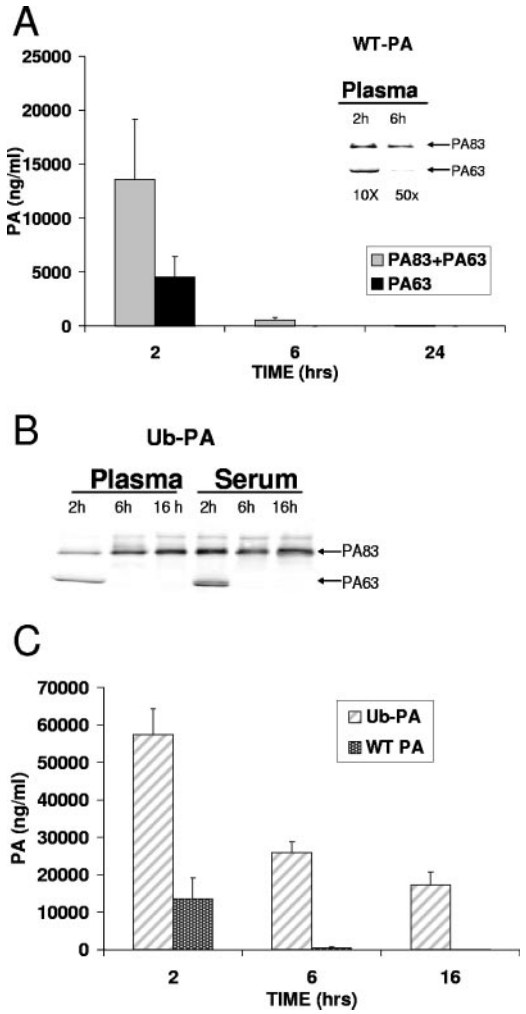


FIG. 3. Cleavage and clearance of WT-PA and Ub-PA. (A) Concentrations of PA83 and PA63 detected in plasma collected at 2, 6, and 24 h after injection of PA (100 μg) measured by 14B7 and 1E9 ELISA ($n = 16$ mice per group). Western blot shows samples from two mice probed with anti-PA polyclonal (1:5,000). For Western blotting, plasma samples were diluted 50-fold (2 h) or 10-fold (6 h) prior to loading 5 μl/well. (B) Ub-PA83 and Ub-PA63 detected in plasma and serum collected at 2 h and 6 h after injection of Ub-PA (100 μg) by Western blots probed with anti-PA polyclonal (1:5,000). Samples were diluted 50-fold prior to loading 5 μl/well. (C) Comparison of PA83 plus PA63 measurements for WT-PA and Ub-PA in plasma at 2, 6, and 16 h after injection of 100 μg (i.v.) into BALB/cJ mice. The combined PA83 and PA63 measurements for samples from mice injected with WT-PA were assessed by 14B7 ELISA ($n = 16$ for 2 h and 6 h and $n = 2$ for 16 h). The combined PA83 and PA63 measurements for samples from mice injected with Ub-PA were assessed by 1G3 ELISA ($n = 8$ for 2 h and 6 h and $n = 4$ for 16 h) and are likely to be underestimates due to the low sensitivity of this ELISA compared to the 14B7 ELISA.

we used Ub-PA, a binding-defective mutant of PA. This mutant was also cleaved to PA63 in both plasma and serum samples (Fig. 3B), indicating that cleavage did not require cell surface proteases. The concentrations of Ub-PA were approximately fourfold higher than wild-type PA after 2 h, 20-fold higher at 6 h, and 50-fold higher at 16 h (Fig. 3C). Surprisingly, however, we could not assess any Ub-PA63 associated with these high concentrations of circulating PA after 6 h (Fig. 3B). This indicates that concentrations of Ub-PA63 and WT PA63 are both below the detection limits of the 1E9-PPT ELISA, as well as the 50 pg/well (5 ng/ml) detection limit of the Western analysis. As it is unlikely that a continuously occurring enzymatic reaction which has adequate substrate available would slow down, the product of this reaction (PA63) is likely being removed from circulation more rapidly, and not simply through binding to PA receptor.

