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Affinity Maturation of an Anti-V Antigen IgG Expressed *In Situ* Via Adenovirus Gene Delivery Confers Enhanced Protection Against *Yersinia pestis* Challenge

Thomas J. Van Blarcom, Ph.D.^{1,2}, Carolina Sofer-Podesta, Ph.D.³, John Ang, B.S.³, Julie L. Boyer, Ph.D.³, Ronald G. Crystal, M.D.³, and George Georgiou, Ph.D.^{1,2,4,5}

¹Department of Chemical Engineering, The University of Texas at Austin, Austin, Texas 78712, U.S.A.

²Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712, U.S.A.

³Department of Genetic Medicine, Weill Cornell Medical College, New York, New York, 10065, U.S.A.

⁴Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, U.S.A.

⁵Section of Molecular Genetics and Microbiology, The University of Texas at Austin, Austin, Texas 78712, U.S.A.

Abstract

Genetic transfer of neutralizing antibodies has been shown to confer strong and persistent protection against bacterial and viral infectious agents. While it is well established that for many exogenous neutralizing antibodies increased antigen affinity correlates with protection, the effect of antigen affinity on antibodies produced *in situ* following adenoviral gene transfer has not been examined. The mouse IgG2b monoclonal antibody 2C12.4 recognizes the *Yersinia pestis* Type III secretion apparatus protein LcrV (V antigen) and confers protection in mice when administered as an IgG intraperitoneally or, following genetic immunization with engineered, replication-defective serotype 5 human adenovirus (Ad)¹. 2C12.4 was expressed as a scFv fragment in *E. coli* and was shown to display a $K_D=3.5$ nM by surface plasmon resonance (SPR) analysis. The 2C12.4 scFv was subjected to random mutagenesis and variants with increased affinity were isolated by flow cytometry using the Anchored Periplasmic Expression (APEX) bacterial display system. After a single round of mutagenesis, variants displaying up to 35-fold lower K_D values (H8, $K_D=100$ pM) were isolated. The variable domains of the H8 scFv were used to replace those of the parental 2C12.4 IgG encoded in the Ad vector, Ad V giving rise to Ad V.H8. The two adenoviral vectors resulted in similar titers of anti-V antigen antibodies 3 days post-immunization with 10^9 , 10^{10} or 10^{11} particle units. Following intranasal challenge with 363 LD₅₀ *Y. pestis* CO92, 54% of the mice immunized with 10^{10} pu of Ad V.H8 survived at the 14 day end point compared to only 15% survivors for the group immunized with Ad V expressing the lower affinity 2C12.4 ($P<0.04$, Ad V versus Ad V.H8). These results indicate that affinity maturation of a neutralizing antibody

Correspondence: Professor George Georgiou, Departments of Chemical Engineering and Biomedical Engineering, and the Institute for Cellular and Molecular Biology, The University of Texas at Austin, 1 University Station, C0800, Austin, Texas 78712-1084. Phone: (512) 471-6975. Fax: (512) 471-7963. gg@che.utexas.edu.

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delivered by genetic transfer may confer increased protection not only for *Y. pestis* challenge but possibly for other pathogens.

Keywords

antibody; affinity maturation; *Yersinia pestis*; adenovirus gene transfer; protection

Introduction

Yersinia pestis is the etiologic agent of the plague and has been responsible for pandemic outbreaks occurring throughout the course of history. Although advances in our current living conditions, public health practices, and antibiotic therapies make future pandemics unlikely, outbreaks of plague resulting from biological warfare are a real threat. The features of *Y. pestis* that make it an attractive option for use as a biological weapon include availability of the organism, capacity for aerosol dissemination, potential for spread of secondary cases, and the high fatality rate of the pneumonic form of plague. In endemic regions of the world, the bacterium survives by causing chronic disease in animal reservoirs. It is spread among these animals and occasionally to humans predominantly through a flea vector, such as *Xenopsylla cheopis*^{2,3}. Without prompt antibiotic therapy, approximately 50% of bubonic plague infections are fatal and can progress to the more dangerous pneumonic plague². Respiratory droplets from a pneumonic infected individual promote rapid spread through a susceptible population. Symptoms develop in 1 to 6 days post-infection and the disease progresses rapidly from a flu-like illness to severe pneumonia with cough, chest pain, and bloody sputum. To be effective, antibiotic therapy must be administered early. If treatment is delayed more than 24 hours following the onset of symptoms, the fatality rate is high⁴. Additionally, the presence of antibiotic-resistant strains of *Y. pestis* renders antibiotic therapy unreliable. For these reasons, *Y. pestis* is a likely agent to be used as a biological weapon since aerosolized bacteria can confer widespread pneumonic plague².

Of the 11 *Yersinia* species, only *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* are human pathogens. *Y. pestis* is a gram-negative, non-motile, non-spore-forming bacterium that replicates intracellularly during the early stages of infection and grows predominantly extracellularly at later stages of the infectious cycle². At present, no plague vaccine has been approved for use in the US. Passive immunization with antibodies specific for the LcrV protein (V antigen) is an attractive alternative to vaccines and have been shown to be effective against lethal challenges with *Y. pestis*^{1,5-7}. The V antigen plays a central role in plague pathogenesis. It activates the type III secretion system and thus mediates translocation of effector proteins (Yops) into host macrophages. It is also released from the bacteria and has immunosuppressive functions manifested by increasing levels of the anti-inflammatory cytokine interleukin 10 and decreasing levels of TNF- α ². A recently developed anti-V antigen monoclonal antibody (mAb) 2C12.4 has been shown to confer protection against lethal challenge with intranasally administered *Y. pestis* in a dose-dependent manner¹.

For several neutralizing antibodies the degree of protection against challenge with pathogen correlates with antigen binding affinity⁸⁻¹¹. For example, while monoclonal antibodies and antibody fragments to the Protective Antigen (PA) of *Bacillus anthracis* with a $K_D=11$ nM fail to confer protection against challenge with the holotoxin or with intranasally administered spores, engineered antibody variants displaying 40- to 200-fold higher affinities were protective in different animal models^{8,12}. Notably, protection appeared to be mediated by blocking the ability of PA to bind to its receptor since PEGylated antibody

fragments exhibiting a $K_D=35$ pM but lacking an Fc domain, and hence incapable of engaging innate immunity mechanisms of pathogen clearance, were protective¹². Engineering antibodies with high affinity has been shown to improve protection for other protein toxins and viruses including Botulism, human immunodeficiency virus (HIV), and human respiratory syncytial virus (RSV) and have increased efficacy when targeting inflammatory cytokines such as TNF- α ^{8-11,13,14}.

In this study, we evaluated whether Ad-mediated delivery of an engineered 2C12.4 IgG exhibiting markedly increased affinity directed towards the V antigen can improve protection against *Y. pestis* challenge in mice¹. Improvement would be demonstrated by conferring a higher level of protection through immunization with an equivalent dose of a recombinant adenovirus encoding the higher affinity neutralizing antibody. However, the mutations needed to confer higher affinity could have unexpected effects on protein expression from the Ad delivered IgG DNA or biodistribution of the IgG, thus making it difficult to predict how protection to pathogen challenge might be affected. A scFv fragment incorporating the V_H and V_L domains of the 2C12.4 IgG was constructed and subjected to affinity maturation by screening a library generated by random mutagenesis. The latter was performed using the *E. coli* display technique Anchored Periplasmic Expression (APEX) coupled with fluorescent activated cell sorting (FACS)¹⁵. In APEX, the protein library is tethered to the periplasmic side of the inner membrane of the bacterium via fusion to the 6 N-terminus amino acids of the native lipoprotein NlpA. The resulting NlpA-scFv fusion is lipidated *in vivo* and thus becomes attached to the inner membrane. Following permeabilization of the *E. coli* outer membrane by chemical and enzymatic means, the cells are labeled with fluorescent antigen and the brightest cells are isolated by FACS. A single round of random mutagenesis and APEX screening resulted in the isolation of a clone, H8, displaying a $K_D=100$ pM which is 35-fold higher affinity than the parental 2C12.4. The H8 V_H and V_L domains were used to exchange those of 2C12.4 IgG in the adenoviral vector Ad V giving rise to Ad V.H8. We show that delivery of the two vectors *in vivo* resulted in approximately the same Ab serum titers and that mice immunized with 10^{10} pu Ad V.H8 conferred a statistically significant increase in protection relative to the parental Ad V.

