PURIFIED BACILLUS ANTHRACIS LETHAL TOXIN COMPLEX FORMED IN VITRO AND DURING INFECTION EXHIBITS FUNCTIONAL AND BIOLOGICAL ACTIVITY

Rekha G. Panchal1,4, Kelly Halverson2, Wilson Ribot1, Tara Kenny1, Teresa G. Abshire2, John W. Ezzell2, Timothy A. Hoover2, Bradford Powell2, Stephen Little2, John J. Kasianowicz3 and Sina Bavari2

1Developmental Therapeutics Program, Target Structure Based Drug Discovery Group, 378 Ware Drive, National Cancer Institute-SAIC, Frederick, MD 21702, USA. 2United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702-5011, USA. 3National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, MD 20899-8120, USA.

Running title: Characterization of purified anthrax lethal toxin complex

Address correspondence to: Sina Bavari or Rekha Panchal, 1425 Porter Street, USAMRIID, Frederick, MD 21702. Tel. 301-619-4246; Fax. 301-619-2348. E-mail: sina.bavari@amedd.army.mil or rekha.panchal@amedd.army.mil

Anthrax protective antigen (PA, 83 kDa), a pore-forming protein upon protease activation to 63 kDa (PA63), translocates lethal factor (LF) and edema factor (EF) from endosomes into the cytosol of the cell. The relatively small size of the heptameric PA63 pore (~12 Å) raises questions as to how large molecules like LF and EF can move through the pore. In addition, the reported high binding affinity between PA and EF/LF suggests that EF/LF may not dissociate but remain complexed with activated PA63. In this study, we found that purified (PA63)7:LF complex exhibited biological and functional activities similar to the free LF. Purified LF complexed with PA63 heptamer was able to cleave both a synthetic peptide substrate and endogenous mitogen-activated protein kinase kinase substrates and kill susceptible macrophage cells. Electrophysiological studies of the complex showed strong rectification of the ionic current at positive voltages, an effect similar to that observed if LF is added to the channels formed by heptameric PA63 pore. Complexes of (PA63)7:LF found in the plasma of infected animals showed functional activity. Identifying active complex in the blood of infected animals has important implications for therapeutic design, especially those directed against PA and LF. Our studies suggest that the individual toxin components and the complex must be considered as critical targets for anthrax therapeutics.

Lethal and edema toxins play key pathogenesis roles as virulence factors produced by Bacillus anthracis, the etiologic agent of anthrax. The toxins show commonality in that both of their enzymatic components, lethal factor (LF, 90 kDa) and edema factor (EF, 88 kDa) require protective antigen (PA) for translocation into host cells. LF, a Zn\(^{2+}\)-dependent metalloprotease cleaves several members of the mitogen-activated protein kinase kinase (MAPKK) family (1-4), and in complex with PA, is responsible for the lethal action of anthrax toxin. EF is a calcium and calmodulin dependent adenylate cyclase that elevates intracellular levels of cyclic AMP, causing deregulation of cellular physiology and resulting in edema (5).

The proposed in vitro model for the binding, assembly, translocation and subsequent killing of the target cells by anthrax lethal toxin (LT) involves a series of steps (6). PA binds to the ubiquitous cellular receptors tumor endothelial marker 8 (TEM8) (7) and capillary morphogenesis protein 2 (CMG2) (8). Proteolytic cleavage of PA by cell-surface proteases like furin (9,10), generates a 63 kDa fragment that oligomerizes and forms a ring-shaped heptameric prepore. The LF and EF proteins bind to the prepore and the whole complex then undergoes receptor-mediated endocytosis. The acidic environment in the
endosomes causes PA to undergo a conformational change that promotes membrane insertion and formation of a transmembrane channel. In a recent study, this process of insertion was shown to occur in the early endosomes, whereas the delivery of the enzymatic moieties required the involvement of the late endosomes (11). LF in the cytosol cleaves MAPKK (3,12,13) and thereby inhibits one or more signaling pathways. Disruption of the signaling cascade by LT impairs the host immune response in macrophages (2-4,14) and dendritic cells (15), thereby allowing the growth and infection of the bacterium. To date the direct causal relation between inactivation of the MAPK signaling and LT-induced cytotoxicity remains unclear.

Under saturating conditions, the PA heptamer in solution binds to three molecules of the ligand (16). Prior studies by Singh et al (17) showed that PA63 in either the monomeric or the oligomeric form can bind LF in a 1:1 ratio. Nevertheless, both the enzymatic moieties EF and LF bind the heptamerized PA63 competitively and with high affinity (Kd ~ 1nM) (18). This interaction is not affected by low pH conditions (pH 5.2) (18). To date there is no direct evidence showing that full-length LF dissociates from the PA63 heptamer complex either during or after translocation through the PA pore or that, LF bound to the PA63 heptamer is capable of cleaving its substrates. In this study, we provide experimental evidence showing that LF bound to the PA63 heptamer is active, and exhibits biological and biophysical properties similar to those of free LF.