Cleavage and clearance of uncleavable PA mutants. We next investigated if the cleavage occurring in the blood required the furin cleavage sequence $_{164}$RKKR$_{167}$ (6, 8, 18). To answer this question we used three PA mutants in which the cleavage site is replaced by sequences that are either uncleavable (PA-U7) or cleaved by the cell-surface proteases, matrix metalloproteinases (PA-L1), or urokinase plasminogen activator (PA-U2) (13–15). Blood and plasma collected from mice injected with these proteins showed no evidence for cleavage to PA63, whether assayed by ELISA or Western blot analysis (Fig. 4A and B). A PA-reactive band of about 75 kDa was seen in the concentrated (50×) loadings at earlier time points (6 h) when more PA83 was present. This band is not a cross-reactive blood protein as it disappears at 12 h and 24 h with the same concentrated gel loading and is more likely to be a PA breakdown or cleavage product unique to the uncleavable PA proteins and only detected when higher PA concentrations are present. Circulating concentrations of the three mutated PA proteins were far higher than wild-type PA at later times, in a manner similar to Ub-PA (Fig. 4A and C). The less than twofold reduction of PA-U7 levels by 6 h (as measured by ELISA) (Fig. 4C) was not even apparent by Western blotting (Fig. 4B). All quantifications of cleavage for mutated PA proteins compared to WT-PA by ELISA were done with plasma samples in order to avoid the possibility that serum collections would result in additional nonspecific differential PA83 cleavage due to activation of clotting cascade proteases. Although the three furin site mutants were always consistently at higher concentrations than WT-PA after 2 h, the difference was most substantial at 6 h, with 10- to 20-fold higher concentrations than WT-PA. By 12 h, however, unlike the high concentrations of Ub-PA which were still measurable at 16 h (Fig. 3B and C), circulating concentrations of all three mutants and WT-PA were below 500 ng/ml (Fig. 4C). This difference in clearance between these mutants and Ub-PA likely reflects cell binding-based removal of these proteins.

Serum- and plasma-mediated cleavage of PA in vitro. To further characterize the cleavage of PA that occurs in blood, we performed controlled in vitro experiments. Mouse plasma samples were collected by both EDTA and heparin methods for comparison with serum. In a 15 mM HEPES–2 mM NaCl buffer at pH 7.5, PA cleavage was strong with serum samples but was observed with heparinized plasma only at higher concentrations (>12%) (Fig. 5A). EDTA-plasma, however, showed poor cleavage in this buffer, indicating a possible requirement for divalent ions. When DMEM was used with 10 mM HEPES as the reaction buffer, cleavage was strong with heparinized plasma, although always slightly less than with serum, independent of the concentration of plasma or serum used (Fig. 5A). In DMEM, cleavage was detected with the EDTA-plasma as well, albeit less than with heparinized plasma and serum, and decreased with increasing concentrations of

