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ARTICLE *in* ANALYTICAL CHEMISTRY · DECEMBER 2007

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Anal. Chem. **2007,** *79,* 8463−8470

Detection and Quantification of Anthrax Lethal Factor in Serum by Mass Spectrometry

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The lethal toxin produced during *Bacillus anthracis* infection is a complex of protective antigen, which localizes the toxin to the cell receptor, and lethal factor (LF), a zinc-dependent endoproteinase whose known targets include five members of the mitogen-activated protein kinase kinase (MAPKK) family of response regulators. We have developed a method for detecting functional LF in serum. Anti-LF murine monoclonal antibodies immobilized on magnetic protein G beads were used to capture and concentrate the LF from serum. The captured LF was exposed to an optimized MAPKK-based peptide substrate, which it hydrolyzed into two smaller peptides. The LF cleavage products were then analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) and quantified by isotope dilution-MS. The entire analytical method can be performed in less than 4 h with detection of LF levels as low as 0.05 ng/mL. The method was used to quantify LF levels in serum from rhesus macaques infected with *B. anthracis*. Serum samples obtained at day 2 postinfection contained 30−250 ng/mL LF and illustrated the clear potential to detect LF earlier in the infection cycle. This method represents a highly specific and rapid diagnostic tool for early anthrax and has a potential additional role as a research tool for understanding toxemia and effects of medical countermeasures for anthrax.

Anthrax is caused by infection with *Bacillus anthracis*, a spore-forming, Gram-positive bacterium. The dormant spore is resistant to extremes of temperature, desiccation, and a variety of chemical treatments.[1] The stability, ease of production, and infectious capacity of the spores confer *B. anthracis* with high potential as a biological weapon.[2] *B. anthracis* spores gain entry through a dermal abrasion or gastrointestinal lesion causing cutaneous or gastrointestinal anthrax, respectively, or are inhaled, causing pulmonary anthrax. Systemic infection from the progression of any of the three forms of anthrax frequently results in secondary shock, multiple organ failure, and death.[3] Early diagnosis is critical for effective treatment of pulmonary (inhalation) anthrax.[4] In the U.S. bioterrorism attacks of 2001, pulmonary anthrax had a 45% fatality rate despite antibiotic treatment and aggressive supportive care of the patients.[5,6]

The two exotoxins of *B. anthracis* are binary combinations of protective antigen (PA) and either edema factor (EF) or lethal factor (LF).[7] The complex of PA and EF forms edema toxin (ETx) and PA complexed with LF forms lethal toxin (LTx). PA is secreted as an 83-kDa protein (PA83), which binds to known receptors TEM8 (tumor endothelium marker 8)[8] and CMG2 (capillary morphogenesis protein 2)[9] on the surface of target cells where it is cleaved by a furin-like cell surface protease to a 63-kDa protein (PA63).[10] PA83 may also be cleaved to the PA63 conformer by serum proteases.[11] Cleavage causes a conformational

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Published on Web 10/12/2007

change that allows PA to form a heptamer, which binds three molecules of either LF, EF, or a mixture of the two. This is followed by endocytosis-mediated entry of the toxin complex LTx and ETx into the cell. The low endosomal pH induces PA to form a pore and translocate EF and LF into the cytosol, where they target their respective substrates.[10] EF is an adenylate cyclase, which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The accumulation of cAMP causes an efflux of fluid from the cell and leads to edema. The chronic excess of cAMP during infection also leads to activation of cAMP-dependent signaling pathways and inhibition of phagocytes.[12] LF is a zinc-dependent endoproteinase, which is known to target the amino terminus of five members of the mitogen-activated protein kinase kinase (MAPKK) family of response regulators.[7] The cleavage of these proteins disrupts a signaling pathway and leads to cytokine dysregulation and immune dysfunction. When administered in the absence of infection, LTx can lyse certain cell lines in vitro and is lethal to some species of laboratory animals.[13]

Diagnosis of *B. anthracis* infection relies on a combination of tests.[14] These include immunofluorescent microscopy and amplification of *B. anthracis* genes.[6,15,16] Methods for definitive phenotypic and genotypic identification of isolates require initial culturing of the organism and at least 16 h. Serological diagnosis is sensitive and specific, but because an antibody response can take up to 8 days to become detectable, it may offer only retrospective confirmation of infection.[17] Production of toxin is dependent on the presence of viable *B. anthracis*. A toxin-based assay with high sensitivity and specificity may provide early evidence of *B. anthracis* infection in a much reduced time frame compared to culture and gene amplification and thus facilitate a reduction in the number of tests and time to confirmatory diagnosis. In addition, the technology will convey valuable quantitative information about the kinetics of toxemia during infection and treatment.

Recent efforts in our laboratory have focused on mass spectrometry (MS)-based detection of bacterial protein toxin endopeptidase activities (Endopep MS).[18−20] Application of Endopep MS for anthrax LF incorporates three levels of specificity; monoclonal antibody-specific capture of LF, the unique enzymatic cleavage properties of the LF endoproteinase for a synthetic peptide substrate, and the application of matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS for LF cleaved product specific mass detection. Detection of mass-specific peaks gives certainty of the cleavage products, which is not possible with fluorescence resonance energy transfer (FRET)-based LF cleavage assays.[21] With FRET cleavage, fluorescence is the cleavage indicator, and cleavage by other proteases at non-LF-specific amino acid residues would also produce fluorescence. Detection of mass-specific peaks is also quantitative. The MS peak intensities correlate with the concentration of LF present and can be measured by isotope-dilution MS. Isotope-dilution MS is a well-established MS quantification technique that is traditionally performed using tandem MS on triple-quadrupole instrumentation[22] or high-resolution MS on a magnetic sector mass spectrometers.[23] It has recently been applied to MALDI-TOF MS and has also been used to quantify enzyme activity,[24,25] which supports its use for this assay. Here, we report a rapid, specific, and quantitative method for detecting functional anthrax toxin lethal factor, and its application to sera from rhesus macaques with pulmonary anthrax. The method limits of detection were 0.05 ng/mL with a 4-h total time to detection, and 10 times lower detection is possible with a 20-h reaction time. The LF MS method represents a highly specific tool for the diagnosis of anthrax.