Materials and methods

Bacterial strains and plasmid vectors

Escherichia coli Jude-1 (*E. coli* DH10B F^- *mcrA* (*mrr-hsdRMS-mcrBC*) 80*dlacZ* M15 *lacX74 recA1 araD139* (*ara leu*)7697 *galU galK rpsL endA1 nupG* harboring an F from *E. coli* XL1-blue introduced by conjugation) were used for all antibody engineering experiments. Plasmid pMoPac16 was used for soluble single-chain antibody fragment (scAb) expression and has been previously described¹⁶. Plasmids pAPEX1 was used for *E. coli* display of soluble single-chain variable fragment antibodies (scFv)¹⁵. pFLAG-APEX was constructed by replacing the polyhistidine and c-myc sequences in pAPEX1 via *Bam*HI and *Not*I restriction sites with the FLAG peptide epitope sequence amplified by PCR using primers TVB100 (5' - GTCGCTGCGGCCGAGATTACAAAGACGACGATGACAAGTAGTGATATCGCAA GCTTGACC-3') and TVB101 (5' - CAGCGAGGATCCGTGACGCAGTAGCGGTAACGGC-3').

Protein expression and purification

Recombinant V antigen was expressed and purified as previously described^{1,17}. Monomeric scAbs were expressed and purified according to published procedures^{15,16}. Briefly, *E. coli* Jude-1 containing pMoPac16 derivatives encoding the desired scAb genes were streaked from frozen stocks onto agar plates containing Luria-Burtani Miller (LB) medium (Becton

Dickinson Difco™, Sparks, MD) supplemented with 2% (wt/vol) glucose (2% glc) and 200 µg/ml ampicillin (Amp200) (20 h, 25 °C). Individual colonies were cultured in 2 ml of Terrific Broth (TB) medium (Becton Dickinson Difco™, Sparks, MD) + 2% glc + Amp200 (8 h, 250 rpm, 30 °C) then 1 ml was used to inoculate 500 ml of TB medium + 2% glc + Amp200 (250 rpm, 30 °C). After overnight growth, cells were pelleted by centrifugation (15 min, 4,400 × g, 4 °C) and resuspended in 500 ml of TB medium + Amp200 + 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO) to induce protein expression. After 4 h of incubation at 25 °C, the cells were pelleted by centrifugation (15 min, 4,400 × g, 4 °C), and the periplasmic fraction from the cell pellet was isolated by osmotic shock according to published procedures¹⁶. scAb proteins were purified from the shockate by immobilized metal affinity chromatography (IMAC) using Ni-NTA Agarose based on the manufacturer's protocol (Qiagen, Hilden, Germany), and monomeric scAbs were further isolated by size-exclusion FPLC (SEC) on a Superdex™ 200 column (GE Healthcare, Uppsala, Sweden) using HEPES buffered saline with surfactant P20 (HBS-P) (GE Healthcare, Uppsala, Sweden). Proteins were quantified based on the A₂₈₀ measured using a NanoDrop™ 1000 (Thermo Fisher Scientific, Wilmington, DE) and the appropriate extinction coefficients calculated using Protein Calculator (<http://www.scripps.edu/~cdputnam/protcalc.html>, Putnam Lab at The Scripps Research Institute, La Jolla, CA). Relative concentrations were verified and purity determined by SDS-PAGE using a 4-20% gel (NuSep, Lawrenceville, GA) stained with GelCode Blue (Thermo Fisher Scientific, Rockford, IL). All scAbs used in this work were at least 90% pure.

Anti-V antigen antibody fragment cloning, expression and analysis

Genes encoding the variable heavy chain (V_H) and the variable light chain (V_K) domains of anti-V antigen 2C12.4 IgG were amplified by PCR from the previously described adenovirus vector plasmid DNA¹ using primers TVB102 (5' - GCAGCGAGGCCAGCCGGCCATGGCGcagtaactctgaaagagtctg-3') and TVB103 (5' - agaGCCGCC GCCGCCgtaCCaCCaCCaCCagaaCCaCCaCCaCctgaggagactgtgagagtgtg-3') for V_H and TVB104 (5' - GGtGGtGGtGGtagcGGCGGCGGGCtctGGCGGCGGGCtccgacattgtgctgacac agtcg-3') and TVB105 (5' -CGAATTCGGCCCCCGAGGCcggtttacttccagcttgctc-3') for V_K¹. The resulting PCR products were combined by overlap extension PCR and cloned into pMoPac16 via the non-compatible *Sfi*I restriction sites for soluble expression as a scAb. The resulting ligation product was transformed into *E. coli* Jude-1, and scAb expression was performed as previously described^{16,18}. Briefly, individual colonies were cultured overnight in 2 ml of TB medium + 2% glc + Amp200 at 30 °C. After overnight growth, cells were pelleted by centrifugation (10 min at 4,600 × g, 4 °C) and resuspended in 2 ml of TB medium + Amp200 + 1 mM IPTG to induce protein expression. After 4 h incubation at 25 °C, 1 ml of cells were pelleted by centrifugation (10 min at 4,600 × g, 4 °C), and the cell pellet was resuspended in 1 ml of lysis buffer which consisted of BugBuster™ HT Protein Extraction Reagent (Novagen, Madison, WI) diluted 1:4 in phosphate buffered saline (PBS)¹⁹. The cells were incubated in lysis buffer while rotating on an inverter (30 min, 60 RPM, 25 °C) then the insoluble fraction was removed by centrifugation (1 min, 10,000 × g, 4 °C).

The soluble fraction was analyzed for the presence of full-length scAb specific for the V antigen by Western blot analysis and enzyme-linked immunosorbent assay (ELISA), respectively. Western blot analysis with anti-polyhistidine (anti-His) peroxidase (HRP) conjugate (Sigma-Aldrich, St. Louis, MO) was performed as previously described²⁰. ELISA was performed by coating Costar® high binding 96-well EIA/RIA plates (Corning, Corning, NY) overnight at 4 °C with 50 µl of 5 µg/ml recombinant V antigen or bovine

serum albumin (BSA) in PBS. The plates were washed three times with PBS followed by blocking with 400 μ l PBS supplemented with 2% milk for 4 h at room temperature. Samples were diluted 1:1 in PBS with 2% milk and incubated for 1 h at room temperature. The plates were washed three times with PBS then an additional three times with PBS containing 0.1% Tween-20. Immunocomplexes were detected using an anti-human kappa light chain (anti-HuC) polyclonal serum HRP conjugate (Sigma, St. Louis, MO) applied at a 1:10,000 dilution and incubated for 30 min at room temperature. The plates were washed as described above and developed using the chromogenic HRP substrate TMB+ (Dako, Glostrup, Denmark) as described by the manufacturer. The A_{450} was measured with a microplate reader (BioTek, Winooski, VT).