Materials and Methods

Purification of (PA63)_7:LF complex - Purified PA63, PA63 and LF proteins (List Biological laboratories Inc.) from Bacillus anthracis were used for these studies. Purified PA63 and LF proteins were mixed either 3:2 or 3:1 molar ratio of PA63 monomer : LF monomer and incubated for 30 min at room temperature (RT). The resulting (PA63)_7:LF complex was purified over a Superose 6 size exclusion column (Amersham Pharmacia, Piscataway, NJ), previously equilibrated with phosphate-buffered saline (PBS), pH 7.4. Total protein concentration of the complex fraction was determined by the Bradford protein assay reagent (Bio-Rad, Hercules, CA). Protein concentration of LF in the complex was determined by ELISA. Briefly, two-fold serially diluted LF protein or the complex was coated on microtiter plates (NUNC) for 16 h at 4°C. The wells were washed four times with Tris-buffered saline (TBS) – Tween-20 (0.1%) and incubated with anti-LF polyclonal antibody (1:500) for 2 h at room temperature (RT). Wells were washed as above and incubated with peroxidase-conjugated goat anti rabbit antibody at 1:2000 dilution for 2 h at RT. Wells were washed and reactivity was developed with one-component ABTS peroxidase substrate (Kirkegaard and Perry Laboratories Inc., MD). Color development was monitored at a wavelength of 405 nm. A calibration curve was established using the LF protein as a standard and LF concentration in the complex was determined based on the standard curve.

LF activity assay - LF activity in the complex was determined using a fluorescence plate-based assay, as previously described (19). Briefly, a reaction mix containing 40 mM Hepes buffer pH 7.2, 0.05% Tween-20 and different concentrations of free LF or complexed LF was added to each well. The reaction was initiated by adding 20 µM of the optimized peptide substrate (20) MCA-KKVYPYPME[dpn]K amide and kinetic measurements were obtained every minute for 30 min using a fluorescence plate reader (Tecan, Safire). The peptide has an excitation peak at 324 nm and an emission peak at 395 nm.

Alternatively, LF activity was also measured after capture of the complex using PA antibody. The PA monoclonal antibody (PAI 3F10) was bound to Nunc Immuno Maxisorp surface (NUNC, Denmark) plates for 2 h at room temperature and then blocked overnight with Tris-buffered saline (TBS) – Tween-20 (0.1%) containing 6% nonfat dry milk at 4°C. The plate was washed three times with TBS-Tween-20 and purified complex (either 200 ng/well or 20 ng/well of LF in complex form) was added to the antibody plate and incubated for 2 h at RT. After extensive washing, the reaction mixture containing 20 µM of fluorogenic LF peptide substrate was added to the appropriate wells and incubated for 30 mins. Cleavage of the peptide by bound LF was monitored by an increase in fluorescence intensity in a plate reader.
A HPLC based LF activity assay was used to determine the kinetic constants (V max and K m values) of both LF in complex and free LF (19). Reaction mixture (30 µl total volume) containing 40 mM Hepes buffer pH 7.2, 0.05% Tween-20 and seven different concentrations of peptide substrate ranging from 2 to 40 µM was incubated with free LF or LF in the complex (1 µg/ml) for 5 min at 30°C. The reaction was stopped by adding 8 M guanidine hydrochloride in 0.3% trifluoroacetic acid (TFA). Substrate and products were separated on a Hi-Pore C18 column (Bio-Rad laboratories, Richmond, CA) using 0.1% TFA (solvent A) and 0.1 % TFA/70% acetonitrile (solvent B). The column effluent was monitored at 365 nm, where the substrate and C-terminal cleavage products had the strongest absorbance.

MAPKK cleavage by western blot - J774A.1 macrophage cells (~1x10^6) seeded in a six well plate were incubated with either anthrax toxin (PA (88 ng/ml) and LF (16 ng/ml)) or purified complex (20 ng/ml or 200 ng/ml of LF in complex form) for 4 h at 37°C. Cells were harvested and lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1% Triton X-100, 10 mM NaF, 1 mM sodium orthovanadate, protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail I and II (Sigma). The cell lysates were incubated for 30 min on ice and then centrifuged for 30 min at 14,000 rpm. Cell extracts (30 µg) were electrophoresed on SDS-PAGE and then subjected to western blotting. Immunoactive proteins were visualized by enhanced chemiluminescence (ECL).

Collection of plasma from infected animals - Rabbits and guinea pigs that appeared moribund were anesthetized with a combination of ketamine, xylazine and Acepromiz under the guidance of staff veterinarians. Blood was drawn directly into tubes containing EDTA to inhibit proteases known to cleave PA83 to PA63, mixed, centrifuged and filtered through a 0.2 µm filter and stored at -70°C. Plasma from the infected animals was then used to detect LF activity in the (PA63)7:LF complex using either the antibody plate capture assay or electrophoresis, in a 4-15% native or SDS gradient gel for immunoblot analysis of the complex.

Antibodies - The antibodies used in this study include MEK1 NT (Upstate Biotechnology, Charlottesville, VA) and MEK2 NT, MEK3 C-19, MEK4 NT (Santa Cruz Biotechnology, Santa Cruz, CA). The PA and LF proteins in the complex were detected with rabbit anti-PA polyclonal antibody and a previously characterized LF monoclonal antibody (LFIII-5D2-1-1) respectively (21).

