Role of the N-terminal Amino Acid of *Bacillus anthracis* Lethal Factor in Lethal Toxin Cytotoxicity and Its Effect on the Lethal Toxin Neutralization Assay

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ABSTRACT

The cytotoxic activity of lethal factor (LF), a critical reagent used in the cell-based lethal toxin neutralization assay to assess anthrax vaccines, was shown to depend on the identity of its N-terminal amino acid which plays a role in the targeting of LF to the proteasome for degradation. These results demonstrate that care must be taken to ensure that LF preparations used in standardized cell-based assays are not altered at their N-terminal ends.
Because of the potential for *Bacillus anthracis* spores to be used as a bioweapon, new generation anthrax vaccines are currently being developed. Most new generation anthrax vaccines are based on protective antigen (PA) (4, 6), a protein produced by *B. anthracis* which combines with either lethal factor (LF) or edema factor (EF) to form lethal toxin (LT) or edema toxin (ET), respectively (9). Because LT and ET are important for virulence of *B. anthracis* (3), neutralization of these toxins by anti-PA antibodies would be expected to provide protection against the disease. In fact, LT neutralizing antibodies have been shown to correlate with protection in animal models (10, 12). Therefore, clinical immunogenicity of new anthrax vaccines will likely be evaluated using the LT neutralization antibody assay (TNA), an assay that measures antibody-mediated protection of mouse macrophage or macrophage-like cells such as J774A.1 cells from LT-induced cytotoxicity (8, 13). This cytotoxicity results from cleavage and inactivation of mitogen-activated protein kinase kinases (MEKs) by LF once it gains access to the interior of the cell (5, 16).

Efforts have been made to standardize the TNA assay reagents since availability of well-characterized and consistent reagents could improve interlaboratory comparability and facilitate comparison of TNA assay data generated in different laboratories. The most critical reagents are LF, PA, J774A.1 cells, and a reference serum. In order to expedite clinical evaluation of new generation anthrax vaccines, the NIH Biodefense and Emerging Infections Research Resources Repository (BEI Resources) acquired these reagents and made them available to those involved in
harmonization of the TNA assay and characterization of critical reagents used in the assay.

As part of this effort, we compared two recombinant LF lots made available to us through BEI Resources for evaluation in both a macrophage cytotoxicity assay and in the TNA assay. The two LF lots were identified as LF NR-142 and LF NR-724. Both lots were produced and characterized by a third party and then deposited into BEI Resources for distribution. When we evaluated LF NR-142 and LF NR-724 head-to-head, LF NR-142 was found to be significantly more cytotoxic than LF NR-724 when each was combined with PA. To assess cytotoxic potency of these lots, various concentrations of the two lots of LF were mixed with a fixed concentration of PA (50 ng/ml). The LF+PA mixture was then added to J774A.1 cells. After 4 hours, cell viability was measured by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (11). As seen in Figure 1A, significantly higher concentrations of LF NR-724 as compared to LF NR-142 were needed to kill J774A.1 cells. As shown in Figure 1B, this difference in activity had a significant effect on the output of the TNA assay conducted essentially as previously described (13). The neutralization curves generated with the less potent LF NR-724 are shifted to the right and exhibit considerably less depth than those generated using the more potent LF NR-142 indicating that less antibody is required for neutralization when LF that has a lower potency is used in the assay. Thus the TNA output is highly dependent on LF potency.