Isolation of high affinity variants of the 2C12.4 scFv

The anti-V antigen 2C12.4 scFv and the anti-digoxin 26.10 scFv were cloned into pAPEX1 and pFLAG-APEX via the non-compatible *SfiI* restriction sites¹⁵. Purified recombinant V antigen was conjugated to BODIPY® using 6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)hexanoic acid succinimidyl ester (Invitrogen, Carlsbad, CA) at a molar ratio of 1:5 as described by the manufacturer. Free BODIPY FL-X SE was removed using a NAP-10 gel filtration column as described by the manufacturer (GE Healthcare, Uppsala, Sweden). The extent of conjugation was determined based on the ratio of A_{280} and A_{504} measured using a NanoDrop™ 1000 and the appropriate extinction coefficients and correction factors as recommended by the manufacturer (Invitrogen, Carlsbad, CA; <http://www.scripps.edu/~cdputnam/protcalc.html>, Putnam Lab at The Scripps Research Institute, La Jolla, CA).

The anti-V antigen 2C12.4 scFv was subjected to random mutagenesis by error-prone PCR²¹, the amplified DNA was cloned into pFLAG-APEX via the non-compatible *SfiI* restriction sites, and the ligation product was transformed into electrocompetent *E. coli* Jude-1¹⁵. Cells were cultured on agar plates containing LB medium + 2% glc + 30 μ g/ml chloramphenicol (Cm30) (22 h, 25 °C). Cells were transferred to LB medium + 2% glc + 15% glycerol (v/v) to A_{600} ~ 10 then stored at -80 °C. Frozen cell stocks were thawed on ice and subcultured to A_{600} 0.1 in TB medium + 2% glc + Cm30 at 37 °C, grown to A_{600} ~ 0.8-1.2, cooled for 30 min to 25 °C, and protein expression was induced with the addition of 1mM IPTG. After 4h induction at 25 °C, 1 ml of cells at A_{600} 5.0 were converted to spheroplasts using Tris/Sucrose/EDTA/lysozyme as previously described^{15,19}. The spheroplasted cells were first labeled with 5 μ g/ml PhycoLink® anti-Flag® R-Phycoerythrin (anti-Flag PE) (Prozyme, San Leandro, CA) in PBS supplemented with 1% BSA (PBSB) (30 min, 150 rpm, 25 °C). The spheroplasts were washed with PBSB and incubated with 500 nM V antigen conjugated to BODIPY (V antigen-BODIPY) in PBSB (1 h, 150 rpm, 25 °C). Labeled spheroplasts were washed with PBSB and analyzed on a FACSaria (Becton Dickinson Biosciences, San Jose, CA) droplet deflection flow cytometer by exciting with a 488-nm laser and measuring the fluorescence emission spectrum of BODIPY and PE with 530/40 and 570/40 band-pass filters, respectively. Fluorescence compensation was performed using the APEX controls as previously described¹⁹. The library population was gated to avoid aggregates as determined by the forward scatter (FSC) and side scatter (SSC) parameters, and the brightest 1-2% of the population based on both the BODIPY and PE emission spectrums was collected^{15,19}. The collected spheroplasts were immediately resorted, and the scFv genes in the resort solution were amplified by PCR using primers BRH06 (5'-GCGGATAACAATTTTCACACAGG-3') and AHX89 (5'-CGCAGTAGCGGTAAACGGC-3')¹⁵. The amplified DNA was cloned into pFLAG-APEX via the non-compatible *SfiI* restriction sites, and the ligation mixture was transformed into electrocompetent *E. coli* Jude-1. At least 10-fold excess of colonies relative to the number of events in the resort were obtained. Colonies were scraped from the agar plates into liquid

media as above and subjected to an additional two rounds of sorting exactly as described above.

The scFv genes from the third round of sorting were subcloned into pMoPac16, and the ligation product was transformed into *E. coli* Jude-1 for expression of soluble scAb similar to above¹⁵. In addition, *E. coli* Jude-1 containing pMoPac16 encoding 2C12.4 scAb was streaked from a frozen stock onto an agar plate containing LB medium + 2% glc + Amp200. Further, the anti-digoxin 26.10 scFv was cloned into pMoPac16 via the non-compatible *Sfi*I restriction sites, and the ligation product was transformed into *E. coli* Jude-1. 72 individual colonies from round III, 8 colonies of 2C12.4 scAb and 4 colonies of 26.10 scAb were picked and cultured overnight in sterile Costar® round bottom 96-well microtiter plates (Corning, Corning, NY) containing 200 µl of TB medium + 2% glc + Amp200 on a microtiter plate shaker. After overnight growth (150 rpm, 30 °C), 4 µl aliquots were transferred to a master microtiter plate well containing 156 µl of TB medium + 2% glc + Amp200, cultured on a plate shaker (8 h, 150 rpm, 30 °C) and stored at 4 °C for up to two weeks. The remaining cells were pelleted by centrifugation (10 min at 4,600 × g, 4 °C) and resuspended in 200 µl of TB medium + Amp200 + 1 mM IPTG to induce protein expression. After 4 h induction at 25 °C, cells were pelleted by centrifugation (10 min at 4,600 × g, 4 °C), the cell pellet was resuspended in 1 ml of lysis buffer for 30 min at 25 °C, and the insoluble fraction was removed by centrifugation (20 min at 4,600 × g, 4 °C). The soluble fraction was further clarified using 96-well MultiScreen® HTS NA clearing filter plates (Millipore, Billerica, MA) as described by the manufacturer. Filtered lysates were transferred to Costar® non-binding 96-well plates (Corning, Corning, NY) and covered with microplate foil (GE Healthcare, Uppsala, Sweden). Relative expression levels of scAb proteins in filtered lysate were obtained using a Minifold® I Dot Blot System (Whatman, GE Healthcare, Uppsala, Sweden) by transferring 10 µl of filtered lysate diluted with 90 µl of PBS to a nitrocellulose membrane following the manufacturer guidelines. Immunological detection of the polyhistidine tagged scAb proteins immobilized on the nitrocellulose membrane was performed with anti-His HRP conjugate as previously described²⁰. The scAbs in filtered lysate were analyzed by SPR as described below.

Surface Plasmon resonance (SPR)

Antigen binding kinetics of scAb proteins in filtered lysate were analyzed via SPR using a Biacore™ 3000 (GE Healthcare, Uppsala, Sweden). Approximately 200 response units (RU) of V antigen in 10 mM sodium acetate, pH 5.0 were coupled to a CM5 chip (GE Healthcare, Uppsala, Sweden) by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxy succinimide chemistry. BSA was similarly coupled to the chip and used for in-line subtraction. Kinetic analysis was performed in HBS-P (GE Healthcare, Uppsala, Sweden) at a flow rate of 25 µl/min at 25 °C unless indicated. Samples were injected for 3 min and dissociation was monitored for 5 min. The surface was regenerated with two 12 sec injections of 50 mM phosphoric acid. The antigen binding kinetics of purified monomeric scAb were determined in HBS-P at a flow rate of 50 µl/min. Samples were injected for 2 min and dissociation was monitored for 10 min. The surface was regenerated with two 12 sec injections of 50 mM phosphoric acid. Four 2-fold dilutions of each antibody beginning at 160 nM were performed in triplicate, double referenced with a blank, and globally fit to a 1:1 Langmuir binding model for the calculation of both k_{on} and k_{off} using BIAevaluation software (GE Healthcare, Uppsala, Sweden)²².