Cell-based cytotoxicity assay - J774A.1 cells were seeded at a density of 6 x 10^4 cells/well in a 96-well plate. The next day, cells were treated with two-fold serially diluted anthrax toxin (PA (500 ng/ml) and LF (500 ng/ml)) or complex (500 ng/ml of LF in complex). After a 4 h incubation with the toxin, 25 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 1 mg/ml) dye was added and the cells were further incubated for 2 h. The reaction was stopped by adding an equal volume of lysis buffer (20% DMF and 20% SDS, pH 4.7). Plates were incubated overnight at 37°C and absorbance was read at 570 nm in a multwell plate reader. Experiments were performed in duplicate and repeated three independent times for the toxin and the complex samples. The results are given as averages with standard deviation.

Electrophysiology - Channel recordings were carried out with planar lipid bilayer membranes as previously described (22). Briefly, solvent-free diphytanoyl phosphatidylycholine membranes were formed on a 50 µm - 100 µm diameter hole in a thin Teflon partition. The partition separated two identical Teflon chambers that each contained ~2 mL of aqueous solution (0.1 M KCl, 5 mM MES, pH 6.6). Voltage was applied across the membrane via an Axon Instruments patch clamp amplifier through Ag-AgCl electrodes. The current was amplified using a patch-clamp amplifier (Axon Instruments 200B), recorded with an analog to digital converter (DigiData 1322) and analyzed off-line. A negative transmembrane potential drove anions from the cis to the trans chamber. Details of this particular method were summarized elsewhere (23).

Channels were formed by adding small aliquots (~100 ng) of the purified (PA63)7:LF complex to the aqueous electrolyte solution bathing one side of the membrane (called cis). The
formation of individual channels was indicated by the stepwise changes in ionic current monitored at -50 mV applied potential. Data acquisition began after the current achieved a steady-state value. We applied brief (0.5 sec) voltage pulses (typically +160 to -160 mV in 10 mV steps) across the membrane, and averaged the first 100 ms of the signal to obtain the instantaneous current-voltage (I-V) relationship.

RESULTS

Purification and characterization

To generate the (PA63):LF complex, activated PA63 was incubated with LF either in 3:2 (Fig. 1A) or 3:1 (Fig. 1B) molar ratio of PA63 monomer to LF monomer, to demonstrate the titration of free LF. The complex was isolated by size-exclusion chromatography. Peak fractions were collected and analyzed by native PAGE (Fig. 1C) and SDS-PAGE (Fig. 1D). As seen in Fig. 1A, a small amount of aggregated protein eluted first, followed by the peak corresponding to the (PA63):LF complex. Unbound or free LF eluted last. The protein peak corresponding to high molecular weight aggregate was not detectable by Coomasie blue staining, however LF activity was detected in this fraction (data not shown), suggesting that this aggregated protein contains some LF. Electrophoresis of PA63 (Fig. 1C, lane 2) on native gel, showed a smear, probably due to complexes of different molecular sizes. However, binding of LF to PA63 resulted in a distinct (PA63):LF complex band (Fig. 1C, lanes 3 and 5). SDS-PAGE of purified (PA63):LF complex shows the presence of LF, PA63 monomer and SDS stable and heat resistant PA63 heptamer (Fig. 1D, lane 3).

Alternatively, complex was also prepared and purified by mixing trypsin cleaved PA63 and LF proteins (data not shown). The peak fraction corresponding to the (PA63):LF complex generated by either of the two methods was tested in different functional assays and displayed similar biological activity and biophysical properties.

Purified (PA63):LF complex can cleave optimized peptide substrate

To test functional activity of LF in the complex, equal concentrations of either bound or free LF forms were incubated with an optimized peptide substrate that had a fluorogenic coumarin group at the N-terminus and a 2, 4-dinitrophenyl (dnp) quenching group at the C-terminus (19). Kinetic measurements were made using a fluorescence plate reader at an excitation and emission maxima of 324 and 395 nm respectively. As shown in Fig. 2A, LF in the complex form exhibits proteolytic activity, as it was able to cleave the peptide substrate and produce an increase in the fluorescence intensity.

To determine the rate of hydrolysis of the peptide substrate by LF in both the free and bound forms, kinetics were studied by HPLC (19). Kinetic constants were obtained from plots of initial rates with seven different concentrations of the substrate. The Vₘₐₓ and Kₘ values for free LF were 1.8 ± 0.9 μmol/min/mg and 18.9 ± 0.14 μM respectively. LF in the bound form had a Vₘₐₓ value of 2.45 ± 0.21 μmol/min/mg and a Kₘ of 25 ± 4.3 μM. Thus, LF showed a similar rate of enzyme catalyzed hydrolysis of the peptide substrate whether in the free or bound form. A slightly higher Kₘ value for bound LF may indicate that the LF active site was not as accessible to the peptide substrate (with respect to free LF).

