ELSEVIER

Journal of Immunological Methods 333 (2008) 89–106

JIM Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

Standardized, mathematical model-based and validated *in vitro* analysis of anthrax lethal toxin neutralization☆

Han Li, Stephen D. Soroka, Thomas H. Taylor Jr., Karen L. Stamey, Kelly Wallace Stinson, Alison E. Freeman, Darbi R. Abramson, Rita Desai, Li X. Cronin, J. Wade Oxford, Joseph Caba, Cynthia Pleatman, Sonal Pathak, Daniel S. Schmidt, Vera A. Semenova, Sandra K. Martin, Patricia P. Wilkins, Conrad P. Quinn *

Microbial Pathogenesis & Immune Response Laboratory, Meningitis and Vaccine Preventable Diseases Branch, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC), Atlanta, GA 30333, United States

Received 22 October 2007; received in revised form 11 January 2008; accepted 17 January 2008
Available online 8 February 2008

Abstract

Quantification of anthrax lethal toxin (LTx) neutralization activity (TNA) is pivotal in assessing protective antibody responses to anthrax vaccines and for evaluation of immunotherapies for anthrax. We have adapted and redesigned the TNA assay to establish a unifying, standardized, quantitative and validated technology platform for LTx neutralization in the J774A.1 murine cell line. Critical design features of this platform are 1) the application of a free-form or constrained 4 parameter logistic (4-PL) function to model neutralization responses within and between boundary limits of 100% cell survival and 95% cell lysis and 2) to exploit innovative assay curve recognition algorithms for interpretive endpoints. The assay was validated using human serum ED50 (dilution of serum effecting 50% neutralization) as the primary reportable value (RV). Intra-operator and intermediate precision, expressed as the coefficient of variation (%CV), were high at 10.5–15.5%CV and 13.5–14.5%CV respectively. TNA assay dilutional linearity was demonstrated for human sera using linear regression analysis of $\log_{10}$ transformed data with slope = 0.99, intercept = −0.03 and $r^2 = 0.985$. Assay accuracy, inferred from the precision and linearity data and using a spike-recovery approach, was high with a percent error (%E) range of only 3.4–20.5%E. The lower limit of detection (LLOD) was ED50 = 12 and the lower limit of quantification (LLOQ) was ED50 = 36. The cell-based assay was robust, tolerating incubation temperatures from 35 to 39 °C, CO_2 concentrations from 3% to 7% and reporter substrate (MTT) concentrations of 2.5–7.5 mg/ml. Strict assay quality control parameters were met for up to 25 cell culture passages. The long term (50 month) assay stability, determined using human reference standards AVR414 and AVR801, indicated high precision, consistent accuracy and no detectable assay drift. A customized software program provided two additional assay metrics, Quantification Titer (QT) and Threshold Titer (TT), both of which demonstrate acceptable accuracy, precision and dilutional linearity. The TT was also used to establish the assay reactivity threshold (RT). The application of the assay to sera from humans, Rhesus macaques and rabbits was demonstrated separately and by aggregate dilutional linearity analysis of the ED50 (slope = 0.98, intercept = 0.003, $r^2 = 0.989$). We propose this TNA assay

☆ The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention.

* Corresponding author. Microbial Pathogenesis & Immune Response Laboratory, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D-11, Atlanta, GA 30333, United States. Tel.: +1 404 639 2858; fax: +1 404 639 2835.
E-mail address: cquinn@cdc.gov (C.P. Quinn).

0022-1759/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/j.jim.2008.01.007

format with a qualified standard reference serum and customized interpretive software as a unifying platform technology for determination of functional serologic responses to anthrax vaccines and for evaluation of anthrax immunotherapeutics.
Published by Elsevier B.V.

Keywords: Anthrax toxin; Neutralization; Validation; 4 Parameter logistic; Standardization; Antibody

1. Introduction

The pathogenicity of *Bacillus anthracis* is due primarily to the separate but complementary actions of the γ-linked poly-D-glutamic acid (γDGA) capsule and the two protein exotoxins, edema toxin (ETx) and lethal toxin (LTx). Production of capsule and toxins parallels the germination and outgrowth of *B. anthracis* spores (Guidi-Rontani et al., 1999). The γDGA capsule protects the multiplying bacilli against phagocytosis and the exotoxins disarm the innate and acquired immune responses thus facilitating bacterial proliferation (Agrawal et al., 2003; Baldari et al., 2006; Xu and Frucht, 2007). The exotoxins comprise binary combinations of protective antigen (PA) with edema factor (EF) to form ETx, or PA with lethal factor (LF) to form LTx (Leppla, 1999). ETx elevates intracellular cyclic-adenosine monophospate (cAMP) levels resulting in cytokine modulation, upregulation of the anthrax toxin receptor and disruption of interstitial fluid balance (Cui et al., 2007; Tessier et al., 2007). LTx inactivates members of the mitogen-activated protein kinase kinase (MAPKK) family to cause an imbalance in the production or release of a range of cytokines (Singh et al., 1999). The combined effects of the toxins are tissue edema and local necrosis in cutaneous anthrax, and hemorrhagic mediastinal necrosis, hypoxic insult and pleural edema in systemic anthrax (Duesbery et al., 1998; Moayeri et al., 2003). Ameliorating the toxin effects is central in host protection against anthrax and much evidence has accumulated that protection is mediated by antibody responses whether actively induced or passively administered. Vaccines and therapies targeting PA are particularly effective (Klein et al., 1968; Little et al., 1997; Pitt et al., 2001; Welkos et al., 2001).

LTx has been shown to cause death in a variety of animal models and can lyse certain cell lines *in vitro* (Vick et al., 1968; Ezzell et al., 1984; Friedlander, 1986). An *in vitro* LTx cytotoxicity assay (Friedlander et al., 1993) has previously been developed and used to evaluate the neutralizing potential of polyclonal and monoclonal antibodies to PA and anthrax vaccines (Pitt et al., 2001; Little et al., 2004; Pittman et al., 2005, 2006). The neutralization assay has been applied and validated in a variety of formats (Hering et al., 2004). A range of LTx sensitive cell types have been used in these assays, most notably the murine monocyte/macrophage J774A.1 and RAW264 cell lines (Hanna et al., 1993; Arora et al., 1994; Tang and Leppla, 1999). The reporter systems have included monitoring release of lactate dehydrogenase (LDH) and use of colorimetric reporters, most commonly the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) or alamarBlue (Hansen et al., 1989; Quinn et al., 1991; Friedlander et al., 1993; Hanson et al., 2006).

Typically, LTx neutralization activity (TNA) has been reported as the ED50 — the dilution of serum neutralizing 50% of total cell cytotoxicity, and the NF50 — the quotient of a reference sample ED50 and the test sample ED50 (Hering et al., 2004). The concentration of purified antibody neutralizing 50% of total cell cytotoxicity is usually reported as the EC50 (Lim et al., 2005).

The TNA assay has become increasingly pivotal as a tool for quantifying functional antibody responses to anthrax vaccines, for evaluation of immunotherapies for anthrax and characterization of anthrax toxin inhibitors (Brossier et al., 2004; Mohamed et al., 2004; Wang et al., 2004; Huber et al., 2005; Hull et al., 2005; Lim et al., 2005; Hanson et al., 2006; Pittman et al., 2006). The assay formats, reportable values and interpretation however, can vary significantly between laboratories, leading to difficulty in comparison of data between studies. It was our objective to address this variability by creating a standardized technology platform for quantifying LTx neutralization, developing this technology to a level of standardization and robustness that facilitates its use in a variety of laboratories, making the technologies and pivotal reagents generally available and thence providing a framework for qualitative and quantitative comparison of different anthrax vaccine and therapeutic studies. This article reports the development, performance characteristics and validation using human serum of a robust and rugged format of the TNA assay and its application in evaluating immune serum from humans, Rhesus macaques and rabbits. This format uses standardized and characterized reagents in conjunction

with customized interpretive software and a novel mathematical algorithm to calculate and extrapolate multiple reportable values, in addition to ED50, with high specificity, analytical sensitivity, accuracy and precision.

2. Materials and methods

2.1. Anthrax lethal toxin

Recombinant anthrax toxin protective antigen (rPA) and lethal factor (rLF) were either supplied by Dr. Stephen Leppla, National Institute of Craniofacial and Dental Research, National Institutes of Health, Bethesda, MD or purchased from List Biologicals, Campbell, CA. Antigens were stored frozen in small aliquots (30 μl, ≥ 1 mg/ml) in 5 mM Hepes, pH 7.5 at ≤ −70 °C. The concentration of LTx (50 ng/ml PA plus 40 ng/ml LF) used in the TNA assay was determined by titration to ensure a potency of approximately 95% cell killing under standard assay conditions. LTx potency was reviewed every 2 months.

2.2. Human reference sera

Human standard reference sera AVR414 and AVR801 were prepared from healthy adult volunteers who had received a minimum of 4 subcutaneous injections of Anthrax Vaccine Adsorbed (AVA, BioThrax, BioPort Corp., Lansing MI) with the licensed regimen (0, 2, 4 weeks, 6, 12 and 18 months). The preparation and characterization of these sera are detailed elsewhere (Semenova et al., 2004). The assigned ED50 values and operational ranges of AVR414 and AVR801 in this assay were determined from the average of 132 and 1045 individual test results, respectively. Human reference serum AVR801 is available from the CDC and BEI Resources (Manassas, VA) under appropriate agreements. The acquisition and use of human serum in this study were approved by the Centers for Disease Control and Prevention (CDC) Human Subjects Institutional Review Board.

2.3. Preparation of pooled Rhesus macaque normal serum and pooled Rhesus macaque anti-AVA antisera

Negative control Rhesus macaque (*Macaca mulatta*) serum was derived from a Rhesus macaque normal serum pool obtained commercially (Valley Biomedical, Winchester, VA) and pre-screened by species specific anti-PA immunoglobulin (Ig) G ELISA to confirm non-reactivity to anthrax toxin PA. An anti-PA Rhesus macaque standard reference serum AVR731 was prepared by pooling serum from 10 different macaques who received 3 intramuscular injections of a full human dose of AVA at 0, 4 and 26 weeks. The sera were collected at 7–14 days after the week 26 injection. To create AVR731, the serum pool was diluted 1/3 with pooled normal Rhesus macaque serum (Research Diagnostics, Inc, Flanders, NJ). An anti-PA IgG concentration of 171.9 μg/ml was assigned to AVR731 by quantitative species specific anti-PA IgG ELISA with affinity purified anti-PA IgG as a standard (V. Semenova, unpublished data). All animal procedures were approved by the CDC Institutional Animal Care and Use Committee (IACUC) and were implemented by the CDC Scientific Resources Program (SRP).

