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## Human anti-anthrax protective antigen neutralizing monoclonal antibodies derived from donors vaccinated with anthrax vaccine adsorbed

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### Abstract

**Background:** Potent anthrax toxin neutralizing human monoclonal antibodies were generated from peripheral blood lymphocytes obtained from Anthrax Vaccine Adsorbed (AVA) immune donors. The anti-anthrax toxin human monoclonal antibodies were evaluated for neutralization of anthrax lethal toxin *in vivo* in the Fisher 344 rat bolus toxin challenge model.

**Methods:** Human peripheral blood lymphocytes from AVA immunized donors were engrafted into severe combined immunodeficient (SCID) mice. Vaccination with anthrax protective antigen and lethal factor produced a significant increase in antigen specific human IgG in the mouse serum. The antibody producing lymphocytes were immortalized by hybridoma formation. The genes encoding the protective antibodies were rescued and stable cell lines expressing full-length human immunoglobulin were established. The antibodies were characterized by; (1) surface plasmon resonance; (2) inhibition of toxin in an *in vitro* mouse macrophage cell line protection assay and (3) *in vivo* in a Fischer 344 bolus lethal toxin challenge model.

**Results:** The range of antibodies generated were diverse with evidence of extensive hyper mutation, and all were of very high affinity for PA83~1 × 10<sup>-10-11</sup>M. Moreover all the antibodies were potent inhibitors of anthrax lethal toxin *in vitro*. A single IV dose of AVP-21D9 or AVP-22G12 was found to confer full protection with as little as 0.5× (AVP-21D9) and 1× (AVP-22G12) molar equivalence relative to the anthrax toxin in the rat challenge prophylaxis model.

**Conclusion:** Here we describe a powerful technology to capture the recall antibody response to AVA vaccination and provide detailed molecular characterization of the protective human monoclonal antibodies. AVP-21D9, AVP-22G12 and AVP-1C6 protect rats from anthrax lethal toxin at low dose. Aglycosylated versions of the most potent antibodies are also protective *in vivo*, suggesting that lethal toxin neutralization is not Fc effector mediated. The protective effect of AVP-21D9 persists for at least one week in rats. These potent fully human anti-PA toxin-neutralizing antibodies are attractive candidates for prophylaxis and/or treatment against Anthrax Class A bioterrorism toxins.

## Background

Unlike diphtheria, tetanus and botulinum, anthrax infection manifests itself due to toxin mediated immune dysfunction, which permits the anthrax bacteria to evade immune surveillance and thus disseminate throughout the body and reach extremely high levels. Very high levels of toxins produced later in the infection may also facilitate subsequent rapid onset of death due to massive organ failure. Hence inhibiting anthrax toxins early may change the course of infection and may allow a vigorous immune response against the bacteria and the toxins; in essence passive immunity against the toxins may facilitate active immunity in a natural exposure. Anthrax toxin, which consists of three polypeptides protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa), is a major virulence factor of *Bacillus anthracis*. The LF and EF components are enzymes that are carried into the cell by PA. The combination of PA and LF forms lethal toxin [1-3]. Anthrax toxin enters cells via a receptor-mediated endocytosis [4,5]. PA binds to the receptor and is processed (PA, 63 kDa), which forms a heptameric ring that delivers the EF or LF to the cytosol. The path leading from PA binding to cells via TEM-8 [5] or CMG2 [6], furin processing, heptamer formation, LF or EF binding to heptamer, or the translocation of EF/LF to the cytosol provides multiple sites for molecular intervention.

The PA plays an elaborate yet critical role in virulence and has been the main target for immune disruption of the anthrax toxins. The role of the PA component in the vaccine was established soon after the discovery of the toxin [7]. In the 1880's it had been demonstrated that inoculation of animals with attenuated strains of *B. anthracis* led to protection [8]. An improved unencapsulated avirulent variant of *B. anthracis* was developed in the late 1930's for veterinary use [9,10]. The observation that exudates from anthrax lesions could provide protection in laboratory animals [11] led to the evaluation of filtrates of culture of *B. anthracis* as vaccines [12]. The current licensed anthrax vaccine developed more than half a century ago is based on *B. anthracis* culture filtrate [13], utilizes *B. anthracis* strains that produce more PA under certain growth conditions [14,15]. The standard immunization schedule with this crude PA preparation with aluminium hydroxide, involves 3 subcutaneous injections at 0, 2 and 4 weeks, and 3 booster at 6, 12 and 18 months, and it is suggested that an annual booster is required to maintain immunity.