To address concerns about free LF contamination in the purified complex, we bound the complex to a PA monoclonal antibody coated onto a Maxisorp microtiter plate. After washing, the reaction mixture containing the optimized LF peptide substrate was added and incubated for 30 min. The increase in fluorescence intensity after peptide cleavage was measured using a fluorescence plate reader. As shown in Fig. 2B, LF activity was indeed observed when the complex was bound to the PA antibody plate, suggesting that LF in complex with PA63 heptamer was active. Furthermore, the activity of LF in the complex form was inhibited by NSC 12155, a previously reported small molecule LF inhibitor (19). Additionally, to show that free LF did not non-specifically bind to the PA antibody, purified LF (10 μg/ml) was added to the PA antibody plate and LF activity was measured (Fig. 2B). Subsequent analysis indicated no LF activity, suggesting that LF does not non-specifically bind to the PA antibody.

Purified complex can cleave MAPKK substrates and kill susceptible macrophage cells
Anthrax LF is known to cleave several different isoforms of MAPKK both in vitro and in macrophage cells. To determine if LF in the complex form could cleave endogenous MAPKK substrates, J774A.1 cells were treated with either the complex or anthrax toxin (PA$_{63}$ and LF), and cellular lysates were prepared and analyzed by western blotting with antibodies against four different isoforms of MAPKK (Fig. 3A). The efficiency of the complex to cleave the four different isoforms of endogenous MAPKK was not significantly different from that of the toxin (PA$_{83}$ and LF). A two to ten fold excess of complexed LF was needed to obtain complete cleavage of endogenous MAPKK (compared to anthrax toxin), raising the possibility that not all the LF active sites in the complexed form were easily accessible to the endogenous substrate.

To test if LF in the complex form was capable of killing susceptible macrophages, J774A.1 cells were treated with different concentrations of either the complex or the anthrax toxin, incubated for 4 h and cell viability measured by the standard MTT assay (Fig. 3B). Above 100 ng of LF, the killing efficiency between the complex and lethal toxin (mixed PA$_{83}$ and LF) was not significantly different.

Purified complex forms functional channels in planar bilayer membranes

Previous electrophysiological studies in planar bilayers demonstrated that PA$_{63}$ heptamer at low pH (~ pH 6.5) forms cation selective channels in lipid bilayers (24,25) and in cell membranes (26). To study the biophysical properties of the (PA$_{63}$)$_7$:LF complex, we first measured the ionic conductance of (PA$_{63}$)$_7$ channels in both the absence and presence of LF. Purified (PA$_{63}$)$_7$ added to the electrolyte solution (0.1 M KCl, 5 mM MES, pH 6.6) spontaneously formed channels in bilayer membranes, as is shown in Fig. 4A, with each step in current (~ 4 pA at -50 mV applied potential) corresponding to the formation of a new channel. Interestingly, under similar conditions, adding purified (PA$_{63}$)$_7$:LF complex also formed channels (Figure 4B). However, the characteristic single channel current was ~ 1.8 pA (V = -50 mV). Thus, even with LF bound to the heptameric PA$_{63}$, it is still possible to form channels.

To mitigate the slow intrinsic gating of the (PA$_{63}$)$_7$:channel (data not shown), we measured the instantaneous current-voltage relationship of membranes containing many channels of either (PA$_{63}$)$_7$ (Fig. 4C without LF; Fig. 4D with LF in the aqueous phase) or (PA$_{63}$)$_7$:LF complex (Fig. 4E) over a wide range of applied potential (e.g., -160 mV < V < 160 mV). In the absence of LF, (PA$_{63}$)$_7$: channels exhibit current-voltage relationship that is only slightly nonlinear. In contrast, when ~ 1 nM LF was added to the cis subphase, the (PA$_{63}$)$_7$: channels became extremely rectifying. Specifically, the conductance for positive potentials was much less than that for negative potentials (compare Figs. 4C and 4D). Channels formed by the purified (PA$_{63}$)$_7$:LF complex also strongly rectified (Fig. 4E) in a manner that is virtually identical to that of (PA$_{63}$)$_7$ with LF added to the subphase (Fig. 4D). The averaged instantaneous $I$-$V$ relationships for these particular experiments are shown in Fig. 4F. The results demonstrate that purified (PA$_{63}$)$_7$:LF preformed complex formed ion conducting channels that are asymmetrically blocked by LF in planar bilayer membranes.

Biologically active complex detected in plasma of infected animals

Our studies suggest that in vitro generated and purified (PA$_{63}$)$_7$:LF complex is functionally and biologically active, but does such an active complex exist in an infected animal? Earlier studies by Ezzell and Abshire (27) showed the presence of a high molecular weight complex in the serum of anthrax-infected guinea pigs. PA was found to exist primarily as a 63 kDa protein in complex with LF, while no PA$_{83}$ protein was detected in the infected blood. To identify a functionally active complex in the blood of infected animals, rabbits (n=3) and guinea pigs (n=2) were infected by aerosol challenge with a fully virulent $B$. anthracis strain. Just before death, the animals were anesthetized and blood was collected in tubes containing EDTA. Plasma was isolated and added to the PA antibody plate, washed to remove non-binding proteins and then incubated in the presence of fluorogenic peptide substrate. Representative data from different infected animal species are shown in Fig. 5A. LF activity was detected in this assay suggesting that there is a (PA$_{63}$)$_7$:LF complex in the plasma of infected animals and that LF in the complex form is catalytically active. Incubation of fluorogenic
peptide substrate with normal serum showed no increase in LF substrate cleavage as measured by fluorescence intensity, suggesting that cleavage of the peptide was not caused by serum proteases.