2.4. Preparation of pooled rabbit normal serum and rabbit anti-PA antisera

Negative control rabbit serum was prepared by pooling equal volumes of normal rabbit serum (Life Technologies, Gaithersburg, MD and Sigma-ALDRICH, Inc., Saint Louis, MO). Rabbit anti-PA standard reference serum AVR819 was prepared by pooling equal volumes of serum from 2 rabbits (New Zealand White, Myrtle's Rabbitry Inc., Thompson's Station, TN) immunized with 7 intramuscular injections (0, 17, 28, 38, 49, 59, and 71 days) of 50 μg rPA in Freund's Incomplete Adjuvant (Sigma-ALDRICH, Inc., Saint Louis, MO). The sera for this reference serum pool were collected at 12 days after the 7th injection. The serum pool was diluted 1/4 with pooled normal rabbit serum (Sigma-ALDRICH, Inc., Saint Louis, MO and Valley Biomedical, Winchester, VA). An anti-PA IgG concentration of 523.8 μg/ml was assigned to AVR819 by quantitative species specific anti-PA IgG ELISA with affinity purified anti-PA IgG as a standard (D. Schmidt, unpublished data).

2.5. Sera for assay development and validation

Human, Rhesus macaque and rabbit sera were used for assay development and performance evaluation. Separate analyses of genus-specific dilutional linearity and inferred accuracy together with analyses of the aggregate data from all three genera were completed. Assay validation was based on human sera because these were the most abundant and best characterized reagents available in terms of anti-PA antibody reactivity. Specifically, the validation sera were the human standard reference serum AVR414, negative control (NE) sera AVR190 and AVR811 and positive control sera AVR216, AVR284 and AVR370. Positive control sera were selected from human AVA vaccinees on the basis of measurable PA-specific IgG reactivity and LTx neutralization activity

as measured by enzyme-linked immunosorbent assay (ELISA) and the TNA assay respectively (Quinn et al., 2002, 2004; Semenova et al., 2004).

2.6. Toxin neutralization activity (TNA) assay standard procedure

The J774A.1 murine monocyte/macrophage cell line (TIB-67) used in this study is available from the American Type Culture Collection (ATCC, Manassas, VA) and from BEI Resources (Manassas, VA). The J774A.1 cells were cultured in D-MEM supplemented with high glucose (4.5 g/l), 4 mM L-glutamine (Gibco BRL, Gaithersburg, MD), 5% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), 10 mM HEPES buffer solution (Gibco BRL), penicillin (50 units/ml), streptomycin sulfate (50 μg/ml) and 1 mM sodium pyruvate (Gibco BRL). All incubations were at 37 °C in a 5% CO_2 atmosphere, 95% relative humidity. Cultures of J774A.1 cells were harvested during late exponential growth into warmed growth medium and plated at 3×10^4 cells/well in 96-well flat bottom microtiter plates, 17–19 h prior to the assay. The standard reference serum, positive neutralization control serum (PNC), negative control serum (NE) and test materials were prepared in a separate 96-well microtiter plate and then incubated with anthrax lethal toxin (50 ng/ml PA and 40 ng/ml LF) at 37 °C for 30 min. This ratio of PA to LF provides a 1.4:1 molar ratio of PA:LF such that LF is in approximately a 1.7-fold functional excess (Mogridge et al., 2002). LTx was titrated to effect 95% cell lysis. The standard reference serum and test materials were each prepared as seven-point $\log_2$ dilution series and plated in 3 adjacent well series ('pseudo triplicates') to control for potential inter-well cell density variance. Matched controls for the test sera were plated in duplicate in the absence of LTx at the lowest dilution of the test serum series (100% viability controls). An additional, independent serum positive neutralization control (PNC) for 100% viability in the presence of LTx was plated in triplicate at 1/100. Spent medium was removed from the J774A.1 cells, the toxin–antiserum mix was then transferred (100 μl/well) to the J774A.1 cell plate and the incubation continued for 4 h. Cell viability was determined by the addition of 25 μl/well of a 5 mg/ml stock solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemicals, St. Louis, MO) dissolved in 0.01 M phosphate buffered saline (PBS) pH 7.4 (Life Technologies, Gaithersburg, MD), and the incubation continued for 2 h. The assay was terminated by addition of 100 μl/well of an aqueous preparation of 20% wt/vol SDS (Sigma Fine Chemicals, St. Louis, MO), 50% vol/vol *N*,*N*,-dimethyl formamide (DMF) (Fisher Scientific, Pittsburg, PA) and incubation at 37 °C for 16 to 20 hours (overnight). Optical density (OD) values were read at 570 nm with a 690 nm reference filter using a MRX Revelation™ microtiter plate reader (Thermo Labsystems, Franklin, MA). The minimum serum dilution used in the assay was 1/50. For development and validation studies the test materials were sera or purified murine monoclonal antibodies. Alternative test materials for evaluation as LTx inhibitors may be substituted at dilution or concentration ranges that generate a sigmoidal dose–response curve.

2.7. Mathematical model for dose–response curves

All TNA assay analyses and endpoint calculations were done in SAS® version 8.0, 8.2, or 9.1 (SAS Institute Inc. Cary, NC) running a customized endpoint calculation algorithm (Taylor et al., 2003). The TNA analysis code used in this study was designated ED50.51. The code used a 4 parameter logistic (4-PL) model to fit a dose–response curve to the data (Fig. 1). For evaluation of sera of low reactivity the code contains a pattern-recognition algorithm which distinguishes among fully formed sigmoid neutralization curves, partially formed curves (upper asymptote not defined by the data) and the absence of curve formation (negative sera). Full curves are estimated using Gaussian or Marquardt methods, which deliver nearly equivalent results if both are convergent. Partial curves are defined algorithmically using the pattern-recognition logic described above. For partial curves in which the upper region of the curve is not well-characterized by the dilution data, the computed curve is constrained to the upper asymptote of either the reference standard or the matched-serum control on the same plate. The choice of constraint is based on the best fit of the data. The resultant fitted curve is the best approximation of the true, full sigmoid curve that would have been estimated if the full range of OD signal had been observable below the minimal starting dilution (Fig. 1.A).

2.8. Assay reportable values

To enhance the analytical sensitivity of the assay the software calculates three reportable values (RV); the reciprocal of the effective dilution of a serum sample providing 50% LTx neutralization (ED50), the Threshold Titer (TT) and the Quantification Titer (QT). Each of these values was defined in the context of the implicit full curve from the 4-PL function, fitted to the 7-point $\log_2$ dilution series of triplicate optical density (OD) values from each independent serum neutralization curve. The ED50 was the primary reportable value and was determined as the reciprocal of the dilution corresponding

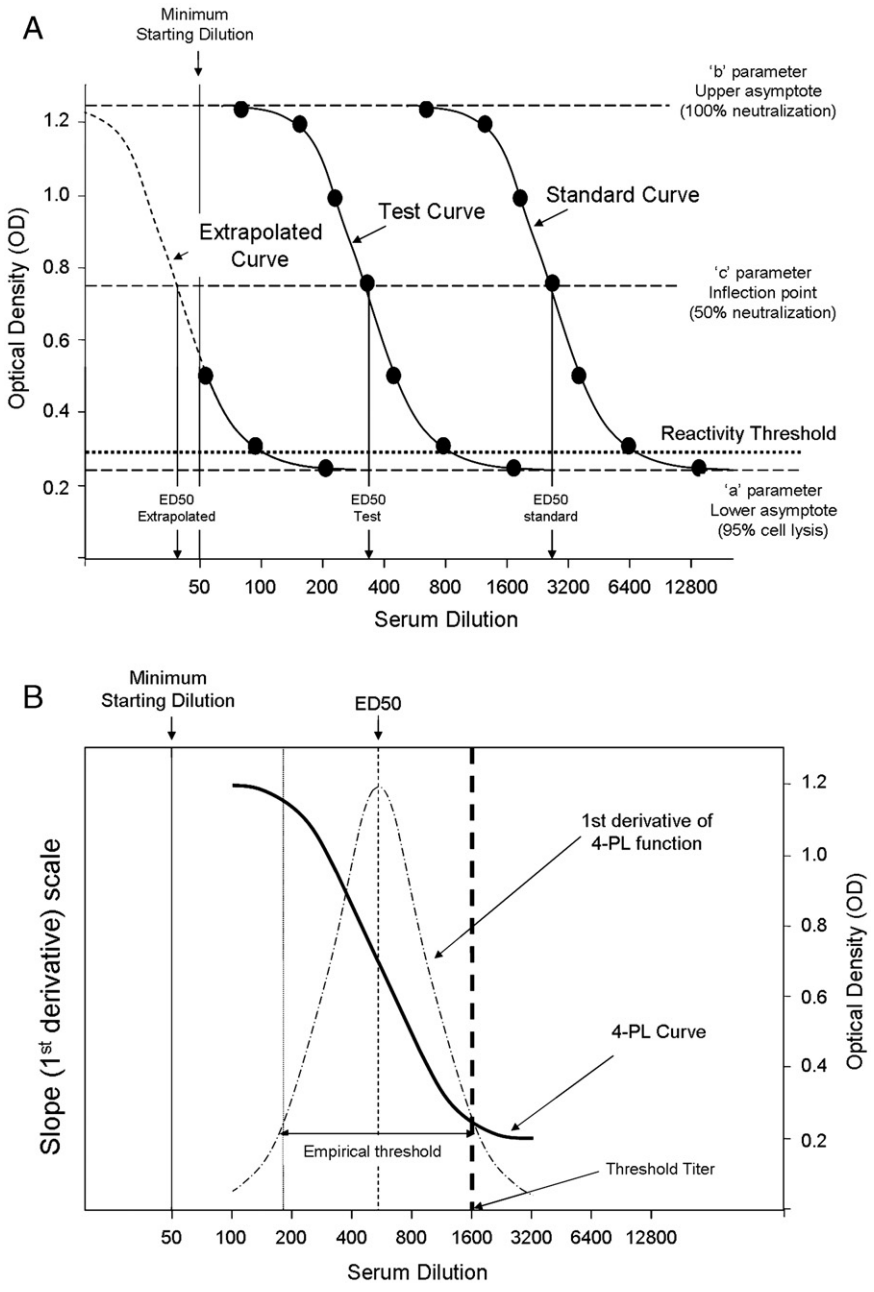

Fig. 1. Schematic representation of the 4-PL model for LTx neutralization in J774A.1 monocyte macrophages (not to scale). A. ED50, reactivity threshold (RT) and curve extrapolation for low reactivity sera. The software used a 4 parameter logistic (4-PL) model to fit a dose–response curve to the data. Full curves are estimated using Gaussian or Marquardt methods, dependent on convergence. Partial curves from sera of low reactivity are defined algorithmically using a pattern-recognition logic which distinguishes among fully formed sigmoid neutralization curves, partially formed curves (upper asymptote not defined by the data) and the absence of curve formation (negative sera). For partial curves the computed curve is constrained to the upper asymptote of either the reference standard or the matched-serum control on the same plate (100% neutralization). The choice of constraint is based on the best fit of the data. The resultant fitted curve is the best approximation of the true, full sigmoid curve that would have been estimated if the full range of OD signal had been observable below the minimal starting dilution. B. Illustration of the relationship between the 1st derivative of the 4-PL model and the Threshold Titer (TT). The TT was defined as the highest serum dilution corresponding to a fixed, empirically determined level of 30% of the maximum slope of the first derivative of the fitted 4-PL curve. C. Illustration of the relationship between the 2nd derivative of the 4-PL model and the Quantificaton Titer (QT). The second derivative of the 4-PL model was used to identify the upper and lower "bends" of the curve. The region between these points was the Quantification Range of the assay curve. The higher-dilution end of the range was interpolated to the dilution axis to provide the QT. The SAS algorithm also used the quantification range to compute mass values for test samples by interpolation to a reference serum with an assigned anti-PA IgG calibration factor.

