Characterization of the Native Form of Anthrax Lethal Factor for Use in the Toxin Neutralization Assay

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The cell-based anthrax toxin neutralization assay (TNA) is used to determine functional antibody titers of sera from animals and humans immunized with anthrax vaccines. The anthrax lethal toxin is a critical reagent of the TNA composed of protective antigen (PA) and lethal factor (LF), which are neutralization targets of serum antibodies. Cytotoxic potency of recombinant LF (rLF) lots can vary substantially, causing a challenge in producing a renewable supply of this reagent for validated TNAs. To address this issue, we characterized a more potent rLF variant (rLF-A) with the exact native LF amino acid sequence that lacks the additional N-terminal histidine and methionine residues present on the commonly used form of rLF (rLF-HMA) as a consequence of the expression vector. rLF-A can be used at 4 to 6 ng/ml (in contrast to 40 ng/ml rLF-HMA) with 50 ng/ml recombinant PA (rPA) to achieve 95 to 99% cytotoxicity. In the presence of 50 ng/ml rPA, both rLF-A and rLF-HMA allowed for similar potencies (50% effective dilution) among immune sera in the TNA. rPA, but not LF, was the dominant factor in determining potency of serum samples containing anti-PA antibodies only or an excess of anti-PA relative to anti-rLF antibodies. Such anti-PA content is reflected in immune sera derived from most anthrax vaccines in development. These results support that 7- to 10-fold less rLF-A can be used in place of rLF-HMA without changing TNA serum dilution curve parameters, thus extending the use of a single rLF lot and a consistent, renewable supply.

Anthrax is caused by contact with Bacillus anthracis spores (via inhalation, ingestion, cutaneous, and injection routes) that germinate and produce a tripartite exotoxin that is the predominant virulence factor of disease (reviewed in reference 1). The toxin consists of a binding moiety, protective antigen (PA), which forms complexes with either lethal factor (LF), to form lethal toxin (LT), or edema factor (EF), to form edema toxin. The mechanism of action of these anthrax toxins is that PA forms a heptamer pore that enables LF, a zinc metalloproteinase, or EF, a calmodulin-dependent adenylate cyclase, to translocate to the cytoplasm (2). In most cell types, the protease activity of LF targets the N terminus of several mitogen-activated protein kinase kinases, thereby disrupting several cellular functions, leading to different types and degrees of toxicity that are cell type dependent (2). An additional cytotoxic mechanism exists in some, but not all, macrophage types (the cell type most sensitive to LT-mediated lysis) in which LF directly activates the inflammasome-activating molecule, Nlrp1b, leading to caspase-1 activation (3, 4).

Medical countermeasures against anthrax include the Food and Drug Administration-licensed BioThrax (anthrax vaccine adsorbed), also referred to asAVA (http://www.cdc.gov/phpr/stockpile/stockpile.htm), which is indicated for active immunization for the prevention of anthrax disease. Investigational anthrax vaccines, including recombinant protein-based vaccines and those with additional adjuvants, are under development (5, 6). Serum titers of anthrax LT-neutralizing antibodies following vaccination have been evaluated in animal models of anthrax disease and in human clinical immunogenicity studies (7–10). While antibodies to all toxin components can be detected via an enzyme-linked immunosorbent assay (ELISA) after vaccination with AVA, serum titers of antibodies to PA are most prevalent and appear to be responsible mainly for LT-neutralizing activity in the anthrax toxin neutralization assay (TNA) (11–14). In fact, most reported TNAs were designed to emphasize this anti-PA antibody neutralizing contribution, which is the best-accepted correlate of immune protection for certain anthrax vaccine formulations (15–17). This is primarily because PA is considered the principal antigen in licensed vaccines and those in development (5–7). Indeed, TNA responses have the potential to be species neutral (10), which is an advantage relative to quantitation via antibody binding assays.

The TNA consists of a monolayer of LT-sensitive cell lines, the most widely used being the murine macrophage cell lines J774A and RAW264, to which optimal concentrations of recombinant PA (rPA) and LF (rLF) are added in the presence of serially diluted immune sera. After a 4-h incubation, the degree of cytotoxicity is measured via a redox viability dye, which allows for a 50% effective dilution (ED50) potency value to be obtained from the serum titration curve (18, 19). The specific rPA and rLF concentrations of 50 and 40 ng/ml, respectively, have been used in most reported TNA validation and immunogenicity studies of animal and human vaccinations (15, 18–24). Although potency among rPA production lots is consistent (our unpublished observation), the potency of rLF is known to vary substantially among lots. This creates a challenge in producing a renewable supply of this critical reagent for validated TNAs; i.e., consistent production of rLF lots of specific potency required for use at 40 ng/ml is difficult (25).

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TABLE 1 Preparations and sources of anthrax lethal toxin components recombinant protective antigen (rPA) and lethal factor (rLF)

| Reagent | Source (lot no.) | Expression vector | % of N-terminal amino acid sequence |
|---------|-----------------|-------------------|-----------------------------------|
| rPA     | Emergent (02-PUR-10-002) | pBP103 | EVKQE... (95) |
| rLF-HMA<sub>C</sub> | Emergent (03-PUR-10-001) | pSI115 | HMAGG... (95) |
| rLF-HMA| List Biologicals (1722BB) | pSI115 | HMAGG... (21) |
| rLF-A   | Emergent (08-PUR-12-001) | pSI115b | ...AGG... (95) |
| rLF-d   | Emergent (011311) | pSI115 | ...AGG... (50) |

<sup>a</sup> rLF-A, native amino acid sequence; rLF-HMA, additional N-terminal His and Met residues; rLF-d, rLF-HMAE digested with N-terminal dipeptidase.

<sup>b</sup> Emergent BioSolutions, Inc. (Gaithersburg, MD), and List Biological Laboratories, Inc. (Campbell, CA).

<sup>c</sup> Protein N-terminal integrity was assessed via Edman degradation sequencing expressed as the percentage of the expected N-terminal amino acid sequence in each preparation.

A total of 4 production lots of rLF-A showed similar results, and at least 7 production lots of rLF-HMAE showed similar results (all lots showed acceptable purity of ≥95% as assessed by N-terminal sequencing).

