Development of an edema factor-mediated cAMP-induction bioassay for detecting antibody-mediated neutralization of anthrax protective antigen

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Abstract

Intoxication of mammalian cells by Bacillus anthracis requires the coordinate activity of three distinct bacterial proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF). Among these proteins, PA has become the major focus of work on monoclonal antibodies and vaccines designed to treat or prevent anthrax infection since neither EF nor LF is capable of inducing cellular toxicity in its absence. Here, we present the development of a sensitive, precise, and biologically relevant bioassay platform capable of quantifying antibody-mediated PA neutralization. This bioassay is based on the ability of PA to bind and shuttle EF, a bacterial adenylate cyclase, into mammalian cells leading to an increase in cAMP that can be quantified using a sensitive chemiluminescent ELISA. The results of this study indicate that the cAMP-induction assay possesses the necessary performance characteristics for use as both a potency-indicating release assay in a quality control setting and as a surrogate pharmacodynamic marker for ensuring the continued bioactivity of therapeutic antibodies against PA during clinical trials.

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Keywords: Anthrax; Protective antigen; Edema factor; cAMP; Neutralization; Monoclonal antibody

Abbreviations: CHO, Chinese hamster ovary; EF, edema factor; PA, protective antigen; LF, lethal factor; LeTx, lethal toxin; cps, counts per second; CV, coefficient of variation; IBMX, 3-isobutyl-1-methylxanthine; cAMP, cyclic adenosine monophosphate; PAmAb, monoclonal antibody against protective antigen; PAmAB (−), non-specific antibody control; RRA, rubidium release assay; ATR, anthrax toxin receptor; PCR, polymerase chain reaction; PATD, translocation-deficient protective antigen.

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1. Introduction

Intoxication of mammalian cells by Bacillus anthracis comprises a number of distinct steps leading to the death of the infected cells, and often that of the host organism as well. Cellular intoxication begins when full-length, monomeric protective antigen (PA$_{83}$)$_1$ binds to either anthrax toxin receptor (ATR; Escuyer and Collier, 1991), or human capillary morphogenesis protein 2, present on the plasma membrane of most mammalian cells (Scobie et al., 2003). Receptor-bound PA$_{83}$ is cleaved by a furin-class protease liberating a 20-Kd peptide from the N-terminus and yielding receptor-bound (PA$_{63}$)$_1$ (Klimpel et al., 1994). Upon subsequent heptamerization of (PA$_{63}$)$_1$ to (PA$_{63}$)$_7$, binding sites for up to three molecules of edema factor (EF) or lethal factor (LF) are generated within this protein complex (Milne et al., 1994; Elliott et al., 2000). Receptor-bound PA–LF/EF complexes are internalized by receptor mediated endocytosis and shuttled to an endosomal compartment (Gordon et al., 1988) where the low pH causes a conformational change in the structure of PA that allows it to insert into the membrane and form a pore (Blaustein et al., 1989; Koehler and Collier, 1991; Milne and Collier, 1993). EF and LF are released into the cytoplasm (Friedlander, 1986; Gordon et al., 1988) where they interact with their cellular targets. EF, a calmodulin- and calcium-dependent adenyl cyclase, causes a dramatic increase in intracellular cAMP (Leppla, 1982), while LF cleaves and inactivates most mitogen-activated protein kinase kinases (Duesbery et al., 1998), a class of proteins responsible for regulating multiple intracellular signaling pathways.

Among the protein components of the anthrax toxin complex, the majority of studies aimed at treating or preventing anthrax infection have centered on PA for its ability to mediate the intoxication pathway (Casadevall, 2002; Kobiler et al., 2002; Reuveny et al., 2001; Pitt et al., 2001; Beedham et al., 2001; Little et al., 1997). These findings have led to an increased focus on the development of highly specific monoclonal antibodies that neutralize PA activity and have necessitated the creation of relevant and reliable bioassays capable of accurately assessing the potency of such molecules. Currently, two bioassay formats are commonly utilized to determine the effects of anthrax toxin components on mammalian cells in vitro, the rubidium release assay (RRA) and the lethal toxin (LeTx)-mediated killing assay. The RRA measures the release of radioactive rubidium through pores formed in the plasma membrane of Chinese hamster ovary (CHO) cells by PA heptamerization following incubation with PA or combinations of PA and anti-PA (Milne and Collier, 1993). Multiple factors contribute to variability in this assay, however, and the range of antibody concentrations over which a response can be quantified is limited (typically <1 log), making the RRA less desirable as a potency-indicating assay in a quality control setting. In comparison, the LeTx-mediated killing assay provides a more biologically relevant assay for measuring intoxication of target cells that is based upon PA binding and shuttling LF into murine macrophages in a manner thought to be consistent with the naturally occurring pathway (Hanna et al., 1993). This assay has been proven capable of detecting antibody-mediated neutralization of PA/LF activity in serum from vaccinated humans and rabbits and provides a useful means by which the titers of vaccinated individuals can be determined in a relevant assay (Hering et al., 2004).