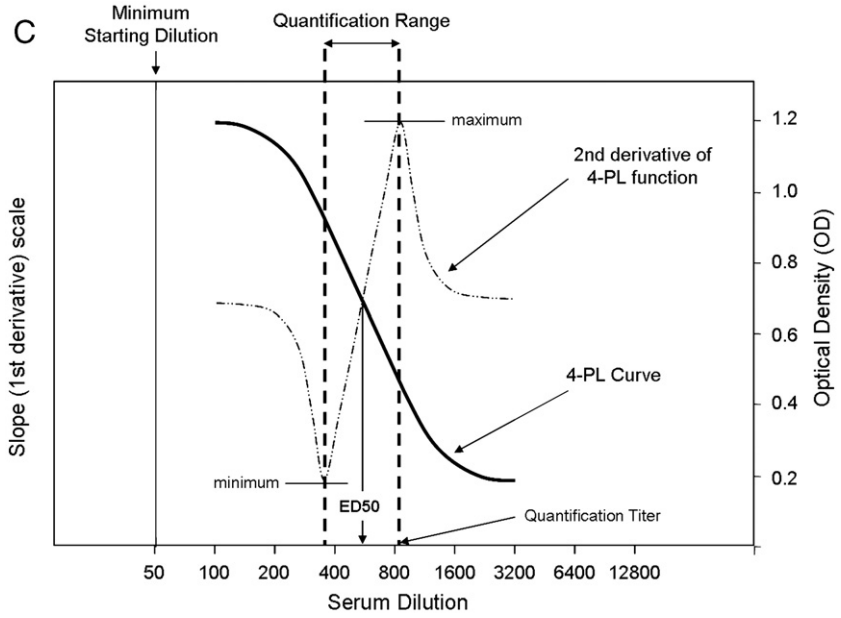

Fig. 1 (*continued*).

to the inflection point ('*c*' parameter) of the 4-PL fit. The TT was defined as the highest serum dilution corresponding to a fixed, empirically determined level of 30% of the maximum slope of the first derivative of the fitted 4-PL curve (Fig. 1.B). The QT was the highest serum dilution in that region defined by the second derivative of the 4-PL model to identify the upper and lower "bends" of the curve. The QT was an extension of the threshold concept to the points where the change in slope was most measurably changing from zero at both the upper and lower bends in the curve. The region between these points was the Quantification Range of the assay curve. The higher-dilution end of the range was interpolated to the dilution axis to provide the QT (Fig. 1.C). The SAS algorithm also computed mass values for test samples by interpolation to a reference serum with an assigned anti-PA IgG calibration factor. The interpolation for mass values was only applied within the Quantification Range of the reference serum.

2.9. Assay acceptance criteria

The acceptance criteria for the each assay plate were designed to be comprehensive and rigorous. They were derived from the performance characteristics of the assay-critical reagents; the anthrax lethal toxin (lethal factor and protective antigen), the J774A.1 cell line and the standard reference reagent. Primary acceptance criteria were based on the essential operational characteristics of the assay; cell density, LTx potency, appropriate dose–response curve formation in the reference standard, and acceptable operator precision. Specifically, these criteria included: 1) the mean OD value for the negative control serum (NE) with LTx ($\geq$95% cell lysis) was required to be $\leq$0.45 OD units; 2) the maximum OD mean triplicate value for the serum standard (100% viability) was required to be $\geq$0.85 (acceptable curve height); 3) within-dilution %CV of OD values for reference standard dilution triplicates was required to be $\leq$20% for 6 of the 7 dilutions (acceptable intra-assay precision); 4) the difference between the maximum OD mean triplicate and the minimum OD triplicate value was required to be $\geq$0.55 OD units (adequate curve depth); 5) the mean OD value for the positive neutralization control (PNC) was required to be $\geq$0.85 (upper asymptote control) and 6) the difference between the mean OD triplicate for the highest dilution and mean OD value for the negative control was required to be $\leq$0.25 OD units (adequate formation of curve lower asymptote). Secondary acceptance criteria were based on specific performance characteristics of the reference standard curve to ensure that the critical reagents and 4-PL model were performing to the required specification. These criteria included: 1) the ED50 value of standard reference serum was required to be within a range of $\pm$2 standard deviations (SD) from the established operational arithmetic mean (*e.g.* AVR414, ED50 = 1128 $\pm$ 542); 2) the 4-PL curve generated from the standard reference serum was required to have a squared correlation coefficient (r^2) value of

≥ 0.945 (adequate goodness of fit to the 4-PL model); 3) at least three OD mean triplicate dilution points were required to be on either side of the inflection (adequate formation and empirical data distribution for a full sigmoid curve from the reference serum). In addition, the test samples were required to have an $r^2 > 0.895$ in the 4-PL model, the percent coefficient of variation (%CV) for all triplicate OD value data points for the test samples was required to be $\leq 20\%$ for 6 of the 7 dilutions and the test sample curve was required to converge using a Gaussian or Marquardt model. All of these criteria were evaluated automatically in the analysis software. The matched-serum controls (no LTx) provided an assessment of potential effects of test serum analytes on the J774A.1 cell viability. Assay development studies were used to evaluate assay ruggedness and robustness and to determine assay tolerances to establish acceptance criteria for validation. Validation focused on intra-operator precision, inter-operator (intermediate) precision, dilutional linearity, lower limit of detection (LLOD) and lower limit of dilutional quantification (LLOQ). In the absence of an independent standard, accuracy was inferred from linearity and precision using assay-assigned ED50 values. The ED50 provided the primary analytical endpoint for assay validation. QT and TT were recorded for additional analysis of assay performance.

2.10. Assay precision, goodness of fit and drift

Precision is a measure of the degree of repeatability of the assay under normal operating conditions. Intra-operator precision was expressed as the coefficient of variation (%CV) of the ED50 for each of 3 different human sera tested $n \geq 4$ on 3 non-consecutive days to generate a minimum of 12 replicates per sample. For validation purposes, the acceptable level of intra-operator precision for the TNA test serum ED50 was $\leq 30\%$CV. Inter-assay (intermediate) precision was expressed as the coefficient of variation (%CV) of the ED50 for each human validation sera tested by 2 different operators over three non-consecutive days to complete a minimum of 24 replicates per sample. Intermediate precision for macaque and rabbit samples was determined from the ED50 endpoints ($n = 50$) from multiple operators over multiple days for each of the macaque and rabbit reference standards AVR731 and AVR819, respectively. The acceptable level of intermediate precision for this TNA assay was a $\leq 30\%$CV. Intermediate precision for both the QT and TT was also recorded.

The 'goodness of fit' of the assay was, for comparative purposes, an indication of how closely the data points of the standard reference serum curve fit the 4-PL model. These data should exhibit a sigmoidal shape when plotted on an optical density–$\log_2$ dilution scale. The 'goodness of fit' was expressed as the estimated non-linear squared correlation coefficient (r^2) of the standard curve and determined by averaging the r^2 values of at least 100 independent standard reference curves for AVR414. An r^2 value that approaches 1.0 is indicative of a 'good fit' for the data to the curve.

Assay stability, longitudinal performance and drift were assessed from the characteristics of reference standards AVR414 and AVR801 over a 50 month period (25 months' AVR414; 26 months' AVR801 with 1 month overlap). Additional parameters reviewed included the Quantification Titer (QT), Threshold Titer (TT), mean r^2 of the 4-PL fit for each reference (RSQRD), mean OD of the negative control (ODNEGMEAN), maximum OD triplicate mean for a sample (ODTRIPMAX), minimum OD triplicate mean for a sample (ODTRIPMIN), maximum CV of the dilution OD values for a sample (ODCVMAX), mean OD of the positive neutralization control (ODPNCMEAN), and the lower asymptote '*a*', upper asymptote '*b*' and slope '*d*' parameters of the 4-PL fit.

2.11. Accuracy and inferred accuracy

Accuracy is a measure of the exactness of the assay, or the closeness of agreement between the measured value and the value that is accepted as a true value or an accepted reference value. Accuracy was expressed as the percent error (%E) between the assay-determined (observed) value and the assigned (expected) value for the validation serum. In this study, accuracy was evaluated in two separate approaches. In the first approach, accuracy was evaluated by repeated analysis of 12 positive control human anti-AVA antisera for which anti-PA IgG concentrations were pre-assigned by independent enzyme-linked immunosorbent assay (Quinn et al., 2002) and one affinity purified IgG1 murine monoclonal anti-PA IgG (AVR1046) with a mass value assigned by absorbance at 280 nm. The observed assay anti-PA IgG values were calculated in the TNA assay by interpolation from the test serum curve to the quantification range of AVR414 reference standard using a calibration factor of 141.2 μg/ml anti-PA IgG (Semenova et al., 2004). For accuracy experiments, each of 12 human sera and 1 murine monoclonal anti-PA IgG was tested a minimum of 3 times by 3 independent operators. In the second approach, accuracy was determined by inference. Inferred accuracy is used where an independently assigned neutralization standard is not available (Food and Drug Administration, Guidance for Industry). In this assay, it

was determined by spike-recovery analysis of 21 positive sera for which the ED50 had been assigned based on the dilution factor of the parental sample. Inferred accuracy required acceptable levels of linearity and precision to be demonstrated. Inferred accuracy was calculated by comparing the %E between the observed ED50 of spiked serum with the expected ED50 for that serum. For inferred accuracy experiments, each of 21 human, 7 rabbit and 7 Rhesus macaque samples was tested a minimum of 3 times by 2 independent operators using the same sample panel as for dilutional linearity.