LF NR-724 was manufactured at the same facility as LF NR-142. The two LF lots were purified from the same recombinant strain of *B. anthracis*, however LF NR-724 was purified using a modified protocol that improved the homogeneity of the preparation.
As shown in Figure 1C, the vast majority of the protein of each of the LF lots migrated as a single protein band of $\approx 90,000$ Da when analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE), although LF NR-142 exhibited a few minor bands of molecular mass less than 50,000 Da and LF NR-724 exhibited a few very faint bands between 40,000 and 70,000 Da. While the $\approx 90,000$ Da band of each LF lot appears to be a single homogeneous species on SDS gels, more sensitive methods apparently could discern heterogeneity in this protein band for LF NR-142 in that the Certificate of Analysis accompanying the product indicates that when LF NR-142 was assessed by electrospray mass spectrometry (EMS), it was found to be composed of 5 components having molecular weights of 88,000 to 91,000 Da (expected molecular weight of LF = 90,496 Da). In contrast, LF NR-724 was reported in its Certificate of Analysis to be a homogeneous preparation comprised of a single species of 90,499 Da as assessed by EMS.

Thus, surprisingly, the more homogeneous preparation (LF NR-724) displays less cytotoxic activity than the more heterogeneous preparation (LF NR-142). We hypothesized that active, yet slightly truncated forms of LF might be generated by proteolysis at the N-terminal end of the protein since up to 23 amino acids can be lost at the N-terminal end of LF without affecting critical functions of that region of the protein (18). In order to determine whether heterogeneity existed at the N-terminal end of the $\approx 90,000$ Da band of the LF lots, we subjected each LF preparation to SDS-PAGE, transferred the $\approx 90,000$ Da band to a polyvinylidene difluoride membrane, and subjected the protein band to Edman degradation and N-terminal amino acid sequence analysis. While the N-terminal amino acid of native LF produced by *B. anthracis* is Ala (Figure
2C), the expected N-terminal amino acid of the recombinant form of LF NR-724 and LF NR-142 is His since an NdeI site encoding His-Met was added to the 5’-end of the lef gene to facilitate cloning (11). Analysis of the ≈90,000 Da band of LF NR-724 yielded primarily His in the first cycle, although a small amount of Ala was also detected. Subsequent cycles yielded Met followed by the expected amino acid sequence for native LF (Figure 2C). In contrast to our findings for LF NR-724, His as well as Ala, Asn, Val, Arg, and Ser were detected in approximately equal amounts in the first cycle analysis of the ≈90,000 Da band of LF NR-142 (data not shown). The presence of His as well as other amino acids in the first cycle suggests the existence of both full length and truncated forms of LF within the ≈90,000 Da band having different N-terminal amino acids.

The possibility exists that the cytotoxicities of LF forms truncated at the N-terminal end of the protein could vary depending on the identity of the N-terminal amino acid since the half-life of LF within the eukaryotic cell, and therefore the window of time in which LF would be available to cleave its target substrate, is believed to follow the N-end rule (17). The N-end rule describes a relationship between the N-terminal amino acid of a protein and the rate at which it is degraded within the cell, a relationship that exists because the N-terminal amino acid of a protein plays an important role in the targeting of the protein to the proteasome for degradation (2, 15). Destabilizing amino acids, i.e., those that impart short half-lives on proteins within the cell because they are recognized by the cell machinery for targeting to the proteasome, are usually positively charged amino acids such as Arg, Lys, and His or bulky hydrophobic residues. Several amino acid residues, including Gln, Glu, Asn, and Asp are thought of as secondary or
tertiary destabilizing amino acids since they are converted to a destabilizing form when they are modified in vivo by the appropriate cell machinery. The extent to which they are modified would be expected to dictate their destabilizing activity. In general, stabilizing N-terminal amino acids, *i.e.*, those that impart longer half-lives on proteins because they are not recognized for targeting to the proteasome, include Met, Val, and Gly. Other amino acids, such as Ala have been demonstrated to be either stabilizing or destabilizing depending in part on cell type, although Ala has been reported to be stabilizing in J774A.1 cells (2, 14, 15).