To address these issues, we have developed and optimized a sensitive and precise EF-mediated, cAMP-induction bioassay capable of quantifying antibody-mediated neutralization of PA activity in vitro based on the initial work of Leppla (1982). By incorporating a chemiluminescent ELISA in place of the subjective CHO cell elongation criteria, or the labor-intensive radioimmunoassay format used previously, we have generated a convenient and reproducible assay for quantifying PA-mediated cellular intoxication in vitro. The results of this study indicate that the cAMP-induction assay possesses the necessary precision, linearity, and reproducibility for use as a potency-indicating release assay in a GMP setting. Additionally, our results indicate that a modified version of the cAMP-induction assay is capable of detecting antibody-mediated neutralization of PA in...
the presence of human serum suggesting a potential usefulness as a surrogate pharmacodynamic marker to ensure the continued efficacy of antibody-based anthrax therapeutics during clinical trials.

2. Materials and methods

2.1. CHO cell culture

CHO-K1 cells (ATTC# CCL-61) were cultured in 75 cm² tissue culture-treated flasks (Costar) with Khaigan’s modified F-12 medium containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Invitrogen, Inc.). Cells were passaged by rinsing once with 10 mL of Ca⁺⁺/Mg⁺⁺-free PBS and then incubated with 2 ml of 0.05% trypsin–EDTA (Clonetics, Inc.) until cells became detached (approximately 4–5 min). Trypsin was neutralized by the addition of 12 ml of pre-equilibrated cell culture medium. Cell density and viability were determined by Trypan Blue staining and counting using a hemacytometer. Cell density was adjusted to 400,000 cells/mL and then 100 μL of cell suspension was added to the inner 60 wells of a 96-well cAMP Screen Direct tissue culture-treated plate (Applied Biosystems, Inc) for a final density of 40,000 cells/well. Cells were incubated at 37 °C in a 5% CO₂ environment with >85% humidity for approximately 24 h before use in the assay.

2.2. Preparation of PA, PATD, EF, and monoclonal antibody to PA (PAmAb)

Protective antigen was produced according to the methods of Laird et al. (2004). Briefly, a synthetic gene encoding residues of the B. anthracis PA protein was constructed by polymerase chain reaction (PCR) using overlapping oligonucleotides. Residues E30-G764 of PA were reverse-translated using codons optimal for expression in the bacterium Escherichia coli. Subsequent amplifications were completed to add heterologous signal peptides to the mature N-terminus of PA. PA was expressed in E. coli and purified using a series of column chromatography steps. A mutant form of PA protein (PATD) was generated by changing two amino acid residues in PA (Lys-397 to Asp and Asp-425 to Lys). This mutant is not able to translocate EF or LF proteins of anthrax toxin (Sellman et al., 2001) and is used as a negative control reagent in assays. The PATD protein was purified from E. coli as described for PA purification. Purified and lyophilized edema factor (EF 173; List Biological Laboratories, Inc.) was diluted to 1 mg/mL with 1% BSA/PBS and stored at −80 °C. Edema factor (EF 1392-4) was produced according to the methods of Cooksey et al. (2004) using a synthetic gene encoding a heterologous signal peptide and residues M34-K800 of the B. anthracis EF protein with codons optimized for expression in E. coli. EF was expressed in E. coli, and then extracted and purified from the cell paste using a series of column chromatography steps.

Human monoclonal antibodies to PA were selected from phage display antibody libraries. One of them, PAmAb (IgG1λ), was chosen to test in assays described in this study. PAmAb was expressed in NS0 cell lines and purified through PROSEP-rA (Millipore) affinity column chromatography followed by SP Sepharose FF (Amersham), Q Sepharose FF (Amersham), and Phenyl Sepharose High Substitution (HS) FF (Amersham) purification steps.