MATERIALS AND METHODS

Monoclonal Antibody Preparation. Monoclonal anti-LF antibodies (mAb) were prepared in the Division of Scientific Resources, NCPDCID, CDC. Two sets of 8−10-week-old Balb/C mice were immunized with recombinant LF. The antigen was prepared with RIBI adjuvant (Ribi ImmunoChem Research, Inc. Hamilton, MT), and 100 μg of the protein/adjuvant was injected subcutaneously with boosts on days 21 and 35. Animals were sacrificed and spleens harvested on day 38. Primary splenocytes (washed with basal IMDM GIBCO 12440-053) were fused with the mouse myeloma cell line SP 2/0 (>95% viability, at a 1:5 myeloma/spleen cell ratio) in the presence of PEG 4000 (Sigma P-2906). Cells were resuspended in fusion medium (IMDM with additional supplements of 20% FBS (Hyclone laboratories), NA amino acids, sodium pyruvate, and 50 units/mL murine recombinant IL-6 + HAT (Boehringer-Mannheim). Fused cells were plated into five 48-well plates at 0.5 mL/well, incubated for 48 h at 37 °C with 5% CO_2, and then transferred into HT fusion media for 7 days. The cell culture supernatants were screened for antibody production, and positive hybridomas were subcloned three times by limiting dilution to isolate a clonal population of antibody producing cells. Monoclonal antibodies were further

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Table 1. Alignment of Known LF Protease Cleavage Sites in Members of the MAPKK Family and Peptide Substrates Used for This Study[a]

Name		Sequence (P1 ↓ P1')	[M + H]$^+$
MAPKK-1_{1-25}	(M1)	MP K K K P T **P** **I** Q L N P A P D G S A V N G T S S	
MAPKK-2_{1-27}	(M2)	M L A R R K P V L **P** **A** L T I N P T I A E G P S P T S E	
MAPKK-$3b_{17-43}$	(M3)	G K S K R K K D L **R** **I** S C M S K P P A P N P T P P R N	
MAPKK-4_{36-62}	(M4a)	S MQ G K R K A L **K** **L** N F A N P P F K S T A R F T L N	
MAPKK-4_{49-75}	(M4b)	A N P P F K S T A **R** **F** T L N P N P T G V Q N P H I E R	
MAPKK-6_{5-31}	(M6)	K G K K R N P G L **K** **I** P K E A F E Q P Q T S S T P P R	
MAPKK-7_{35-61}	(M7a)	S P Q R P R P T L **Q** **L** P L A N D G G S R S P S S E S S	
MAPKK-7_{67-93}	(M7b)	P P A R P R HM L **G** **L** P S T L F T P R S M E S I E I D	
Consensus		S P A R R K K T L $^{P}/_{K}$ $^{L}/_{I}$ P L $^{N}/_{A}$ $^{N}/_{P}$ $^{P}/_{F}$ $^{P}/_{T}$ P A S T P S P T S	
LF-1 (Core)		(R R K K V Y **P** **Y** P M E) P T I A	1751.96
LF-2		R R K K V Y **P** **Y** P M E P T I A K	1880.06
LF-3		S P A R R K K V Y **P** **Y** P M E N P T P R S T P S P T	2857.47
LF-4		S K A R R K K V Y **P** **Y** P M E N F P P S T A R P T	2821.50
NT4		S K A R R K K V Y **P**	1232.75
CT4		**Y** P M E N F P P S T A R P T	1607.74
NT-ISTD		S K (A+7) R R K K V Y **P**	1239.75
CT-ISTD		**Y** P M E N F P P S T (A+7) R P T	1614.74

[a] The eight known LF protein targets were aligned by their cleavage sites and a floating consensus was derived based on the one or two most abundant amino acids at a position. LF hydrolyzes these sequences between the P1 and P1′ residues in boldface print. MAPKK sequences for M1 (P29678), M2 (P36506), M3 (P46734), M4a-b (P47809), M6 (P52564), and M7a-b (O14733) were obtained from the national Center for Biotechnology Information Website (www.ncbi.nlm.nih.gov). Substrate and internal standard (ISTD) peptide sequences and [M + H]$^+$ masses are listed.

screened for their ability to neutralize lethal toxin activity in a cell-based lethal toxin neutralization assay.[26] Two non-neutralizing anti-LF mAbs were identified, of which one was selected for further assay development.

Materials. Dynal Magnetic protein G beads were used to bind and cross-link mAbs according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Recombinant LF, monomeric PA (PA83), and activated heptameric PA (PA63) were purchased from List Biological Laboratory (Campbell, CA). All other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) except where indicated. A Dynal magnetic bead retriever (Invitrogen Corp.) and a Kingfisher 96 magnetic particle processor (Thermo Electron Corp., Waltham, MA) were used for automated sample preparation.