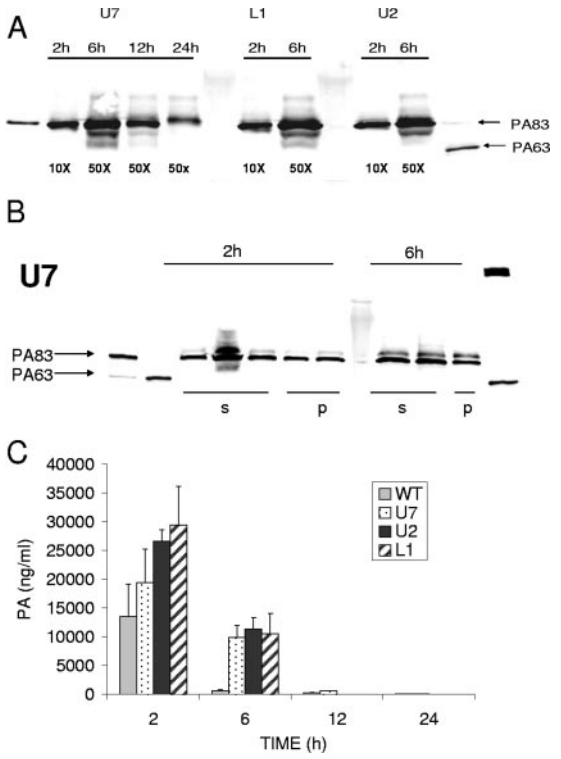


FIG. 4. Cleavage and clearance of uncleavable PA mutants. (A) Western blots of three uncleavable PA mutants detected by polyclonal anti-PA (1:5,000) in plasma collected at various times after injection of 100 μg (i.v.) into BALB/cJ mice. Samples were diluted 50-fold (2 h) or 10-fold (6 h, 12 h, and 24 h) prior to loading 5 μl/well. Purified PA83 and PA63 controls are shown in first and last lanes. The PA63 oligomer is also seen in the last lane. (B) Western blots showing levels of the PA-U7 uncleavable mutant detected by polyclonal anti-PA (1:5,000) in plasma and serum collected at 2 h and 6 h after injection of 100 μg (i.v.) into BALB/cJ mice. Samples were diluted 50-fold prior to loading 3 μl/well. Purified PA83 and PA63 controls are in first, second, and last lanes. (C) Comparison of PA83 and PA63 concentrations as detected by 14B7 ELISA for WT-PA and three uncleavable PA mutants in plasma collected after injection of 100 μg into BALB/cJ mice. For WT-PA, $n = 16$ at 2 h, $n = 16$ at 6 h, $n = 2$ at 12 h, and $n = 4$ at 24 h, For PA-U7, $n = 6$ at 2 h, $n = 6$ at 6 h, $n = 2$ at 12 h, and $n = 2$ at 24 h. For PA-U2 and PA-L1, $n = 4$ at 2 and 6 h and $n = 2$ at 12 and 24 h.

plasma (and thus EDTA), supporting an inhibitory role for EDTA. Since DMEM allowed the cleavage with concentrations as low as 1.5% heparinized plasma, it appeared to supply a condition essential to the cleavage reaction. Two components of DMEM that were considered as potential candidates for enhancing protease function were 100 mM NaCl and 2 mM $CaCl_2$. We performed add-back experiments with HEPES buffer supplemented with these components and restored cleavage with both individually (Fig. 5B). Addition of EDTA to serum or heparinized plasma inhibited cleavage (Fig. 5B). We then tested a panel of protease inhibitors. Cleavage was assessed by Western blotting and quantified by ELISA. We found that leupeptin and an inhibitor cocktail which contained leupeptin were able to inhibit cleavage by greater than 50% (Fig. 5C and D), indicating that serine proteases are responsible for the observed cleavage.

PA cleavage in LT-sensitive and LT-resistant mice and rats. Mouse strains differ in sensitivity to bolus injections of LT. To determine whether differences in PA cleavage or clearance might in part account for this difference, these processes were examined in LT-sensitive BALB/cJ and LT-resistant DBA/2J mice. Both groups of mice processed PA similarly (Fig. 6). We next tested the highly LT-sensitive Fischer rat, which dies in 45 to 70 min when administered LT doses lower than those that kill LT-sensitive mice. Surprisingly, injections of 5 to 100 μg of PA led to rather similar concentrations of circulating PA (200 to 600 ng/ml) after 2 h, with little loss over 6 h (Fig. 7A). PA63 was not detected at any time by ELISA. However, because the blood volume of the rat is approximately 10-times that of the mouse, the concentration of PA at 2 h was approximately 10-fold lower, and it is possible that the PA63 was below the detection limit of the ELISA. Therefore, we tested for PA63 by Western blotting but again could find no evidence for PA cleavage (Fig. 7B). Additionally, injection of 250 μg of PA did not yield detectable PA63 by Western blotting, while 20-μg injections in mice did (data not shown). The same result was obtained in examination of the LT-resistant Lewis rats (Fig. 7B). Surprisingly, the blots showed that concentrations of circulating PA83 in Lewis rats after injection of 5 and 100 μg of PA were similar, and a decrease in circulating PA83 concentrations was hardly discernible between 2 h or 6 h in either rat strain (Fig. 7B). In clear contrast to the results for mice, PA was still easily detected after 18 h by both ELISA and Western blotting (Fig. 7C). The slower clearance of PA83 in the rats likely parallels the slow clearance associated with uncleavable PA in mice. Finally, we performed in vitro cleavage experiments with Fischer rat heparinized plasma in the DMEM buffer and observed no cleavage of PA83 to PA63 (data not shown).