2.12. Dilutional linearity

The dilutional linearity of an assay is its ability to elicit results that are directly, or by a well-defined mathematical transformation, proportional to the neutralizing efficacy of a test sample. Dilutional linearity of ED50, QT and TT and inferred accuracy of the ED50 were evaluated from the same set of experiments using stock sera from three different animal genera; human, Rhesus macaque and rabbit, for which the ED50 was assigned from multiple tests ($n \geq 50$) over a range of 9–35 months. For construction of spike-recovery samples, a human negative serum pool (AVR190), a macaque negative serum pool (AVR815) and a rabbit negative serum pool (AVR820) were each spiked with their genus-specific anti-PA IgG positive control or reference serum at a range of different dilutions. The human positive control sera AVR216, AVR284 and reference standard AVR414 were each spiked into human negative serum at six separate dilutions between 1/2 and 1/64 to create a panel of 21 positive sera that represented a range of expected ED50 values from 10.8 to 940.8. Macaque positive serum pool AVR731 and rabbit positive serum pool AVR819 were spiked into their respective negative sera, AVR815 and AVR820, at six different dilutions between 1/2 and 1/128 to create a panel of 14 sera that represented a range of expected ED50 values from 25.3 to 3237.2 for macaque and rabbit inclusive. All spiked samples were tested at a 1/50 starting dilution. Partial curves were constrained to an upper asymptote as described above. Dilutional linearity was calculated by weighted least squares regression analysis of $\log_{10}$ transformed observed vs. expected values for the ED50, QT and TT for each of the spiked serum pools. A two-sided *t*-test was used to determine whether the intercepts of regression data were significantly different from the origin. The r^2 statistic in combination with the slope and the *y*-intercept was used as a measure of goodness of fit for the regression. All serum tests were done in triplicate in a minimum of two separate assays. For assay validation, the acceptance criteria for dilutional linearity were 1) the slope of the regression for the ED50 values was between the range 0.7 and 1.3 and 2) a squared correlation coefficient (r^2) ≥ 0.900.

2.13. Lower limit of detection and lower limit of quantification

For assay validation using human sera, the empirical lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were determined from the highest dilution of a known positive serum for which a Threshold Titer (TT) was calculated and for which at least one data point on the constructed curve was at or above the TT. The LLOQ was the lowest ED50 calculated with an acceptable level of precision ($\leq 30\%$CV) and accuracy ($\leq 30\%$E). LLOD and LLOQ were calculated from the same data set as for dilutional linearity.

2.14. Reactivity threshold

The reactivity threshold (RT) (Fig. 1.A) was used to categorize a serum as reactive or non-reactive and thence to determine the diagnostic sensitivity (DSN) and diagnostic specificity (DSP) of the assay. For this study the RT required that the OD values of 2 or more data points from the test serum curve were greater or equal to the TT for that curve. If only one data point was greater or equal to the TT optical density (OD) reading or a TT could not be calculated, then the sample was considered non-reactive.

2.15. Diagnostic sensitivity and specificity

Diagnostic sensitivity (DSN) is the ability of the assay to identify a true positive (TP) result. Diagnostic specificity (DSP) is the ability of the assay to identify a true negative (TN) result. For calculation of DSP and DSN, sera were first classified either as true positives (TP) or true negatives (TN) on the basis of their clinical or anthrax vaccination status. DSN was calculated as $(TP/(TP+FN)) \times 100$. DSP was calculated as $(TN/(TN+FP)) \times 100$ where FN and FP are false negatives and false positives, respectively. The DSN test panel comprised 100 sera obtained from humans with either clinically confirmed anthrax (Quinn et al., 2004) or a known history of vaccination with anthrax vaccine adsorbed (AVA). The DSP panel comprised 195 sera assembled from samples provided by the CDC Occupation Health Service (CDC, Atlanta, GA), the National Health and Nutrition Examination Survey (NHANES,

CDC, Atlanta, GA) and individual patient sera with clinically confirmed non-anthrax-related illnesses or vaccinations. The clinical sera were from acute *Haemophilus influenzae* infection, staphylococcal infection, legionellosis, *Chlamydia pneumoniae* infection, *Mycobacterium pneumoniae* infection and from recipients of non-anthrax related vaccines (influenza, hepatitis-B, tetanus toxoid and botulinum toxoid). For determination of DSN and DSP, sera were tested once with a starting dilution of 1/50. FP and FN were identified using the assay RT. The acquisition and use of human serum were approved by the CDC Human Subjects Institutional Review Board.

2.16. Assay robustness

Robustness is a measure of the assay's capacity to remain unaffected by small but deliberate variations in the method parameters. Robustness evaluations provide an indication of assay reliability by simulating the inadvertent variations that might occur in normal use. The parameters tested to evaluate the robustness of the TNA assay were: incubation temperature (35 °C, 37 °C and 39 °C), intoxication period (3.5 h, 4.0 h and 4.5 h), incubator CO_2 concentration (3%, 5% and 7%), MTT concentration (2.5 mg/ml, 5 mg/ml, and 7.5 mg/ml), MTT incubation period (1.5 h, 2.0 h, and 2.5 h), and cell passage number. The cell passage number (P) was defined as the number of times that a cell preparation had been subcultured. Cell stocks received from the American Type Culture Collection (ATCC) were considered as passage number 1. The effect of cell passage number on the ED50 was determined by repeated analysis of characterized test samples on cells that had undergone between 6 and 15 cell passages (P≤15), 20 cell passages (P20), and 25 cell passages (P25). Cell passages P≤15 were considered the basic procedure as these are the minimum number of passages required to produce sufficient cell densities for routine serological testing. The effect of cell passage was determined by comparison of ≥20 ED50 at each passage number value using the standard reference serum and each of 3 test sera, together with the 'goodness of fit' of the standard reference curve to the 4-PL model. For all robustness test conditions, 3 operators each tested 6 plates per day on each of 3 days. The acceptance criteria for robustness included the requirement that all test plates meet all quality control criteria.

2.17. Statistical analysis of test conditions

All statistical analyses were done using SAS® version 9.0 or higher (SAS Institute Inc. Cary, NC). Summary procedures in SAS® were used to capture the mean, standard deviation (SD), standard error (SE) and coefficient of variance (%CV) of the ED50, QT and TT values for the standard reference serum and each test sample, where appropriate. Robustness was evaluated by inter-condition %CV of the ED50 values of all samples for each experimental condition. Dilutional linearity was evaluated by a weighted least squares regression analysis of $\log_{10}$ transformed data. A two-sided *t*-test was used to determine whether the intercepts of regression data were significantly different from the origin. The r^2 statistic in combination with the slope and the *y*-intercept was used as a measure of goodness of fit for the regression. Analysis of covariance (ANCOVA) was used to compare the dilutional linearity regression analyses between human, Rhesus macaque and rabbit data where the slopes of the regression lines were first shown to be equivalent (*t*-test). Where levels of statistical significance were considered, the *p* values were set as $p=0.05$. Rounding of the reported values was done after statistical analyses.

3. Results

3.1. Precision, goodness of fit and assay drift

Intra-operator precision was determined by the repeated analysis of 3 human validation sera that generated full sigmoid dilution–response curves and represented a range of high, medium and low ED50 values (AVR216, ED50=714; AVR284, ED50=345; AVR370, ED50=225). Intra-operator precision was high and ranged from 10.5 to 15.5%CV and met the acceptance criteria of ≤30% (Table 1). Intermediate (inter-operator) precision was also high, with 13.5%–14.5%CV (Table 1). The longitudinal performance of the assay, as determined from 3116 independent AVR414 standard reference curves tested over a 25 month period, was ED50=976 (SE=4.1) and intermediate precision was 23.2%CV. Goodness of fit (mean r^2) for these data was $r^2=0.990$ (Table 2). The proportion of ED50 values >2SD from the assigned value were 7.5% and 1.2% for AVR414 and AVR801 respectively. These proportions comprise all assays including those not meeting the QC criteria (QC failures). The low frequencies of discrepant plates were distributed similarly between high and low ED50 values (data not shown). Performance ranges and intermediate precision of the QT and TT for these data were QT=1739 (SE=10.7) with 34.3%CV and TT=2875 (SE=20.4) with 39.6%CV (Table 2). Similar evaluation of the assay determined from 3130 independent AVR801 standard reference

Table 1
Precision and intermediate precision of the TNA assay using human sera

	Sample	*n*	Mean observed ED50	Std dev	%CV	Pass/fail acceptance criteria
Operator 1 precision	AVR216	12	688	107	15.5%	Pass
	AVR284	12	315	38	11.9%	Pass
	AVR370	12	210	28	13.2%	Pass
Operator 2 precision	AVR216	12	614	65	10.5%	Pass
	AVR284	12	282	37	13.1%	Pass
	AVR370	12	187	24	13.0%	Pass
Intermediate precision	AVR216	24	651	94	14.5%	Pass
	AVR284	24	299	40	13.5%	Pass
	AVR370	24	199	28	14.2%	Pass

Precision and intermediate precision were assessed by two independent operators testing 3 samples a minimum of 4 times on each of three non-consecutive days. Intra-operator precision was expressed as the %CV of the ED50 values for each sample for each operator. Intermediate (inter-operator) precision was expressed as the overall %CV of the ED50 values for each sample for both operators.
n = number of observations.

curves tested over a 26 month period was ED50 = 548 (SE = 2.0) and intermediate precision of 20.6%CV. Goodness of fit (mean r^2) for these data was $r^2 = 0.994$. Performance ranges and intermediate precision of the QT and TT for these data were QT = 902 (SE = 4.5) with 27.6%CV and TT = 1405 (SE = 9.1) with 36.3%CV (Table 2). Macaque and rabbit positive control sera tested ($n = 50$) by multiple operators also showed acceptable levels of intermediate precision for the ED50 of 27.7%CV and 20.5%CV, respectively (data not shown). The combined data indicate a very high agreement of the assay with the 4-PL model, high precision and the assay did not drift in extended use.

