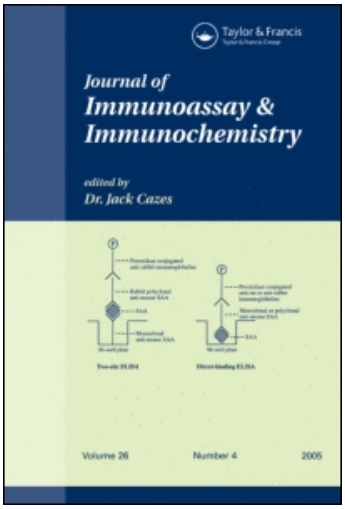

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Abstract: Vaccination with anthrax vaccine adsorbed (AVA) results in the production of protective antigen (PA) specific antibodies, which play an important protective role against anthrax toxins. Analyzing the specificity of serum antibodies generated in response to AVA vaccination can provide insight into the mechanisms of protective immunity against this important pathogen. The goal of this study was to develop a competitive enzyme linked immunosorbent assay (cELISA) to test human immune serum for antibodies specific for a known lethal toxin neutralizing epitope in PA. PA-specific antibodies in sera from individuals who received the six-dose AVA vaccine series competed for binding to immobilized PA with monoclonal antibody F20G75, which binds to a linear epitope in domain 2 of PA and neutralizes lethal toxin activity in

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vitro. These results suggest that antibodies in human AVA vaccinee serum recognize the same epitope as F20G75, or one in close proximity to it, and may serve a protective role against anthrax lethal toxin. This assay may be used for serological confirmation of successful immunization against anthrax and for the identification of antibodies in human vaccinee serum that recognize protective epitopes on PA.

Keywords: Anthrax, Competitive ELISA, Epitope mapping, Protective antigen, Vaccine

INTRODUCTION

Bacillus anthracis, the Gram positive, spore-forming bacterium that causes anthrax, expresses three extracellular toxin protein components, all of which are encoded on its large pXO1 plasmid.[1] During infection, a functional binary toxin is formed when protective antigen (PA), bound to one of its receptors on the surface of target cells,[2–4] combines with either lethal factor (LF) or edema factor (EF). The heptameric toxin:receptor complex is then internalized via clathrin-mediated endocytosis,[5] followed by endosome acidification, structural rearrangements in the PA prepore heptamer, pore formation and membrane insertion,[6,7] and subsequent release of LF and/or EF through the pore into the cytosol (reviewed in Abrami et al., 2005).[8]

Currently licensed human anthrax vaccines include anthrax vaccine adsorbed (AVA) in the U.S., produced under the trade name Biothrax™ by the Bioport corporation, and anthrax vaccine precipitated (AVP) in the U.K., produced under license by the Health Protection Agency. The primary antigenic determinant in both vaccines is PA,[9–12] and the human antibody response to PA resulting from vaccination with AVA has been well characterized.[13,14] Numerous animal studies have determined that a protective immune response to anthrax is associated with a significant humoral response to PA.[15–18] Lethal toxin (LeTx) neutralizing antibodies are readily detectable in human AVA-vaccinee serum,[19,20] and PA-specific IgG levels correlate with toxin neutralization both in response to AVA vaccination[13] and after anthrax infection.[21]

Numerous MAbs that neutralize LeTx in vitro by targeting different regions of PA have been described. Some MAbs target epitopes in regions spanning domains 1 and 2 and domains 3 and 4, and block LF interaction with PA at the cell surface.[22] Some MAbs bind epitopes in domain 2, blocking cleavage of PA83 to PA63[23] or neutralizing LeTx by as yet unidentified mechanisms.[24,25] Other MAbs target epitopes in domain 4, neutralizing the toxin via blocking PA:receptor binding.[22,23,26] Given that multiple regions on PA can potentially serve as targets for antibodies in human serum, we sought to identify antibodies that can bind to a single linear epitope in domain 2 of PA that is the target of a LeTx neutralizing murine

MAb, F20G75.[24] Two different competitive ELISA formats were developed using this MAb. The first employed soluble peptides to identify the minimal epitope recognized by F20G75. The second cELISA was designed to identify serum antibodies in AVA vaccinees that recognize the same epitope as F20G75. Our analyses indicate that human AVA vaccinee serum does indeed compete with this MAb for binding to the same epitope in PA. These results suggest that this cELISA can be readily adapted to assay AVA vaccinee serum to identify a range of antibody epitope specificities, which could prove valuable in the design and assessment of next generation PA-based anthrax vaccines.