PA clearance, PA receptor clearance, and LF toxicity in mice. The ability of PA to productively internalize LF depends on a complex interplay involving PA and LF concentrations in the blood, PA conversion to PA63, receptor number, receptor occupancy by PA and PA63, receptor recycling, and new receptor synthesis. Some information about these processes can be obtained by timed injections of PA and LF. For example, one can hypothesize that injection of larger amounts of PA will occupy and cause internalization of all the available cell surface receptors. Saturation and depletion of receptors in this way would be consistent with PA retention in the blood in detectable concentrations, as shown in Fig. 2A for doses of >50 μg. An animal might then be resistant to subsequent injections of either LF or LT until such time as newly synthesized receptors reach the cell surface in numbers adequate to internalize a toxic amount of LF. To examine whether these and similar considerations have validity, doses of PA were administered to mice, followed at intervals by LF or LT. PA doses of ≥50 μg resulted in full lethality of animals when LF alone was provided (100 μg/i.p. injection) up to 8 h later (Fig. 8A and data not shown). At 10 h and later, injection of LF did not cause lethality, indicating that sufficient PA concentrations were not available for toxicity (Fig. 8A). Lethality occurred during the first 8 h in spite of the fact that circulating PA concentrations were <1 μg/ml (Fig. 2A) and the known bolus LD_{100} for the BALB/cJ strain is 100 μg/mouse by i.p. injection. This finding argues against the hypothesis that receptors or