Ad vectors

Ad V is a replication-defective human adenovirus (Ad) serotype 5, E1-E3⁻ gene transfer vector constructed to direct the expression of the heavy and light chains of the anti-V antigen monoclonal antibody 2C12.4 from a single promoter¹. The expression cassette

contains (5' to 3') the cytomegalovirus (CMV) immediate early promoter/enhancer, the antibody IgG2b heavy chain coding sequence, a 4 amino acid furin cleavage site, the 24 amino acid self-cleaving 2A peptide, the kappa light chain coding sequence, and the simian virus (SV) 40 polyadenylation signal. Ad V.H8 was constructed by assembling the genes encoding the variable heavy chain (V_H) and variable light chain (V_K) domains of the high affinity anti-V antigen 2C12.4 variant, H8, by overlap PCR using the Ad V IgG2b expression cassette as a template and the resulting fragment was used to replace the 2C12.4 coding sequence in Ad V. Ad PA is a similarly constructed gene transfer vector encoding an unrelated antibody (14B7-1H) against *Bacillus anthracis* protective antigen (PA) and was used as a negative control²³. Ad V, Ad V.H8, and Ad PA were produced in human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA) and purified by centrifugation twice through a CsCl gradient, as described²⁴. The titer of each recombinant Ad preparation was determined spectrophotometrically and expressed as particle units (pu) as described²⁵.

Assessment of AdV.H8 *In Vivo*

Male C57BL/6 mice (n=5/group) (The Jackson Laboratory, Bar Harbor, ME) were housed under specific-pathogen-free conditions and used at 6 to 8 weeks of age. Mice were administered Ad V or Ad V.H8 (10^9 , 10^{10} , or 10^{11} pu) via the intravenous route. Naïve mice or mice injected with Ad PA (10^{11} pu) were used as negative controls. Ad vectors were diluted with saline to the specified dose. Serum was collected via the tail vein three days following vector administration, centrifuged at $8000 \times g$ for 20 min, and stored at -20°C . Anti-V antigen serum titers were assessed by ELISA using flat bottomed 96-well EIA/RIA plates (Corning, New York, NY) coated with $100 \mu\text{l}$ $5 \mu\text{g/ml}$ recombinant V antigen in 0.05 M carbonate buffer, pH 7.4 overnight at 4°C . The plates were washed three times with PBS and blocked with $200 \mu\text{l}$ PBS supplemented with 5% milk for 1 hr at 23°C . Serial serum dilutions were added to each well and incubated for 1 hr at 23°C . The plates were washed three times with PBS containing 0.05% Tween-20 (PBST). Immunocomplexes were detected using a sheep anti-mouse IgG HRP conjugate (Sigma, St. Louis, MO) applied at a 1:10,000 dilution in PBS supplemented with 1% milk incubated for 1 hr at 23°C . The plates were washed four times with PBST and once with PBS, developed using $100 \mu\text{l}$ of peroxidase substrate (Bio-Rad, Hercules, CA) incubated for 15 min at 23°C , and the reaction quenched by the addition of $100 \mu\text{l}$ 2% oxalic acid. The A_{415} was measured with a microplate reader (Bio-Rad, Hercules, CA). Antibody titers were calculated based on $\log(\text{optical density}) - \log(\text{dilution})$ normalized with purified anti-V antigen antibodies to account for affinity differences. Mice challenge studies were conducted at The Public Health Research Institute (PHRI) at the International Center for Public Health (Newark, NJ) under biosafety level 3 (BSL3) conditions. *Y. pestis* CO92 was grown aerobically in Heart Infusion Broth (Becton Dickinson Difco™, Sparks, MD) at 30°C and diluted in saline solution for a challenge dose of 2×10^4 cfu which corresponds to 363 LD₅₀. Twenty-five μl of bacterial suspension was used for intranasal infection of mice; bacterial dose was controlled by plating on *Yersinia* Selective Agar (YSA) (Oxoid, Hampshire, UK) and counting colonies for colony-forming units (cfu) determination. Survival was monitored daily for 14 days.

Statistical Analyses

The data are presented as mean \pm standard error of the mean. Statistical analyses were performed using the non-paired two-tailed Student's t-test, assuming equal variance. Survival evaluation was carried out using Kaplan-Meier analysis. Statistical significance was determined at $p < 0.1$.

Results

Engineering high affinity anti-V antigen antibodies

The genes encoding the variable heavy chain (V_H) and light chain (V_L) domain of the *Y. pestis* neutralizing mAb 2C12.4 were used to create a single chain variable fragment antibody (scFv). This scFv gene was cloned into pMoPac16 for soluble expression of the antibody fragment in the single chain antibody (scAb) format¹⁶. The scAb format is comprised of a scFv in which the C termini of the variable light chain domain is fused to the human kappa constant domain, resulting in improved expression in *E. coli* without altering the antibody binding affinity²⁶. Lysates from cells expressing the 2C12.4 scAb were confirmed to express full length protein via Western blotting and displayed binding activity as determined via ELISA using purified *Y. pestis* V antigen.

The gene encoding the scFv version of 2C12.4 was subjected to one round of random mutagenesis by error-prone PCR. Amplified DNA was cloned into pFLAG-APEX and the ligation product was transformed into *E. coli* Jude-1 cells yielding $>2 \times 10^6$ independent transformants. DNA sequencing of 10 clones selected at random revealed an average of 1.75% nucleotide substitutions with a standard deviation of 0.75%. Cells were grown in liquid culture, protein expression was induced, and 4 hours later, the cells were harvested and converted into spheroplasts. The spheroplasted cells were then labeled with anti-Flag-PE and V antigen-BODIPY to determine full-length scFv expression levels and antigen binding, respectively (Figure 1a). A total of 8×10^7 cells (40-fold library coverage) were subjected to high-throughput fluorescence activated cell sorting (FACS) and the top 2% events in terms of PE and BODIPY fluorescence emissions were collected (Figure 1b). The collected spheroplasts were immediately resorted as above without additional labeling to maximize the isolation of antibodies with slow dissociation rate constants toward V antigen¹⁵. The scFv genes in the spheroplasts collected during the resort were amplified by PCR, and the DNA was subcloned back into the pFLAG-APEX vector. Following transformation, the cells were subjected to two additional rounds of sorting using increasingly stringent collection criteria, namely a higher fluorescence threshold. After the third round of sorting, the number of events falling within the window used for the 1st round increased from 2% to 23% (Figure 1b).

The scFv gene pool from the third round of sorting was amplified by PCR and cloned into pMoPac16 for expression in the scAb format. SPR was used to rank order the dissociation rate constants of individual clones (Supplementary Figure 1). Of the 72 colonies tested, 41 (57%) expressed scAbs that displayed significant binding towards V antigen immobilized on the Biacore chip. Of these 41 clones, four clones exhibited dissociation rate constants at least 10-fold slower than those expressing the parental antibody (Supplementary Figure 1e and 1f).

These four scAbs, as well as the 2C12.4 and 26.10 scAbs, were expressed at the 500 ml scale in shake flasks, the periplasmic fraction was isolated by osmotic shock, and monomeric scAb proteins were purified by immobilized metal affinity chromatography (IMAC) and size exclusion FPLC. Consistent with the isolation of the respective clones based on increased PE fluorescence, the protein yields for all four scAbs was higher than that of the parental 2C12.4 scAb (Table 1). All antibodies exhibited at least 10-fold lower equilibrium dissociation constants (K_D) relative to the parental 2C12.4 scAb (Table 1) which is consistent with the measurements obtained using crude cell lysates (Supp. Fig 1F). The highest affinity clone, H8, exhibited an affinity of 100 pM that translates to a 35-fold improvement compared to the parental antibody 2C12.4. The improved affinity for all clones was almost exclusively the result of a decrease in the dissociation rate constant whereas the association rate constant remained essentially unchanged. The four variants, H8,

E4, F4 and G8, contained either 7 or 9 amino acid mutations compared to the parental antibody (Table 2) of which two were common: L49S in framework 2 of the V_H, which is immediately prior to complementary determining region 2 (CDR2); and Y32F in CDR1 of the light chain²⁷. Further, three of the variants contained an additional four common mutations (D1N, T5S, R24K, T97R) in the light chain.