EXPERIMENTAL

Human Serum

AVR801 human standard anthrax reference serum was generously provided by Dr. Conrad Quinn, from the Centers for Disease Control and Prevention in Atlanta, and is described in detail elsewhere.[21] Test serum was collected from five individuals who received the three primary vaccinations and at least three booster vaccinations, as per the recommended course of immunization with AVA.[27] These individuals received the vaccine as part of a voluntary vaccination program, and provided plasma as part of a voluntary donation program under an approved human use protocol. All samples were labelled with a code number and donors remained anonymous to all investigators. Control serum was obtained from Pulse Scientific (Burlington, ON, Canada) and is not known to contain anthrax-specific antibodies. All sera were stored in aliquots at -20°C prior to use.

Indirect ELISA to Detect Human Serum Binding to Recombinant PA (rPA)

rPA was a generous gift from Dr. Jeremy Mogridge at the University of Toronto, and was produced as described.[28] rPA and bovine serum albumin (BSA) were diluted appropriately in phosphate buffered saline (PBS; pH 7.2) and were coated, in triplicate, at 200 ng or 1 μg/well, respectively, in 96-well ELISA plates (Nunc-Immuno^{TM} 96 MicroWell^{TM} MaxiSorp^{TM}, Nalge Nunc International, Rochester, NY) at 4°C overnight. The plates were then blocked with 10% skim milk (Difco^{TM}, BD Diagnostics^{TM}, Franklin Lakes, NJ) in PBS for 90 minutes at 37°C, followed by three washes with 0.9% NaCl/0.05% Tween-20 (Fisher Scientific, Fair Lawn, NJ). Human sera were diluted (1:10 to $1:10^{10}$) in 10% skim milk in PBS, applied to the wells, and then incubated at 37°C for 90 minutes. The wells were then washed four times, and incubated with the secondary antibody,

horseradish peroxidase (HRP) conjugated goat anti-human IgG $F(ab')_2$, (Jackson ImmunoResearch, West Grove, PA) diluted 1:5000 in 10% skim milk in PBS, at 37°C for 90 minutes. The wells were washed four times, and then colour development was monitored for 15 to 60 minutes after the addition of 200 μL of ABTS developing solution (Roche Diagnostics, Indianapolis, IN), followed by scanning at 405 nm on a Multiskan Ascent® ELISA plate reader (Thermo Electron Corporation, Waltham, MA).

Serum cELISA

Murine MAb F20G75 was chosen as the PA-specific MAb against which the human AVA sera compete for binding to rPA in the cELISA format. This LeTx neutralizing MAb is specific for a linear epitope in domain 2 of PA, and is described in detail elsewhere.[24] Using the same ELISA procedure outlined above, but employing HRP-conjugated goat anti-mouse IgG $F(ab')_2$ (Jackson ImmunoResearch) as the secondary antibody, various dilutions of F20G75 were assayed for binding to different amounts of rPA coated on 96 well ELISA plates to optimize the MAb dilution and rPA coating amount for the cELISA. Coating with 125 ng of rPA/well and employing a 1:1000 dilution (final concentration 0.001 mg mL^{-1}) of MAb F20G75 resulted in OD_{405} readings of approximately 1 after 30 minutes of colorimetric development; these conditions were chosen for the cELISA. Aliquots (100 μL) of F20G75 were mixed and pre-incubated with 100 μL of diluted (1:5 to $1:10^5$) non-immune control serum or AVA-vaccinee serum for 15 minutes at room temperature. Following this incubation step, 100 μL of each mixture was added to blocked, rPA coated ELISA plates (prepared as described above) and incubated for ninety minutes at 37°C. Washing, incubation with HRP-conjugated goat anti-mouse IgG $F(ab')_2$, colorimetric development, and ELISA plate scanning were performed as described above. Controls included no pre-incubation of MAb F20G75 with any serum, pre-incubation with 100 μL of soluble rPA (1.0 mg mL^{-1} stock, diluted 1:5 to $1:10^5$), and pre-incubation with 100 μL of rPA-inoculated rabbit immune serum (diluted 1:5 to $1:10^5$). Loss of detectable signal indicates competition between the sera and MAb F20G75. All experiments were performed at least twice, with every dilution of each control or experimental condition being tested at least in triplicate.