Materials and Methods

Production and purification of rPA and rLF preparations. The anthrax LT components rPA, rLF-A, rLF-HMA<sub>C</sub> (rLF containing additional N-terminal His and Met residues), and rLF-d (rLF-HMAE N-termially digested with diaminopropaeptide) were produced at Emergent BioSolutions, Inc. (Gaithersburg, MD) (<table 1>). Both of the vectors expressing rLF-A and rLF-HMA were licensed from the National Institutes of Health (Bethesda, MD). Another lot of rLF-HMA, rLF-HMA<sub>E</sub>, was produced by List Biological Laboratories, Inc. (Campbell, CA) (<table 1>). The fermentation process of rPA, rLF-A, and rLF-HMAE was based on a method described elsewhere (26), in which the fermentation product was harvested by centrifugation at 2 to 8°C and the supernatant was concentrated using tangential-flow filtration. The purification process was performed at 2 to 8°C and consisted of a hydrophobic interaction capture column, an ion-exchange purification column, and a final hydrophilic polishing column. All purification columns were operated using standard linear salt gradients per the manufacturer’s recommendations. Minor modifications to linear salt gradients were used for rLF purification columns.

Generation of rLF-d was performed by subjecting rLF-HMAE to N-terminal dipeptidase activity of dipeptidylaminopeptidase I (DAPase I; Qiagen, Valencia, CA) for 1.5 h at 37°C in 10 mM Tris, 1 mM EDTA, pH 8.0. DAPase I was removed by Ni-agarose resin (Qiagen) per the manufacturer’s recommendations.

SDS-PAGE. Integrity and purity of rLF were analyzed via SDS-PAGE using precast NuPAGE 4 to 12% Bis-Tris gels (Invitrogen, Grand Island, NY). rLF samples were prepared with a final concentration of 1× NuPAGE LDS sample buffer (Invitrogen) and 2-mercaptoethanol (2.5%) in which 1 μg of protein was loaded on the gel. Electrophoresis was performed for 35 min with a constant voltage of 200 V. Gels were stained with microwave blue stain (Protiga, Frederick, MD) and destained with water. Gel images were captured and analyzed by a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA).

Western blotting. Western blotting was performed with rLF samples separated by SDS-PAGE (as described above) with 0.1 μg of protein. The gel was transferred to an immunoblot polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and a polyclonal anti-rLF mouse serum pool was used to tag the transferred LF protein on the membrane. The tagged LF protein was detected with an alkaline phosphatase-labeled anti-mouse IgG conjugate (KPL, Gaithersburg, MD). The anti-rLF sera were pooled from female CD-1 mice that received two 0.5-ml intraperitoneal (i.p.) injections of 100 μg rLF-A with 750 μg Alhydrogel (Alhydrogel 2%; Brenntag Biosector, Frederikskund, Denmark) and 100 μg CPG 7909 on days 0 and 14. Sera were obtained on day 28. Immunoreactive positive bands were visualized with addition of 5-bromo-4-chloro-3-indolylphosphosphate–nitroblue tetrazolium (BCIP/NBT) substrate (KPL), and Western blot images were captured via a GS-800 calibrated densitometer.

Protein N-terminal sequencing. N-terminal sequences of rPA and rLF proteins were determined with the Edman degradation method (Midwest Analytical Inc., St. Louis, MO). Approximately 35 μg of each protein was transferred to a ProSorb PVDF cartridge (Applied Biosystems, Foster City, CA) and sequenced on a model 477 protein sequencer (Applied Biosystems). Phenylthiohydantoin (PTH) amino acids were separated on a Perkin-Elmer Sphero-5 ODS column (2.1 by 220 mm), and their relative amounts were used to determine percentages of each protein present in a mixture.

Generation of immune serum samples from anthrax vaccines. Animal studies were conducted in compliance with the Animal Welfare Act and followed the principles of the Guide for the Care and Use of Laboratory Animals from the National Research Council (NRC). These animal procedures were approved by Institutional Animal Care and Use Committee (IACUC). Immune sera were obtained via immunization of different animal species with various anthrax vaccines, in which all species received either one or two 0.5 ml intramuscular (i.m.) or i.p. injections of various dilutions (1/2.5 to 1/20 in normal saline) of AVA, AVA with 250 μg CPG 7909 (Girindus AG, Cincinnati, Ohio) (28), or 75 μg rPA (Emergent BioSolutions Inc.) containing 750 μg Alhydrogel. Sera were obtained 3 to 74 weeks after a single injection or 2 weeks after a second injection. Nonhuman primates (NHP) were challenged with virulent <i>B. anthracis</i> spores (Ames strain) 70 days after two immunizations on days 0 and 28. NHP sera were collected 2 months after challenge (<table 2>). The following are characteristics of animals used for immunization: female CD-1 mice 5 to 8 weeks of age (Charles River, Raleigh, NC), female New Zealand White rabbits (Spring Valley Laboratories, Inc., Woodbine, MD), and male and female cynomolgus macaques (1.5 to 2.6 kg of body weight; Vietnamese origin; Covance Research Products, Alice, TX). Human subjects were described previously (28). Aliquots of all sera were stored at −20°C and thawed immediately prior to use.