In order to test whether the N-terminal amino acid of LF affects the cytotoxic action of LT on J774A.1 cells, we produced several recombinant forms of LF that differed only in their N-terminal amino acid (Figure 2). We produced recombinant forms with stabilizing N-terminal amino acids (Met, Val, Ala) or destabilizing amino acids (His, Gln, Glu). LF expression plasmids dictating the synthesis of these LF derivatives were constructed as follows. Using PCR and standard cloning techniques, a modified *lef* gene was cloned into pET22b (Novagen). In this construct, the initiation codon of the *lef* gene was the ATG of the CATATG NdeI cloning site, and the 3' end of the gene was bounded by a NotI site. The sequence of the gene was altered, without changing the encoded amino acid sequence, to introduce an SpeI site at codons 29 and 30, and an AatII site at codons 39 and 40. The different expression constructs used in this study were then created by the subsequent addition of annealed complementary oligonucleotides to introduce, between the signal sequence and the mature LF coding sequence, an optimized (Ala-Gln-Ala) signal peptide cleavage site, a specified N-terminal amino acid, and six histidine residues. DNA sequencing was performed to verify the sequence.
For expression of different recombinant forms of LF, *E. coli* ER2566 (see Table 1 for a list of strains and plasmids used in this study) harboring individual plasmids described above was grown in LB broth overnight at 28°C. The overnight cultures were diluted in fresh LB medium (1:50) and expression of the recombinant proteins was induced by 1mM IPTG at OD\textsubscript{595} of 1.0 for 4 hrs. Cultures were harvested, cells were lysed, and each LF form was purified to homogeneity by Ni\textsuperscript{2+}-NTA chromatography (Qiagen, Inc.), anion exchange chromatography (Biosuite\textsuperscript{TM} Q 10 µm, 7.5 X 75 mm, Waters), and size exclusion chromatography (Biosuite\textsuperscript{TM} 250, 5 µm HR SEC, 7.8 X 300 mm, Waters). Two fold serial dilutions of the LF preparations were subjected to SDS-PAGE and were compared to known amounts of LF standard (LF NR-142) which was run simultaneously on the gel. Protein concentration was determined by densitometric analysis. N-terminal sequencing was performed to verify the identity of the N-terminal amino acid of each LF form. In all cases, the expected N-terminal amino acid was obtained (data not shown).

The cytotoxic activities of the different LF forms were examined using J774A.1 cells. As shown in Figure 3, the activities of the different LF forms followed the pattern predicted by the N-end rule with Met-LF, Val-LF, and Ala-LF being the most cytotoxic and His-LF and Glu-LF being the least cytotoxic. Gln-LF exhibited an intermediate cytotoxicity. Thus the N-terminal amino acid of LF appears to affect its cytotoxicity, likely due to altered stability of the protein as dictated by the N-end rule. These differences in cytotoxicity were reflected in the TNA assay also. Toxin neutralizing curves generated with a fixed concentration of the less potent His-LF are shifted to the
right and exhibit considerably less depth than those generated using the same
centration of the more potent Ala-LF (data not shown).