Epitope Mapping via Peptide cELISA

The peptide competitive ELISA was performed using the same methods as outlined for the serum competitive ELISA, with the following alteration. Aliquots (100 μL) of 1:1,000 diluted F20G75 were pre-incubated with 100 μL of soluble test or control peptides (1.0 mg mL^{-1} stock, diluted 1:5

to 1:10^5) prior to transferring 100 μL of the pre-incubated mixture to the rPA coated ELISA plate. Loss of detectable signal indicates that the peptide is able to compete with rPA for binding to F20G75, suggesting that any such peptides serve as an epitope for MAb binding. All experiments were performed at least twice, with every dilution of each control or experimental condition being tested at least in triplicate.

Peptide Design

The target epitope of MAbs F20G75, which was identified and described in detail elsewhere[24] is contained within the region extending from Ser-301 to Ser-325 in domain 2 of *B. anthracis* PA (SEVHGNAEVHASFF DIGGSVSAGFS). Test peptides derived from this target epitope included PA-7 (ASFFDIGGSVSAGFSNSNSS), PA-8 (VHASFFDIGGSV), PA-9 (NAEVHASFFDIGG), Pep-16 (VHASFFDIGEEEC), and Pep-18 (ASFFDI EEEC). The C-terminal sequence "EEEC" in Pep-16 and Pep-18 was added simply as a linker. PA-D (CAGERTWAETMGLNTADTA) is derived from Domain 2 of PA, from a region upstream of the identified target epitope of F20G75, and was chosen as a PA-derived negative control, since our previous study did not detect any binding of MAb F20G75 to this region of PA.[24] The final peptide, P3 (CNKVGSTK), was derived from PorB of *Neisseria meningitides*,[29] and served as a non-anthrax negative control peptide. All peptides were obtained from United Biochemical Research (Seattle, WA, USA).

Statistical Analysis

All OD values generated from the cELISA experiments were transferred to the GraphPad Prism® 4.0 software suite (GraphPad Software Inc., San Diego, CA) for graphing and analysis. Values obtained from the test sera or peptides and control serum or peptides, at each dilution, were compared to each other using a two-way ANOVA included in the program. The resulting data were subjected to a Bonferroni post-test, allowing an assessment of each dilution series of each test serum or peptide to be made against their respective negative controls to indicate whether statistically significant levels of competition occurred at any given dilution. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

MAb F20G75 binds to a predicted surface exposed linear epitope in domain 2 of PA, located within the following amino acid sequence: 301-SEVHG-NAEVHASFFDIGGSVSAGFS-325,[24] As discussed in Gubbins et al.,[24]