MLA. The J774A.1 mouse macrophage cell line (ATCC; TIB-67, Manassas, VA) was cultured in 75-cm² flasks in Dulbecco’s modified Eagle’s medium with high glucose (DMEM; Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM l-glutamine, 1 mM sodium pyruvate, 0.11 mM sodium bicarbonate, 50 μM of penicillin G, and 50 μg/ml of streptomycin sulfate (complete DMEM). Endotoxin was not detected in the reagents.
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and media described above. Cells were harvested with a sterile plastic scraper and suspended in complete DMEM at a density of 3 × 10^5 viable cells/ml (assessed via trypan blue exclusion assay); 3 × 10^5 cells were seeded in 100 μl per well of polystyrene 96-well round-bottom tissue culture plates (BD, Franklin, NJ) and incubated for 16 to 24 h at 37°C, 5% CO₂. Culture medium was discarded before incubation with rPA and rLF. The macrophage lysis assay (MLA) using different concentrations of rPA and rLF was performed by diluting reagents in appropriate volumes of complete DMEM. One hundred μl of toxin mixture was transferred to appropriate wells containing adherent cells and incubated for 4 h at 37°C, 5% CO₂. Cell viability was assessed via the addition of 25 μl per well of the redox viability dye, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml in phosphate-buffered saline [PBS]; Sigma), for 2 h at 37°C and 5% CO₂ that was dissolved with addition of 100 μl of solubilization buffer and overnight incubation at 37°C (20% [w/vol] SDS [Sigma] dissolved in 50% N,N-dimethyl formamide [Sigma] in deionized water with the pH adjusted to 4.7 using 1 M HCl). Absorbance was measured at 570 and 690 nm, and the 690-nm optical density (OD) values were subtracted from the 570-nm values using a calibrated Molecular Devices Versamax plate reader with SoftMax Pro software (version 5.4.1; Sunnyvale, CA). Cells in the negative-control wells containing only complete DMEM without toxin showed maximal cell viability (0% cytotoxicity), whereas cells cultured with the maximal concentrations of rPA and rLF demonstrated complete (100%) cytotoxicity. The percent cytotoxicity of different combinations of rPA and rLF was calculated as (OD sample − OD negative control)/OD negative control × 100%. The cytotoxic potency of rLF (50% effective concentration [EC_{50}] ) was calculated via the sigmoidal dose-response variable slope fit analysis using log_{10}(rLF) (GraphPad Prism 5.04 software; GraphPad Software, Inc., La Jolla, CA).

TNA. The TNA was performed as previously described (18, 19). Briefly, the J774A.1 mouse macrophage cell line was cultured and seeded in 96-well round-bottom plates as described for the MLA. Test and reference sera were diluted in a 2-fold serial manner in complete DMEM in a fresh 96-well flat-bottomed dilution plate yielding a total of 7 dilutions. LT was prepared at specified concentrations using the appropriate lots of rPA and rLF by diluting reagent stocks in appropriate volumes of complete DMEM. Equal volumes of the diluted LT preparation were added to serial dilutions of sera, and the mixture was incubated for 60 min, at which time 100 μl of the LT and serum mixtures were transferred to appropriate wells containing adherent cells. Culture medium was discarded before addition of LT and serum mixtures. Control wells containing diluted serum in complete DMEM without LT (no toxin controls) and naive mouse sera with LT (negative control) were included. Cells were incubated for an additional 4 h at 37°C with 5% CO₂. Cell viability was assessed via MTT reduction assay as described above.

The neutralizing potency (i.e., ED_{50}) of immune sera was calculated via a 4-parameter logistic (4PL) fit curve (SoftMax Pro 5.4.1 software) as the reciprocal of the dilution at the curve inflection point representing 50% LT neutralization. In some instances in which samples of low neutralizing activity did not achieve complete neutralization, the upper and lower asymptotes of the test sample neutralization curves were constrained to the upper and the lower asymptotes of the reference serum curve so that accurate ED_{50} values could be determined. ED_{50} values of test sera were reported if the respective positive-control, negative-control, and reference serum samples passed all plate acceptance criteria. Plate acceptance criteria included the following: a reference serum curve coefficient of determination (R^2) of >0.985, upper asymptote OD value of >0.70, a difference in OD values of the wells containing the lowest and highest serum dilutions of >0.55 OD units, two negative-control OD values of ≤ 20% of the coefficient of variance, 6th and 7th serial dilution OD values of the positive-control and reference serum samples were within 0.25 OD units of the negative-control average, the positive-control curve upper asymptote was within 0.297 OD units of that for the reference serum, and positive-control and reference serum OD values were monotonic between the 3rd, 4th, and 5th serial dilutions. Test serum ED_{50} values were reportable if the dilution curve showed an R^2 of >0.969 and ED_{50} of >30, if OD values were monotonic among 3rd, 4th, and 5th serial dilutions, if the OD value of the lowest possible dilution was >0.601 (limit of detection [LOD]), if the test sera full-curve upper asymptote was within 0.297 OD units of that for the reference serum, and if the curve depth was >0.661 OD units. The 50% neutralization factor (NF_{50}) is the quotient of the ED_{50} of a test sample serum and the ED_{50} of reference sera.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software, Inc.) as well as SAS version 9.2 or JMP version 9.0 (SAS Institute, Inc., Cary, NC). For comparisons between two groups, the unpaired, two-tailed Student’s t test was used. When necessary, Welch’s correction for unequal variance was applied. For comparisons between three or more groups, either analysis of variance (ANOVA) (parametric) or Kruskal-Wallis ANOVA (nonparametric) was used depending on the distribution of the data set. For comparisons to control groups within the multiple groups, Dunnett’s (ANOVA) multiple-comparison posttest was used. For comparisons between all groups within the multiple groups, Dunn’s (Kruskal-Wallis) multiple-comparison posttest was used. The two one-sided t test (TOST) was used to determine equivalence between TNA curve parameters generated from rLF-HMA2 or rLF-A used in the TNA. Equivalence bounds were selected for each curve parameter using 20% of the respective mean values for all samples tested under the standard (rLF-HMA2) conditions. For all analyses described above, the level of statistical significance was established as α = 0.05. Lin’s concordance coefficient was used to assess the degree of agreement in ED_{50} and NF_{50} values of the test samples measured in the TNA using various LT conditions, and Deming linear regression was used to obtain the equivalence line slope and intercept estimates (see Fig. 4 to 6).