2.3. Detection of antibody-mediated neutralization of cAMP induction

PA and EF were diluted to a final concentration of 1200 ng/mL PA and 100 ng/mL EF 173 by addition to cell culture medium containing 250 μM of 3-isobutyl-1-methylxanthine (IBMX; Sigma) to prevent cAMP degradation. Seventy-five microliters of PA/EF solution was added to the inner 60 wells of three 96-well polypropylene dilution plates. In separate dilution plates, PAmAb neutralizing antibody at an initial concentration of 60 μg/mL was serially diluted 1:3 with cell culture medium containing 250 μM IBMX. Seventy-five microliters of each PAmAb dilution was then added to the corresponding wells of the PA/EF dilution plate, mixed, and allowed to incubate for 1 h at 37 °C. After addition of the PAmAb solutions to the PA/EF dilution plate, the final concentrations of PA and EF were 600 ng/mL and 50 ng/mL, respectively. The final concentration of PAmAb across the plates was 30, 10, 3.3, 1.1, 0.37, 0.12, 0.041, 0.014, 0.005, and 0.0015 μg/mL. Following this pre-incubation
step, cell culture medium was removed from the CHO cells and 100 μL of the neutralization reaction mixtures was added to the corresponding wells of the cell plate and incubated for 1 h at 37 °C. Cells were then lysed by addition of 60 μL of lysis/assay buffer from the cAMP Direct ELISA kit (Applied Biosystems, Inc) followed by incubation for 30 min at 37 °C. Alkaline phosphatase-labeled cAMP was then added to each well along with anti-cAMP antibody according to manufacturer’s instructions and plates were incubated for 1 h at room temperature with shaking. The plates were rinsed 5 times with wash buffer and then incubated with 100 μL of chemiluminescent substrate for 30 min at ambient room temperature. For experiments in which the molar induction of cAMP was determined, cAMP concentrations were estimated based on the cAMP standard curve provided with the cAMP Direct ELISA kits. Luminescence was measure using a MicroBeta 1450 Liquid Scintillation and Luminescence Counter (Perkin–Elmer). A 1/y transformation was performed on the luminescence counts per second (LCPS) so that an increase in LCPS corresponded to an increase in cAMP production. These transformed values are represented as 1/LCPS. Resultant data were then plotted by 4-parameter logistical curve fit using Prism graphing software to determine IC₅₀ values. Relative potencies were calculated by dividing the IC₅₀ of the Standard by the IC₅₀ of the sample ×100%.

3. Results

3.1. cAMP induction is directly proportional to the concentrations of PA and EF

The cAMP-induction assay is based upon the ability of PA to bind EF and facilitate the internalization of the protein complex into target CHO cells. Once inside the cells, the EF-mediated increase in intracellular cAMP is measured using a sensitive chemiluminescent ELISA. Because PA and EF act synergistically during intoxication, and since neither protein is thought to be capable of generating a specific increase in intracellular cAMP on its own, it should be possible to titrate both PA and EF against one another and produce coordinate alterations in cAMP production. Two experiments were performed to test this hypothesis: in the first, PA was titrated from 10 μg/mL to 2 ng/mL against a fixed concentration of EF 173 (1000 ng/mL). In the second, PA was held constant at 600 ng/mL while EF was titrated from 5 μg/mL to 0.08 ng/mL.

When PA was titrated against a fixed concentration of EF, a sigmoidal dose–response curve of cAMP production was generated that was linear between approximately 10 and 1100 ng/mL of PA (R²=0.995; Fig. 1A). No changes in cAMP levels were detected when PA was added to cells in the absence of EF (Fig. 1A). When EF was titrated against a fixed amount of PA, a linear response was observed from approximately 5 to 5000 ng/mL of EF (R²=0.974; Fig. 1B). In the absence of PA, no appreciable cAMP production was observed up to approximately 185 ng/mL of EF (Fig. 1B). At EF concentrations greater than 185 ng/mL, however, a linear increase in cAMP was detected in the absence of PA (R²=0.973; Fig. 1B; see Discussion). Because of this non-specific cAMP induction at high concentrations of EF, a final concentration of 50 ng/mL EF173 was utilized in subsequent experiments, leading to an increase in cAMP of approximately 3–10% compared to non-treated Control cells.