the use of epitope mapping employing peptides coupled to a solid support matrix indicated that the central residues ASFFD were important for MAb binding. To make a more accurate determination of the minimal epitope recognized by the MAb, soluble peptides derived from this larger epitope were synthesized, and used to compete with immobilized rPA for binding to MAb F20G75 in a cELISA format. The use of this assay format has been used successfully to map epitopes of multiple MAbs, demonstrating its utility for this application.[30–32] Given the large difference in molecular weights of the peptides (approximately 0.84 kDa to 2.0 kDa) compared to rPA (83 kDa), mass/volume equivalents of peptides and PA were compared in the cELISA, as outlined in Figure 1. Significant competition was defined as that peptide concentration which resulted in OD_{405} readings reduced by 3-fold or 6-fold, relative to the OD_{405} signal obtained from a control reaction in the absence of any competitor. As shown in Figure 1, the negative control peptides P3 and PA-D were completely unable to compete with immobilized rPA for binding to F20G75, at concentrations ranging to highs of 119 and 50 μM, respectively (Table 1). However, the positive control, soluble rPA, was able to cause a 3-fold decrease in binding signal at 60 nM, and a 6-fold decrease in binding signal at 120 nM (Figure 1 and Table 1). Thus, it was evident that differentiating between targets able to compete with immobilized rPA for binding to F20G75 and those that could not was straightforward.

Several peptides derived from the identified region in domain 2 containing the epitope bound by MAb F20G75 were tested for their ability to compete with immobilized rPA for binding to F20G75. Pep-18 was the smallest, containing the residues ASFFDI, coupled to a short linker sequence of EEEC. As shown in Figure 1, this peptide competed poorly for MAb binding, even at a concentration of 100 μg mL^{-1} (84 μM). This observation suggests that although the residues ASFFD are important for MAb binding,[24] F20G75 is

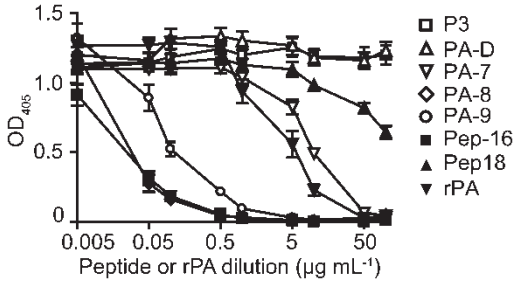

Figure 1. cELISA epitope mapping using soluble peptides. All peptides were diluted as indicated, and pre-incubated with MAb F20G75 prior to being added to ELISA plates coated with 125 ng of rPA. Soluble rPA (stock concentration 1.0 mg mL^{-1}) was diluted as indicated and included as a positive control. Mean values from triplicate experiments are shown. Error bars represent standard error of the mean.

Table 1. Epitope mapping via soluble peptide inhibition of MAb F20G75 binding to immobilized rPA via cELISA

Competitor	Peptide sequence	Concentration of peptide required to inhibit MAb binding in cELISA (nM)[a]	
		3-fold decrease in signal	6-fold decrease in signal
rPA		60	120
PA-7	ASFFDIGGSVSAGFSNSNSS	5155	25,773
PA-8	VHASFFDIGGSV	40	81
PA-9	NAEVHASFFDIGG	74	370
Pep-16	VHASFFDIGEEEC	34	68
Pep-18	ASFFDIEEEC	N.A.[b]	N.A.
PA-D	CAGERTWAETMGLNTDADTA	N.A.	N.A.
P3	CNKVGSTK	N.A.	N.A.

[a]The mean OD_{405} values of replicates (at least six) of each concentration of each peptide (or rPA) used as competitors in the cELISA were compared to the mean value obtained when performing the assay in the absence of any competitor. Inhibition of MAb binding was defined as a 3-fold or 6-fold decrease in the OD_{405} value compared to the no-competitor control. The values reported are statistically significant ($p < 0.05$ via ANOVA) compared to the no-competitor control.

[b]N.A. indicates no competition occurred, as defined by no statistically significant decrease in MAb F20G75 binding to immobilized rPA in the presence of a given competitor.