3.2. Accuracy and inferred accuracy

Accuracy of anti-PA IgG concentration measurements was determined by repeated analysis of 12 positive control human anti-AVA antisera for which the 'expected' anti-PA IgG concentration was assigned by ELISA and a purified murine monoclonal anti-PA IgG1 (AVR1046) whose protein concentration was assigned by absorbance at 280 nm. The 'observed' values were calculated by interpolation to the reference standard on the TNA assay plate. For accuracy of calculated IgG concentrations the data show a range of percent errors for all test sera of 2.4–51.2%E, only two of which were in an acceptable range of <25%E (Table 3). Precision for these data ranged from 6.1 to 19.5%CV except for one sample (AVR386) which had a 39.6%CV. The low accuracy together with the generally high precision indicated that neutralization efficacy of sera with

Table 2
Evaluation of assay performance with extended use

Variable	AVR414 mean performance data by time period (months)			Combined AVR414 data and precision (%CV) $n = 3116$			AVR801 mean performance data by time period (months)			Combined AVR801 data and precision (%CV) $n = 3130$		
	1–8 ($n = 1102$)	9–20 ($n = 1466$)	21–25 ($n = 548$)	Mean	Standard error	%CV	25–32 ($n = 1015$)	33–44 ($n = 1106$)	45–50 ($n = 1009$)	Mean	Standard error	%CV
ED50 ('*c*' parameter)	961	1025	878	976	4.1	23.2%	519	558	565	548	2.0	20.6%
QT	1730	1822	1536	1739	10.7	34.3%	864	961	875	902	4.5	27.6%
TT	2903	2999	2489	2875	20.4	39.6%	1349	1561	1291	1405	9.1	36.3%
RSQRD	0.991	0.989	0.991	0.990	<0.015	0.9%	0.994	0.994	0.994	0.994	<0.015	0.5%
ODNEGMEAN	0.249	0.225	0.247	0.237	<0.015	26.4%	0.224	0.233	0.234	0.230	<0.015	17.3%
ODTRIPMAX	1.457	1.474	1.474	1.468	<0.015	19.8%	1.372	1.312	1.355	1.345	<0.015	17.7%
ODTRIPMIN	0.303	0.284	0.285	0.291	<0.015	29.6%	0.239	0.258	0.254	0.251	<0.015	20.4%
ODCVMAX	7.3%	7.9%	6.9%	7.5%	<0.015	N/A	6.9%	6.5%	6.4%	6.6%	<0.015	N/A
ODPNCMEAN	1.431	1.404	1.395	1.412	<0.015	18.3%	1.324	1.264	1.303	1.296	<0.015	17.6%
'*a*' parameter	0.290	0.270	0.280	0.279	<0.015	29.1%	0.243	0.260	0.262	0.255	<0.015	20.4%
'*b*' parameter	1.460	1.469	1.473	1.466	<0.015	20.2%	1.386	1.320	1.355	1.353	<0.015	18.1%
'*d*' parameter	2.282	2.384	2.412	2.353	<0.015	19.9%	2.627	2.520	3.093	2.739	<0.015	26.9%

Assay stability, longitudinal performance and drift were assessed from the characteristics of reference standards AVR414 and AVR801 over a 50 month period (25 months' AVR414; 26 months' AVR801 with 1 month overlap). Additional parameters reviewed included the Quantification Titer (QT), Threshold Titer (TT), mean r^2 of the 4-PL fit for each reference (RSQRD), mean OD of the negative control (ODNEGMEAN), maximum OD triplicate mean for a sample (ODTRIPMAX), minimum OD triplicate mean for a sample (ODTRIPMIN), maximum CV of the dilution OD values for a sample (ODCVMAX), mean OD of the positive neutralization control (ODPNCMEAN), and the lower asymptote '*a*', upper asymptote '*b*' and slope '*d*' parameters of the 4-PL fit. N/A = not applicable.

Table 3
Assessment of accuracy using anti-PA IgG mass values

Sample	*n*	Expected IgG (μg/ml)	Observed mean IgG (μg/ml)	Observed std dev	% CV	% Error
AVR216	12	102.0	99.6	14.4	14.5	2.4
AVR284	12	100.9	49.4	7.3	14.7	51.0
AVR286	12	34.0	16.6	3.2	19.3	51.2
AVR329	11	380.8	267.6	52.2	19.5	29.7
AVR342	11	31.6	20.1	3.7	18.5	36.4
AVR350	11	151.0	126.9	16.5	13.0	16.0
AVR353	12	43.1	29.9	2.7	9.2	30.7
AVR368	13	326.8	220.7	38.5	17.4	32.5
AVR370	13	59.8	32.5	2.4	7.4	45.7
AVR382	12	263.1	185.7	22.2	12.0	29.4
AVR386	12	137.1	90.4	35.8	39.6	34.1
AVR401	12	131.4	88.8	11.4	12.9	32.4
AVR1046	54	5200.0	2828.6	171.2	6.1	45.6

Accuracy was expressed as the percent error (%E) between the assay-determined (observed) value and the assigned (expected) value for the validation sera ($n \geq 11$). Accuracy was evaluated by repeated analysis of 12 positive control human anti-AVA antisera using anti-PA IgG concentrations assigned by independent ELISA and one murine monoclonal anti-PA IgG with a mass value assigned by absorbance at 280 nm. Samples were tested 3 times by 3 independent operators. The observed anti-PA IgG values were calculated in the TNA assay by interpolation to the AVR414 reference standard with a calibration factor of 141.2 μg/ml anti-PA IgG.

predetermined anti-PA IgG mass values may not be determined by interpolation to a standard reference serum with a fixed anti-PA IgG calibration factor.

Inferred accuracy of the TNA assay ED50 however, was high for samples above the LLOQ. Inferred accuracy was determined using 21 spiked positive human sera that represented a range of expected ED50 values from 10.8 to 940.8. All validation human sera with an ED50 ≥36 (LLOQ) had low percent errors (%E) ranging from 3.4 to 20.5%E with precision ranging from 7.4 to 26.4%CV (Table 4), thus demonstrating high levels both of accuracy and precision for the ED50 for a cell-based assay (Hering et al., 2004; Wei et al., 2007).

3.3. Dilutional linearity

Dilutional linearity of the assay is the ability to elicit results that are directly, or by a well-defined mathematical transformation, proportional to the neutralizing efficacy of a test sample. Data below the empirical LLOQ were included in these analyses. For validation of this method, the fit of all data for the regression analysis of $\log_{10}$ observed vs. expected ED50 values was required to have a mean $r^2 \geq 0.900$ and a slope between 0.7 and 1.3 (unpublished development data). The ED50

Table 4
Assessment of inferred accuracy using assigned ED50 values and spike-recovery of human sera

Sample	*n*	Expected ED50	Observed mean ED50	Observed range	Observed std dev	%CV	% Error	Pass/fail acceptance criteria
AVR284-32	4	11	0[1]	0–0	0	N/A	100.0[2]	Fail
AVR414-64	4	15	0[1]	0–0	0	N/A	100.0[2]	Fail
AVR216-64	4	11	3[1]	0–11	6	200.0[2]	75.4[2]	Fail
AVR414-48	4	20	3[1]	0–12	6	200.0[2]	84.7[2]	Fail
AVR284-24	4	14	6[1]	0–24	12	200.0[2]	58.2[2]	Fail
AVR216-48	4	15	12[1]	0–24	10	80.1[2]	17.7	Pass
AVR284-16	4	22	15[1]	0–32	17	116.3[2]	31.6[2]	Fail
AVR414-32	4	29	17[1]	0–27	12	69.7[2]	43.0[2]	Fail
AVR216-32	4	22	21[1]	13–29	8	40.6[2]	7.0	Fail
AVR216-16	4	45	36*	30–40	5	13.5	20.5	Pass
AVR284-8	4	43	39	31–49	8	19.5	9.5	Pass
AVR414-16	4	59	53	44–60	7	13.3	10.7	Pass
AVR284-4	3	86	82	61–104	22	26.4	5.3	Pass
AVR216-8	4	89	86	74–100	12	14.2	3.4	Pass
AVR414-8	4	118	105	97–113	8	7.5	10.5	Pass
AVR284-2	4	172	145	124–163	16	11.3	15.9	Pass
AVR216-4	4	179	160	141–173	14	8.6	10.5	Pass
AVR414-4	4	235	213	193–231	18	8.2	9.4	Pass
AVR284	12	345	301	259–343	26	8.5	12.8	Pass
AVR216	12	714	676	580–794	65	9.7	5.4	Pass
AVR414	12	941	821	734–920	61	7.4	12.8	Pass

Inferred accuracy was determined by spike-recovery analysis of 21 human positive sera for which the ED50 had been computed from the dilution factor of the parental sample. Samples were tested by two operators on non-consecutive days. Assay-based ED50 was calculated and compared to an expected ED50 and %E was reported. For all accuracy experiments, each of 21 human, 7 rabbit and 7 Rhesus macaque samples was tested a minimum of 3 times by at least 2 independent operators. *Lower limit of quantification (LLOQ). [1] = below LLOQ; [2] = out of acceptance range.

dilutional linearity regression characteristics for human sera ($n=83$ observations) were $r^2=0.985$, slope=0.99 and intercept=−0.03 (Fig. 2.A, Table 5). For Rhesus macaque sera the dilutional linearity regression data ($n=35$) were $r^2=0.993$, slope=0.95 and intercept=0.06. For rabbit sera the regression data ($n=35$) were $r^2=0.990$, slope=0.98, and intercept=0.005. Regression intercepts for all genera were not significantly different from 0 ($p=0.05$).

The QT and TT regression data from each of these data sets were also evaluated (Table 5). The regression intercepts for QT and TT using Rhesus macaque and rabbit sera were not significantly different from 0 ($p=0.05$). For human sera, the QT regression intercept was not significantly different from 0 ($p>0.05$), but only if values below LLOQ were excluded (data not shown). The human TT regression intercept was significantly different.