3.2. Detection of antibody-mediated neutralization of PA

The ability of the cAMP-induction assay to detect antibody-mediated neutralization of PA activity was tested by titrating a fully human monoclonal antibody against PA (PAmAb) into a fixed concentration of PA (600 ng/mL) and EF (50 ng/mL) and then assaying for changes in cAMP production. PAmAb-mediated neutralization of PA activity produced a sigmoidal dose–response curve with clearly defined upper and lower asymptotes (Fig. 2A). A broad, linear response of cAMP production was observed at antibody levels between approximately 50 and 3500 ng/mL (R²=0.982). Among the 6 replicate curves generated, the average IC₅₀ value was 479 ng/mL±26 with a coefficient of variation (CV) of 5%. Levels of cAMP in cells incubated with PA/EF and the highest concentration of PAmAb were identical to those of cells incubated with EF and PAmAb in the absence of PA, indicating 100% neutralization of PA activity. Similar dose–response curves were generated when
primary human macrophages were used in place of the CHO cells in the assay (data not shown).

Inter-plate precision and repeatability were demonstrated by two analysts testing six identical preparations of PAmAb on three different assay plates, respectively. A single PAmAb preparation (present in Row B) on each plate was used to determine the potencies of the other 5 rows. When the average potency for each row was compared (repeatability), the CV of the calculated potency values was 10% for Analyst 1 and 4% for Analyst 2 (Table 1). When the potencies of individual rows were compared across the three assay plates (inter-plate precision), the CV of the potency values ranged from 3 to 15% for Analyst 1 and 13 to 23% for Analyst 2.

Assay reproducibility was demonstrated by testing three independent preparations of PAmAb (Lots M9, M16 and M17), manufactured during different pro-
duction runs, in three independent experiments. On each assay plate, PAmAb Lot M13R was tested in two rows, the first of which was used to determine the potencies of the remaining test rows, while the second represented a sample that should yield a result of 100%. When compared to PAmAb Lot M13R, all samples had a CV of less than 11% across three independent experiments (Table 2) indicating that the cAMP-induction assay possesses the ability to determine the potency of different antibody preparations reproducibly with high levels of precision across independent assays.

3.3. Linearity of response and range of detection

The ability of the cAMP-induction assay to detect differences in the neutralizing activity of different preparations of antibody was demonstrated by testing PAmAb prepared at 50% (15 μg/mL), 75% (22.5 μg/mL), 100% (30 μg/mL), 125% (37.5 μg/mL), and 150% (45 μg/mL) of the normal starting concentration of antibody in the assay. Three replicate assay plates were run per experiment and three independent experiments were run in total. On each plate, the 100% point (30 μg/mL PAmAb Lot M13R) was run twice with one row serving as a standard from which the potencies of the other rows were determined.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Plate 1 (%)</th>
<th>Plate 2 (%)</th>
<th>Plate 3 (%)</th>
<th>Potency (%)</th>
<th>SD</th>
<th>%CV (inter-plate)</th>
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<tr>
<td>Mean</td>
<td>95%</td>
<td></td>
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<td></td>
<td>4.2</td>
<td>4%</td>
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</table>

Two analysts tested identical PAmAb preparations on all 6 test rows of three independent assay plates. A single PAmAb preparation (present in Row B) was used to determine the potency of the other test rows. Inter-plate precision was determined by comparing the potency values for a single sample across three different assay plates. Repeatability was determined by comparing the average potency values for all samples across three assay plates.

3.4. Reproducibility and assay precision

The reproducibility of the cAMP-induction assay was determined using three separate experiments, each performed on three replicate plates. The CV values for each experiment were calculated and averaged across all experiments. The mean CV value for all experiments was 10.3% with a standard deviation of 2.3%.

3.5. Stability of antibody potency

The stability of antibody potency was assessed by testing PAmAb Lot M13R at 30 μg/mL at different time points: 1, 3, 7, and 14 days. The CV values were calculated for each time point and averaged across all experiments. The mean CV value for all time points was 7.5% with a standard deviation of 1.2%.