To visualize the banding pattern of the in vivo complex, infected plasma samples were subjected to native PAGE and analyzed by western blotting using PA (Fig. 5B) and LF (Fig. 5C) antibodies. Infected plasma from three different rabbits (Figs. 5B and 5C, lanes 3-5) and two different guinea pigs (Fig. 5B and 5C, lanes 6 and 7) showed high molecular weight complexes of different sizes that were greater in size to the in vitro generated (PA63):LF complex (Fig. 5B and 5C, lane 1). The complex in all the three rabbits appears as a smear (Fig. 5B, lanes 3-5). The complexes in the infected animals may have interacted and bound to blood-borne proteins, thereby affecting their mobility on native PAGE. Immunoblot with LF antibody showed some free LF in two of the infected rabbit plasma (Fig. 5C, lanes 3 and 4). No free PA83 was detected in the infected samples, as analyzed by western blotting of both native PAGE (Fig. 5B) and SDS-PAGE (Fig. 5D, Top panel). Control serum from untreated guinea pig (Figs. 5B and 5C, lane 2), did not react with the PA or LF antibody. Plasma from representative infected animals when subjected to SDS-PAGE and analyzed by immunoblotting with the PA antibody showed PA63 monomer and SDS resistant and heat stable PA63 oligomer, but no PA63 (Fig. 5D, Top panel). The 90 kDa LF protein was detected with LF antibody (Fig 5D, Bottom panel) in the infected samples.

DISCUSSION

The widely accepted in vitro cell intoxication model explains how PA and LF might act in concert to disable the target. However, biochemical and cellular studies by different groups have posed several interesting questions. First, the reported high-affinity interaction between LF and PA63 heptamer (18) suggests that LF may not dissociate from the PA63 heptamer, even under low pH (pH 5.2). Second, the relatively small size of the PA pore (inner diameter ~12 Å to 17 Å), estimated by single channel recordings (24,28,29) and from molecular modeling of the PA pore (30) suggest that the energy barrier to LF and EF translocation through the channel lumen may be formidable. However, pH-induced translocation studies in either CHO or L6 cells revealed efficient translocation of a truncated LF fragment (LFN, ~ 30 kDa) that contained the N-terminal PA binding domain (31). Their studies suggests that partial unfolding of the protein molecule is required during its passage through the PA channel (31). Electrophysiological measurements of PA63 channel blockade by EFN (28) and LFN (32) have also led to the hypothesis that EF and LF are translocated through the PA channels at pH 5.5. To date, there are no reported studies verifying the physical translocation of physiologically relevant full-length LF or EF molecules through the PA pore. The small size of the PA pore suggests that in order for the translocation process to occur, either EF/LF must be completely unfolded or the PA pore and the lipid bilayer must undergo major conformational changes or a phase transition (33). To begin to address some of these questions, we purified (PA63):LF complex and tested the biological activity of LF in the complex form.

In this study, we have clearly shown that purified LF complexed with PA63 heptamer exhibits biological and functional activity. LF in the complex form cleaved both the synthetic peptide and endogenous MAPKK substrates and killed susceptible macrophages. Moreover, there was no significant difference in the efficiency of cleavage and killing of susceptible cells by LF in complex or in the free LF form. These results suggest that LF does not have to dissociate from PA to exhibit biological activity, and indeed can impart equivalent activity while still bound to PA. However, there is no direct evidence to support the presence of intact complex inside the cells.

In planar bilayer membranes, (PA63), form ionic channels and LF converts the channels to extreme rectifiers. We found that (PA63):LF complex also formed channels, and that its conducting properties were virtually identical to those of (PA63)7 channels in the presence of LF in the aqueous phase. The ionic channel recordings (Figs. 4A and 4B) show that at low applied potential (~50 mV), the conductance of (PA63)7 channels was approximately twice that of channels formed by (PA63):LF complex (see also Fig. 4F). Thus, these results suggest that the channels formed by the purified complex were partially blocked by LF at small negative applied potentials,
as were \((\text{PA}_{63})_7\) channels with LF in the subphase (Fig. 4F, \(-80 \text{ mV} < V < 0\)). Similar observations were made by Zhao and coworkers who used a cell-based approach (34). Under acidic pH conditions (pH 5.5), LF caused a partial blockade of \(\text{PA}_{63}\) channels on the plasma membrane of CHO-K1 cells (34). Thus, our data suggests that the \textit{in vitro} preformed complex has functional properties similar to the complex formed by adding LF to \text{PA}_{63} heptamer in lipid bilayers or in cell based studies, but it does not directly address the issue of LF translocation.