In order to verify that differences in activities observed with the various LF forms
were due to differential targeting of the individual LF forms to the proteasome for
degradation as dictated by their N-terminal amino acid, we examined the ability of His-
LF and Ala-LF to cleave the substrate MEK1 within J774A.1 cells in the presence and
absence of the proteasome inhibitor lactacystin. These LF species were chosen for study
because N-terminal His is a destabilizing amino acid that is recognized by the cell
machinery for targeting to the proteasome whereas N-terminal Ala, at least in J774A.1
cells, is a stabilizing amino acid that would serve as a poor recognition signal or no
recognition signal at all (2, 14, 15). J774A.1 cells were exposed to either Ni\(^{2+}\)-NTA
affinity-purified His-LF or Ala-LF (80 ng/ml) which had been mixed with PA (100
ng/ml) in the presence or absence of lactacystin. Exposure of cells to lactacystin included
a 1 hour pretreatment with the proteasome inhibitor before addition of the toxin
components. At various times, the cells were harvested and lysed. Equal amounts of cell
protein, as determined using Quant-iT protein kit (Molecular Probes) were then subjected
to SDS-PAGE followed by immunoblot analysis using a monoclonal antibody to the N-
terminus of MEK1, NT clone C12T (Upstate Biotechnology), to visualize cleavage of
MEK1 as manifested by loss of signal on the immunoblot, or using MEK1 (61B12)
mouse monoclonal antibody (Cell Signaling Technology) to visualize total MEK1
(cleaved + full length). As seen in Figure 4, in the absence of lactacystin, no significant
cleavage of MEK1 was observed until 120 minutes after exposure of the cells to His-LF
+ PA. In contrast, cleavage of MEK1 by Ala-LF + PA was seen as early as 60 minutes
with most of MEK1 being cleaved by 90 minutes. In the presence of lactacystin, cleavage of MEK1 by His-LF was accelerated, suggesting that inhibition of proteasomal degradation results in an apparent increase in activity of His-LF. Lactacystin had little effect on the rate of Ala-LF cleavage, as might be expected since Ala is a stabilizing amino acid in J774A.1 cells (14) and therefore would not be recognized or would be poorly recognized for targeting of LF to the proteasome. These results are consistent with our interpretation that the N-terminal amino acid of LF can affect the cytotoxicity of LF by dictating the stability of LF within the cell. We note that while the rate at which His-LF cleaved MEK1 was increased in the presence of lactacystin, this rate was still slightly slower than that observed for Ala-LF. A possible explanation for this small residual difference might be the inability of lactacystin to completely abolish degradation of LF by the proteasome. In this regard, lactacystin has previously been shown to inhibit, but not abolish, degradation of the N-terminal domain of LF by the proteasome in L6 cells (17). Alternatively, this small residual difference might be due to alterations in the structure of His-LF such that it is less able to perform one of the functions of LF, e.g., bind to PA, traverse the PA channel, or catalyze cleavage of MEK1.

These results illustrate the importance of ensuring that LF preparations to be used in standardized TNA assays are prepared in a manner such that the N-terminal amino acid is not altered. Proteolytic nicking of LF could occur during purification especially since \textit{B. anthracis} is known to produce a number of extracellular proteases (1). Such proteolytic nicking might be the genesis of the heterogeneity observed at the N-terminal end of LF NR-142. As shown in this study, LF preparations with ragged N-terminals, such as LF NR-142, can have significantly different activity than more intact forms of
LF. In addition, these results suggest that care should be taken when designing recombinant forms of the protein to be used in standardized TNA assays. The N-terminal sequence of LF has been altered to facilitate either cloning of the *lef* gene or purification of the protein (7, 11). Our results would predict that such changes could significantly alter the activity of the protein in cell-based assays such as the TNA.

In summary, the N-terminal amino acid of LF plays an important role in the activity of LF in cell-based assays. Therefore, when preparations of LF are produced that are to be used in standardized cell-based assays such as the TNA assay, care must be taken to ensure that the N-terminal amino acid is not altered.
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| Name     | Description                                                                                                                                                                                                 | Source or Reference |
|----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| ER2566   | *E. coli* host strain used for protein production from pET22b constructs                                                                                                                                    | New England Biolabs |
| pET22b   | Expression cloning vector, driven by T7 promoter                                                                                                                                                           | Novagen             |
| pSS4438  | pET22b with *lef* gene cloned *NdeI* – *NotI*, *SpeI* & *AatII* sites flanking w.t. VQG signal peptidase recognition site and Ala-His6 N-terminus                                                              | This study          |
| pSS4440  | pSS4438 substituted between *SpeI* and *AatII* sites with synthetic DNA to change signal peptidase recognition site to AQA (see Figure 2).                                                                 | This study          |
| pSS4442  | As for pSS4440 but with Met as the N-terminal residue                                                                                                                                                     | This study          |
| pSS4443  | As for pSS4440 but with His as the N-terminal residue                                                                                                                                                     | This study          |
| pSS4444  | As for pSS4440 but with Val as the N-terminal residue                                                                                                                                                     | This study          |
| pSS4464  | As for pSS4440 but with Gln as the N-terminal residue                                                                                                                                                      | This study          |
| pSS4465  | As for pSS4440 but with Glu as the N-terminal residue                                                                                                                                                      | This study          |
Figure Legends

