NOTES

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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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 to form lethal toxin (LT) or edema toxin, respectively (9). Because LT and edema toxin 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 by LF once it gains access to the interior of the cell (5, 16).

Efforts have been made to standardize the TNA reagents since availability of well-characterized and consistent reagents could improve interlaboratory comparability and facilitate comparison of TNA 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 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 both in a macrophage cytotoxicity assay and in the TNA. 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 potencies 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 h, cell viability was measured by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as previously described (11). As seen in Fig. 1A, significantly higher concentrations of LF NR-724 than of LF NR-142 were needed to kill J774A.1 cells. As shown in Fig. 1B, this difference in activity had a significant effect on the output of the TNA, conducted essentially as described previously (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 Fig. 1C, the vast majority of the protein of each of the LF lots migrated as a single protein band when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), although LF NR-142 exhibited a few minor bands with molecular masses less than 50,000 Da and LF NR-724 exhibited a few very faint bands with masses between 40,000 and 70,000 Da. While the ≈90,000-Da band of each LF lot appears to be a single homogeneous species on SDS gels, more-sensitive methods appar-

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ently could discern heterogeneity in this protein band for LF NR-142 in that the Certificate of Analysis accompanying the product indicated that when LF NR-142 was assessed by electrospray mass spectrometry, it was found to be composed of five components having molecular masses of 88,000 to 91,000 Da (expected molecular mass of LF /H11005/ 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 electrospray mass spectrometry.

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 LF NR-142 band of the LF lots, we subjected each LF preparation to SDS-PAGE, transferred the 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 (Fig. 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 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 (Fig. 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 LF NR-142 (data not shown). The presence of His and other amino acids in the first cycle suggests the existence of both full-length and truncated forms of LF within the LF NR-142 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

FIG. 1. Behavior of LF NR-142 and NR-724 in a cytotoxicity assay (A) or TNA (B). (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 that of untreated cells, is shown. Samples were analyzed in duplicate with ranges indicated by error bars. Results are representative of three independent experiments. (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. (C) three µg of LF NR-142 (lane 1) or LF NR-724 (lane 2) was subjected to SDS-PAGE and stained with Bio-Safe Coomassie stain (Bio-Rad, Hercules, CA).
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 to 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 the 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 (Fig. 2). We produced recombinant forms with stabilizing N-terminal amino acids (Met, Val, and Ala) or destabilizing amino acids (His, Gln, and Glu). LF expression plasmids dictating the synthesis of these LF derivatives were constructed as follows. Using PCR and standard cloning techniques, a modified \textit{lef} gene was cloned into pET22b (Novagen). In this construct, the initiation codon of the \textit{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, \textit{Escherichia 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 1 mM isopropyl-\(\beta\)-thiogalactopyranoside at an optical density at
and each LF form was purified to homogeneity by Ni^{2+}-2-nitrotriacetic acid chromatography (Qiagen, Inc.), anion exchange chromatography (Biosuite Q, 10 μm, 7.5 by 75 mm; Waters), and size exclusion chromatography (Biosuite 250, 5-μm high-resolution size exclusion chromatography column, 7.8 by 300 mm; Waters). Twofold 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 concentrations were 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 Fig. 3, the activities of the different LF forms followed the pattern predicted by the N-terminus, 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-terminus rule. These differences in cytotoxicity were reflected in the TNA also. Toxin neutralizing curves generated with a fixed concentration of the more-potent 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-h pretreatment with the proteosome 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 a MEK1 (61B12) mouse monoclonal antibody (Cell Signaling Technology) to visualize total MEK1 (cleaved plus full length). As seen in Fig. 4, in the absence of lactacystin, no significant cleavage of MEK1 was observed until 120 min after exposure of the cells to His-LF plus PA. In contrast, cleavage of MEK1 by Ala-LF plus PA was seen at as early as 60 min, with most of MEK1 being cleaved by 90 min. In the presence of lactacystin, cleavage of MEK1 by His-LF was accelerated, suggesting that inhibition of proteosomal degradation results in an apparent increase in the 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., binding to PA, traversing the PA channel, or catalyzing cleavage of MEK1.

These results illustrate the importance of ensuring that LF preparations to be used in standardized TNAs 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 *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-terminal ends, 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 TNAs. 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, care must be taken to ensure that the N-terminal amino acid is not altered.

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**REFERENCES**