Detecting \((\text{PA}_{63})_7\):LF complex but not \(\text{PA}_{63}\), in the blood of infected animals suggests that the \(\text{PA}_{63}\) protein released by \textit{B. anthracis} was efficiently activated by serum proteases. This is consistent with the studies by Ezzell and Abshire (27) which showed that only \text{PA}_{63} was detected in the blood of infected animals and appeared to be complexed with LF (and presumably with EF as well). In their studies, they found no free \text{PA}_{63} and proposed that the lethal/edema toxin are preformed in the blood at some point between the release of toxin components through the membrane of the bacillus and before binding to the host cell. Cleavage of the \text{PA}_{63} protein is catalyzed by a calcium-dependent, heat-labile protective antigen protease found in the serum of a wide range of animal species (27). EDTA inhibits this protease activity. As blood drawn from infected animals was collected in the presence of EDTA, cleavage of \text{PA}_{63} by proteases activated by the clotting cascade can be ruled out. An interesting observation in this study was the detection of functionally active \((\text{PA}_{63})_7\):LF complex in the blood of infected animals. Electrophysiological studies of purified \((\text{PA}_{63})_7\):LF complex from the plasma of infected guinea pigs strongly rectified the ionic current similar to that observed with \textit{in vitro} purified complex (data not shown). Although the complex in the blood could be detected only during the septicemia or the late stage of infection, unpublished data by Ezell and coworkers (personal communication) suggests that the \text{PA}_{63} within \textit{B. anthracis} is cleaved to \text{PA}_{63} on the bacterium surface by serum proteases. The \text{PA}_{63} appears to resist release from the bacteria and the mechanism by which the predicted \((\text{PA}_{63})_7\):LF complex is released in the blood of infected animals has yet to be elucidated.

These findings raise questions about the role of the complex in anthrax pathogenesis. During the early stages of infection, sublethal dose of lethal toxin protects the bacteria by inactivating the immune system through MEK cleavage, thus preventing cytokine responses (4,35,36) and impairing adaptive immunity of dendritic cells (15). Whether the preformed complex has a similar role during the early infection process remains to be determined. In the late stage of infection, when a critical bacterial load or “point of no return” stage is reached, antibiotics kill the bacteria, but the animal still dies (37); therefore design of a adjunct anti-toxin therapeutics should consider the possibility that preformed complex is circulating in the blood of the terminal animals. If indeed the complex is formed in the early stages of infection, on the surface of the bacteria, then the timing of PA-directed therapeutics could be crucial. The individual toxin components and the complex must be considered as critical targets for anthrax therapeutics.

ACKNOWLEDGEMENTS

We thank Rosemary Cushman for help in purifying the complex, Dr. James Schmidt and Martin Misakian for their useful comments. This work was supported in part by the Defense Advance Research Projects Agency (to J. Ezzell), NIST ATP, Defense Threat Reduction agency/JSTO-CBD and by USAMRIID project numbers 0242C012 (to S. Bavari) and 02-4-CC-010 (to B. Powell).