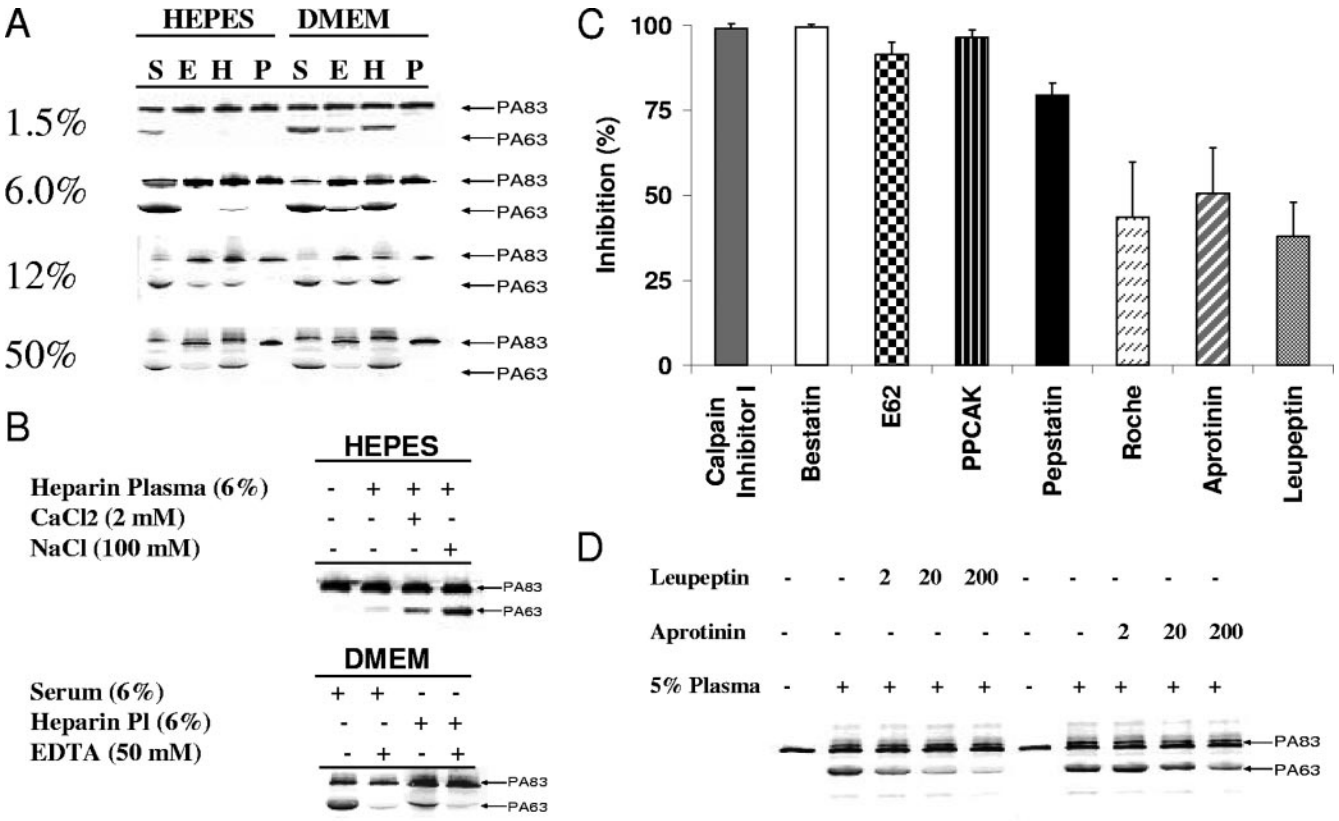


FIG. 5. Serum- and plasma-mediated cleavage of PA in vitro. (A) PA83 cleavage to PA63 in 15 mM HEPES–2 mM NaCl (4 left lanes) compared to DMEM–10 mM HEPES (four right lanes) using 1.5%, 6%, 12%, and 50% concentrations of BALB/cJ serum (S), EDTA-plasma (E), and heparin-plasma (H). Control lanes represent PBS treatment (P). (B) PA83 cleavage levels in 15 mM HEPES in the presence of $CaCl_2$ (2 mM) and NaCl (100 mM) using 6% heparin-plasma (upper panel) or in DMEM with EDTA (50 mM)) using 6% heparin plasma and serum (lower panel). (C) Inhibition of PA83 cleavage to PA63 as quantified by PA63 ELISA using various protease inhibitors. Results shown are for the highest tested inhibitor doses listed in Materials and Methods and represent averages from two independent experiments. PPACK, D-phenylalanyl-L-arginine chloromethylketone. (D) Representative Western blots showing levels of inhibition of plasma-mediated PA83 cleavage (in DMEM) by leupeptin and aprotinin at various doses (shown in μg/ml).

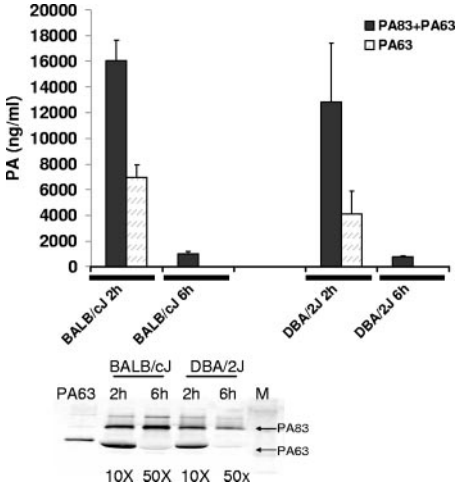


FIG. 6. Comparison of PA cleavage and clearance in BALB/cJ and DBA/2J mice. (A) Concentrations of PA83 and PA63 detected in plasma samples collected at 2 h and 6 h after injection of PA (100 μg) into BALB/cJ or DBA/2J mice measured by 14B7 and 1E9-PPT ELISAs (n = 4 mice per time group). Western blot shows samples from four mice probed with anti-PA polyclonal (1:5,000) antibody. Samples were diluted 50-fold (2 h) or 10-fold (6 h) prior loading 5 μl/well. PA63 control protein is shown in first well.

bound PA can be depleted to levels that make animals resistant to subsequent toxin challenge within this 8-h window, despite very low levels of circulating PA. Evidence that a sufficient number of receptors is present 10 h after PA injection is provided by an experiment in which LT (LF and PA) was injected at intervals after PA (Fig. 8B), with the result that all animals died. The delay in time to death seen with LT injections at 10 h compared to 16 h after PA injection may be due to partial receptor clearance at 10 h as higher numbers of receptors may become available by 16 to 24 h.

Mutant PA therapy against LT lethality. One consequence of the finding that PA83 can be cleaved to PA63 in the blood is that the PA63 heptamers in plasma might bind LF in nonproductive complexes. This suggested that Ub-PA might have therapeutic value by blocking toxin action, since it would be predicted that cleaved, oligomerized Ub-PA63 could remove LF from circulation. Injections of 2 LD_{100}s (60 μg of PA plus 20 μg of LF, i.v.) were done in BALB/cJ mice, either alone or combined with 60, 120, or 240 μg of Ub-PA. The 60-μg Ub-PA dose protected 50% of the mice, while the two higher doses resulted in full protection (Fig. 9A), with no symptoms of malaise appearing in the mice. Next, we tested the effects of pretreatment and posttreatment with the Ub-PA using an