### Table 2: Immune sera generated from different species and anthrax vaccines

| Species | Total no. of samples | rPA + Alhydrogel | AVA | AVA + CPG 7909 | No. of immunizations | Injection route |
|---------|----------------------|------------------|-----|----------------|----------------------|----------------|
| NHP     | 15                   | 0                | 15  | 0              | 2                   | i.m.           |
| Rabbit  | 45                   | 0                | 8   | 37             | 1 or 2              | i.m.           |
| Human   | 12                   | 0                | 12  | 2              | 2                    | i.m.           |
| Mouse   | 32                   | 1                | 12  | 1              |                      | i.p.           |

a Serum samples were obtained from nonhuman primates (NHP), rabbits, humans, and mice that were immunized with one or two injections of vaccine via intraperitoneal (i.p.) or intramuscular (i.m.) injections.

b NHP subjects were exposed to a lethal aerosolized dose of *Bacillus anthracis* spores after the second injection.

c Fifteen samples were derived from one immunization and 30 from two immunizations (7 AVA and 23 AVA/CPG 7909 sera were derived from animals that received two immunizations).
RESULTS

Biochemical characterization of rLF lots. Most commercially available sources of rLF (e.g., List Biological Laboratories) contain additional N-terminal His and Met residues (rLF-HMA), which are by-products of the expression vector system (26) and cause a reduction in cytotoxic potency (25, 27). By measuring the proportion of N-terminal sequences via Edman degradation, we showed that rLF-HMA material produced by Emergent BioSolutions (rLF-HMAE) contained ≥95% of the expected N-terminal sequence. The same protein produced by List Biological Laboratories, Inc. (rLF-HMAL), showed 21% of this expected sequence (Table 1), demonstrating substantial N-terminal degradation. Presumably, this degradation most likely is attributed to differences in trace levels of endogenous protease activity during the production and purification processes. This possibility was supported by a brief (90-min) digestion of rLF-HMAE with the dipeptidase, DAPase I, resulting in the removal of N-terminal His and Met residues that yielded two rLF digestion products (rLF-d) that varied in length in the first two or four amino acids (Table 1). Such rLF-d material showed an expected profile similar to that of rLF-A by SDS-PAGE and Western blotting (Fig. 1). Note that the rLF-A preparation showed a highly pure profile of the expected sequence and material (Table 1 and Fig. 1). In addition, differences in N-terminal sequence content between rLF-HMA preparations correlated with expected degradation profiles in SDS-PAGE and Western blot analyses (i.e., rLF-HMAE yielded a single band demonstrating a high degree of purity that is consistent with N-terminal sequencing results, whereas that of rLF-HMAL yielded multiple bands that were consistent with degradation). Note that the SDS-PAGE and Western blotting bands do not resolve differences among the relatively pure materials that differed by 1 or 2 N-terminal amino acid residues (i.e., rLF-A, rLF-d, and rLF-HMAE) (Fig. 1).

Association of cytotoxic potency with N-terminal residue content of different rLF preparations. Cytotoxic potency of dif-
further rLF expression variants was evaluated using MLA. Different concentrations of rLF-A, rLF-d, rLF-HMAE, and rLF-HMAE were combined with different concentrations of a single rPA lot, and the resulting mixtures were added to monolayers of the LT-sensitive macrophage cell line, J774A.1, for assessment of cytotoxicity. Concentration-response curves of rLF cytotoxicity in the MLA demonstrated that different threshold concentrations of rPA and the respective rLF preparation were necessary to achieve complete cytotoxicity (maximum lower plateau of curve) (Fig. 2). In addition, increased potencies of rLF preparations correlated with increased rPA concentration, such that rLF concentration-response curves shifted to the left (more potent) in the presence of rPA concentrations as low as 15 ng/ml, whereas the less potent rLF-HMAE and rLF-HMAE materials allowed for a significantly lower effective concentration of rPA in combination with different rLF materials showed a pattern of reagent concentrations that achieved 95 to 99% cytotoxicity in which increases in rPA concentrations required concomitant decreases in rLF concentrations (Table 4). In addition, lower effective concentrations of rPA were associated with more potent rLF preparations. For example, the potent rLF-A and rLF-d materials allowed for 95 to 99% cytotoxic activity in the presence of rPA concentrations as low as 15 ng/ml, whereas the less potent rLF-HMAE and rLF-HMAE materials allowed for a similar degree of cytotoxicity, with rPA concentrations only as low as 50 and 100 ng/ml, respectively (Table 4).

rLF-A and rLF-HMAE effects on serum dilution curve parameters in the TNA. We identified the optimal concentration of rLF-A that showed cytotoxic activity most similar to that of 40 ng/ml rLF-HMAE by testing a range of rLF-A concentrations that passed the 95 to 99% cytotoxicity criteria in the presence of 50 ng/ml rPA. rLF-A concentrations of 5 and 6 ng/ml showed similar

tocytotoxic potency (25), and that increased potency of the N-terminally degraged rLF-HMAE material relative to that of the highly pure, nondegraded rLF-HMAE is consistent with the N-terminus sequence integrity of rLF has a substantial effect on

cytotoxicity (maximum lower plateau of curve) (Fig. 2). In addition, increased potencies of rLF preparations correlated with increased rPA concentration, such that rLF concentration-response curves shifted to the left (more potent) in the presence of rPA concentrations as low as 15 ng/ml, whereas the less potent rLF-HMAE and rLF-HMAE materials allowed for a significantly lower effective concentration of rPA in combination with different rLF materials showed a pattern of reagent concentrations that achieved 95 to 99% cytotoxicity in which increases in rPA concentrations required concomitant decreases in rLF concentrations (Table 4). In addition, lower effective concentrations of rPA were associated with more potent rLF preparations. For example, the potent rLF-A and rLF-d materials allowed for 95 to 99% cytotoxic activity in the presence of rPA concentrations as low as 15 ng/ml, whereas the less potent rLF-HMAE and rLF-HMAE materials allowed for a similar degree of cytotoxicity, with rPA concentrations only as low as 50 and 100 ng/ml, respectively (Table 4).