Synthetic Peptide Design. Synthetic peptides LF-1, -2, -3, and -4, NT-ISTD, and CT-ISTD were synthesized and purified by Los Alamos National Laboratory, Los Alamos, NM (Table 1). LF-1 was based on an optimized peptide developed by Turk et al. but without the fluorogenic labels.[21] LF-2 is identical to LF-1 except that it includes an additional C-terminal lysine. LF-3 and LF-4 maintain the central core of LF-1 and have longer sequences based on an extended consensus of known MAPKK substrates aligned by their cleavage sites (Table 1). The NT-ISTD (amino-terminal internal standard) and CT (carboxy-terminal)-ISTD peptides have amino acid sequences identical to that of the LF-4 amino-terminal (NT4) and carboxy-terminal (CT4) LF cleavage products, respectively, except that the alanine residue in each peptide was replaced with an isotopically labeled derivative, containing $^{13}C_3$, 3-deuterium (D), and one ^{15}N. This gave the NT- and CT-ISTD peptides a total mass that was 7 mass units higher than NT4 and CT4, respectively (Table 1). These two peptides served as isotopically labeled internal standards for isotope dilution quantification of the LF-4 cleavage products.

Animal Infection Sera. Rhesus macaque sera were obtained from the Microbial Pathogenesis & Immune Response (MPIR) Laboratory, Division of Bacterial Diseases, Meningitis & Vaccine Preventable Diseases Branch, NCIRD, CDC. Sera from infected animals were filter sterilized (0.2 μm) and stored frozen at −70 °C in aliquots of 100 μL. Sera were used without heat inactivation of complement. The animal protocol for this study (1174ASHMONX) was approved by the CDC Institutional Animal Care and Use Committee (IACUC). All serum samples were handled under BSL-2 requirements in a biological safety cabinet with appropriate personnel protective equipment.

LF Activity Assay. For determining assay performance characteristics, the activity of LF was assayed in 40 μL total volume of reaction buffer (RB), except where indicated otherwise, containing 20 mM Hepes buffer pH 7.3, 1 mM DTT, 20 μM $CaCl_2$, 10 mM $MgCl_2$, 20 μM $ZnCl_2$, protease inhibitor mix,[20] and 2 nmol of substrate peptide LF-4. LF in a sample was incubated with RB and substrate at 37 °C for 2 h or the time indicated, and a 1-μL aliquot of the reaction was used for MALDI-TOF MS analysis.

MALDI-TOF MS Analysis. For MALDI-TOF MS detection, a 1- or 2-μL aliquot of the LF reaction mixture was removed and added respectively to 9 or 18 μL of α-cyano-4-hydroxycinnamic acid (CHCA) at 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, and 1 mM ammonium phosphate (CHCA matrix), with or without 1 pmol of NT- and CT-ISTD where indicated, mixed, and 0.5 μL was spotted in triplicate onto a 192-spot stainless steel MALDI plate (Applied Biosystems, Framingham, MA), and then mass spectra were collected from 750 to 3200 mass/charge (m/z) or as described, in MS positive ion reflectron mode on the

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Applied Biosystems 4700 Proteomics Analyzers (Framingham, MA). This instrument uses a nitrogen laser at 337 nm, and each final mass spectrum was an average of spectra obtained from 2400 laser shots. All spectra shown were smoothed once with a correlation factor of 0.7.

Relative and Absolute Quantification of LF Activity by Isotope Dilution MALDI-TOF MS. The isotopically labeled NT-ISTD and CT-ISTD peptides were added to the CHCA MALDI matrix at 1 pmol each, along with 1 μL of each LF reaction mixture, and analyzed as described above. Since the ISTD peptides were at the same concentration in each CHCA−sample mixture to be analyzed, they normalize the spot-to-spot intensity differences typical with MALDI-TOF MS analysis. The spectra acquired were converted to T2D files (custom Applied Biosystem MS program files) from which clustal isotopic peak areas were obtained. For each spectrum, the response factor for the two LF cleaved products, NT4 and CT4, were obtained by dividing the area of the NT4 product peak by the area of the NT-ISTD peak, and the CT4 peak area by the CT-ISTD peak area. The response factors generated were referred to as the area ratios. For most relative and quantitative analyses in this study, the CT4/CT-ISTD area ratio was used, except for the timed reaction comparing four substrates for which the NT4/NT-ISTD area ratios were compared for reasons explained.

Comparison of Four Peptide Substrates. The relative cleavage properties were compared for four peptide substrates, LF-1, LF-2, LF-3, and LF-4 (Table 1), by performing a timed LF cleavage reaction at 37 °C and analyzing 1 μL at 5, 10, 15, 30, 60, 90, 120, and 240 min of incubation (Table 1). The 200-μL reactions for each peptide included the same RB above, 5 nmol of each substrate, and 10 ng of LF, for a concentration of 532 pM LF. For relative quantification of the cleaved product peptides, the NT- and CT-ISTD's were included in the MALDI analysis (described below). An additional 1-μL aliquot was analyzed at 120 min to obtain spectra without ISTD, removing a maximum of 4.5% reaction volume over a 4-h reaction time.

Extraction of LF from Buffer and Serum Using Magnetic Antibody Beads Followed by LF Cleavage Reaction. Magnetic antibody beads for capturing LF (LF MABs) were prepared by covalently linking anti-LF mAbs to Dynal protein G magnetic beads at 40 μg of protein/100 μL of beads following the manufacturer's protocol. LF MABs were preblocked with 1% casein in phosphate-buffered saline with 0.05% Tween 20 (PBS/TW), and then 25 μL of the beads was mixed with either recombinant LF-spiked serum in PBS/TW at the concentrations indicated or serum from rhesus macaques with pulmonary anthrax for a final volume of 500 μL or 1 mL where indicated. The Dynal bead retriever was programmed for automatic sample handling, which included gentle mixing of the antibody beads with the serum samples for 1 h (antibody binding) and two washes each in 1 mL of PBS/TW, followed by a wash in 200 μL of dH_2O and an elution in 100 μL of dH_2O. The beads were then reconstituted in 40 μL of RB containing 2 nmol of substrate LF-4, were incubated at 37 °C for 2 h, and then analyzed by MALDI-TOF MS with ISTD as described.