REFERENCES

1. Pellizzari, R., Guidi-Rontani, C., Vitale, G., Mock, M., and Montecucco, C. (2000) Int J Med Microbiol 290, 421-427
2. Vitale, G., Bernardi, L., Napolitani, G., Mock, M., and Montecucco, C. (2000) Biochem J 352 Pt 3, 739-745
3. Duesbery, N. S., Webb, C. P., Leppla, S. H., Gordon, V. M., Klimpel, K. R., Copeland, T. D., Ahn, N. G., Oskarsson, M. K., Fukasawa, K., Paull, K. D., and Vande Woude, G. F. (1998) *Science* **280**, 734-737
4. Pellizzari, R., Guidi-Rontani, C., Vitale, G., Mock, M., and Montecucco, C. (1999) *FEBS Lett* **462**, 199-204
5. Leplla, S. H. (1982) *Proc Natl Acad Sci U S A* **79**, 3162-3166
6. Duesbery, N. S., and Vande Woude, G. F. (1999) *Cell Mol Life Sci* **55**, 1599-1609
7. Bradley, K. A., Mogridge, J., Mourez, M., Collier, R. J., and Young, J. A. (2001) *Nature* **414**, 225-229
8. Scobie, H. M., Rainey, G. J., Bradley, K. A., and Young, J. A. (2003) *Proc Natl Acad Sci U S A* **100**, 5170-5174
9. Klimpel, K. R., Molloy, S. S., Thomas, G., and Leppla, S. H. (1992) *Proc Natl Acad Sci U S A* **89**, 10277-10281
10. Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R., and Thomas, G. (1992) *J Biol Chem* **267**, 16396-16402
11. Abrami, L., Lindsay, M., Parton, R. G., Leplla, S. H., and van der Goot, F. G. (2004) *J Cell Biol* **166**, 645-651
12. Duesbery, N. S., and Vande Woude, G. F. (1999) *J Appl Microbiol* **87**, 289-293
13. Vitale, G., Pellizzari, R., Recchi, C., Napolitani, G., Mock, M., and Montecucco, C. (1998) *Biochem Biophys Res Commun* **248**, 706-711
14. Park, J. M., Greten, F. R., Li, Z. W., and Karin, M. (2002) *Science* **297**, 2048-2051
15. Agrawal, A., Lingappa, J., Leplla, S. H., Agrawal, S., Jabbar, A., Quinn, C., and Pulendran, B. (2003) *Nature* **424**, 329-334
16. Mogridge, J., Cunningham, K., and Collier, R. J. (2002) *Biochemistry* **41**, 1079-1082
17. Singh, Y., Klimpel, K. R., Goel, S., Swain, P. K., and Leplla, S. H. (1999) *Infect Immun* **67**, 1853-1859
18. Elliott, J. L., Mogridge, J., and Collier, R. J. (2000) *Biochemistry* **39**, 6706-6713
19. Panchal, R. G., Hermone, A. R., Nguyen, T. L., Wong, T. Y., Schwarzenbacher, R., Schmidt, J., Lane, D., McGrath, C., Turk, B. E., Burnett, J., Aman, M. J., Little, S., Sausville, E. A., Zaharevitz, D. W., Cantley, L. C., Liddington, R. C., Gussio, R., and Bavari, S. (2004) *Nat Struct Mol Biol* **11**, 67-72
20. Turk, B. E., Wong, T. Y., Schwarzenbacher, R., Jarrell, E. T., Leplla, S. H., Collier, R. J., Liddington, R. C., and Cantley, L. C. (2004) *Nat Struct Mol Biol* **11**, 60-66
21. Little, S. F., Leplla, S. H., and Cora, E. (1988) *Infect Immun* **56**, 1807-1813
22. Montal, M., and Mueller, P. (1972) *Proc Natl Acad Sci U S A* **69**, 3561-3566
23. Kasianowicz, J. J., and Bezrukov, S. M. (1995) *Biophys J* **69**, 94-105
24. Blaustein, R. O., Koehler, T. M., Collier, R. J., and Finkelstein, A. (1989) *Proc Natl Acad Sci U S A* **86**, 2209-2213
25. Koehler, T. M., and Collier, R. J. (1991) *Mol Microbiol* **5**, 1501-1506
26. Milne, J. C., and Collier, R. J. (1993) *Mol Microbiol* **10**, 647-653
27. Ezzell, J. W., Jr., and Abshire, T. G. (1992) *J Gen Microbiol* **138** (Pt 3), 543-549
28. Finkelstein, A. (1994) *Toxicology* **87**, 29-41
29. Blaustein, R. O., and Finkelstein, A. (1990) *J Gen Physiol* **96**, 905-919
30. Nguyen, T. L. (2004) *J Biomol Struct Dyn* **22**, 253-266
31. Wesche, J., Elliott, J. L., Falnes, P. O., Olsnes, S., and Collier, R. J. (1998) *Biochim Biophys Acta* **1397**, 15737-15746
32. Zhang, S., Udho, E., Wu, Z., Collier, R. J., and Finkelstein, A. (2004) *Biophys J* **87**, 3842-3849
33. Di Marzio, E. A., and Kasianowicz, J. J. (2003) *Journal of Chemical Physics* **119**, 6378-6387
34. Zhao, J., Milne, J. C., and Collier, R. J. (1995) *J Biol Chem* **270**, 18626-18630
35. Popov, S. G., Villasmil, R., Bernardi, J., Grene, E., Cardwell, J., Popova, T., Wu, A., Alibek, D., Bailey, C., and Alibek, K. (2002) *FEBS Lett* **527**, 211-215
Erwin, J. L., DaSilva, L. M., Bavari, S., Little, S. F., Friedlander, A. M., and Chanh, T. C. (2001) *Infect Immun* 69, 1175-1177

Dixon, T. C., Meselson, M., Guillemin, J., and Hanna, P. C. (1999) *N Engl J Med* 341, 815-826

FIGURE LEGENDS

**Fig. 1.** Purification and characterization of (PA63)7:LF complex. (*A & B*) HPLC profiles of the preformed (PA63)7:LF complex purified over a Superose 6 size exclusion chromatography column. (*A*) A 3:2 molar ratio of PA63 monomer to LF monomer was used for the formation and isolation of the complex (*B*) To demonstrate titration of LF, a 3:1 molar ratio of PA63 monomer to LF monomer was used for the formation and isolation of the complex (*C*) Coomassie blue staining of native PAGE. Samples were separated on a 4 to 15% gradient gel (PhastGel) and stained with coomassie blue. Purified LF (lane 1), purified PA63 (lane 2), PA63-LF mix before loading onto size exclusion column (lane 3), aggregated protein complex (lane 4), purified (PA63):LF complex (lane 5) and unbound or free LF (lane 6). (*D*) Coomassie blue staining of SDS-PAGE gels. Samples were solubilized in SDS sample buffer, heated at 95°C for 5 mins, and electrophoresed on 4-12% gradient gel. Purified LF (lane 1), purified PA63 (lane 2) and purified (PA63):LF complex (lane 3).

**Fig. 2.** LF complexed with PA63 heptamer can cleave synthetic peptide substrate. (*A*) Representative data of fluorescent plate reader assay showing LF in the complex form exhibits similar activity to the free LF. Equal concentrations of either free LF or LF in complex form were incubated with 20 µM of the optimized peptide substrate (19) and kinetic measurements were obtained every min for 30 min at excitation and emission maxima of 325 and 394 nm respectively. (*B*) LF activity was observed when the complex was bound to the PA antibody plate. PA monoclonal antibody (PAI3F10) was bound to maxisorp plate, blocked overnight, washed and then incubated with the purified complex (either 200 ng/well or 20 ng/well of LF in complex form). After extensive washing, a reaction mixture containing 20 µM of fluorogenic LF peptide substrate was added to the appropriate wells and incubated for 30 min. Cleavage of the peptide by bound LF was monitored by an increase in fluorescence intensity in a plate reader. As control, free LF (10 µg/ml) did not show any non-specific binding to the PA mAb. LF activity in the bound form could be inhibited by a previously identified small molecule LF inhibitor NSC 12155 (10 µM) (19).