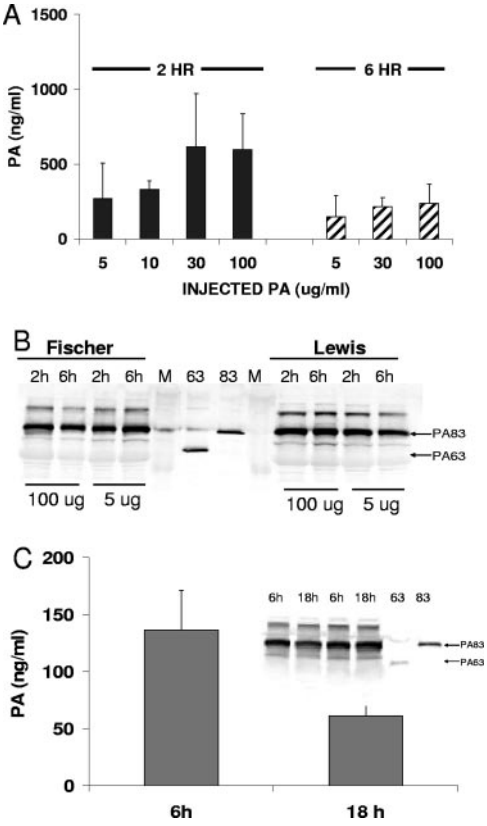


FIG. 7. PA cleavage and clearance in rats. (A) Concentrations of PA83 detected by 14B7 ELISA in plasma samples collected at 2 and 6 h from Fischer rats injected with various doses of PA ($n = 4$ for each group). (B) Western blotting with plasma samples from Fischer or Lewis rats collected 2 or 6 h after injection of PA (100 μg or 5 μg). Western blot detection used anti-PA polyclonal antibody (1:5,000). (C) Concentration of PA83 detected by 14B7 ELISA and by Western blotting (anti-PA polyclonal antibody used at 1:5,000) in plasma samples collected at 6 and 18 h after injection of PA (100 μg) ($n = 4$ for each group in ELISAs).

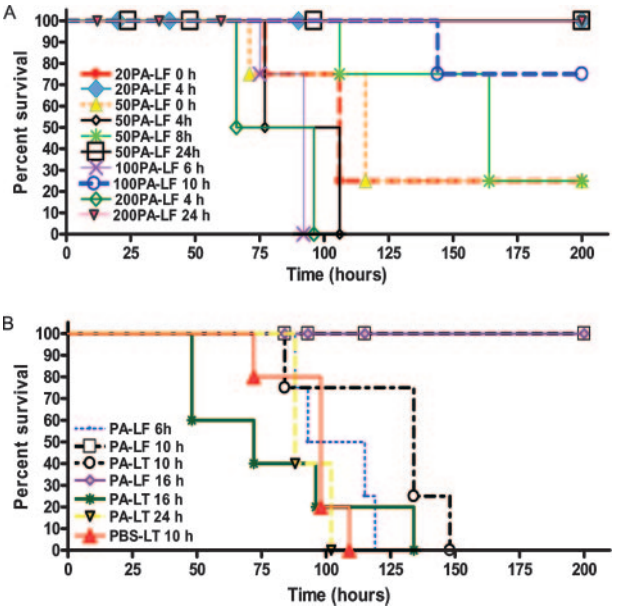


FIG. 8. PA clearance and LF toxicity in mice. (A) BALB/cJ mice were injected (i.v.) with various doses of PA (20, 50, 100, or 200 μg) followed by an immediate (0 h) or delayed (2, 4, 6, 8, 10, or 24 h) i.p. injection of LF alone (100 μg) and were monitored for survival ($n = 4$ mice per group). (B) BALB/cJ mice were injected (i.v.) with PA (100 μg), followed by an immediate (0 h) or delayed (6, 10, 16, or 24 h) i.p. injection of LF alone (100 μg) or LT (100 μg of PA plus 100 μg of LF) and were monitored for survival ($n = 4$ mice per group).

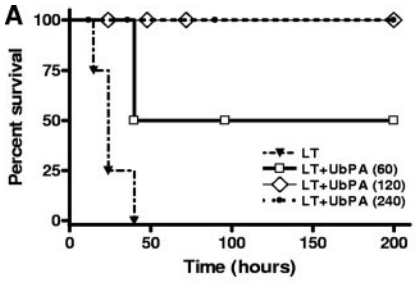
LD_{100} challenge dose (i.v.) of 35 μg of PA plus 15 μg of LF. We administered a sixfold excess of Ub-PA (210 μg, i.p.) 30 min prior to or after the lethal challenge and found that all the mice pretreated with the mutant PA survived. Not surprisingly, only 50% of the mice given Ub-PA 30 min after LT survived. Full protection with simultaneous administration of a 2× (70 μg) or 4× (140 μg) excess dose of Ub-PA was again verified (Fig. 9B).