Effect of PA on LF Antibody Binding and Activity. The effect of PA on LF capture by LF MABS and LF enzymatic activity was evaluated. LF was mixed with PA_{83} and PA_{63} at a PA/LF ratio 4:1 (w/w) PA:LF. Specifically, 1 μg of LF alone (1), with 4 μg of PA_{83} (2), or with 4 μg of PA_{63} (3) was diluted in 20 μL of PBS and the resultant mixtures incubated at 20 °C for 2 h. These three mixtures were diluted to 1 ng/μl LF, and from them, aliquots containing 2 ng of LF were assayed three ways. One was assayed directly for LF activity in a total volume of 100 μL of RB described above (21 pM LF), containing LF-4 substrate in the reaction. Aliquots of the three LF mixtures were also spiked in buffer and serum, captured by LF MABs as described above, and reconstituted in a total volume of 100 μL of RB (21 pM LF), containing LF-4 substrate included for the cleavage reaction. After a 2 h incubation at 37 °C, 1 μL aliquots from all reactions were spiked into CHCA with ISTD peptides. Mass spectra were acquired with CT-ISTD included to visualize differences.

Characterization of Range of Detection for LF MS Method in Human Serum. Recombinant LF was diluted in pooled normal human serum (Interstate Blood Bank, Memphis, TN) from 0.005 to 800.0 ng/mL and 5, 20, and 200 μL of spiked serum was subjected to LF MAB capture, a 2-h substrate cleavage reaction with LF-4 peptide (Table 1), and MALDI-TOF MS analysis with ISTD. The CT4 product peak/CT-ISTD area ratios were determined for triplicate spots at each concentration. To cover this broad range of concentrations and to effectively characterize the upper and lower asymptotes, the log concentration to log area ratio was plotted over the entire range for all three serum volumes.

Quantification of LF in Rhesus Macaque Serum. Recombinant LF was spiked into pooled rhesus macaque control serum containing from 10 to 400 ng/mL LF to generate a standard calibration curve. A 5-μL aliquot of each serum sample was used for the LF capture assay with a 2- h cleavage reaction and MALDI-TOF MS analysis for quantification. LF CT4 product/CT-ISTD area ratios were determined and were plotted versus LF concentration. The concentration of LF in infection sera were calculated from the equations generated for the standard curve (ng/mL). For this volume and reaction time, the method was linear over this range.

RESULTS AND DISCUSSION

Concept and Peptide Design for an Anthrax LF MS Method. We have implemented MALDI-TOF MS for detecting LF activity by targeted cleavage of a peptide substrate and the production of two specific peptide products. To illustrate the diagnostic concept and specificity of MS for LF activity, MALDI-TOF MS spectra are shown for reactions with peptide LF-4 both in the absence of LF and with 1 ng of LF (Figure 1). MALDI-TOF MS displays the peptides as peaks, which are separated on the *x*-axis by mass to charge ratio (*m/z*) and *y*-axis by peak intensity. The substrate and two predicted LF cleaved peptide sequences and their expected singly charged *m/z* values are shown (Figure 1A). In the absence of LF, two main peaks were visible that represent the singly and doubly charged substrate peptide at 2821.8 and 1411.5 *m/z*, respectively (Figure 1B). After a 2-h reaction with 1 ng of LF, two peaks matching the predicted singly charged masses of LF cleaved product peptides were present at 1232.9 and 1607.9 *m/z*. The substrate peak heights were also reduced, indicating its consumption resulting from cleavage by LF (Figure 1C).

The quantity of peptide product generated in a given time frame is proportional to the quantity of LF in a sample, and the products can be measured by MS. Likewise, the quantity of cleavage products increases with continued incubation time. The

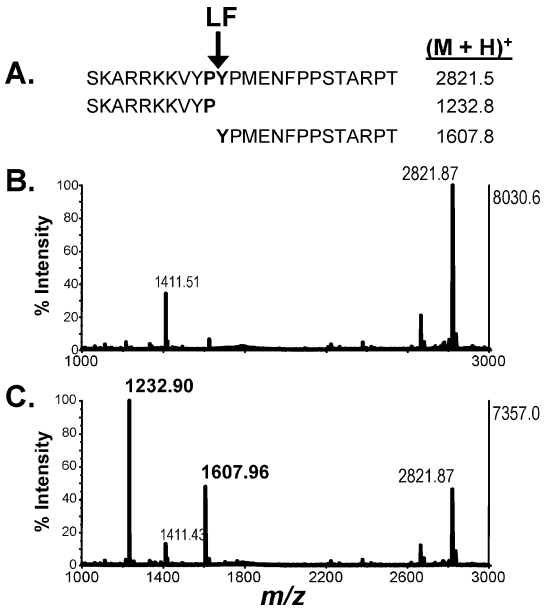

Figure 1. MALDI-TOF MS detection of LF activity. LF hydrolyzes a synthetic peptide substrate to yield two smaller peptide products. The substrate LF-4, cleaved product sequences, and expected $[M + H]^+$ of each are shown (A). MALDI-TOF MS mass spectra are shown for a 2-h cleavage reaction at 37 °C with 2 nmol of LF-4 and reaction buffer, one without LF (B) and with 1 ng of LF (C).