Fig. 2. Antibody-mediated neutralization of PA activity in the cAMP-induction assay. PAmAb was titrated into a fixed amount of PA and EF and incubated for 1 h at 37 °C to allow for neutralization. Upon addition of the PAmAb–PA/EF solutions to CHO cells, a linear dose–response range of neutralizing activity was present from approximately 50 to 3500 ng/mL of antibody with little variability among the 6 sample replicates.
3.4. Assay specificity

Assay specificity was demonstrated by testing closely related, yet functionally incompetent, proteins in place of the original assay components. A translocation-deficient mutant PA protein (PATD) unable to shuttle EF or LF into the cytosol, and a class-matched monoclonal antibody incapable of binding PA (PAmAb(−)) were generated for this purpose.

When PAmAb was incubated with fully functional PA and EF, a typical sigmoidal dose–response curve was generated with an IC50 of 509 ng/mL (Fig. 4). When PAmAb was incubated with PATD, no increase in cAMP was observed, indicating the absence of PA/EF complex internalization at any concentration of neutralizing antibody. When PAmAb (−) was incubated with functional PA and EF, full stimulation of cAMP production was observed at all antibody levels. Lastly, when PAmAb (−) was incubated with PATD, no increase in cAMP levels was observed at any antibody concentration, as expected. These results demonstrate that the cAMP assay is capable of discerning between closely related, yet functionally distinct, proteins.

3.5. Comparison of different Edema Factor proteins in the cAMP-induction assay

The results presented thus far were obtained using a commercially available EF protein (EF 173; List Biological Laboratories, Inc.). To compare the activity of EF 173 to a recombinant EF protein expressed in E. coli (EF 1394-2; Human Genome Sciences, Inc.), we titrated both proteins against a fixed concentration of PA (600 ng/mL) and determined the effect on cAMP production at each EF concentration in the presence, or absence, of PA over a range of EF spanning from 98 pg/mL to 300 ng/mL. Our results show that EF 1394-2 is significantly more potent than EF 173 (P<0.0001 at all EF concentrations tested) with a maximum signal:noise ratio of 18:1 obtained at 26 ng/mL of EF 1394-2 (Fig. 5A; Table 3). In contrast, the maximum signal:noise ratio for EF 173 was 3:1 at 40 ng/mL of EF (Fig. 5A; Table 3).

When the two EF proteins were compared by molar induction of cAMP, EF 1394-2 generated a maximum 654 pmol of cAMP when used at 111 ng/mL in the assay compared to 30 pmol of cAMP using the same concentration of EF 173. Basal levels of cAMP ranged from 0.2 to 0.8 pmol for control wells with cells only (no PA or EF). Higher concentrations of either EF 1394-2 or EF 173 showed increased cAMP production in the absence of PA, as mentioned previously. For both proteins a maximum increase in cAMP of three to fourfold was observed between cell-only controls and cells treated with the highest concentrations of EF in the absence of PA.

To further characterize EF 1394-2, we titrated PAmAb against 600 ng/mL PA using EF 1394-2 at a final concentration of 2, 5, or 10 ng/mL (Fig. 5B). The results indicate that EF 1394-2 produces dose-
response curves for antibody-mediated neutralization of PA that are highly similar to those produced using the EF 173 protein, albeit with an increased range of cAMP production. At 2, 5, and 10 ng/mL EF 1394-2, maximum signal:noise ratios of 4:1, 7:1, and 9:1 were achieved, respectively, with IC_{50} values of 92, 99, and

![Graph showing linearity and range of the cAMP-induction assay.](image)

**Fig. 3.** Linearity and range of the cAMP-induction assay. PAmAb was prepared at 50%, 75%, 100%, 125%, and 150% of the normal starting concentration of antibody and tested in the cAMP-induction assay. Linear regression analysis of the resultant data indicated a high degree of correlation between expected and observed potency values with an average $R^2$ value of 0.98 among three independent experiments. Dashed lines represent the 95% confidence intervals of the data averaged across three independent experiments.

![Graph showing effects of a translocation deficient PA mutant and a non-specific human antibody in the cAMP-induction assay.](image)

**Fig. 4.** Effects of a translocation deficient PA mutant and a non-specific human antibody in the cAMP-induction assay. Translocation-deficient PA (PATD) and a class-matched non-specific human antibody (PAmAb (-)) were substituted into the cAMP-induction assay in place of wild-type PA (PA(wt)) and PAmAb, respectively. Control curves generated with PAmAb and PA(wt) displayed a typical sigmoidal dose–response curve characteristic of the assay. When PAmAb(-) was incubated with PA(wt) full stimulation of cAMP production was observed indicating no neutralization of PA activity. In contrast, when either antibody was incubated with PATD, no increase in cAMP production was observed indicating lack of internalization of PA/EF complexes.
147 ng/mL. These results indicate that EF 1394-2 possesses significantly greater enzymatic activity than EF 173, a property that may serve to increase the sensitivity and range of detection of the cAMP-induction assay.