Figure 1. Behavior of LF NR-142 and NR-724 in a cytotoxicity assay (A) or TNA assay (B). Panel A. PA (50 ng/ml) was combined with the indicated concentrations of LF NR-142 (●) or LF NR-724 (■). Cytotoxicity of the PA/LF mixture was assessed using J774A.1 cells as described in the text. Viability of the cells, normalized to untreated cells, is shown. Samples were analyzed in duplicate with ranges indicated by error bars. Results are representative of three independent experiments. Panel B. PA (50 ng/ml) was combined with either LF NR-142 (●) or LF NR-724 (■), each at a concentration of 40 ng/ml. The PA/LF mixture was added to the indicated dilutions of serum from a rabbit immunized with recombinant PA. Neutralization of the PA/LF mixture was assessed as described in the text. Similar results were obtained with additional rabbit serum samples. Panel C. 3 µg of LF NR-142 (lane 1) or LF NR-724 (lane 2) were subjected to SDS-PAGE and stained with Bio-Safe Coomassie stain (BioRad, Hercules, CA).

Figure 2. Generation of LF with different N-terminal residues. a) Schematic of LF N-terminal amino acid sequence encoded by, and key nucleotide sequences of, pSS4438, the immediate predecessor used to derive the individual expression constructs. Nucleotides altered to create SpeI and AatII restriction sites, without changing the encoded amino acid sequence, are shown in upper case. The site of signal peptidase cleavage is indicated by an arrowhead. Non-native amino acid residues are shown in gray text. b) N-terminal sequence of LF precursor protein encoded by plasmids
pSS4440, pSS4442, pSS4443, pSS4444, pSS4464, and pSS4465 in which the N-terminal amino acid, post signal-peptidase cleavage is Ala, Met, His, Val, Gln, and Glu, respectively, as denoted by “X”. In all cases the signal peptidase recognition site has been changed from VQG to AQA. c) N-terminal sequence of mature LF proteins. The six different N-terminal residues examined in this study are indicated by “X”. w.t. LF = wild-type LF.

Figure 3. Cytotoxicity of LF forms differing in N-terminal amino acid. Cytotoxicity of the indicated LF forms was determined by combining the indicated concentrations of LF with PA (50 ng/ml). Cytotoxicity was assessed using J774A.1 cells as described in the text. Viability of the cells, normalized to untreated cells, is shown. Samples were analyzed in duplicate with ranges indicated by error bars. Results are representative of three independent experiments.

Figure 4. Immunoblot analysis of MEK1 in cells treated with His-LF or Ala-LF. Equal amounts of protein from J774A.1 cells that had been treated with either His-LF or Ala-LF (80 ng/ml) + PA (100 ng/ml) for the indicated times in the presence or absence of lactacystin (20 µM) were subjected to electrophoresis followed by immunoblot analysis using a monoclonal antibody against the N-terminal peptide of MEK1 which is lost upon cleavage with LF (anti-MEK1-NT) or a monoclonal antibody that reacts with both cleaved and full-length MEK1 (anti-MEK1-Total) to visualize total MEK1. Results are representative of three independent experiments.
Figure 1
Figure 2

a: M [amino acids 2-27] I P L V Q G A H H H H H H G G H G D V G •••
cat atg ••••••••••• tag ccA ctA gta cag ggg gct cat cac cat cac cat cac ggc ggt gat ggt gac gtc ggt
Nde I Spe I

b: M [amino acids 2-27] I P L A Q A X H H H H H H G G H G D V G •••

X-LF: X H H H H H H G G H G D V G •••
LF NR-724: H M A G G H G D V G •••
w.t. LF: A G G H G D V G •••
Figure 4