relative cleavage efficiency of any given substrate by LF can be measured by comparing the relative amount of cleaved products observed in MALDI-TOF MS snapshots taken over time. This property was used to investigate and design potential LF substrates that were suitable for MS analysis. Four substrates were compared for use in the LF activity assay. LF-1 is based on an optimized peptide developed by Turk et al. that showed an improved rate of cleavage with two tyrosine residues on either side of the P1 proline residue, but it does not have fluorogenic labels (Table 1).[21] However, LF-1 was not entirely suitable for MS analysis since the carboxyl (C)-terminal cleavage product of LF-1 lacks an amino acid with a free higher-order amine, such as found on an arginine or lysine, required to carry the MALDI ionization charge and would not be visible by MS. Therefore, a second peptide (LF-2) was designed, which included an additional C-terminal lysine (Table 1). In order to design and investigate substrates with longer sequences, the sequences of the eight known MAPKK cleavage locations, designated M1−M7b, were aligned by their cleavage sites and the extended sequences were analyzed (Table 1). These eight extended regions show moderate sequence similarity but not identity. It is notable that six of eight of the extended C-terminal sequences contain two or more prolines, and seven of eight contain two or more of the structurally and functionally similar serines(S)/threonines(T). There are double prolines found in M3, M4, and M6 and doublets serine/threonine, occurring as TS, ST, or SS, found in all the sequences except M3 and M4b. M7a has a total of six serines. A floating consensus was derived as shown and was used to design two additional longer substrates (Table 1). LF-3 consists of the 11-amino acid core of LF-1 with an additional 3 amino (N)-terminal and 7 C-terminal amino acids that match the consensus, except for arginine (R_{19}), which was included for MS detection. LF-4 also includes the core, but extended sequences do not directly match the floating consensus. For LF-4, a total of three prolines (one PP doublet), three serine/threonines (one ST doublet), one alanine, and an arginine for MS charge were included in a less ordered sequence to determine whether the exact ordering of these amino acids is critical (Table 1).

Cleavage reactions were performed for each peptide substrate, and mass spectra were acquired at timed intervals for each reaction. Substrate sequences, expected products, and masses are shown alongside representative mass spectra for the 120-min incubation time (Figure 2A−D). The spectrum for LF-1 shows that the substrate peak at 1750.9 m/z is dominant, and the lower intensity LF cleaved N-terminal product peak at 946.5 m/z is present (Figure 2A). As anticipated, the C-terminal product with a predicted mass of 824.3 m/z was not sufficiently ionized and, therefore, was not visible in the spectrum. Incorporation of the lysine in LF-2 renders the C-terminal product visible, and peaks for both the expected products at 946.5 and 951.4 m/z are present along with the dominant substrate peak of 1879 m/z (Figure 2B). Since these two cleavage products are only ~5 mass units apart, the x-axis was expanded to easily distinguish them and the isotopic detail associated with both peaks (inset spectrum, Figure 2B). For substrate LF-3, the spectrum shows that the additional 10 amino acids considerably enhance LF cleavage of this substrate. The two product peaks are dominant at 1201.6 and 1674.7 m/z, and the substrate peak intensity at 2857.4 m/z is minor (Figure 2C). The spectrum for LF-4 also shows higher product peaks at the expected masses of 1232.7 and 1607.6 m/z and a low-intensity substrate peak at 2821.4 m/z (Figure 2D).

The relative efficiency of cleavage for all four substrates was compared by plotting the N-terminal cleavage product/NT-ISTD area ratios of each versus reaction time (ratios of the N-terminal product to NT-ISTD peaks, from spectra acquired for each peptide at each reaction time) (Figure 3A). The N-terminal cleavage products were preferred for this comparison since this product peptide was visible by MS in all four peptides. The focused spectrum shows the quantitative MS targets for LF-4, NT4 product, and associated isotopes at 1232.7 m/z and the NT-ISTD at 1239.7 m/z for the 15-min reaction time point (Figure 3B). In support of the visual differences observed in the spectra from Figure 2, the relative rate plots in Figure 3 show that the shorter substrates, LF-1 and LF-2, had slower relative cleavage rates compared to the longer substrates, LF-3 and LF-4. In reactions with the shorter substrates, there was no further increase in product peptides after 120 min, even though there was a considerable amount of substrate remaining as seen in Figure 2A and B. Ongoing analysis of reaction kinetics may determine whether this is due to reaching zero-order kinetics, or the shorter peptides have an inhibitory effect on LF activity. Consistent with the observations in this report for LF-1 and LF-2, it has been reported previously that LF activity was inhibited by some shorter FRET LF substrates.[27] This phenomenon may limit the detection of LF in FRET-based assays for which shorter substrates are preferred. Cleavage of the longer peptides LF-3 and LF-4 continued to accumulate product for more than 4 h (Figure 3A). Including consensus-like amino acids downstream of the cleavage sites in MAPKK substrates appears to provide a better structure for binding and cleavage by LF. The

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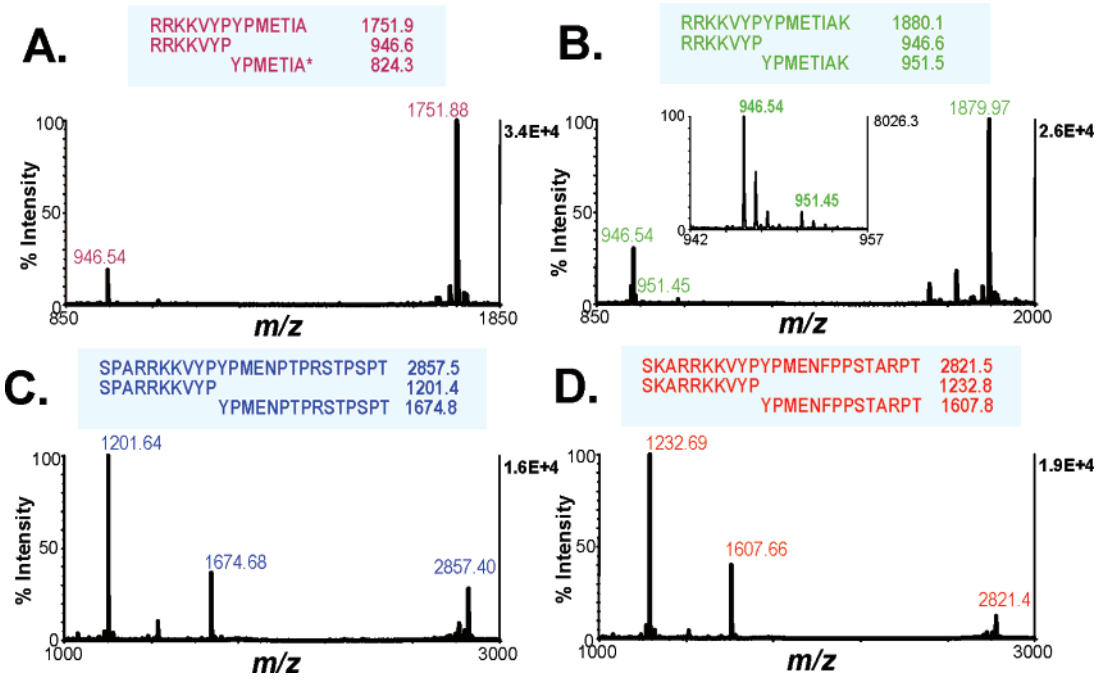