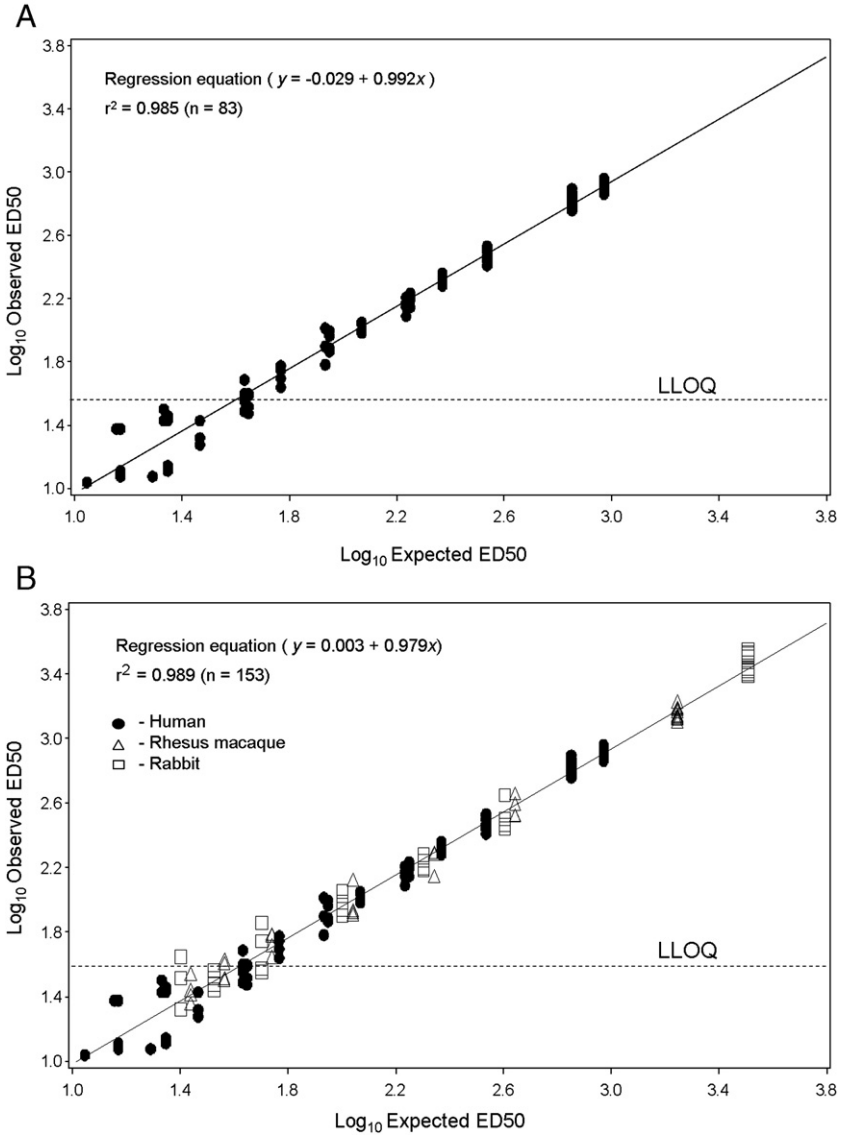

Fig. 2. Dilutional linearity regression analysis of human, Rhesus macaque and rabbit sera. Plot of weighted least squares regression analysis of $\log_{10}$ transformed dilutional linearity data for (A) human sera and (B) combined human (●), Rhesus macaque (Δ) and rabbit (○) sera. Sera were prepared as described in the text. Partial curves were constrained to an upper asymptote as described in the text. All serum tests were done in triplicate in a minimum of two separate assays. Assay validation acceptance criteria for dilutional linearity were 1) the slope of the regression for the ED50 values was between the range 0.7 and 1.3 and 2) a squared correlation coefficient (r^2) ≥0.900.

Table 5
Dilutional linearity regression statistics for human, Rhesus macaque and rabbit sera

Genus	Reportable value	r^2	Slope	Intercept	Observations (n)
Human	ED50	0.985	0.99	−0.03	$n=83$
	QT	0.984	0.91	0.13*	
	TT	0.980	0.86	0.22*	
Rhesus macaque	ED50	0.993	0.95	0.06	$n=35$
	QT	0.990	0.96	0.05	
	TT	0.983	0.96	0.04	
Rabbit	ED50	0.990	0.98	0.005	$n=35$
	QT	0.986	0.97	0.02	
	TT	0.978	0.96	0.04	
Combined	ED50	0.989	0.98	0.003	$n=153$
	QT	0.985	0.95	0.06	
	TT	0.975	0.92	0.09	

Dilutional linearity of human, Rhesus macaque and rabbit sera was evaluated using a weighted regression analysis of $\log_{10}$ transformed observed vs. expected data. A two-sided t-test was used to determine whether the intercepts of regression data were significantly different from the origin. The r^2 statistic in combination with the slope and the y-intercept was used as a measure of goodness of fit for the regression. For assay validation, the fit of all data for the regression analysis of $\log_{10}$ observed vs. expected ED50 values was required to have a mean $r^2 \geq 0.900$ and a slope between 0.7 and 1.3. * = value not significantly different from 0 ($p=0.05$).

Combining data from humans, macaques and rabbits, the ED50 dilutional linearity data ($n=153$) demonstrated an $r^2=0.989$, slope=0.98 and an intercept of 0.003 (Fig. 2.B, Table 5). For the ED50 data, the slopes of the linear regression equations for each of human, Rhesus macaque and rabbit data were not significantly different from each other or from 1.0 ($p>0.05$). Analysis of covariance (ANCOVA) demonstrated that these data may be interpreted as equivalent. This is a strong indicator of the intergenus application of the assay using ED50 as the RV. For the QT and TT however, although the intergenus aggregate dilutional linearity data had good characteristics for slope, intercept and regression coefficient (Table 5), ANCOVA demonstrated that there was a difference between genera. We interpret this to mean that neither QT nor TT may be used as an intergenus RV in this assay. The differences in the regression data for the QT and TT may be attributable to differences in the slope of each genus' data in the 4-PL model which become accentuated at the low and high response regions of the curve. Additional implications of this are that multipoint interpolation from a test curve to the standard curve should also be avoided unless adequate parallelism is demonstrated (Plikaytis et al., 1994).

In this study, the dilutional linearity r^2 statistic, in combination with the slope and the y-intercept, was used as a measure of goodness of fit of these data. Because the slopes were very close to 1.0 and the intercepts close to 0, a high level of agreement between the measured and the expected values is indicated. In addition, the high positive values of the r^2 statistics for all genera and RVs tested demonstrated that this agreement was very precise.

3.4. ED50 lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

LLOD and LLOQ were calculated from the same data set as for dilutional linearity. Data indicated that the empirical LLOD for this assay under normal conditions was an ED50=12 and the LLOQ was ED50=36 (13.5% CV, 20.5%E; Table 4). These limits were reaffirmed by partial revalidation of the assay using reference standard AVR801, the data for which indicated an LLOD ED50=11 and an LLOQ ED50=36 (27.4%CV, 4.4%E; data not shown).

The human serum LLOQ for QT and TT with precision criteria of ≤40%CV and ≤50%CV respectively and accuracy criteria of ≤40%E and ≤50%E respectively, were QT=41 (21%CV, 0%E, $n=4$) and TT=59 (10.3%CV, 17.5%E, $n=4$). The lowest dilution of serum used in the assay is 1/50, thus the empirical LLOQ using the QT within authentic data points is 50. Both the QT and TT are therefore suitable intra-genus reportable endpoints titers for this assay.

3.5. Diagnostic sensitivity and specificity

The DSP was determined from a panel of 195 serum samples from individuals with clinically confirmed non-anthrax related illnesses, individuals receiving non-anthrax related vaccines and normal sera. The DSN test panel comprised 100 sera obtained from humans with either clinically confirmed anthrax or a known history of vaccination with anthrax vaccine adsorbed (AVA). No false negatives or false positives were detected, indicating that this assay has 100% DSP and 100% DSN under the conditions described.

3.6. Robustness

Robustness is a measure of the assay's capacity to remain unaffected by small but deliberate variations in the method parameters. The standard operational conditions for this TNA assay are CO_2 concentrations of 5% and a 2 h incubation using a stock MTT solution of 5 mg/ml. Changing incubator temperature conditions from 35 °C to 39 °C demonstrated an inter-condition %

CV range from 0.1 to 6.6%CV, indicating that the assay tolerated this range of temperatures. Comparison of CO_2 concentrations of 5% vs. 3% (vol/vol) and 3% vs. 7% (vol/vol) demonstrated ED50 values within an acceptable range of 2 standard deviations from the established mean of the samples tested and had acceptable inter-condition precision of 5.8%CV and 8.6%CV respectively. On the basis of inter-condition CVs, these data indicate that CO_2 concentration is an important parameter but that the assay will tolerate fluctuations in CO_2 concentrations from 3% to 7% (vol/vol). Similarly, when comparing cell intoxication incubation times of 3.5 h vs. 4.5 h, the ED50 values of all test samples were within an acceptable range of 2SD and the inter-condition %CV for this comparison was 8.4%. Thus the assay can tolerate variations in cell intoxication incubation times between 3.5 and 4.5 h. MTT incubation times of 1.5 h vs. 2.5 h generated low inter-condition % CV, ranging from only 5.5 to 8.3%. Comparison of MTT concentrations of 5 mg/ml vs. 7.5 mg/ml and 2.5 mg/ml vs. 7.5 mg/ml generated inter-condition CVs ranging from 7.6% to 15.5%. On the basis of these data, the assay tolerated MTT incubation times of 1.5–2.5 h and MTT concentrations of 2.5–7.5 mg/ml.

3.7. Cell passage number

The effect of cell passage number (P) on the reportable ED50 was determined by evaluation of inter-condition %CVs at P≤15, 20 and 25 cell passages. Inter-condition %CVs, when comparing P≤15 vs. P20, P≤15 vs. P25 and P20 vs. P25, ranged from 0.2 to 12.1%CV, 15.7 to 25.3%CV and 17.9 to 32.3%CV, respectively (Table 6). On the basis of inter-condition CVs, the assay was qualified to test serum with cells that have undergone up to 25 cell passages. In practice, however, the recommended number of cell passages is ≤20. By limiting the passage number in this way, the intention is to maintain longitudinal assay performance characteristics and avoid adjusting key parameters such as cell plating density.

Table 6
Evaluation of the effect of differing cell passage numbers on ED50 endpoints

Cell passage	Mean ED50			
	AVR216	AVR284	AVR370	AVR414
P≤15	714 (*n*=102)	345 (*n*=156)	225 (*n*=140)	941 (*n*=460)
P20	602 (*n*=24)	313 (*n*=24)	226 (*n*=24)	938 (*n*=24)
P25	959 (*n*=24)	431 (*n*=24)	292 (*n*=24)	1352 (*n*=24)
Inter-condition comparison	**Inter-condition %CV**			
P≤15 vs. P20	12.1%CV	6.8%CV	0.3%CV	0.2%CV
P≤15 vs. P25	20.7%CV	15.7%CV	18.2%CV	25.3%CV
P20 vs. P25	32.3%CV	22.4%CV	17.9%CV	25.6%CV

The precision of 3 test samples and the standard reference serum AVR414 was evaluated at cell passage numbers P≤15, P20 and P25. For each cell passage number, a minimum of 24 tests were completed for each sample. Mean ED50 values were compared using inter-condition precision (%CV).