3.6. Detection of PAmAb in the presence of human serum

While the cAMP-induction assay was originally developed as a release assay to quantify the potency
of PAmAb, we also sought to determine if the assay could tolerate the presence of human serum and, therefore, allow for its use as a surrogate pharmacodynamic marker to monitor levels of antibodies against PA, molecules for which no human efficacy data can be obtained, during clinical trials. The effects of serum on the performance of the cAMP-induction assay were examined by titrating PAmAb into a constant concentration of 10%, 7.5%, 5%, 2.5%, 1.25%, or 0% pooled human serum diluted with cell culture medium. Unlike earlier experiments using EF 173, the significant increase in activity of EF 1394-2 was utilized to provide a broader detection range of antibody-mediated neutralization of PA, and therefore, increase the sensitivity of the assay in the presence of serum. Here, EF 1394-2 was utilized at a final concentration of 10 ng/mL in place of EF 173.

Our results indicate that a characteristic sigmoidal shaped dose–response curve is generated in the presence of different levels of human serum highly similar to that produced in cell culture medium alone. As the level of serum increased in the assay, the overall amount of cAMP produced decreased proportionally causing the dose–response curve to shift toward the left. When compared to the dose–response curve generated in the absence of human serum, the 10% human serum curve showed a 20% average reduction in signal, corresponding to a reduction in cAMP production, at all points along the curve. Likewise, an average change of 16%, 15%, 5%, and

### Table 3

<table>
<thead>
<tr>
<th>[EF] ng/mL</th>
<th>300</th>
<th>200</th>
<th>133</th>
<th>89</th>
<th>40</th>
<th>18</th>
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<tr>
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<td>6.7</td>
<td>4.5</td>
<td>3.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Two different EF protein preparations (EF 173 and EF 1394-2) were titrated against a fixed amount of PA (600 ng/mL) to compare cAMP production for each protein. The signal:noise ratio obtained at each concentration of EF protein is shown for both EF 1394-2 and EF 173.

![Fig. 6. Dilutional linearity of the cAMP-induction assay in the presence of human serum. PAmAb was spiked into a final concentration of 10% human serum at 5 different starting concentrations to generate 5 experimental blocks on a single assay plate. Within each block, the stock PAmAb preparation was twice diluted 1:10 to generate two additional points consisting of 1 and 0.1% serum concentrations, respectively. Values for percent neutralization of PA activity were determined by averaging triplicate wells and then plotted by linear regression analysis. In each instance, dose-dependent neutralization of cAMP induction was observed regardless of the concentration of human serum present in the samples. Dashed lines represent the 95% confidence interval of the data averaged from three independent experiments.](image-url)
2% was observed at the 7.5%, 5%, 2.5%, and 1.25% serum concentrations, respectively.

The dilutional linearity of the cAMP-induction bioassay in the presence of human serum was then examined by spiking PAmAb into 10% human serum at 5 different starting concentrations and then twice diluting these preparations 1:10 to generate 5 different experimental blocks on each of three assay plates, respectively. Within an experimental block, each concentration of PAmAb was represented in triplicate wells in final serum concentrations of 10, 1, and 0.1%. The percent neutralization at each concentration of PAmAb was calculated based on PA/EF and EF-only control wells and the results analyzed by linear regression. For each concentration of serum, dose-dependent dilutional linearity was observed with $R^2$ values of 0.98, 0.95 for the 10 and 1% human serum curves, respectively (Fig. 6). The 0.1% curve possessed an $R^2$ value of 0.78 due to the loss of detection sensitivity at approximately 50 ng/mL of PAmAb.

Together these results indicate that the cAMP-induction assay is capable of detecting PAmAb in the presence of human serum and may provide a useful means of monitoring the biological activity of monoclonal antibodies against PA during clinical studies.