Figure 2. Comparison of spectral and cleavage quality of substrate peptides. Four substrate peptides were compared for potential use in this assay, LF-1 (A), LF-2, (B), LF-3 (C), and LF-4 (D). Full length and expected LF cleaved peptide sequences and masses are shown for each substrate. Cleavage reactions with each substrate were performed using 10 ng of LF, The 5 nmol of substrate in 200 μL of reaction buffer was incubated for 2 h at 37 °C after which MALDI-TOF MS spectra were acquired without ISTD (A−D). The *X*-axis was narrowed to visualize both cleavage products for LF-2, which are separated only 5 mass units.

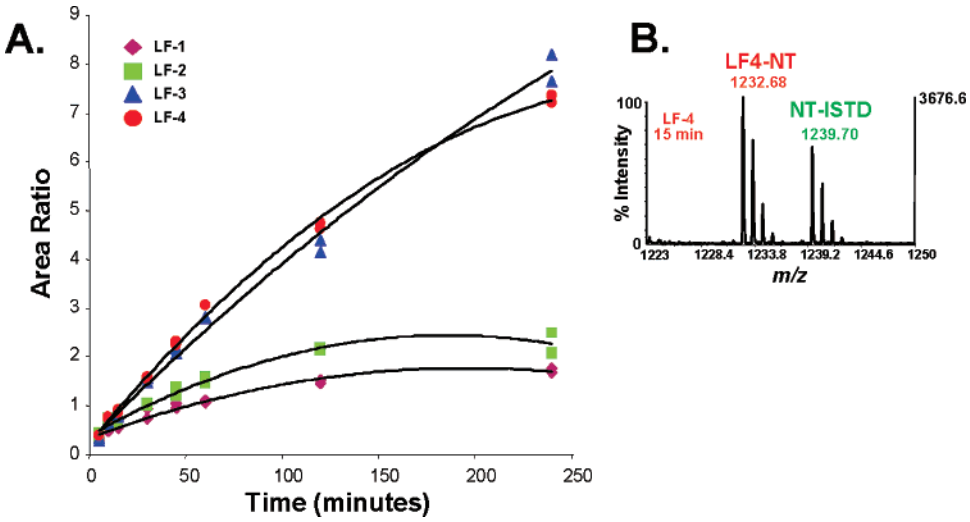

Figure 3. Relative cleavage rates of substrate peptides. The relative cleavage efficiency was compared for four substrate peptides LF-1, LF-2, LF-3, and LF-4 with timed reactions for each in 200 μL of buffer with 10 ng of LF, The 5 nmol of substrate was at 37 °C. Mass spectra were acquired with ISTDs at times 5, 10, 15, 30, 45, 60, 90, 120, and 240 min. The area ratio of the NT to NT-ISTD peptide peaks was plotted for each peptide and time point and fitted to a quadratic equation, which depicted the relative rates of reaction for each substrate (A). All R^2 values were greater than 0.975. The *X*-axis was narrowed for a representative spectrum from LF-4 cleavage at 15 min, which shows the LF-4 quantitative target NT4 and NT-ISTD peaks (B).

extended sequences include a number of serines, threonines, and prolines (Table 1). The prolines may impart regional structural stability, and the functional homologues serine and threonine might be important for hydrogen-bonding contacts within the substrate binding groove.[28] We expect that the longer peptides may more closely reflect the cleavage properties observed for the full-length native substrates. Although the relative cleavage rate for both LF-3 and LF-4 was similar, LF-4 gave lower limits of detection and was more stable over longer incubations (data not shown). This explains its targeted use in Figure 1 and remaining experimentation.

Effect of PA on LF Antibody Binding and Activity. During *B. anthracis* infection, LF and monomeric PA83 are produced as independent proteins that interact to form the LTx complex of PA63 and LF at the cell surface.[10] PA83 may also be activated to

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PA63 by serum protease activity, and the LTx complex is present in blood from *B. anthracis* infected animals.[11,29] Since the two proteins are so closely associated in vivo, it was important to test whether the presence of PA83 and PA63 interferes with detecting and measuring LF in this assay.