We next compared the protective efficacy of Ub-PA to that of other mutated PA proteins that have been reported to block toxin action through other mechanisms. We tested the previously mentioned PA-U7, which cannot be proteolytically activated, as well as the previously described PA-DN (24). PA-DN has two point mutations which result in nonfunctional oligomers when a single DN PA63 is present in oligomers, such that the LF cannot be translocated into the cytosol (25). WT-PA was also included. The proteins were administered i.p. at four different doses (70, 105, 210, and 280 μg) at 12, 6, and 2 h prior to the i.v. lethal challenge (Fig. 9C). We found that PA-DN when given at 6× and 8× molar excesses at 12, 6, and 2 h prior to challenge provided protection levels similar to Ub-PA (Fig. 9C). Injections of the uncleavable PA-U7 at 8×, 6×, and even at 3× molar excess totally protected against the lethal i.v. challenge, even when given up to 12 h prior to challenge, making it the most effective PA variant in protecting against LT (Fig. 9C). Even WT-PA was able to protect against lethal challenge at these doses given up to 6 h prior to challenge but not at 12 h. These findings suggest that receptor blocking or clearance is likely to account for much of the protection afforded by all of these proteins, with the obvious exception of Ub-PA.

DISCUSSION

We report here findings on PA83 and PA63 concentrations in the blood following bolus injection of various doses of PA in mice and rats. Our results support an earlier report which found PA in the blood of infected animals, primarily in the PA63 form, and postulated a PA protease that was not cell associated but free in serum and fully active in heparin-plasma (5). We find that after injection, PA83 is rapidly cleaved in mice but not in rats and that this cleavage is independent of overall sensitivity to LT and occurs via a leupeptin- and calcium-sensitive protease. Cleavage does not require binding of PA to receptors, as evidenced by efficient cleavage of Ub-PA, a binding-defective PA mutant. This result was further supported by the ability of Ub-PA to protect against LT challenge through binding and removal of LF.

Rapid clearance of PA required binding and cleavage, as evidenced by the slow clearance of several uncleavable PA mutants as well as Ub-PA. The slower clearance of these pro-



C

Protective Antigen (IV)	Lethal Toxin (35 μg PA+ 15 μg LF)(IV)		
70 μg	-12 h	-6 h	-2 h
WT			1/5
DN			5/5
U7			4/5
Ub			4/5
PBS			0/5
105 μg			
WT		2/5	8/8
DN		6/9	5/5
U7		4/5	8/8
Ub		7/9	5/5
PBS		0/9	0/5
210 μg			
WT	1/5	8/9	2/4
DN	4/5	7/9	4/4
U7	5/5	9/9	0/4
Ub	5/5	6/9	8/8
PBS	0/10	0/9	0/4
280 μg			
WT	1/5	4/5	
DN	5/5	14/14	
U7	5/5	5/5	
Ub	5/5	12/14	
PBS	0/10	0/10	

FIG. 9. PA mutant protection against LT challenge. (A) BALB/cJ mice were injected i.v. (200 μl) with LT (60 μg of PA plus 20 μg of LF) alone or with Ub-PA included at various doses (60, 120, or 240 μg) and monitored for survival ($n = 4$ for each group). (B) BALB/cJ mice were injected i.v. (200 μl) with LT (35 μg of PA plus 15 μg of LF) alone or with Ub-PA included at 70 or 140 μg. In two groups of mice, Ub-PA (210 μg) was administered i.p. 30 min before or after the LT (35 μg of PA plus 15 μg of LF) injection ($n = 4$ for each group). (C) Groups of BALB/cJ mice were injected i.p. at 2, 6, or 12 h prior to LT challenge (i.v. injection of 35 μg of PA plus 15 μg of LF) with various doses of the following: PBS, WT-PA (WT), Ub-PA (Ub), PA-DN (DN), or U7-PA (U7). Mice were monitored for survival. Numbers in the table refer to the number of surviving mice relative to the number of mice in each treatment group.

teins from the blood provides benefits for their therapeutic use in the retargeting of PA to cancer cells, for which they were developed (13–15). Interestingly, one of these mutants, PA-U7, provided the best protection against LT challenge even when injected 12 h prior to LT. This protection is likely due to the slower receptor uptake/clearance associated with (uncleaved) PA83 (1), resulting in prolonged blocking of receptors at the cell surface. PA-DN, a binding- and cleavage-competent mutant which is unable to translocate LF, although cleared at the more rapid rate similar to WT-PA (data not shown), could provide protection at three levels: competition for LF binding with its circulating PA63 form, receptor clearance through its binding function, and finally, disabling WT-PA63 oligomers for LF translocation into cells.