Genetic immunization using IgG encoding adenovirus vectors and challenge with *Y. pestis*

The V_H and V_K domains of the 2C12.4 mouse IgG2b antibody were replaced with those from the highest affinity antibody variant, H8, and engineered into a replication-defective adenovirus serotype 5 E1-E3- gene transfer vector to create Ad V.H8¹. Mice were immunized with varying doses of Ad V and Ad V.H8 [10⁹ – 10¹¹ particle units (pu)], or, as controls, the highest dose (10¹¹ pu) of Ad PA, an adenovirus vector encoding a mouse IgG mAb specific for the *B. anthracis* PA or PBS. Serum anti-V antigen antibody titers were measured via ELISA three days post immunizations (Figure 2). A similar dose dependent response was observed for both Ad V and Ad V.H8. The titers for equivalent immunization levels were within 10-fold for both Ad V and Ad V.H8. Both Ad PA and PBS controls did not result in any measurable anti-V antibody titers. Three days after immunization, the mice were challenged intranasally with 363 LD₅₀ of the fully virulent *Y. pestis* CO92 and survival monitored for two weeks (Figure 3). All mice immunized with the highest dosage (10¹¹ pu) of Ad V and Ad V.H8 survived (Figure 4). However, at a 10-fold lower dose (10¹⁰ pu), only 15% of the mice immunized with Ad V survived while 54% of the mice immunized with this dose of Ad V.H8 survived ($P < 0.04$, Ad V versus Ad V.H8). In addition, 15% of the mice immunized with an additional 10-fold lower dose (10⁹ pu) of Ad V.H8 survived, but none survived with the same dosage level of Ad V.H8 ($P < 0.1$, Ad V versus Ad V.H8).

Discussion

Passive immunization with engineered antibodies displaying enhanced antigen affinity has been shown to increase the neutralization efficacy of a number of bacterial or viral pathogens and toxins^{8-11,13,14}. However, to the best of our knowledge, neither the effect of antigen affinity for antibodies expressed *in situ* following viral transfer of the antibody gene, nor the relationship between V antigen affinity and protection to the potential biowarfare agent *Y. pestis*, have been examined. Here we found that increasing the affinity of the *Y. pestis* neutralizing antibody 2C12.4 from 3.5 nM to 100 pM (35-fold) could confer significant protection against a lethal challenge with *Y. pestis* at a 10-fold lower dose of a recombinant adenovirus encoding the neutralizing antibody.

The affinities of antibodies generated by the natural immune system are constrained by the kinetics of *in vivo* selection²⁸. Most high affinity mouse monoclonal antibodies display nanomolar affinities and this is the case for the anti-V antigen antibody 2C12.4 which displays a K_D=3.5 nM by surface Plasmon resonance (SPR). Combinatorial mutagenesis and library screening by phage display or other high throughput techniques has been employed for affinity maturation of the variable domains of monoclonal antibodies^{8-11,13,14}. In earlier studies, we used random mutagenesis and screening of *E. coli* displayed libraries by APEX to isolate picomolar affinity antibodies to the *B. anthracis* PA which in turn proved to have markedly improved neutralization potency *in vitro* and in various animal models^{8,12,29}. Key advantages of this strategy are the ease with which libraries of random mutants can be constructed in *E. coli* and screened by FACS. FACS is a high-throughput screening technique that enables real-time quantitative multi-parameter analysis on a cell by cell basis. Therefore, multiple properties can be monitored simultaneously to specifically isolate antibodies with desired characteristics. In this work antibody fragments were isolated from a library on the basis of both antigen affinity and expression level (Figure 1). Subsequently,

clones isolated from the last round of FACS were rank-ordered by comparing the dissociation rate constants in crude lysates (Supplementary Figure 1). This approach significantly expedited the analysis of the clones obtained after screening, a process that often represents the rate limiting step in antibody affinity maturation. Thus, a single round of mutagenesis followed by three rounds of FACS screening resulted in the isolation of antibodies displaying affinities in the 100 pM range and also higher expression yields in *E. coli* (Figure 1b, Table 1). In addition, the conversion of these monovalent antibody fragments to full-length antibodies can further enhance their potency due to the avidity associated with these bivalent molecules leading to an even higher apparent affinity.

Genetic delivery of full-length antibodies *in vivo* has been carried out using adenovirus, adeno-associated virus, and vaccinia virus vectors³⁰⁻³² and is an attractive alternative to the administration of antibody protein which requires production, purification, and formulation prior to injection into the patient^{31,33}. The administration of antibodies as injectable therapeutics is complicated by bioavailability issues, and by the potential for increased immunogenicity resulting from large dosages required for therapeutic purposes and the ensuing aggregation issues³⁴. A common side effect is infusion reactions with symptoms ranging from mild to life-threatening and typically occur near the time of administration³⁵. Following infusions, complicated pharmacokinetics result in a continual decrease in serum concentrations which effects the bioavailability and the pharmacodynamic properties of the therapeutic antibody^{34,36}. However, genetic delivery can provide immediate and sustained serum levels for several months from a single treatment with both adenovirus (Ad) and adeno-associated virus (AAV) vectors encoding the same monoclonal antibody³⁷. This approach has been demonstrated with a neutralizing antibody and offered almost immediate protection against anthrax toxin challenge *in vivo* which was sustained for 6 months²³. This approach circumvents many of the issues associated with conventional methods and has the additional benefits of increasing patient quality of life and protecting soldiers entering combat for extended periods.