TABLE 4 Inverse relationship of rPA and rLF concentrations achieving 95 to 99% cytotoxicity in the MLAa

| rLF preparationb | rLF concn (ng/ml) causing 95 to 99% MLA cytotoxicity at the indicated rPA concn (ng/ml)5: | 10 | 15 | 20 | 30 | 50 | 80 | 100 | 120 |
|-----------------|---------------------------------------------------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| rLF-A           | ND                                                                              | 3   | 3   | 3   | 3   | 4–6 | 8   | 12–16 | 24–64 |
| rLF-HMAEc       | 64                                                                              | 64–80 | 80–112 | NA  | NA  | NA  | NA  | NA  | NA  |
| rLF-HMAEb       | ND                                                                              | 16  | 16  | 24  | 32–40 | NA  | NA  | NA  | NA  |
| rLF-d           | ND                                                                              | ND  | ND  | ND  | 6–8 | 10  | 13–16 | 24  | NA  |

a Evaluation of different rLF concentrations in combination with those of rPA demonstrated unique rPA and rLF combinations that achieved optimal cytotoxicity between 95 and 99% for use in the anthrax toxin neutralization assay (TNA).
b rLF-A, native LF sequence. rLF-HMAE and rLF-HMAE, containing additional N-terminal Met and His residues, were produced by Emergent BioSolutions and List Biological Laboratories, respectively. rLF-d was derived from rLF-HMAE digested with N-terminal dipeptidase, which removed 2 or 4 N-terminal residues.
c ND, not determined; NA, not applicable (rLF concentration did not induce at least 95% cytotoxicity).
cytotoxicity (i.e., cell viability and OD) relative to rLF-HMAL, whereas 4 ng/ml rLF-A showed significantly ($P < 0.05$) lower cytotoxicity than rLF-HMAL (Fig. 3A). This cytotoxicity profile correlated with the profile of serum ED$_{50}$ values, in that ED$_{50}$ values of two mouse immune serum samples (derived from pooled sera of mice that received a single i.p. injection of AVA plus CPG 7909) obtained in the presence of 4 ng/ml rLF-A were greater than that of rLF-HMAL, whereas those obtained in the presence of 5 and 6 ng/ml rLF-A were similar to those obtained with rLF-HMAL (Fig. 3B and C). While both 5 and 6 ng/ml rLF-A yielded similar cytotoxicity results, the lower concentration of 5 ng/ml was selected to ensure that potential excessive cytotoxicity would be avoided. These two immune serum samples were further evaluated in three independent TNA experiments, each with 4 (lot MS122010) or 5 (lot MS011211) replicate measurements, in the presence of 50 ng/ml rPA with either 5 ng/ml rLF-A or 40 ng/ml rLF-HMAL. The four parameters, A, B, C, and D (upper asymptote, slope, ED$_{50}$, and lower asymptote, respectively), of serum dilution 4PL fit curves generated with 5 ng/ml rLF-A and 40 ng/ml rLF-HMAL were similar (Table 5).

The selection of 5 ng/ml rLF-A was confirmed by evaluation of a panel of immune sera obtained from mice immunized with different anthrax vaccines (Table 2) in the TNA using 50 ng/ml rPA in combination with rLF-HMAL (40 ng/ml) or three concentrations of rLF-A (4, 5, and 6 ng/ml). As previously described for Fig. 3A, 4 ng/ml rLF-A showed significantly less cytotoxicity, while 5 and 6 ng/ml rLF-A showed cytotoxicity similar to that of rLF-HMAL (40 ng/ml). Such modest but significant differences in the degree of cytotoxicity among rLF-A concentrations correlated with modest shifts in serum ED$_{50}$ values; i.e., 4 ng/ml rLF-A resulted in slightly greater serum ED$_{50}$ values, while 5 ng/ml rLF-A resulted in serum ED$_{50}$ values most similar to those obtained with 40 ng/ml rLF-HMAL (Fig. 4).

Although 5 ng/ml rLF-A was identified as the optimal concentration for TNA serum potency comparisons to the standard conditions with 40 ng/ml rLF-HMAL, all three concentrations of rLF-A, 4, 5, and 6 ng/ml, qualified for use in the TNA, because (i) the three concentrations passed the 95 to 99% MLA cytotoxicity criteria using 50 ng/ml rPA and (ii) differences in cytotoxicity and serum ED$_{50}$ values among these concentrations (notably, 4 ng/ml rLF-A) were relatively small (Fig. 3 and 4). In fact, normalization of each test sample ED$_{50}$ value to an NF$_{50}$ value using the serum reference ED$_{50}$ as the denominator predictably led to normalization of the differences between 4 ng/ml rLF-A and 40 ng/ml rLF-HMAL (Fig. 4D). Moreover, the TNA performed with immune sera from mice, humans, NHP, and rabbits vaccinated with different anthrax vaccines showed that potency values (ED$_{50}$ and NF$_{50}$) obtained with 5 ng/ml rLF-A were similar to those obtained with 40 ng/ml rLF-HMAL.

![FIG 3](http://cvi.asm.org/)

**FIG 3** Identification of optimal rLF-A concentration relative to rLF-HMAL at 40 ng/ml for comparison of TNA ED$_{50}$ values of immune sera. Two lots of pooled sera from mice immunized with AVA plus CPG 7909 (Table 5 describes the serum samples) were evaluated in the TNA using 50 ng/ml rPA in the presence of rLF-HMAL at 40 ng/ml or rLF-A at 4, 5, or 6 ng/ml. The lowest rLF-A concentration that yielded cytotoxicity and an ED$_{50}$ most similar to that of rLF-HMAL was considered optimal (i.e., 5 ng/ml). Each serum lot was evaluated with 13 replicate measurements, and the means ± standard deviations (SD) are reported. *, OD (A) and ED$_{50}$ (B and C) values obtained with 4 ng/ml rLF-A were significantly different from those obtained with rLF-HMAL ($P < 0.05$ by Student’s t test with Dunn’s multiple-comparison posttest).