unable to bind a minimal peptide epitope containing residues ASFFDI. Peptide PA-7, which also contains ASFFDI, but an additional 14 residues at its C-terminus, was able to compete with immobilized PA for MAb binding albeit poorly, with concentrations of 5 μM or 26 μM required to cause a 3-fold or 6-fold reduction in binding signal, respectively (Figure 1 and Table 1). These observations suggested that residues located N-terminally to ASFFDI are likely important for F20G75 binding. Peptides PA-8 and Pep-16 exhibited a closely matched ability to compete with immobilized rPA for binding. PA-8 inhibited binding by 3-fold and 6-fold at 40 and 80 nM, respectively, while Pep-16 caused the same levels of binding inhibition at 34 and 68 nM (Figure 1 and Table 1). Since Pep-16 contains the same short linker sequence (EEEC) as Pep-18, and apart from containing that sequence is essentially identical to PA-8, it is unlikely that this linker sequence affects MAb binding. Interestingly, PA-9, which is very similar in sequence to PA-8, does not compete as effectively for MAb binding as PA-8 (Table 1). This observation suggests that subtle differences in the number of residues both N- and C-terminal to the core ASFFDI residues influences

MAb binding. Based upon these combined observations obtained from the cELISA epitope mapping experiments, the specific epitope of F20G75 is likely minimally VHASFFDIG, which supports the solid-phase epitope mapping results previously reported.[24]

The successful assessment of the cELISA employed for epitope mapping suggested that this assay format could be adapted to identify epitope-specific antibodies in human serum. To confirm that the human reference and test sera contained significant, detectable levels of PA-specific antibodies, all samples were first tested in a standard indirect ELISA against immobilized rPA. The AVR801 reference serum and the sera collected from five individual AVA vaccinees reacted strongly with rPA in ELISA, but showed no significant reaction with the negative control antigen (BSA) included in the assay (Figure 2 and data not shown). Similarly, the non-immune human control serum demonstrated no detectable reaction to rPA. Collectively, this panel of sera was used to develop and test a cELISA platform for epitope-specific serum antibody detection.

A representative example of the results of a serum cELISA experiment is shown in Figure 3. A given dilution of a serum sample was defined as able to compete with F20G75 for binding to immobilized rPA when it caused a minimal 2-fold decrease in OD_{405} readings compared to a control condition in which no serum was present. Human standard anthrax reference serum AVR801 and all five of the individual AVA vaccinee sera were able to compete with F20G75 for binding to immobilized rPA in a dose-dependent manner. Non-immune control serum was unable to compete with F20G75 for binding, while serum from a New Zealand White rabbit immunized with highly purified rPA was able to compete more effectively than any of the

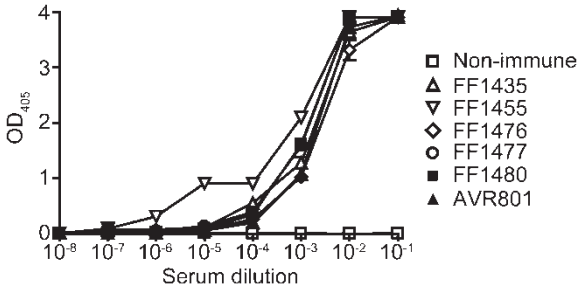

Figure 2. Indirect ELISA confirms reactivity of AVA vaccinee sera with immobilized rPA. All sera were diluted as indicated and assessed for their ability to bind to rPA coated at 200 ng/well in standard 96-well ELISA plates. AVR801 is a standard reference serum pooled from multiple AVA vaccinees; non-immune serum was obtained from a commercial source, and is not known to contain any anthrax-specific antibodies. The remaining five serum samples were obtained from individuals who received at least the first six inoculations of the standard AVA vaccination schedule. Mean values from triplicate experiments are shown. Error bars represent standard error of the mean.

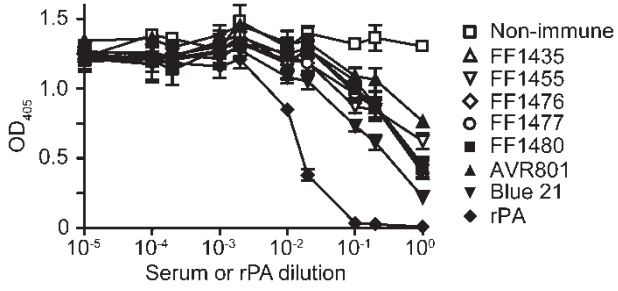

Figure 3. cELISA demonstrates human AVA vaccinee sera competing with MAb F20G75 for binding to rPA. All sera were diluted as indicated, and pre-incubated with MAb F20G75 prior to being added to ELISA plates coated with 125 ng of rPA. Sample Blue 21 is serum obtained from a New Zealand White rabbit inoculated with purified rPA; the remaining serum samples are labelled as indicated in the legend for Figure 2. Soluble rPA (stock concentration 1.0 mg mL^{-1}) was diluted as indicated and included as a positive control. Mean values from triplicate experiments are shown. Error bars represent standard error of the mean.