Here we demonstrate that the affinity of antibodies produced *in situ* following adenoviral gene transfer correlates with protection. Although human Ad serotype 5(Ad5) has been effectively used in a variety of animal models for genetic delivery, it has limited use because approximately 35 to 50% of humans have preexisting neutralizing antibodies against it³⁸. However, several non-human primate adenovirus vectors for which no natural immunity exists in humans have been described and can be deployed to circumvent the neutralization or clearance of the vector³⁷. There are many pathogens to which an effective vaccine has yet to be produced and neutralizing antibodies remain the only means for prophylaxis and therapy. The potency of neutralizing antibodies can benefit from engineering their affinity, stability and neutralization potency (for instance through mutations that increase antibody-dependent cell-mediated cytotoxicity³⁹). The *in situ* synthesis of optimized neutralizing antibodies by low immunogenicity genetic delivery vectors could represent a promising alternative for the treatment of a number of infectious diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Sofer-Podesta C, et al. Adenovirus-mediated Delivery of an Anti-V Antigen Monoclonal Antibody Protects Mice Against a Lethal *Yersinia pestis* Challenge. *Infect Immun*. 2009; 77:1561–1568. [PubMed: 19124600]
2. Smiley ST. Immune defense against pneumonic plague. *Immunol Rev*. 2008; 225:256–271. [PubMed: 18837787]
3. Brouqui P, Raoult D. Arthropod-borne diseases in homeless. *Ann N Y Acad Sci*. 2006; 1078:223–235. [PubMed: 17114713]
4. Zhou D, Han Y, Yang R. Molecular and physiological insights into plague transmission, virulence and etiology. *Microbes Infect*. 2006; 8:273–284. [PubMed: 16182593]
5. Hill J, et al. Administration of antibody to the lung protects mice against pneumonic plague. *Infect Immun*. 2006; 74:3068–3070. [PubMed: 16622253]
6. Hill J, et al. Synergistic protection of mice against plague with monoclonal antibodies specific for the F1 and V antigens of *Yersinia pestis*. *Infect Immun*. 2003; 71:2234–2238. [PubMed: 12654847]
7. Hill J, et al. Regions of *Yersinia pestis* V antigen that contribute to protection against plague identified by passive and active immunization. *Infect Immun*. 1997; 65:4476–4482. [PubMed: 9353022]
8. Maynard JA, et al. Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. *Nat Biotechnol*. 2002; 20:597–601. [PubMed: 12042864]
9. Wu H, et al. Development of motavizumab, an ultra-potent antibody for the prevention of respiratory syncytial virus infection in the upper and lower respiratory tract. *J Mol Biol*. 2007; 368:652–665. [PubMed: 17362988]
10. Marks JD. Deciphering antibody properties that lead to potent botulinum neurotoxin neutralization. *Mov Disord*. 2004; 19(Suppl 8):S101–108. [PubMed: 15027061]
11. Barbas CF 3rd, et al. In vitro evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. *Proc Natl Acad Sci U S A*. 1994; 91:3809–3813. [PubMed: 8170992]
12. Mabry R, et al. Passive protection against anthrax by using a high-affinity antitoxin antibody fragment lacking an Fc region. *Infect Immun*. 2005; 73:8362–8368. [PubMed: 16299334]
13. Riano-Umbarila L, et al. A strategy for the generation of specific human antibodies by directed evolution and phage display. An example of a single-chain antibody fragment that neutralizes a major component of scorpion venom. *FEBS J*. 2005; 272:2591–2601. [PubMed: 15885107]
14. Rajpal A, et al. A general method for greatly improving the affinity of antibodies by using combinatorial libraries. *Proc Natl Acad Sci U S A*. 2005; 102:8466–8471. [PubMed: 15939870]
15. Harvey BR, et al. Anchored periplasmic expression, a versatile technology for the isolation of high-affinity antibodies from *Escherichia coli*-expressed libraries. *Proc Natl Acad Sci U S A*. 2004; 101:9193–9198. [PubMed: 15197275]
16. Hayhurst A, et al. Isolation and expression of recombinant antibody fragments to the biological warfare pathogen *Brucella melitensis*. *Journal of Immunological Methods*. 2003; 276:185–196. [PubMed: 12738372]
17. Chiuchiolo MJ, et al. Protective immunity against respiratory tract challenge with *Yersinia pestis* in mice immunized with an adenovirus-based vaccine vector expressing V antigen. *J Infect Dis*. 2006; 194:1249–1257. [PubMed: 17041851]
18. Chen G, et al. Isolation of high-affinity ligand-binding proteins by periplasmic expression with cytometric screening (PECS). *Nat Biotechnol*. 2001; 19:537–542. [PubMed: 11385457]
19. Mazor Y, Van Blarcom T, Iverson BL, Georgiou G. E-clonal antibodies: selection of full-length IgG antibodies using bacterial periplasmic display. *Nat Protoc*. 2008; 3:1766–1777. [PubMed: 18948976]
20. Ribnicky B, Van Blarcom T, Georgiou G. A scFv antibody mutant isolated in a genetic screen for improved export via the twin arginine transporter pathway exhibits faster folding. *J Mol Biol*. 2007; 369:631–639. [PubMed: 17462668]
21. Fromant M, Blanquet S, Plateau P. Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. *Anal Biochem*. 1995; 224:347–353. [PubMed: 7710092]

22. Myszka DG. Improving biosensor analysis. *J Mol Recognit.* 1999; 12:279–284. [PubMed: 10556875]
23. De BP, Hackett NR, Crystal RG, Boyer JL. Rapid/sustained anti-anthrax passive immunity mediated by co-administration of Ad/AAV. *Mol Ther.* 2008; 16:203–209. [PubMed: 18059375]
24. Rosenfeld MA, et al. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell.* 1992; 68:143–155. [PubMed: 1370653]
25. Mittereder N, March KL, Trapnell BC. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol.* 1996; 70:7498–7509. [PubMed: 8892868]
26. Hayhurst A. Improved expression characteristics of single-chain Fv fragments when fused downstream of the Escherichia coli maltose-binding protein or upstream of a single immunoglobulin-constant domain. *Protein Expr Purif.* 2000; 18:1–10. [PubMed: 10648163]
27. Martin AC. Accessing the Kabat antibody sequence database by computer. *Proteins.* 1996; 25:130–133. [PubMed: 8727325]
28. Foote J, Eisen HN. Kinetic and affinity limits on antibodies produced during immune responses. *Proc Natl Acad Sci U S A.* 1995; 92:1254–1256. [PubMed: 7877964]
29. Mohamed N, et al. A high-affinity monoclonal antibody to anthrax protective antigen passively protects rabbits before and after aerosolized Bacillus anthracis spore challenge. *Infect Immun.* 2005; 73:795–802. [PubMed: 15664918]
30. Fang J, et al. Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol.* 2005; 23:584–590. [PubMed: 15834403]
31. Jiang M, et al. Gene therapy using adenovirus-mediated full-length anti-HER-2 antibody for HER-2 overexpression cancers. *Clin Cancer Res.* 2006; 12:6179–6185. [PubMed: 17062695]
32. BenAmmar-Ceccoli S, et al. Recombinant vaccinia viruses expressing immunoglobulin variable regions efficiently and selectively protect mice against tumoral B-cell growth. *Cancer Gene Ther.* 2001; 8:815–826. [PubMed: 11687905]
33. Daugherty AL, Mersny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. *Adv Drug Deliv Rev.* 2006; 58:686–706. [PubMed: 16839640]
34. Samaranyake H, et al. Challenges in monoclonal antibody-based therapies. *Ann Med.* 2009;1–10.
35. Chung CH. Managing premedications and the risk for reactions to infusional monoclonal antibody therapy. *Oncologist.* 2008; 13:725–732. [PubMed: 18586928]
36. Joshi A, et al. An overview of the pharmacokinetics and pharmacodynamics of efalizumab: a monoclonal antibody approved for use in psoriasis. *J Clin Pharmacol.* 2006; 46:10–20. [PubMed: 16397279]
37. Boyer JL, Kobinger G, Wilson JM, Crystal RG. Adenovirus-based genetic vaccines for biodefense. *Hum Gene Ther.* 2005; 16:157–168. [PubMed: 15761256]
38. Hashimoto M, et al. Induction of protective immunity to anthrax lethal toxin with a nonhuman primate adenovirus-based vaccine in the presence of preexisting anti-human adenovirus immunity. *Infect Immun.* 2005; 73:6885–6891. [PubMed: 16177368]
39. Lazar GA, et al. Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci U S A.* 2006; 103:4005–4010. [PubMed: 16537476]
40. Wu H, et al. Ultra-potent antibodies against respiratory syncytial virus: effects of binding kinetics and binding valence on viral neutralization. *J Mol Biol.* 2005; 350:126–144. [PubMed: 15907931]