### TABLE 5

| Serum sample* | rLF preparation ([rPA]/[rLF], both in ng/ml) | Mean (± SD) P | Mean (± SD) P | Mean (± SD) P | Mean (± SD) P |
|---------------|------------------------------------------|---------------|---------------|---------------|---------------|
| MS011211      | rLF-A (50/5)                             | 1.88 (0.10)   | 0.45          | 2.27 (0.14)   | 0.10          | 2.130 (214)   | 0.11          | 0.47 (0.04)   | 0.14          |
|               | rLF-HMAL (50/40)                         | 1.89 (0.16)   |               | 2.06 (0.19)   |               | 1.914 (106)   |               | 0.43 (0.03)   |               |
| MS122010      | rLF-A (50/5)                             | 2.00 (0.18)   | 0.45          | 2.30 (0.15)   | 0.18          | 10.363 (949)  | 0.15          | 0.46 (0.04)   | 0.20          |
|               | rLF-HMAL (50/40)                         | 1.98 (0.10)   |               | 2.19 (0.12)   |               | 9.586 (57)    |               | 0.44 (0.02)   |               |

* Anthrax LT neutralization activity of two different lots (MS122010 and MS011211) of pooled immune sera from CD-1 female mice (immunized with a single injection of 0.5 ml of a 5-fold dilution in normal saline of AVA containing 500 µg/ml CPG 7909) were evaluated in the TNA using 50 ng/ml rPA in the presence of 40 ng/ml rLF-HMAL or 5 ng/ml rLF-A. The rLF-A preparation was expressed from vector constructs containing the native LF sequence, whereas that of rLF-HMAL (produced by List Biological Laboratories, Inc., Campbell, CA) contained two additional N-terminal residues, His and Met.

* Serum dilution 4-parameter logistic fit curve parameter values were obtained from 3 independent experiments, each with 4 (MS122010) or 5 (MS011211) replicate measurements. The 3 mean values from the respective experiments were used to derive the composite mean ± SD values for each curve parameter, in which no significant differences were found between the composite means of curve parameters obtained with rLF-A or rLF-HMAL ($P > 0.05$ by Student’s t test with Welch’s correction for unequal variance).
ng/ml rLF-HMA₅ (Fig. 5). Other TNA curve parameters (upper and lower asymptotes and slopes) obtained with rLF-A and rLF-HMA₅ were also similar (data not shown). Note that some of these samples were derived from NHP challenged with B. anthracis spores after immunization and from species that received one or two immunizations. These results demonstrate the robustness of the rLF-A reagent concentration variable in the TNA, especially via serum NF₅₀ values.

Impact of total rLF concentration on TNA curve parameters. The observation that serum potency obtained with 40 ng/ml rLF-HMA₅ was similar to serum potency obtained with 4, 5, or 6 ng/ml rLF-A suggested that the degree of cytotoxicity, rather than total concentration, of rLF was a primary factor that directly influenced the TNA outcome. This was confirmed by comparing serum TNA potency obtained with rLF-A to that obtained with a blend of rLF-A and rLF-HMA₅, such that a total of 40 ng/ml was used (32 ng/ml rLF-HMA₅ plus 8 ng/ml rLF-A, which showed 95 to 99% cytotoxicity in the MLA). This 40 ng/ml concentration of the rLF blend was designed to be in molar equivalence to that used for rLF-HMA₅, but it allowed for evaluation of a defined amount of rLF-A in the presence of a molar excess of less active rLF-HMA molecules. rLF-A alone was used at 4 ng/ml, which showed similar cytotoxicity to that of the rLF blend (Fig. 6). As expected, highly correlative ED₅₀ values of immune sera were observed when either the rLF blend or rLF-A alone was used (Fig. 6). In addition, no significant differences were observed between rLF-A and the rLF blend regarding curve upper asymptotes, lower asymptotes, and slopes, and all curves passed test sample criteria as described in Materials and Methods (data not shown). These results confirmed that a molar excess of rLF molecules did not affect TNA ED₅₀ values. Collectively, these results of the rLF blend confirm that the potent rLF-A used at a 7- to 10-fold lower concentration (4 to 6 ng/ml) than the rLF-HMA material (40 ng/ml) could be used with the standard 50 ng/ml rPA in the TNA and maintain serum dilution curve parameters.
Dominance of the rPA component of LT in the TNA. The observation that rLF-A and rLF-HMA₅ cytotoxic potency and concentration can vary without affecting serum neutralization curve parameters suggested that the concentration of 50 ng/ml rPA was the critical factor in maintaining these TNA parameters. This importance of 50 ng/ml rPA is based on a concept of rPA dominance in the TNA. To demonstrate the dominant contribution of rPA (relative to rLF), different combinations of rPA and rLF concentrations that achieved 95 to 99% cytotoxicity were evaluated in the TNA to determine the relative importance of each component in the neutralization capacity of immune sera. To evaluate the widest range of rPA concentrations in the TNA, the more potent rLF-A was used at various concentrations, such that an increase in rPA concentration required a concomitant decrease in rLF concentration to maintain 95 to 99% cytotoxicity (Table 4). Serum samples from mice immunized with AVA containing the oligodeoxynucleotide immunostimulatory sequence CPG 7909 (a Toll-like receptor-9 agonist) showed an inverse correlation between ED₅₀ values and rPA concentration and a positive correlation with rLF-A and rLF-d concentration in the TNA (Fig. 7). This inverse relationship between serum ED₅₀ values and rPA concentrations is predictable, in that a serum sample dilution curve would be expected to shift to the right (greater ED₅₀) in the presence of a lower concentration of the target molecule (i.e., toxin). Only the rPA, but not the rLF, concentration followed this principle of inverse correlation with serum potency, suggesting that the rPA concentration, but not that of rLF, would be important in maintaining serum dilution curve parameters.

This hypothesis of rPA concentration dominance in the TNA is further supported with results of the less potent rLF-HMA mater.
rial used at optimal cytotoxic concentrations with 50 or 100 ng/ml rPA (95 to 99% in MLA). Mouse serum (MSO11211; the same sample lot as that used for Fig. 7) ED₅₀ values derived using rLF-A and rLF-HMA materials (rLF-HMA₉ or rLF-HMAₓ) were similar, in which 50 ng/ml rPA with 5 ng/ml rLF-A or with 40 ng/ml rLF-HMAₓ yielded ED₃₀ values (means ± standard errors of the means [SEM]) of 2.13 ± 214 and 1.91 ± 106 (n = 3), respectively, and 100 ng/ml rPA with 2 ng/ml rLF-A or with 100 ng/ml rLF-HMAₓ showed ED₃₀ values of 1.53 ± 67 or 1.51 ± 57 (n = 10), respectively. Different rPA concentrations were required because of the substantial differences in cytotoxic potency between rLF-HMAₓ and rLF-HMA₉. That is, rPA concentrations lower than 50 or 100 ng/ml were not sufficient to achieve 95 to 99% cytotoxicity of the rLF-HMAₓ or rLF-HMA₉ lots, respectively (Table 4).