In the event of an intentional or inadvertent exposure to *B. anthracis* aerosolized spore [16], immediate immunity is required. This may be attained by passive immunization. Passive immunity against *B. anthracis* has been demonstrated with polyclonal antibodies in laboratory animals [17,18]. More recently several groups have demonstrated passive efficacy of recombinant antibody frag-

ments derived and optimized by phage display directed against PA in Fisher 344 rats challenged with lethal toxin [19,20]. Neutralizing anthrax toxins immediately may allow the immune system to recognize components of the *B. anthracis* bacteria and mount an appropriate response and significantly alter the course of infection. Secondly, toxin neutralization may also prevent death.

A passive immunization approach would provide immediate immunity, which would complement antibiotic therapy.

Here we describe the generation of a panel of potent human monoclonal antibodies derived from anthrax vaccine adsorbed immune donors. Protection against anthrax toxin challenge in an *in vitro* cell culture assay correlates well with affinity, with the highest affinity antibody AVP-21D9 (K<sub>d</sub> = 82 pM) exhibiting the most potent toxin inhibition. Moreover, we report a panel of fully human antibodies generated from AVA vaccinated donors using Xenex<sup>TM</sup> technology protect Fisher 344 rats from anthrax intoxication *in vivo*.

## Methods

### Selection of donors

We have an Institutional Review Board (IRB) approved protocol to obtain units of blood from donors at Avanir Pharmaceuticals. Volunteer donors informed consent were obtained. Volunteers serum obtained at the time of blood collection by venipuncture from anthrax-vaccinated donors were pre-screened against a panel of antigens (including components of the anthrax exotoxin PA and LF) in an ELISA for both IgG and IgM. An internal calibrator was incorporated into each assay consisting of a control antiserum containing both IgG and IgM anti-tetanus toxoid. The IgG and IgM titres were compared across assays performed on different days, thereby permitting more robust comparisons of the entire donor panel.

### Engraftment of SCID mice with human PBMC from pre-selected AVA immune donors

Peripheral blood mononuclear cells were separated from whole blood of AVA immune donors by density gradient using Histopaque (Sigma, St Louis, MO). Twelve-week-old SCID/bg mice were each engrafted (via intra peritoneal ip injection) with  $2.5 \times 10^7$  human PBMC. They were treated with 0.5 ml of conditioned media, which contains 0.2 mg of the anti-CD8 monoclonal antibody. After 2 hours, the mice were immunized (ip) with the recombinant PA and LF (List Laboratories Inc, Campbell, CA) 2 µg each adsorbed to Alum (Imject<sup>®</sup>, Pierce, Rockford, IL) and subsequently boosted (ip) 8 and 28 days later. Mice were inoculated with 0.5 ml of EBV obtained from B95-8 cells spent conditioned culture medium at day 15 following engraftment. Test bleeds

were obtained from the orbital sinus, on days 15 and 30. Two consecutive iv and ip boosts with the appropriate toxins were administered (typically, 5 µg each on day 35 and day 36; both in saline) prior to harvesting cells for fusion on day 37. The total IgG and specific anti-PA IgG assays combined with potency in the RAW 264.7 cell bioassay (as described below) were determined.

#### Generation of human hybridomas

Splenocytes, peritoneal washes, as well as lymphoblastoid cell line (LCL) tumors transformed by EBV, were harvested on day 37 from those mice showing positive test bleeds in PA specific ELISA and the appropriate bioassay. Human hybridomas were generated from these in separate fusions using a mouse myeloma cell line P3X/63Ag8.653 [21] with PEG-1500 (Sigma, St Louis, MO). Double selection against the EBV transformed LCL and the un-fused fusion partner was carried out using a combination of HAT and Ouabain.