Potential interferences by PA were tested for the LF activity component alone and for the entire assay including antibody binding, extraction, and enzymatic activity. We first tested whether PA83 or PA63 had a direct effect on LF activity irrespective of antibody binding, by comparing mass spectra from 2-h reactions with LF, LF + PA83, or LF + PA63. The CT4 to CT-ISTD area ratios reflected the relative activity of LF under each condition. We did not observe any inhibition of LF activity in the presence of PA83 or PA63 (data not shown). Cleavage of the substrate was very similar for LF and LF + PA83, and there was a ~6-fold enhancement in cleavage (higher area ratios) for LF + PA63. Reactions for LF captured by antibody (LF + LF mAb) also showed a 6-fold increase over LF without antibody capture. Second, we tested whether PA83 or PA63 had any effect on the entire capture-activity assay for both buffer and serum spiked with LF, LF + PA83, or LF + PA63. Even in the presence of a large excess of either PA83 or PA63, there was no decrease in the amount of LF mediated cleavage, indicating that PA83 and PA63 do not interfere with antibody binding or the cleavage reactions. Specifically, under entire assay conditions captured from serum, relative cleavage from LF + PA83 was similar to that for LF, but a slight enhancement was observed for LF + PA63 (data not shown).

These results indicate that neither PA83 nor PA63 has a negative impact on any component of the assay. The similar enhancement of LF activity for LF + PA63 and LF + LF mAb capture was surprising and deserves further exploration. Since both PA63 and the LF mAb bind to LF, the two may induce similar structural changes in the substrate binding site that enhance catalysis. In fact, an important component of this assay is the activity enhancement conferred with antibody capture. This feature improves detection of LF in this capture activity assay compared to capture by other LF antibodies targeted to different epitopes (data not shown).

Range of Detection for LF Activity. To determine the range of detection for LF activity, standards with LF spiked in human serum were prepared over a 5 order of magnitude concentration range, from 800 to 0.005 ng/mL. Three volumes, 5, 20, and 200 μL, of each standard were subjected to the 4-h LF assay; antibody capture of LF, a 2-h cleavage reaction, and MALDI-TOF MS analysis. The CT4/CT-ISTD area ratios for triplicate analyses were plotted versus the standard concentration, and because of the large concentration range analyzed, a $\log_{10}/\log_{10}$ conversion and sigmoidal curve fit was used to evaluate the results (Figure 4). The optimum ranges are approximately 2.5−400, 0.5−100, and 0.05−10 ng/mL for 5, 20, and 200 μL volumes, respectively. The estimated limits of detection (LODs) for each volume (5, 20, and 200 μL) are 2.5, 0.5, and 0.05 ng/mL, respectively, because the area ratios of standards below these fall within the asymptote of each curve (Figure 4). Data provided as Supporting Information confirm these LODs (Figure S1). It shows spectra focused on the

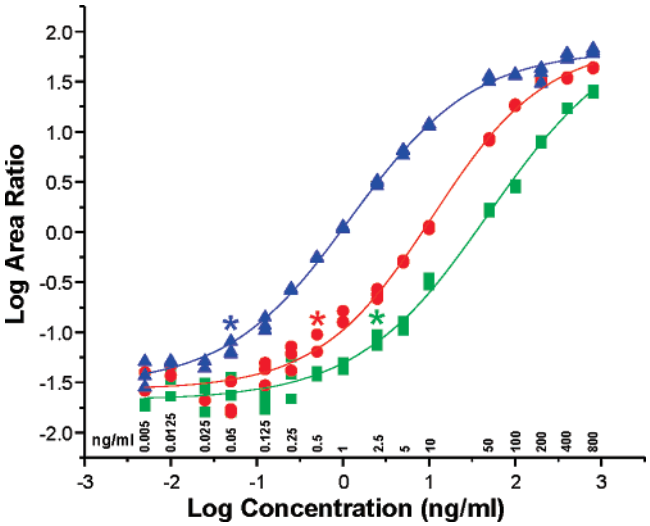

Figure 4. Range of detection and quantification of the LF activity assay in serum. Recombinant LF was spiked in pooled normal human serum from 800 to 0.005 ng/mL, and 5 (■), 20 (●), and 200 μL (▲) of spiked serum was subjected to antibody capture, 2-h substrate cleavage reaction with LF-4, and MALDI-TOF MS analysis with ISTD. The CT4/CT-ISTD area ratios were determined for triplicate spots, and the log concentration to log area ratio was plotted over the entire range for all three serum volumes. The actual concentrations for each $\log_{10}$ LF value plotted are given along the *X*-axis. The lowest standard above the lower asymptote of each curve is indicated with an asterisk (*). Curves were modeled with a Boltzmann equation with no weighting. R^2 values were 0.99 and higher.

C-terminal cleavage peptide peaks for the two lowest standards in each range, which have signal-to-noise ratios of 3:1−4:1 for the lowest LOD standard (S-1). In cases where adequate sample is available for analysis, the limit of detection of ≥0.05 ng/mL for the 200-μL volume is 400 times lower than the 20 ng/mL limit of detection reported for an LF ELISA.[30] Tenfold lower detection limits of 0.25, 0.05, and 0.005 ng/mL LF for 5, 20, and 200 μL, respectively, were achieved with 21-h reaction incubations of these standards, and volumes and will be described in future work (data not shown).

Quantification of LF in Experimental Inhalation Anthrax: Proof of Concept. The ability to quantify LF activity was tested using experimental serum from three rhesus macaques with pulmonary (inhalation) anthrax (IA). Eight samples available for analysis included preinfection (day 0) and day 2 postinfection sera for all three animals (animals A, B, and C), and serum from the day of demise, day 2 for animal A, day 3 for animal B, and day 4 for animal C. Volumes of 5 μL of each sample were analyzed along with 5-μL spiked macaque control serum to generate standards for LF with concentrations ranging from 10 to 400 ng/mL LF. Spectra for the lowest three standards from the 2-h cleavage reactions were focused on the CT4 and CT-ISTD peaks (mass spectra may be viewed as Supporting Information, Figure S-2). The CT peak intensity decreases with decreasing concentration of LF relative to the CT-ISTD and visually illustrates the capacity of MALDI-TOF MS to differentiate concentrations and to quantify by isotope dilution MS (Figure S-2). The lowest concentration used for this high-level analysis was 10 ng/mL, an equivalent of 50 pg