**Fig. 3.** Purified (PA63)7:LF complex exhibits biological activity. (*A*) Proteolytic cleavage of endogenous MAPKK by LF in the complex. J774A.1 macrophages were either not treated (lane 1) or treated with anthrax toxin (88 ng/ml PA63 and 16 ng/ml LF) (lane 2), complex (20 ng/ml of LF in complex) (lane 3), complex (200 ng/ml of LF in complex) (lane 4) and incubated for 4 h at 37°C. Cell lysates were prepared and analyzed by immunoblotting with antibodies specific to four different isoforms of MAPKK. A housekeeping transferrin protein was used to show uniform loading of the lysates. (*B*) LF in the complex kills macrophages. J774A.1 cells were incubated for 4 h with two fold serial dilutions of anthrax toxin (starting with 0.5 µg/ml PA63 and 0.5 µg/ml LF) or complex (starting with 0.5 µg/ml LF in complex). Cell viability was then measured by an MTT assay.

**Fig. 4.** (PA63)7 and purified (PA63):LF complex form functional channels in planar bilayer membranes. The discrete steps in ionic current in (*A*) and (*B*) correspond to the spontaneous formation of single channels of (PA63)7 and purified (PA63):LF complex, respectively. The applied potential was -50 mV. The representative data of instantaneous current-voltage (*I-V*) relationship for (PA63)7 in the absence (*C*) and presence of ~1 nM LF (*D*) demonstrate that LF added to the *cis* compartment converted the (PA63)7 *I-V* relationship from a slightly nonlinear to extremely rectifying. (*E*) Similar experiments show that channels formed from purified (PA63):LF complex (< 200 ng of LF in the complex) also exhibited a highly rectifying *I-V* relationship. (*F*) A plot of the *I-V* relationship of (PA63)7, (PA63)7 + 1 nM LF, and the
purified \((PA_{63})_7:\text{LF}\) complex, show that at positive applied potentials, LF blocked the pore whether it was initially bound to the channel as a complex, or added subsequently to the subphase bathing \((PA_{63})_7\) channels. The aqueous solutions on both sides of the membrane contained 0.1 M KCl, 5 mM MES at pH 6.6.

**Fig. 5.** Detection of \((PA_{63})_7:\text{LF}\) complex in plasma of infected animals. (A). Representative data showing LF activity in \((PA_{63})_7:\text{LF}\) complex from plasma of infected animals. Plasma (50 µl) from infected animals or normal serum was bound to the PA antibody plate, washed to remove any non-specific proteins, and then incubated with 20 µM of fluorogenic peptide substrate for 30 min at room temperature. Cleavage of the peptide by LF in complex form was monitored by an increase in fluorescence intensity at an excitation and emission maxima of 325 and 394 nm, respectively. LF activity was detected suggesting that \((PA_{63})_7:\text{LF}\) complex in plasma of infected animals is functionally active. (B and C). High molecular weight \((PA_{63})_7:\text{LF}\) complex detected in the plasma of different infected animals. *In vitro* generated and purified \((PA_{63})_7:\text{LF}\) complex (lane 1), control untreated guinea pig serum (lane 2), plasma from three different infected rabbits (lanes 3-5), plasma from two different infected guinea pigs (lanes 6-7) and purified PA or LF protein (lane 8) were subjected to native PAGE, transferred onto nitrocellulose membranes and then immunoblotted (IB) with either PA (B) or LF (C) antibody. (D) PA was not detected in plasma of infected animals. Representative data of samples subjected to SDS-PAGE and immunoblotted with anti PA (Top panel) and anti LF (Bottom panel) antibodies. (Top panel): PA (lane 1), PA (lane 2), *in vitro* purified complex (lane 3), plasma from infected guinea pig (lane 4) and plasma from infected rabbit (lane 5). (Bottom panel): LF (lane 1), *in vitro* purified complex (lane 2), plasma from infected guinea pig (lane 3) and plasma from infected rabbit (lane 4).
Figure 1
Figure 4

A (PA$_{63}$/7)

B (PA$_{63}$/7; LF)

C (PA$_{63}$/7)

D (PA$_{63}$/7 + LF)

E (PA$_{63}$/7; LF)

F I (pA) vs V (mV)

-160 -80 80 160

-160 -100 100 200

Time (ms)

Time (ms)
Figure 5
Purified bacillus anthracis lethal toxin complex formed in vitro and during infection exhibits functional and biological activity

Rekha G. Panchal, Kelly Halverson, Wilson Ribot, Douglas Lane, Tara Kenny, Teresa G. Abshire, John W. Ezzell, Timothy A. Hoover, Bradford Powell, Stephen Little, John J. Kasianowicz and Sina Bavari

J. Biol. Chem. published online January 11, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M412210200

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