#### Variable region IgH and IgL cDNA cloning and expression

Total RNA was prepared from hybridoma cells using RNeasy Mini Kit (Qiagen, Valencia, CA). Mixture of VH and VL cDNAs were synthesized and amplified in a same tube using One-Step RT-PCR Kit (Qiagen, Valencia, CA). Cycling parameters were 50°C for 35 min, 95°C for 15 min, 35 cycles of 94°C for 30 sec, 52°C for 20 sec and 72°C for 1 min 15 sec, and 72°C for 5 min.

Primers used for RT-PCR were:

#### For VH $\gamma$

Forward

- CVH2 TGCCAGRTCACCTTGARGGAG
- CVH3 TGCSARGTGCAGCTGKTGGAG
- CVH4 TGCCAGSTGCAGCTRCAGSAG
- CVH6 TGCCAGGTACAGCTGCAGCAG
- CVH1257 TGCCAGGTGCAGCTGGTGSARTC

Reverse (located at 5' of CH1 region)

- C $\gamma$ II GCCAGGGGAAGACSGATG

#### For VL $\kappa$

Forward

- VK1F GACATCCRGDTGACCCAGTCTCC
- VK36F GAAATTGTRWTGACRCAGTCTCC

- VK2346F GATRTTGTGMTGACBCAGWCTCC

- VK5F GAAACGACACTCACGCAGTCTC

Reverse (located in constant region)

- Ck543 GTTTCTCGTAGTCTGCTTTGCTCA

#### For VL $\lambda$

Forward

- VL1 CAGTCTGTGYTGACGCAGCCGCC
- VL2 CAGTCTGYCTGAYTCAGCCT
- VL3 TCCTATGAGCTGAYRCAGCYACC
- VL1459 CAGCCTGTGCTGACTCARYC
- VL78 CAGDCTGTGGTGACYCAGGAGCC
- VL6 AATTTTATGCTGACTCAGCCCC

Reverse (located in constant region)

- CL2 AGCTCCTCAGAGGAGGGYGG

The RT-PCR was followed by nested PCR with High Fidelity Platinum PCR Mix (Invitrogen, Carlsbad, CA). An aliquot (1 µl) of RT-PCR products were used for VH $\gamma$ , VL $\kappa$  or VL $\lambda$  specific cDNA amplification in a separate tube. At the same time restriction enzyme sites were introduced at both ends. Cycling parameters were 1 cycle of 94°C for 2 min, 60°C for 30 sec and 68°C for 45 sec, 35 cycles of 94°C for 40 sec, 54°C for 25 sec and 68°C for 45 sec, and 68°C for 5 min.

Each specific PCR product was separately purified, digested with restriction enzymes, and subcloned into appropriate mammalian full-length Ig expression vectors as described below.

Primers for nested PCR were:

#### For VH $\gamma$

Forward (adding BsrGI site at 5' end)

- BsrGIVHF2 AAAATGTACAGTGCCAGRTCACCTTGARGGAG
- BsrGIVHF3 AAAATGTACAGTGCSARGTGCAGCTGKTGGAG
- BsrGIVHF4 AAAATGTACAGTGCCAGSTGCAGCTRCAGSAG

d. BsrGIVHF6 AAAATGTACAGTGCCAGGTACAGCT-GCAGCAG

e. BsrGIVHF1257 AAAATGTACAGTGCCAGGTGCAGCT-GGTGSARTC

Reverse (including native ApaI site)

a. C $\gamma$ ER GACSGATGGGCCCTTGGTGGGA

VH $\gamma$ PCR products were digested with BsrG I and Apa I and ligated into pEEG1.1 vector that is linearized by Sph I and Apa, I double digestion.