4. Discussion and conclusions

As a result of the anthrax bioterrorism attacks in 2001 (Jernigan et al., 2001), the Department of Health and Human Services (DHHS) prioritized the development and acquisition of medical countermeasures against biological threat agents, including *B. anthracis* (Russell, 2007). As a measure of functional activity, the TNA assay is of increasing importance for evaluation and comparison of current and next generation anthrax vaccines and development of LTx countermeasures for inhalation anthrax. Comparison and selection of countermeasures using standardized assay technologies is therefore a high priority. The TNA assay was developed previously, validated in a variety of formats and used to evaluate the toxin neutralizing potential of polyclonal and monoclonal antibodies raised against anthrax vaccines and anthrax toxin components (Friedlander, 1986; Little et al., 1997; Hering et al., 2004). Standardization of the test reagents, the test procedure, interpretation of performance characteristics and a specific method for data reduction to ensure comparability of data from different sources has however, not previously been accomplished. Given the importance of biological assay standardization in vaccine research and product development (Rymer et al., 1999; Borrow et al., 2006), it was our objective to create a unifying technology platform with broad application for *in vitro* quantification of LTx neutralization activity and to facilitate its widespread use as a framework for standardized comparison of licensed and newly developed anthrax vaccines, therapeutics and toxin inhibitors (Casadevall, 2002). To meet this objective we have developed and validated a highly standardized version of the TNA assay in the J774A.1 murine cell line. We have established and made available a comprehensive package of detailed protocols, procedures, cells, reagents and customized analytical software. The primary reportable value is the dilution of serum that provides 50% protection (ED50) against a fixed concentration of anthrax lethal toxin (LTx). The ED50 is calculated from the inflection point of a 4-PL model fit

of the serum neutralization curve. A curve recognition algorithm in the analytical software permits extrapolation of neutralization response curves with incomplete upper asymptote formation (low potency) and facilitates accurate determination of ED50 values below the starting dilution of the assay (1/50). This approach was substantiated by dilutional linearity spike-recovery studies in which ED50 values could be calculated as low as ED50=12. In addition, the customized analytical software uses the 1st and 2nd derivatives of the 4-PL fit to report additional parameters, the Threshold Titer (TT) and Quantification Titer (QT).

The assay as presented has high inferred accuracy and precision with 100% diagnostic sensitivity and diagnostic specificity. The TNA assay is also rugged and robust, withstanding variation in a range of key operational procedures and conditions. Nonetheless, the successful implementation of the standardized assay format is dependent on achieving a balance of several key parameters of cell growth, cell density, LTx protein concentration, LTx potency to meet the QC and performance criteria of the standard reference serum. These key parameters include a well-defined upper asymptote constraint (100% cell survival) and a maximum allowable lower asymptote (minimum level of LTx-mediated cell killing) with sufficient depth of curve between asymptotes to fit the 4-PL dilution–response model to the standard reference serum data. Operational assessment of these has been incorporated into the assay quality control (QC) criteria of the customized analytical software. Critical steps to meeting these criteria and the successful execution of the standardized assay include harvesting of the target cells at late exponential growth phase, plating of sufficient cell density to maximize reporter signal and robustly measure changes in neutralization, and the titration of the appropriate concentration of anthrax lethal toxin proteins to achieve 95% cell lysis. We validated the TNA assay for human sera using ED50 and demonstrated its intergenus application by analysis of covariance (ANCOVA) of aggregate dilutional linearity data from human, Rhesus macaque and rabbit sera.

There is generally a high positive correlation between anti-PA IgG antibody levels and LTx neutralization efficacy in serum from clinical anthrax cases and recipients of anthrax vaccines (Pittman et al., 2002; Quinn et al., 2004; Gorse et al., 2006; Hanson et al., 2006; Semenova et al., 2007). Empirical spike-recovery analyses in this study, however, demonstrated that although anti-PA IgG mass values could be calculated from the TNA assay standard curve with high precision, the accuracy of this approach was generally low, with only 2 of 13 samples tested, including one monoclonal anti-PA, having a %E <25%. This finding may be attributed to the presence of additional anti-PA Ig isotypes in polyclonal antiserum, inherent differences in anti-PA Ig epitope recognition relative to neutralization potential and the undefined epitope interactions of antibodies with different conformers of LTx. This is also the case for purified monoclonal antibodies, the neutralizing potential of which are dependent on the epitope and the affinity of the antibody–antigen interaction. This interpretation has important impact on the characterization of antibody-based medical countermeasures for anthrax. The data provided by this study strongly suggest that the neutralizing efficacy of an antibody preparation should not be expressed using solely either antigen specific binding or assignment of neutralization units by interpolation to a standard curve in the TNA assay. For polyclonal antiserum this is primarily because quantitative antigen binding may not fully reflect neutralization and simple interpolation to a neutralizing standard discounts the antigen-binding but non-neutralizing constituents of the antibody population. For monoclonal antibodies, depending on the recognized epitope, high affinity binding may occur in the absence of neutralization (Mohamed et al., 2004). These data and their interpretation lead us to suggest that neutralizing efficacy of antibody preparations, and other inhibitors of LTx, should be expressed as a specific neutralizing activity such as the effective concentration providing 50% neutralization (EC50) under standardized conditions (Lim et al., 2005).

The TNA assay as described here has been in use at CDC for more than 4 years. This format of the TNA assay uses standardized and characterized reagents, in conjunction with customized interpretive software and a novel mathematical algorithm, to calculate and extrapolate multiple reportable values, in addition to ED50, with high specificity, analytical sensitivity, accuracy and precision. This format has been tested extensively and applied broadly to evaluate antibody responses in clinical anthrax, for vaccine evaluation in animal models and human clinical trials and to provide *in vitro* characterization of anthrax immunotherapeutics (Quinn et al., 2004; Semenova et al., 2004, 2007; Williamson et al., 2005; Gorse et al., 2006; Albrecht et al., 2007). The extended inter-laboratory precision and ruggedness of the TNA assay, together with development of a high-throughput format and an evaluation of the NF-50 as a reportable value, are currently under evaluation in an independent study (F. Lynn, NIAID, personal communication). The assay validation master file is on record with the Food and Drug Administration (1999) (FDA; BB MF 12964) and the detailed protocols, standard

reference serum and the interpretive software are available from CDC. This highly precise, accurate and robust version of the anthrax TNA assay reduces the variance associated with these types of assays (Zmuda et al., 2005), establishes a point of reference for standardized analysis of antibody responses to anthrax vaccines and develops the critical link between pre-clinical animal models of vaccine efficacy and humoral antibody responses in human vaccinees (Pitt et al., 2001; Little et al., 2004; Williamson et al., 2005).

Acknowledgements

We thank Dr. Stephen Leppla (NIAID, NIH) for providing recombinant PA and LF for the initiation of this study. We acknowledge LTC. Phillip R. Pittman (USAMRIID) for providing a panel of negative control and AVA vaccinee sera, Dr. Barry Fields (NCIRD, CDC) for providing sera from clinical cases of legionellosis, *Chlamydia pneumoniae* infection and *Mycoplasma pneumoniae* infection, Dr. Patrick Schlievert (University of Minnesota, Minneapolis MN) for providing sera from clinical cases of staphylococcal toxic shock syndrome and group A streptococcal infections, Sandra Bragg (CDC) for providing sera from clinical cases of brucellosis, Miriam Alter, Ian Williams and Wendi Kuhnert (CDC) for providing sera from clinical cases of hepatitis-A, hepatitis-B and influenza antisera, Henrietta Hall (CDC) for providing antisera from hepatitis and influenza vaccinees. We thank Ed Nuzum, Judy Hewitt, Scott Winram, Freyja Lynn, Emily Kough, Eileen Flynn (NIAID, DMID, NIH) for enthusiastic support and interagency collaboration and Brian D. Plikaytis (DBD, NCIRD, CDC) for critical review and suggestions for statistical analyses. SDS, LXC, JWO, JC and CP were funded through the Atlanta Research and Education Foundation (AREF), Atlanta, GA.

References

Agrawal, A., Lingappa, J., Leppla, S.H., Agrawal, S., Jabbar, A., Quinn, C., Pulendran, B., 2003. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. Nature 424, 329.

Arora, N., Williamson, L.C., Leppla, S.H., Halpern, J.L., 1994. Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells. J. Biol. Chem. 269, 26165.

Albrecht, M.T., Li, H., Williamson, E.D., Lebutt, C.S., Flick-Smith, H.C., Quinn, C.P., Westra, H., Galloway, D., Mateczun, A., Goldman, S., Groen, H., Baillie, L.W., 2007. Human monoclonal antibodies against anthrax lethal factor and protective antigen act independently to protect against *Bacillus anthracis* infection and enhance endogenous immunity to anthrax. Infect. Immun. 11, 5425.

Baldari, C.T., Tonello, F., Paccani, S.R., Montecucco, C., 1996. Anthrax toxins: a paradigm of bacterial immune suppression. Trends Immunol. 27, 434.

Borrow, R., Carlone, G.M., Rosenstein, N., Blake, M., Feavers, I., Martin, D., Zollinger, W., Robbins, J., Aaberge, I., Granoff, D.M., Miller, E., Plikaytis, B., van Alphen, L., Poolman, J., Rappuoli, R., Danzig, L., Hackell, J., Danve, B., Caulfield, M., Lambert, S., Stephens, D., 2006. *Neisseria meningitidis* group B correlates of protection and assay standardization—international meeting report Emory University, Atlanta, Georgia, United States, 16–17 March 2005. Vaccine 24, 5093.

Brossier, F., Levy, M., Landier, A., Lafaye, P., Mock, M., 2004. Functional analysis of *Bacillus anthracis* protective antigen by using neutralizing monoclonal antibodies. Infect. Immun. 72, 6313.

Casadevall, A., 2002. Passive antibody administration (immediate immunity) as a specific defense against biological weapons. Emerg. Infect. Dis. 8, 833.

Cui, X., Li, Y., Li, X., Laird, M.W., Subramanian, M., Moayeri, M., Leppla, S.H., Fitz, Y., Su, J., Sherer, K., Eichacker, P., 2007. *Bacillus anthracis* edema and lethal toxin have different hemodynamic effects but function together to worsen shock and outcome in a rat model. J. Infect. Dis. 195, 572.

Duesbery, N.S., Webb, C.P., Leppla, S.H., Gordon, V.M., Klimpel, K.R., Copeland, T.D., Ahn, N.G., Oskarsson, M.K., Fukasawa, K., Paull, K.D., Vande Woude, G.F., 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280, 734.

Ezzell, J.W., Ivins, B.E., Leppla, S.H., 1984. Immunoelectrophoretic analysis, toxicity, and kinetics of in vitro production of the protective antigen and lethal factor components of *Bacillus anthracis* toxin. Infect. Immun. 45, 761.

Food and Drug Administration, 1999. Guidance for Industry: Validation of Analytical Procedures: Methodology: Final Guidance. Found at http://www.fda.gov/cvm/Guidance/guida64.htm.

Friedlander, A.M., 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. 261, 7123.

Friedlander, A.M., Bhatnagar, R., Leppla, S.H., Johnson, L., Singh, Y., 1993. Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. Infect Immun. 61, 245.

Gorse, G.J., Keitel, W., Keyserling, H., Taylor, D.N., Lock, M., Alves, K., Kenner, J., Deans, L., Gurwith, M., 2006. Immunogenicity and tolerance of ascending doses of a recombinant protective antigen (rPA102) anthrax vaccine: a randomized, double-blinded, controlled, multicenter trial. Vaccine 24, 5950.

Guidi-Rontani, C., Weber-Levy, M., Labruyère, E., Mock, M., 1999. Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol. Microbiol. 31, 9.