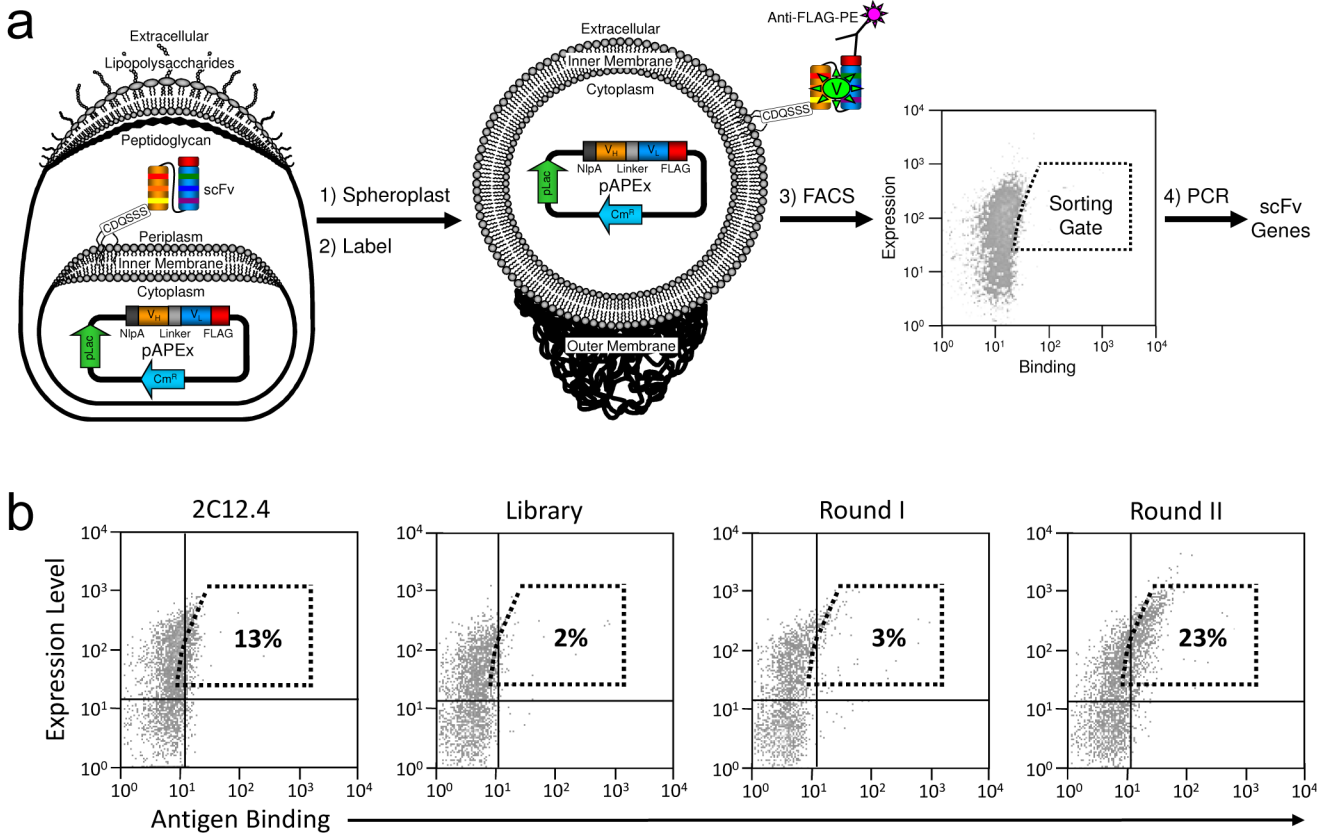


Figure 1. Affinity maturation of the anti-V antigen scFv 2C12.4 using APEX. (a) Schematic diagram of anchored periplasmic expression (APEX) (b) FACS analysis of *E. coli* spheroplasts expressing 2C12.4 scFv, the library of random mutants and the populations recovered following two rounds of FACS. Spheroplasts were labeled with 500 nM V antigen-BODIPY and 5 μ g/ml Anti-FLAG-PE. The dashed box represents the gate used to isolate cells by FACS from the original library. The percentage of the spheroplast in this gate for each population is indicated.

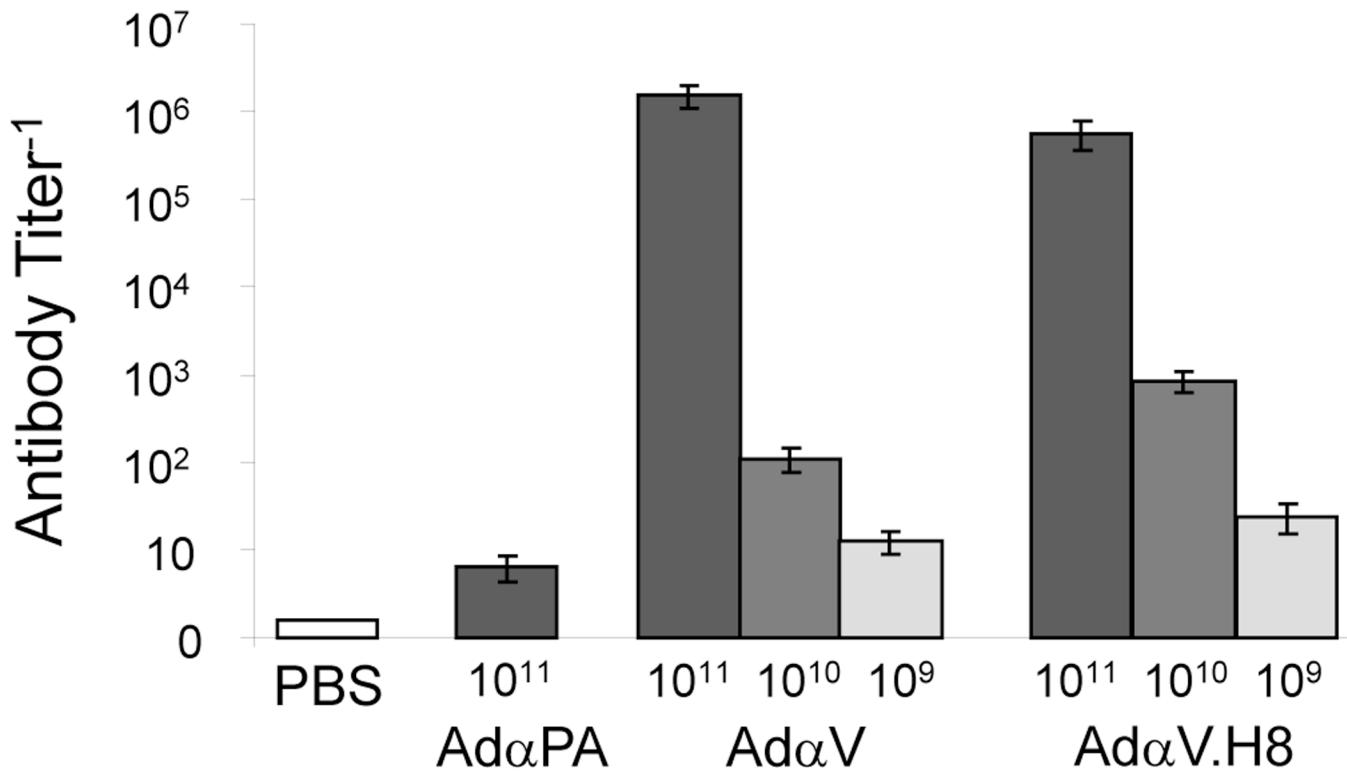


Figure 2.

Anti-V antigen IgG titers in mice following immunization with Ad vectors. C57BL/6 mice ($n = 13$ /group for Ad V and Ad V.H8; $n = 1$ /group for Ad PA and PBS) were administered intravenously with the indicated Ad vectors or PBS. Serum anti-V antigen antibody titers were measured three days post immunization via ELISA. PBS, negative control; Ad PA, vector encoding an IgG specific for the *B. anthracis* PA; Ad V, vector encoding the 2C12.4 IgG; Ad V.H8, vector encoding the 2C12.4 IgG mutant H8.