We hypothesized that the reason for the rPA component of LT in the TNA being the determining factor of serum potency (ED₅₀) is that the anti-PA antibody content in sera derived from AVA immunization was represented in substantially greater levels than anti-LF (i.e., the natural anti-PA/anti-LF ratio of >10:1 [11, 12, 14]), causing a dominance of rPA activity in the TNA. Accordingly, there would be rLF dominance in the TNA if the anti-LF antibody fraction was in excess or a codominance of rPA and rLF activities if similar levels of both antibody fractions were present. To test this hypothesis, we generated mouse immune sera that contained only anti-rPA antibodies (immunization with adjuvanted rPA) or only anti-rLF antibodies (immunization with adjuvanted rLF-A) to evaluate the pattern of LT component dominance in the TNA. Accordingly, sera that contained only anti-rPA antibodies showed a PA dominant pattern of LT neutralization, whereas serum containing only anti-rLF antibodies showed an rLF dominant pattern of LT neutralization (Fig. 8A). Note that the increase in ED₃₀ values with decreasing rLF-A concentration was only apparent between 16 and 4 ng/ml rLF-A but not between 4 and 3 ng/ml, presumably due to the relatively small difference between the latter concentrations. Anti-rLF and anti-rPA serum sample mixtures were based on the neutralization activity (i.e., ED₃₀) of the individual anti-rPA and anti-rLF sera, such that a sample of a 1:1 ratio reflected serum content of equivalent ED₃₀ values. When evaluated in the TNA using different combinations of optimal rPA and rLF-A concentrations (i.e., 95 to 99% cytotoxicity in the MLA), there was an additive contribution of the anti-rPA and anti-rLF fractions when both were represented in equal proportions or in an excess of anti-rPA activity of up to 5-fold (Fig. 8B to D). This additive contribution of each antibody fraction was not significant when the ratio of anti-rPA to anti-rLF serum activity was increased to the natural ratios of >10:1 ob-

FIG 8 Additive neutralization activity of anti-rPA and anti-rLF mouse serum mixtures in the TNA, The anthrax LT neutralization capacity of anti-rPA (lot rPA-QC-H1) or anti-rLF-A (lot rLF-QC-2) mouse sera alone (A) or in combination (at ratios of 1:1, 1:2, 1:5, 1:10, or 1:20 anti-rLF to anti-rPA) (B to F) was evaluated using the TNA. (Anti-rPA sera were derived from a single immunization with rPA plus Alhydrogel, and that of anti-rLF was from two immunizations with rLF-A plus Alhydrogel and CPG 7909.) Serum mixture ratios were based on the neutralization capacity of each sample, in which the final 1:1 mixture contained equal ED₃₀ values for the anti-rPA and anti-rLF serum samples. Accordingly, each serum sample evaluated alone was diluted in an equivalent manner with naive mouse serum. LT was composed of 20, 50, or 100 ng/ml rPA with 16, 4, and 3 ng/ml rLF-A, respectively. Data are the ED₃₀ values (means ± SD) of either 2 (1:2 and 1:5 mixtures) or 4 (1:1, 1:10, and 1:20 mixtures) replicate evaluations. (A) A number sign indicates significantly different ED₃₀ values obtained with 20/16 and 100/3 ng/ml rPA/rLF combinations (anti-rPA sera; P < 0.05, two-tailed Student’s t test). A dagger indicates a significantly different value from the ED₃₀ value obtained with the 20/16 ng/ml (rPA/rLF) combination (anti-rLF sera; P < 0.05 by two-tailed Student’s t test). (B to D) An asterisk indicates significant difference in ED₃₀ value of the anti-rPA serum alone (control; white bar) from that of the respective anti-rLF/anti-rPA serum mixture (gray bars) (P < 0.05 by two-tailed Student’s t test).
served after AVA immunization (11, 12) (Fig. 8E and F). These results support that the TNA can be sensitive to either LT component, but the excess content of the anti-PA antibody fraction that exists in sera derived from rPA-based or AVA vaccinations favors rPA dominance in the TNA.

**DISCUSSION**

This study demonstrated that the potent rLF-A material with the native LF amino acid sequence (of high purity) can be used in place of the less potent rLF-HMA material (of variable purity) in the TNA while maintaining serum neutralization curve parameters. Because of the increased cytotoxic potency of rLF-A relative to that of rLF-HMA, rLF-A can be used at a 7- to 10-fold lower concentration in the TNA (4 to 6 ng/ml rLF-A versus 40 ng/ml rLF-HMA), significantly extending the use of a single rLF lot. Such increased longevity of a single rLF-A lot appears to address the supply challenge of a renewable critical reagent for validated TNAs. Indeed, the native sequence characteristic of rLF-A may lend itself to greater biochemical relevancy in the TNA used for critical immunogenicity endpoints in anthrax vaccine and antibody therapeutic development studies.

The precise inverse correlation of rPA and rLF concentrations to maintain the cytotoxic level at 95 to 99% appears to be dictated by the fixed density of anthrax PA receptors expressed by the fixed number of J774A.1 cells in the TNA culture well (29). Therefore, equilibrium should exist between the two LT component concentrations, such that a specific concentration of the resulting rPA and rLF cytotoxic complex is achieved that engages a specific number of receptors to maintain 95 to 99% cytotoxicity. The similarity of 95 to 99% cytotoxicity between 5 ng/ml rLF-A and 40 ng/ml rLF-HMA, is likely due to a similar number of active rLF molecules that efficiently bind the finite number of PA heptamers (heptamer formation is limited by rPA concentration; i.e., 50 ng/ml). Furthermore, it is likely that competitive binding to PA heptamers occurs between active and inactive (due to extensive degradation) or less active (due to instability mediated by N-terminal His and Met) rLF molecules in the rLF-HMA preparation. Therefore, a greater concentration of active rLF molecules would be expected in rLF-HMA relative to that of rLF-A to induce a similar degree of PA binding and cytotoxicity. This rLF competitive binding concept between active and less active rLF molecules is evident in our rLF blend results (Fig. 6), in which the degree of cytotoxicity mediated by 4 ng/ml rLF-A required 8 ng/ml rLF-A in the presence of 32 ng/ml of very-low-potency rLF-HMA, Note also that the 7:3 molar ratio of PA to LF of the receptor-mediated cytotoxic LT complex on the cell surface (30) is not reflected in the stoichiometry of rPA and rLF concentrations, because different ratios of rPA and rLF concentrations other than 7:3 achieved a similar degree of cytotoxicity in the MLA (e.g., 95 to 99%) (Table 4).