Surprisingly, preinjections of WT-PA were also quite effective in protecting against LT challenge, most probably through receptor clearance alone, although less effectively than PA-U7 over longer periods (12 h) due to the slow clearance of that mutant. The protection provided by WT-PA through receptor clearance supports a 1963 study performed in Fischer rats using PA and LF before the actual functions of these two proteins were known (19). In those studies, Molnar and Altenbern injected rats with PA or LF and then with PA, LF, or LT at 30, 50, 90, 120, 180, 240 min or 20 to 24 h later. PA injected 3 h before LT delayed death and injected 4 h before LT prevented death, but it had no effect when the interval was 24 h. This led to the conclusion that "the observation that lethality of the toxic mixtures can be inhibited by large doses of PA suggests that both are fixed at the same tissue site and that toxic mixtures are fixed through their PA component. The toxin continuously released during anthrax infection might well be antagonized by repeated injections of PA to occupy all the available tissue sites" (19). In the study cited, providing LF alone at any time point later than 2 h did not result in animal death, indicating that functional PA oligomers were no longer available for LF transport. Our mouse study, however, showed that despite very low PA concentrations (<1 μg/ml) in circulation 2 h after injection of a 50-μg dose, providing LF up to 8 h later still resulted in 75% lethality in mice. This implies availability of active, cleaved PA at cell or tissue surfaces for long periods after clearance from the bloodstream. Thus, the timing of therapies which bind or deactivate circulating PA must also consider the activity and availability of PA already bound to tissues.

It remains to be seen if the bulk of PA63 generated in circulation is inefficient in the toxic process and is simply cleared nonproductively (without internalizing LF) when it is not generated at the cell surface. Studies on PA endocytosis have shown that cleavage of PA83 to PA63 and subsequent heptamerization are required for movement of PA receptors into rafts and that this raft association is required for toxin uptake (1). It is unclear if soluble monomeric PA63 produced in blood would first bind individual receptors, resulting in receptor clustering driven by its heptamerization, or if it would rapidly associate to form heptamers prior to receptor binding, resulting in less effective endocytosis for most of the precleaved PA63. Most analyses of PA63 have shown it to exist almost entirely in heptamer rather than monomer form. LF bound to preformed PA63 oligomers as a complex was shown to have equivalent cellular MEK cleavage activity but decreased cytotoxic activity compared to PA83 plus LF (20). Other studies have suggested that oligomers containing mix-

tures of PA83 and PA63 can form and endocytose LF effectively (3). Thus, the evidence indicates that cell surface formation of the heptamer may be the most efficient route for LF internalization.

The identity of the protease cleaving PA in the blood is unknown. Almost all blood proteases are bound by inhibitors or present in inactive proforms in physiologically normal states. In vitro studies of the PA-active protease activity in plasma indicate it is inhibited by EDTA and requires $CaCl_2$ and higher salt concentrations for optimal activity. The earlier report on the proposed PA protease also found it to be inhibited by EDTA and EGTA and high concentrations of antipain (1 mg/ml). Leupeptin at 100 μg/ml, however, did not inhibit PA proteolysis in the previous studies, whereas it was the only effective inhibitor in our studies. The early study also reported in vitro conversion of PA83 to PA63 in serum from a wide range of species including rats (5). However, blood from rats may have been collected as serum, which could account for the observed cleavage due to clotting cascade enzyme activation, while guinea pig studies were repeated with heparinized plasma. Our experiments indicate that PA protease activity is not detectable in plasma collected from Fischer and Lewis rats, and after a large initial drop in circulating PA concentrations to <1 μg/ml by 2 h (independent of injected dose), PA concentrations change very slowly over time compared to results in mice. The relevance of the PA protease, therefore, may vary among species.

Finally, an interesting tool used in our studies was a previously unreported PA63 ELISA. While PA-specific ELISAs similar to our 14B7 immunoassay have recently been used to measure terminal PA concentrations in infected rabbits and guinea pigs (9, 16), the serendipitous discovery of the CCM1-PPT's PA63-specific binding and capture allowed development of a PA63-specific ELISA. We are currently investigating the component in this medium responsible for PA63 capture.

ACKNOWLEDGMENTS

This research was supported by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases.

We thank Rasem Fattah for purification of toxin proteins, Larry Lantz for purification of monoclonal antibodies, and Jiamo Lu for help with antibody precipitation.

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Editor: J. B. Bliska