human sera tested. Not unexpectedly, soluble rPA, employed in the assay as a non-immunoglobulin positive control competitor, most efficiently competed with F20G75 for binding to immobilized rPA (Figure 3). The apparent level of competition exhibited by the human sera did not vary widely, although it does appear that the pooled AVR801 reference serum was not as efficient a competitor as any of the individual AVA vaccinee serum samples. This result is supported by the observation that AVR801 serum exhibited statistically significant competition, compared to a non-immune serum control, only when it was not diluted prior to testing in the assay. In contrast, serum samples 1435, 1455, 1477, and 1480 exhibited statistically significant competition at dilutions of 1:10, while sample 1476 exhibited statistically significant competition at a 1:5 dilution. This slight difference in competitive ability might be because AVR801 serum is pooled from multiple individuals who received a minimum of four doses of the initial six-dose vaccination series,[21] while the individual test sera were obtained from subjects who received a confirmed minimum of all six initial doses. However, considering the many factors that influence an individual's response to any vaccination, and the observed variability of the humoral response to human anthrax vaccines[14] it is very difficult to determine an exact cause of such a subtle difference in the results obtained in the assay described here. Regardless, it is clear that antibodies present in all of the AVA vaccinee serum samples tested compete with MAb F20G75 for binding to PA, either by interacting with the same epitope, or a nearby epitope, thereby hindering MAb binding.

Development of this cELISA methodology clearly allowed for detection of epitope specific antibodies present in human AVA serum. Undoubtedly, a wide variety of epitopes exist on PA that serve as targets for LeTx neutralizing

antibodies, as evidenced by the generation of neutralizing murine MAbs with differing target specificity by multiple research groups.[22–24,26] Exhaustive identification of every neutralizing epitope on PA that is targeted by human antibodies in response to AVA vaccination would likely be difficult, labour intensive, and impractical. However, identification of single PA epitopes that are targeted by both neutralizing MAbs and serum antibodies is achievable. Epitope specific IgG antibodies purified from the serum of individuals immunized with the U.K. licensed vaccine (AVP) have been identified via a competitive-binding ELISA employing a MAb specific for an epitope spanning the border between domains 3 and 4 of PA.[11,33] In the data presented in this report, antibodies specific for a completely different epitope were identified in unpurified serum obtained from AVA vaccinees. Thus, it is evident that this method is useful for the identification of PA epitope specific antibodies in recipients of the U.S. licensed human anthrax vaccine, without the need to purify any antibodies out of the serum.

CONCLUSIONS

The data reported here indicate that individuals receiving the initial full six-dose course of the U.S. licensed AVA vaccine produce antibodies that recognize a linear epitope in domain 2 that is targeted by a neutralizing murine MAb. The potential contribution these antibodies make to LeTx neutralization is currently unknown, and a separate study would be required to make that assertion. However, antibodies present in all of the human AVA vaccinee sera, and in immune serum from a rabbit vaccinated with highly purified rPA, tested in this report can recognize the same domain 2 epitope in PA. When coupled with our previous report of this epitope being the target of LeTx neutralizing murine MAbs,[24] these observations suggest that this is an immunologically important epitope in domain 2 of PA. The cELISA employed for identifying these antibodies can be adapted to identify other PA epitopes in AVA vaccinees, which could aid in the identification of human serum antibodies that contribute to neutralization of anthrax infection and contribute to a better understanding of the humoral response to AVA vaccination. As such, this assay could also serve as a useful tool in the design and assessment of future anthrax vaccines.

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