LT component dominance in the TNA was identified by correlating changes in rPA and rLF concentration (while maintaining 95 to 99% cytotoxicity) with the predicted changes in serum ED$_{50}$ values; i.e., an inverse correlation between toxin concentration and serum potency is predictable, in that a serum sample dilution curve would be expected to shift to the right (greater ED$_{50}$) in the presence of a lower concentration of the target molecule (i.e., toxin). Because LT contains two toxin components, we used this principle to show that changes in rPA, but not rLF, concentration followed this inverse correlation with ED$_{50}$ of all test sera (except that of anti-rLF sera, which showed an expected inverse correlation with rLF concentration). The rPA component was the determining factor of neutralizing potencies of all test sera because those samples contained either anti-PA antibodies only (i.e., from rPA plus Alhydrogel vaccination) or substantially greater anti-PA neutralizing content relative to that of anti-LF that is expected in sera derived from AVA vaccinations with or without CPG 7909 (11, 12). Indeed, most anthrax vaccines in development are designed to induce a strong neutralizing anti-PA antibody titer as the predominant protective mechanism (11–14), in which case the rPA concentration, rather than the rLF concentration, would be the dominant component in those respective TNAs. However, the rLF concentration would be the dominant or determining component in the TNA if the anti-LF antibody neutralization titer was represented either in a pure form or in excess of anti-PA, as with a sample containing a therapeutic anti-LF monoclonal antibody (31, 32) or that derived from an rLF-based vaccine. In summary, the dominant LT component of the TNA is determined by the proportional anti-PA and anti-LF neutralizing antibody content of a serum sample.

We showed that the combined neutralizing content of anti-PA and anti-LF sera yielded an additive effect, which is consistent with results of others using anti-PA and anti-LF monoclonal antibodies (32). Interestingly, this additive neutralizing activity of anti-PA and anti-LF polyclonal sera suggests that multiple neutralizing epitopes on PA and LF can be simultaneously recognized by anti-PA and anti-LF antibodies without causing a synergistic neutralizing effect. The threshold ratio of anti-PA to anti-LF neutralizing content at which anti-LF neutralizing activity cannot be detected was 10-fold (Fig. 8). This anti-PA/anti-LF threshold ratio is not dependent on specific anti-LF antibody concentration, because the extensive predilution of serum necessary to generate a full-neutralization TNA curve would result in overdilution of the anti-LF neutralizing fraction if it were at least 10-fold less than that of the anti-PA fraction. Therefore, the anti-LF neutralizing fraction was undetectable in our TNA. That anti-PA content is >10-fold higher than that of anti-LF antibodies in AVA immune sera (11, 12) explains why there was no detectable effect on TNA serum potency upon desorption of the anti-LF fraction from sera (14), despite neutralizing activity of anti-LF antibodies isolated from human serum derived from AVA immunization having been demonstrated (11).

One of the first TNA validation reports described the selection of a molar functional excess of 40 ng/ml rLF-HMA relative to that of rPA to skew toward an anti-rPA-selective assay in which saturation binding of the anti-LF fraction with excess rLF molecules favored detection of anti-PA-mediated neutralization of LT (11, 19). This conclusion appears to have resulted in the use of the standard concentrations of 50 and 40 ng/ml for rPA and rLF-HMA, respectively, by the majority of laboratories reporting TNA validation and immunogenicity studies (15, 18–24). Our results are in agreement with such a concept, in that increasing rLF-A concentration decreased the measured potency (and, most likely, sensitivity) of anti-LF antibody neutralizing activity (Fig. 8A). The idea of using an elevated concentration of low-potency rLF-HMA material is an attractive approach in creating an anti-PA-selective assay, which would be useful in evaluating the specific anti-PA neutralizing contribution in serum samples that contain substantial levels of anti-LF content. The objective of our study was to demonstrate that the use of a low concentration of the highly potent rLF-A material would not affect serum potency relative to
that obtained with the higher concentration of lower potency rLF-HMA₂ in the presence of 50 ng/ml rPA. While the use of lower concentrations of rLF-A relative to rLF-HMA₂ may pose a theoretical risk of affecting serum ED₅₀ values, this would be unlikely to occur because, in most cases, the serum anti-LF neutralizing fraction is expected to be below the level of detection in the current TNA method (i.e., at least 10-fold lower than that of anti-PA). Indeed, we showed that ED₅₀ values of all sera derived from AVA vaccination with or without CPG 7909 (from multiple species, including infection with spores) that are expected to contain anti-LF antibodies were not affected by changes in rLF concentrations (i.e., rLF-A at 5 ng/ml versus rLF-HMA₂ at 40 ng/ml). However, a comprehensive evaluation of anti-PA and anti-LF neutralization ratios in such sera is required to determine the frequency or conditions under which anti-LF content could be elevated to detectable levels in the TNA.

In conclusion, we showed that the standard 50 ng/ml rPA concentration used in most developed TNAs reported was important in maintaining curve parameters for sera derived from most anthrax vaccines in development (rPA or AVA based). In addition, the specific rLF potency, preparation (N-terminal residue content), or concentration did not appear to be important in ED₅₀ determination of such vaccines as long as the cytotoxic level of LT was maintained at 95 to 99% and the anti-LF fraction was substantially smaller (i.e., at least 10-fold) than that of the anti-PA fraction in test sera. Our conclusions are consistent with guidance presented in a commentary for B. anthracis toxin component consensus standards in which it was suggested that concentrations of LT components should be empirically derived per lot via checkerboard MLA (22).

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