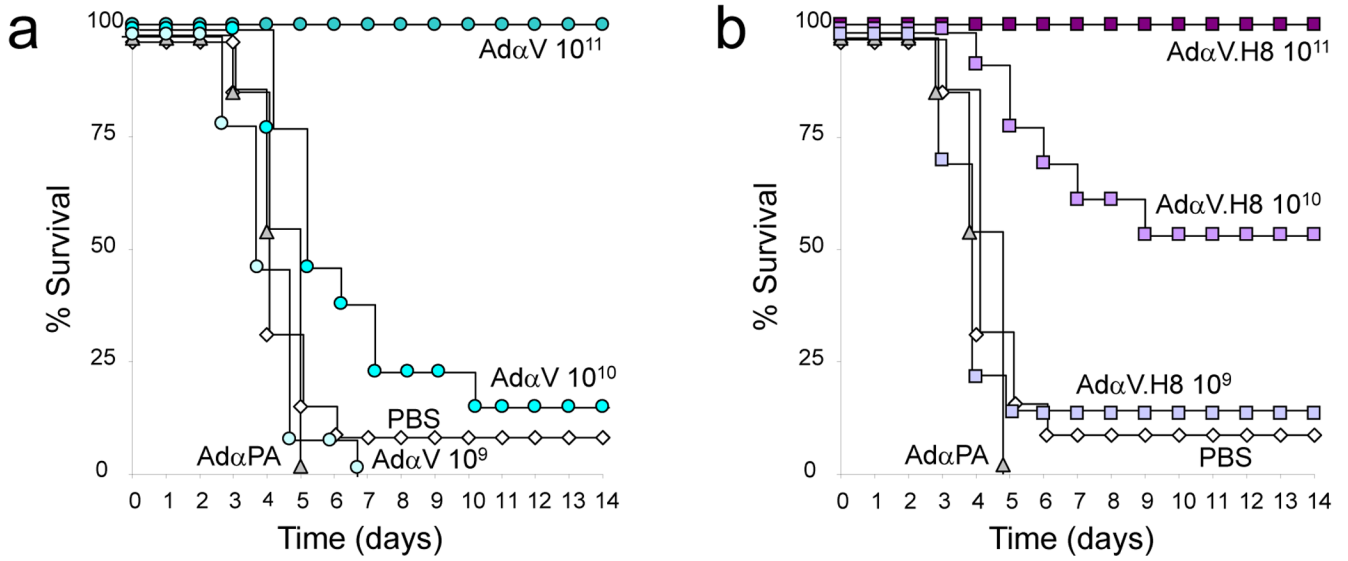


Figure 3. Survival of mice challenged with *Y. pestis* following prophylactic administration of Ad vectors. Three days following intravenous administration of Ad vectors or PBS, C57BL/6 mice ($n = 13/\text{group}$ for Ad V and Ad V.H8; $n = 13/\text{group}$ for Ad PA and PBS) were challenged with a lethal dose of *Y. pestis* intranasally. Survival of the mice was monitored for 14 days following challenge. (a) Immunization with Ad V compared to Ad PA and PBS. (b) Immunization with Ad V.H8 compared to Ad PA and PBS.

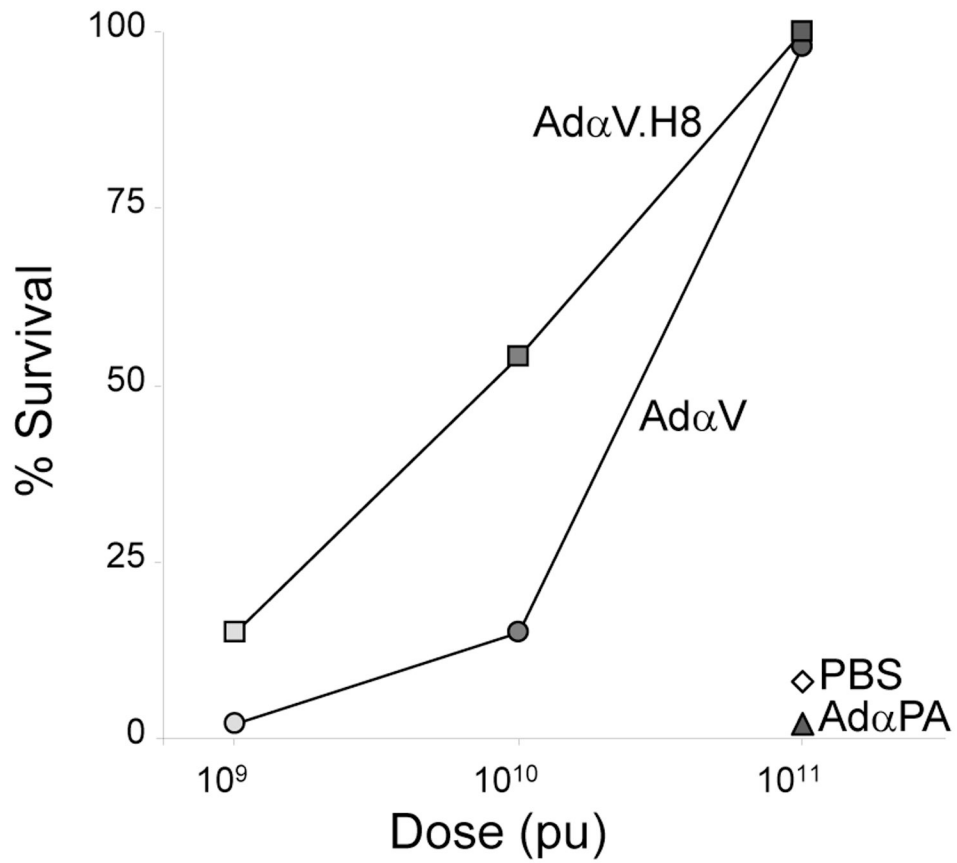


Figure 4. Net survival of mice challenged with *Y. pestis* following prophylactic administration of Ad vectors. Percentage of C57BL/6 mice ($n = 13/\text{group}$ for Ad V and Ad V.H8; $n = 13/\text{group}$ for Ad PA and PBS) surviving 14 days following intranasal challenge with a lethal dose of *Y. pestis*.

Table 1

Kinetic analysis of anti-V antigen antibody fragments

Antibody	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (pM)	Affinity Improvement ^a	Expression Improvement ^b
2C12.4	9.2×10^4	3.2×10^{-4}	3500	1x	1x
H8	1.2×10^5	1.2×10^{-5}	100	35x	4x
E4	1.0×10^5	1.8×10^{-5}	180	20x	5x
F4	1.2×10^5	2.6×10^{-5}	220	16x	3x
G8	9.8×10^4	2.4×10^{-5}	250	14x	2x

^aAffinity improvement relative to the parental antibody 2C12.4

^bYield of purified scAb (mg/L A600) compared to 2C12.4

Table 2

Sequence analysis of anti-V antigen antibody fragments

Clone	Variable Heavy Chain ^a					Variable Light Chain ^a												
	FW1	CDR1	FW2	CDR2	FW3	FW1	CDR1	FW2	CDR2	FW3	CDR3							
3	11	32	45	49	51	65	67	82	1	5	24	32	39	53	72	84	97	
2C12.4	T	I	S	L	L	I	S	L	I	D	T	R	Y	K	N	T	A	T
H8			S	V				N	S	K	F							R
E4	V	P	S					F	R	S								T
F4	A		P	S		V		N	S	K	F							R
G8			S	G		T	N	S	K	F					A			R

^a Amino acid substitutions based on Kabat numbering. FW, framework; CDR, complementary determining region