4. Discussion

The development of a simple and standardized bioassay capable of detecting antibody-mediated neutralization of PA activity as both a release assay and as a pharmacodynamic assay would be of significant benefit in the successful production and comparison of antibody-based therapeutics to treat and prevent anthrax infection. In the present study, we described the performance characteristics of a cAMP-induction bioassay capable of performing both of these tasks with high levels of precision, sensitivity, and reproducibility. Our results indicate that the cAMP-induction assay is a desirable format for use as either a potency-indicating release assay or as a pharmacodynamic assay in number of important regards. Firstly, the cAMP-induction assay possesses a large linear range from which accurate antibody potency determinations can be made using standard statistical methods that depend on sample parallelism as an acceptance criterion. Secondly, the cAMP-induction bioassay is biologically relevant, as it mimics the steps of cellular intoxication by PA and EF in vitro in both process and response. Thirdly, the cAMP-induction assay employs an extremely sensitive chemiluminescence-based ELISA that can detect small changes in intracellular cAMP. Fourthly, the cAMP-induction assay employs a minimal number of rinsing and transfer steps as all procedures occur in a single assay plate under physiological conditions. Lastly, the cAMP-induction bioassay is capable of detecting antibody-mediated neutralization of PA activity in the presence of human serum allowing for its use as a surrogate pharmacodynamic marker during clinical trials to ensure the continued activity of antibody-based anthrax treatments. Indeed, to date, this assay has proven to be a viable alternative to the RRA or LeTx-mediated killing assay as (1) a GMP release assay for PAmAb, a monoclonal antibody against PA (Zmuda et al., 2003), (2) a surrogate pharmacodynamic marker for monitoring the biological activity of PAmAb during human clinical trials (Zmuda et al., 2004a), and (3) a relevant bioassay for detecting host-generated neutralizing antibody titers against PA in cynomolgus monkeys 6 months after surviving lethal anthrax spore challenge following passive administration of PAmAb, as well as 28 days following secondary challenge in the absence of PAmAb (Zmuda et al., 2004b).

4.1. Performance characteristics of the cAMP-induction bioassay

In order for a bioassay to be suitable for use as a potency-indicating release assay, it is necessary that a number of key performance characteristics are present. Minimally, a release assay must demonstrate sufficient inter- and intra-plate precision, linearity, accuracy, specificity, and possess a suitable dose–response range from which potency determinations can be made. In this study, we have characterized the performance characteristics of the cAMP-induction bioassay with regard to these parameters and found it to be acceptable in all regards. Indeed, the cAMP-induction assay possesses a number of important characteristics that not only make it acceptable as a potency-indicating assay, but also make it desirable from a validation and quality control standpoint due to the simple format and sensitive detection methods.
used. For detecting cAMP, the ELISA portion of the assay is performed in the same plates in which the cells are seeded, and the bioassay performed, thus eliminating transfer steps that might increase assay variability. Additionally, this ELISA possesses a number of distinct advantages over traditional detection techniques in that chemiluminescence offers significantly increased sensitivity compared to either fluorescence or colorimetric-based analyses and since chemiluminescence emission is detected using a luminometer without the need for an incident light source, there is less concern regarding changes in the excitation light source that might affect the results of the assay.

When the data produced in the cAMP-induction assay were analyzed by 4-parameter logistical fit, the curves generated exhibited the necessary characteristics of a potency-indicating assay. In each instance, the cAMP-induction assay possessed clearly defined upper and lower asymptotes as well as multiple points within the linear portion of the curve spanning from approximately 50 ng/mL to 3500 ng/mL of PAmAb. Linearity was exhibited between 50 and 150% potency with an overall $R^2$ value of 0.98 across three experiments. Indeed, the three independent experiments that were averaged to generate this result provided $R^2$ values of 0.99, 0.98, and 0.97, respectively, indicating that individual experiments are sufficient to generate an accurate result. Lastly, excellent repeatability and reproducibility were observed when identical preparations of PAmAB were tested on individual assay plates (repeatability) or when independent preparations of PAmAb were compared across multiple, independent assays (reproducibility). Together, these results suggest that the cAMP-induction assay possesses the necessary biological relevance, performance characteristics, and simplicity of design for successful implementation as a potency-indicating product release assay for monoclonal antibodies against PA.