Hanna, P.C., Acosta, D., Collier, R.J., 1993. On the role of macrophages in anthrax. Proc. Natl. Acad. Sci. U. S. A. 90, 10198.

Hansen, M.B., Nielsen, S.E., Berg, K., 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119, 203.

Hanson, J.F., Taft, S.C., Weiss, A.A., 2006. Neutralizing antibodies and persistence of immunity following anthrax vaccination. Clin. Vaccine Immunol. 13, 208.

Hering, D., Thompson, W., Hewetson, J., Little, S., Norris, S., Pace-Templeton, J., 2004. Validation of the anthrax lethal toxin neutralization assay. Biologicals 32, 17.

Huber, M., Vor Dem Esche, U., Grunow, R., Bessler, W.G., 2005. Generation of mouse polyclonal and human monoclonal antibodies against *Bacillus anthracis* toxin. Drugs Exp. Clin. Res. 31, 35.

Hull, A.K., Criscuolo, C.J., Mett, V., Groen, H., Steeman, W., Westra, H., Chapman, G., Legutki, B., Baillie, L., Yusibov, V., 2005. Human-

derived, plant-produced monoclonal antibody for the treatment of anthrax. Vaccine 23, 2082.
Jernigan, J.A., Stephens, D.S., Ashford, D.A., et al., 2001. Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States. Emerg. Infect. Dis. 7, 933.
Klein, F., Lincoln, R.E., Dobbs, J.P., Mahlandt, B.G., Remmele, N.S., Walker, J.S., 1968. Neurological and physiological responses of the primate to anthrax infection. J. Infect. Dis. 118, 97.
Leppla, S.H., 1999. The bifactorial *Bacillus anthracis* lethal and oedema toxins, In: Alouf, J.E., Freer, J.H. (Eds.), The Comprehensive Sourcebook of Bacterial Protein Toxins, 2nd edition. Academic Press, London, p. 243.
Little, S.F., Ivins, B.E., Fellows, P.F., Friedlander, A.M., 1997. Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea pigs. Infect. Immun. 65, 5171.
Little, S.F., Ivins, B.E., Fellows, P.F., Pitt, M.L., Norris, S.L., Andrews, G.P., 2004. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. Vaccine 22, 422.
Lim, N.K., Kim, J.H., Oh, M.S., Lee, S., Kim, S.Y., Kim, K.S., Kang, H.J., Hong, H.J., Inn, K.S., 2005. An anthrax lethal factor-neutralizing monoclonal antibody protects rats before and after challenge with anthrax toxin. Infect. Immun. 73, 6547.
Moayeri, M., Haines, D., Young, H.A., Leppla, S.H., 2003. *Bacillus anthracis* lethal toxin induces TNF-α-independent hypoxia-mediated toxicity in mice. J. Clin. Invest. 112, 670.
Mogridge, J., Cunningham, K., Collier, R.J., 2002. Stoichiometry of anthrax toxin complexes. Biochemistry 41, 1079.
Mohamed, N., Li, J., Ferreira, C.S., Little, S.F., Friedlander, A.M., Spitalny, G.L., Casey, L.S., 2004. Enhancement of anthrax lethal toxin cytotoxicity: a subset of monoclonal antibodies against protective antigen increases lethal toxin-mediated killing of murine macrophages. Infect. Immun. 72, 3276.
Pitt, M.L., Little, S.F., Ivins, B.E., Fellows, P., Barth, J., Hewetson, J., Gibbs, P., Dertzbaugh, M., Friedlander, A.M., 2001. In vitro correlate of immunity in a rabbit model of inhalational anthrax. Vaccine 19, 4768.
Pittman, P.R., Kim-Ahn, G., Pifat, D.Y., Coonan, K., Gibbs, P., Little, S., Pace-Templeton, J.G., Myers, R., Parker, G.W., Friedlander, A.M., 2002. Anthrax vaccine: immunogenicity and safety of a dose-reduction, route-change comparison study in humans. Vaccine 20, 1412.
Pittman, P.R., Leitman, S.F., Oro, J.G., Norris, S.L., Marano, N.M., Ranadive, M.V., Sink, B.S., McKee Jr., K.T., 2005. Protective antigen and toxin neutralization antibody patterns in anthrax vaccinees undergoing serial plasmapheresis. Clin. Diagn. Lab. Immunol. 12, 713.
Pittman, P.R., Norris, S.L., Barrera Oro, J.G., Bedwell, D., Cannon, T.L., McKee Jr., K.T., 2006. Patterns of antibody response in humans to the anthrax vaccine adsorbed (AVA) primary (six-dose) series. Vaccine 24, 3654.
Plikaytis, B.D., Holder, P.F., Pais, L.B., Maslanka, S.E., Gheesling, L.L., Carlone, G.M., 1994. Determination of parallelism and nonparallelism in bioassay dilution curves. J. Clin. Microbiol. 32, 2441.
Quinn, C.P., Singh, Y., Klimpel, K.R., Leppla, S.H., 1991. Functional mapping of anthrax toxin lethal factor by in-frame insertion mutagenesis. J. Biol. Chem. 266, 20124.
Quinn, C.P., Semenova, V.A., Elie, C.M., Romero-Steiner, S., Greene, C., Li, H., Stamey, K., Steward-Clark, E., Schmidt, D.S., Mothershed, E., Pruckler, J., Schwartz, S., Benson, R.F., Helsel, L.O., Holder, P.F., Johnson, S.E., Kellum, M., Messmer, T., Thacker, W.L., Besser, L., Plikaytis, B.D., Taylor Jr., T.H., Freeman, A.E., Wallace, K.J., Dull, P., Sejvar, J., Bruce, E., Moreno, R., Schuchat, A., Lingappa, J.R., Martin, S.K., Walls, J., Bronsdon, M., Carlone, G.M., Bajani-Ari, M., Ashford, D.A., Stephens, D.S., Perkins, B.A., 2002. Specific, sensitive, and quantitative enzyme-linked immunosorbent assay for human immunoglobulin G antibodies to anthrax toxin protective antigen. Emerg. Infect. Dis. 8, 1103.
Quinn, C.P., Dull, P.M., Semenova, V., Li, H., Crotty, S., Taylor, T.H., Steward-Clark, E., Stamey, K.L., Schmidt, D.S., Stinson, K.W., Freeman, A.E., Elie, C.M., Martin, S.K., Greene, C., Aubert, R.D., Glidewell, J., Perkins, B.A., Ahmed, R., Stephens, D.S., 2004. Immune responses to *Bacillus anthracis* protective antigen in patients with bioterrorism-related cutaneous or inhalation anthrax. J. Infect. Dis. 190, 1228.
Russell, P.K., 2007. Project BioShield: What It Is, Why It Is Needed, and Its Accomplishments So Far, vol. 45, p. S68. C.I.D.
Rymer, J.C., Sabatier, R., Daver, A., Bourleaud, J., Assicot, M., Bremond, J., Rapin, J., Salhi, S.L., Thirion, B., Vassault, A., Ingrand, J., Pau, B., 1999. A new approach for clinical biological assay comparison and standardization: application of principal component analysis to a multicenter study of twenty-one carcinoembryonic antigen immunoassay kits. Clin. Chem. 6, 869.
Semenova, V.A., Steward-Clark, E., Stamey, K.L., Taylor Jr., T.H., Schmidt, D.S., Martin, S.K., Marano, N., Quinn, C.P., 2004. Mass value assignment of total and subclass immunoglobulin G in a human standard anthrax reference serum. Clin. Diagn. Lab. Immunol. 11, 919.
Semenova, V.A., Schmidt, D.S., Taylor Jr., T.H., Li, H., Steward-Clark, E., Soroka, S.D., Ballard, M.M., Quinn, C.P., 2007. Analysis of anti-protective antigen IgG subclass distribution in recipients of anthrax vaccine adsorbed (AVA) and patients with cutaneous and inhalation anthrax. Vaccine 25, 1780.
Singh, Y., Klimpel, K.R., Goel, S., Swain, P.K., Leppla, S.H., 1999. Oligomerization of anthrax toxin protective antigen and binding of lethal factor during endocytic uptake into mammalian cells. Infect. Immun. 67, 1853.
Taylor, T., Quinn, C., Schmidt, D., Freeman, A., Li, H., Semenova, V., Hoekstra, M., Plikaytis, D., 2003. Novel mathematical approach to TNA endpoints. Abstracts of the 5th International Meeting on Anthrax, Nice, France, March 31 to April, vol. 4, p. 2003.
Tang, G., Leppla, S.H., 1999. Proteasome activity is required for anthrax lethal toxin to kill macrophages. Infect. Immun. 67, 3055.
Tessier, J., Green, C., Padgett, D., Zhao, W., Schwartz, L., Hughes, M., Hewlett, E., 2007. Contributions of histamine, prostanoids, and neurokinins to edema elicited by edema toxin from *Bacillus anthracis*. Infect. Immun. 75, 1895.
Vick, J.A., Lincoln, R.E., Klein, F., Mahlandt, B.G., Walker, J.S., Fish, D.C., 1968. Neurological and physiological responses of the primate to anthrax toxin. J. Infect. Dis. 118, 85.
Wang, F., Ruther, P., Jiang, I., Sawada-Hirai, R., Sun, S.M., Nedellec, R., Morrow, P.R., Kang, A.S., 2004. Human monoclonal antibodies that neutralize anthrax toxin by inhibiting heptamer assembly. Hum. Antibodies 13, 105.
Welkos, S., Little, S., Friedlander, A., Fritz, D., Fellows, P., 2001. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology 147, 1677.
Williamson, E.D., Hodgson, I., Walker, N.J., Topping, A.W., Duchars, M.G., Mott, J.M., Estep, J., Lebutt, C., Flick-Smith, H.C., Jones, H.E., Li, H., Quinn, C.P., 2005. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect. Immun. 73, 5978.
Wei, X., Grill, D.S., Heatherington, A.C., Swanson, S.J., Gupta, S., 2007. Development and validation of a quantitative cell-based bioassay for

comparing the pharmacokinetic profiles of two recombinant erythropoietic proteins in serum. J. Pharm. Biomed. Anal. 43, 666.

Xu, L., Frucht, D.M., 2007. *Bacillus anthracis*: a multi-faceted role for anthrax lethal toxin in thwarting host immune defenses. Int. J. Biochem. Cell. Biol. 39, 20.

Zmuda, J.F., Zhang, L., Richards, T., Pham, Q., Zukauskas, D., Pierre, J.L., Laird, M.W., Askins, J., Choi, G.H., 2005. Development of an edema factor-mediated cAMP-induction bioassay for detecting antibody-mediated neutralization of anthrax protective antigen. J. Immunol. Methods 298, 47.