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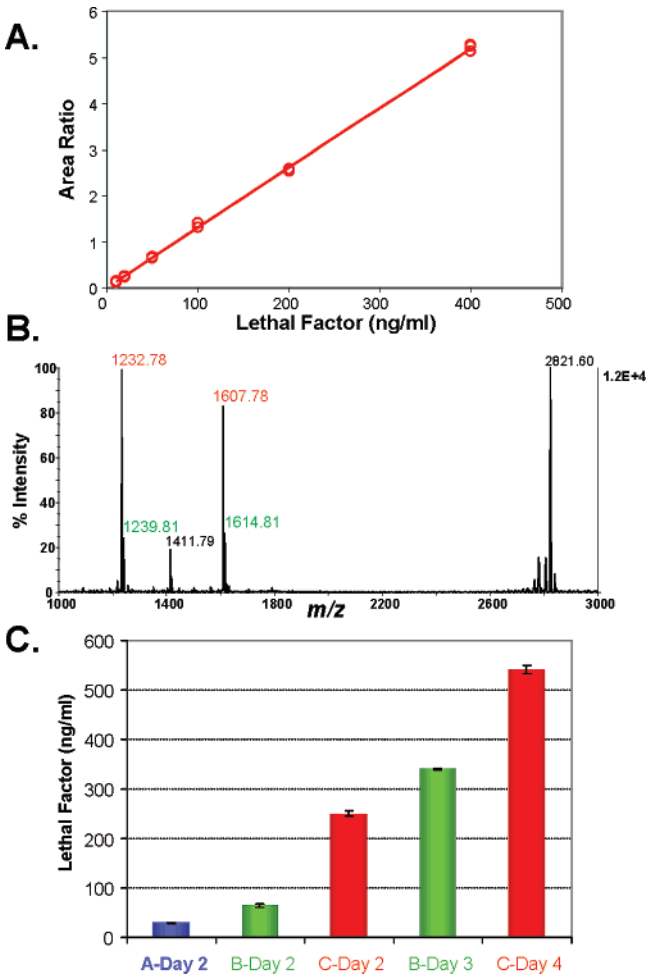

Figure 5. Detection and quantification of LF in rhesus macaque inhalation anthrax infection sera. Serum samples were analyzed for three macaques, A, B, and C, with inhalation anthrax and included samples prior to infection and on day 2 for all three animals, and on the day of demise, day 2 for animal A, day 3 for animal B, and day 4 for animal C. LF spiked serum from 10 to 400 pg/mL LF was analyzed (5 μL) to form a high range standard curve, along with 5 μL for each infection sample. All were captured by LF MABs, reacted with substrate for 2 h, and analyzed in triplicate by MALDI-TOF MS with ISTD. The standard curve, generated by plotting the CT product/CT-ISTD area ratios against the LF concentration, was fitted to a linear equation, with an R^2 of 0.9993 (A). A representative spectrum for animal C on day 2 of infection is shown (B). The LF concentrations calculated from the linear equation, for each triplicate MS analysis, were averaged and plotted with standard error bars (C).

of LF in 5 μL of serum. CT4/CT-ISTD area ratios were plotted versus concentration yielding a standard curve, which was linear in this range with an R^2 of 0.9993 (Figure 5A). Preinfection sera tested for all three animals using LF MABs showed no evidence of LF substrate hydrolysis (data not shown). LF activity was easily detectable in 5 μL for all postinfection samples. A representative spectrum for animal C on day 2 postinfection shows high-intensity product peaks and yields a calculated value of 250 ng/mL (Figure 5B). Representative spectra for animals A and B on day 2 (available as Supporting Information, Figure S-3), also show product peaks with high signal-to-noise ratio even at the lowest concentration of 30 ng/mL for animal A. LF activity levels were quantified from the linear equation and compared for all five postinfection sera (Figure 5C). LF levels on day 2 were variable and proportionally higher in each animal that survived longer, Animal C >B > A. As might be expected, levels were also higher later in infection for animals B and C. The analysis of LF for three animals with IA shows the utility of the method, and the results provide a preliminary evaluation of the range of LF expected in the acute phase (day 2) of the disease. The lowest day 2 LF value of 30 ng/mL is well above the faster 4-h total time to detection limit of 0.05 ng/mL for a 200-μL sample; clearly detection earlier in the disease course is feasible. The variable day 2 levels and time to death may simply reflect variations in animals or gender and may be clarified with larger studies that assess several variables including toxemia and bacteremia.

CONCLUSIONS

Inhalation anthrax in the United States is rare. Until the most recent case in February 2006,[17] the last human case of naturally acquired inhalation anthrax occurred in 1976, 30 years earlier.[2] However, the deliberate release of *B. anthracis* spores through the mail in 2001 illustrated the need for rapid diagnostics, methods to study the toxemia of infection, and therapies to combat toxicity. The highly specific and sensitive MS method for LF activity developed here could be used for all these purposes. The combination of LF-specific antibody capture, LF-specific peptide cleavage, and MS peptide detection combines three layers of specificity for an unambiguous diagnostic result for anthrax LF. We showed here that it confirmed the presence of *B. anthracis* LF in rhesus macaque serum as early as 2 days postinfection with results in less than 4 h. The automated purification and 96-well plate format provides a robust and high-throughput platform. Future work will include method validation and animal studies to track the appearance of toxemia in the earliest stages of infection and to correlate toxemia with clinical status as infection progresses. This method is the first of its kind to implement layers of specificity and to quantify low levels of LF in experimental infection. It provides a rapid anthrax diagnostic tool and has considerable potential for advancing our understanding of *B. anthracis* infection and toxemia.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 17, 2007. Accepted September 21, 2007.

AC701741S