4.2. Comparison of different EF proteins in the cAMP-induction assay

Throughout the majority of this study, a commercially available EF protein was utilized (EF 173) that was purified from a non-virulent strain of anthrax. When the activity of EF 173 was compared to an in-house EF preparation made in E. coli (EF 1394-2), the results of these experiments disclosed a number of important factors that could impact the performance of the assay. At 26 ng/mL, EF 1394-2 exhibited its maximum signal:noise ratio of 18:1 compared to approximately 3:1 for the EF 173 at the same concentration. It is possible that the lyophilization process of EF 173 may detract from its potency while EF 1394-2 is purified and then frozen immediately at $-80^\circ$C until the time of use. This difference in potency is important since the precision of the assay decreased slightly at very high levels of cAMP production, and since our results showed that in the absence of PA, higher levels of either EF protein induced detectable levels of cAMP production proportional to the amount of EF added to the cells. The reason for this non-specific increase in cAMP production in the absence of PA, however, is unclear. EF 173 was generated by the introduction of a plasmid encoding the EF sequence into a non-sporulating, avirulent strain of anthrax that possessed none of the other toxin components (PA or LF), thus reducing the possibility of cross-contamination of PA, a protein of roughly the same size as EF, in the preparation. Similarly, EF 1394-2 was generated in E. coli using only the coding sequence for EF, eliminating the possibility of PA cross-contamination that could lead to the internalization of EF and increased cAMP. These findings, while consistent with one another, are in apparent contrast to those described by Leppla (1982) whereby EF concentrations of up to 1 \( \mu \)g/mL produced nearly no increase in cAMP production compared to controls possessing PA only. Indeed, the EF used by Leppla was purified from a non-virulent strain of anthrax that produced all three toxin components rather than by the more specific recombinant DNA techniques used to express EF 173 and 1394-2. Thus, it would not be unlikely that this type of purification from whole bacterial extract might possess low levels of PA cross-contamination in the EF fraction, which would presumably be absent in EF 1394-2 and EF 173, that could lead to the production of cAMP in the absence of PA added to the system. While EF 1394-2 appears to be more potent than the preparation tested by Leppla (EF 1394-2 continues to display significant production of cAMP at levels as
low as 390 pg/ml compared to approximately 2–5 ng/mL for the preparation tested by Leppla), increased potency alone cannot be responsible for the observed difference between EF preparations since EF 173, which possesses lower activity than EF 1394-2, shows similar stimulation of cAMP in the absence of PA. Additional functional studies will be required to determine the mechanism by which these two EF preparations, produced by two different recombinant techniques, are able to cause a small, but detectable, increase in cAMP levels in the absence of PA.

4.3. Detection of biologically active PAmAb in patient serum samples using the cAMP-induction assay

The ability of the cAMP-induction assay to be adapted to allow for detection of biologically active antibodies against PA in patient serum offers an additional benefit during the development of antibody-based anthrax therapeutics. In this capacity, a modified version of the cAMP-induction assay has proven valuable as a surrogate pharmacodynamic assay to monitor the biological activity of PAmAb during Phase I dose escalation studies (Zmuda et al., 2004a). Compared to traditional ELISA analysis, the cAMP-induction assay generated results that were statistically similar to those of the ELISA in terms of antibody concentration, terminal-phase slope and half-life. Here, the availability of a surrogate bioassay may prove useful in detecting differences in antibody efficacy that are not readily attainable using traditional ELISA-based methods. Unlike an ELISA, that might detect the presence of the therapeutic antibody regardless of certain changes that could affect its bioactivity in vivo, the use of a bioassay would come closer to confirming that the observed levels of circulating antibody are indeed biologically active. Proteolytic degradation of the antibody, binding and inactivation by host proteins, and/or aggregation are all events that might not be readily identified using ELISA-based methods depending on the detection technique utilized. If these alterations resulted in a change in the biological activity of the molecule, a bioassay would be more likely to detect these differences than a traditional ELISA and may prove more useful in monitoring the fate of the molecule post-injection.

5. Conclusions

In the present study, we have presented data on the development of an EF-mediated cAMP-induction bioassay for determining the potency of antibodies against anthrax protective antigen. Our results suggest that the cAMP-induction assay possesses the necessary biological relevance and performance characteristics for use as a potency-indicating release assay and that this assay may also prove useful as a standardized assay by which the in vitro efficacy of different antibody-based anthrax therapeutics could be compared. Additionally, the ability of the cAMP-induction assay to reliably detect therapeutic antibodies against PA in human serum may also prove valuable in ensuring the sustained biological activity of antibodies against PA following administration during clinical trials.

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References