#### For VL $\kappa$

Forward (adding AgeI site, Cys and Asp at 5'end)

a. AgeIVK1F TTTTACCGGTGTGACATCCRGDTGAC-CCAGTCTCC

b. AgeIVK36F TTTTACCGGTGTGAAATTGTRWT-GACRCAGTCTCC

c. AgeIVK2346F TTTTACCGGTGTGATRTTGTGMTGACB-CAGWCTCC

d. AgeIVK5F TTTTACCGGTGTGAAACGACACTCAGC-CAGTCTC

Reverse (adding SphI site, located between FR4 and 5' of constnt region)

a. SplKFR4R12 TTTCGTACGTTTGAYYTCCASCTTGGTC-CCYTG

b. SplKFR4R3 TTTCGTACGTTTSAKATCCACTTTGGTC-CCAGG

c. SplKFR4R4 TTTCGTACGTTTGATCTCCACCTTGGTC-CCTCC

d. SplKFR4R5 TTTCGTACGTTTAATCTCCAGTCGTGTC-CCTTG

VL $\kappa$ PCR products were digested with Age I and Spl I and ligated into pEEK1.1 vector linearized by Xma I and Spl I double digestion.

#### For VL $\lambda$

Forward (adding ApaI site at 5' end)

a. ApaIVL1 ATATGGGCCCAGTCTGTGYTGACG-CAGCCGCC

b. ApaIVL2 ATATGGGCCCAGTCTGYCTGAYTCAGCCT

c. ApaIVL3 ATATGGGCCCAGTATGAGCTGAYRCAGCY-ACC

d. ApaIVL1459 ATATGGGCCCAGCCTGTGCTGACT-CARYC

e. ApaIVL78 ATATGGGCCCAGDCTGTGGTGACYCAG-GAGCC

f. ApaIVL6 ATATGGGCCCAGTTTTATGCTGACT-CAGCCCC

Reverse (adding Avr II site, located between FR4 and 5' of constant region)

a. AvrIIVL1IR TTTCCTAGGACGGTGACCTTGGTCCCAGT

b. AvrIIVL237IR TTTCCTAGGACGGTCAGCTTGGTSC-CTCCKCCG

c. AvrIIVL6IR TTTCCTAGGACGGTCACCTTGGTGCCACT

d. AvrIIVLmixIR TTTCCTAGGACGGTCARCTKGGTBC-CTCC

VL $\lambda$ PCR products were digested with Apa I and Avr II and ligated into pEELg vector linearized by Apa I and Avr II double digestion.

The positive clones were identified after transient co-transfection by determining expression in the supernatants by indirect ELISA on PA coated plates. CHO K1 cells were transfected with different combinations of IgG and IgK cDNAs using Lipofectamine-2000 (Invitrogen, Carlsbad, CA). The supernatants were harvested 48 – 72 h after transfection. Multiple positive clones were sequenced with the ABI 3700 automatic sequencer (Applied Biosystems, Foster City, CA) and analyzed with Sequencher v4.1.4 software (GeneCodes, Ann Arbor, MI).

#### Stable cell line establishment

Ig heavy chain or light chain expression vector were double digested with Not I and Sal I, and then both fragments were ligated to form a double gene expression vector. CHO-K1 cells in 6 well-plate were transfected with the double gene expression vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 hrs, transfected cells were transferred to 10 cm dish with selection medium (D. MEM supplemented with 10% dialyzed FBS, 50  $\mu$ M L-methionine sulphoximine (MSX), penicillin/streptomycin, GS supplement). Two weeks later MSX resistant transfectants were isolated and expanded. High anti-PA antibody producing clones were selected by measuring the antibody levels in supernatants in a PA specific ELISA

assay. MSX concentration was increased from 50 to 100  $\mu\text{M}$  to enhance the antibody productivity.

#### **Antigen specific ELISA**

The presence of antibody to anthrax toxin components in human sera, engrafted SCID mouse sera, supernatants of hybridomas or transiently transfected CHO-K1 cells were determined by ELISA. Briefly, flat bottom microtiter plates (Nunc F96 Maxisorp, Rochester, NY) were coated with the appropriate component of the *Bacillus anthracis* tripartite exotoxin, such as PA or LF, diluted sera was added to the wells for one hour at room temperature. Plates were washed and secondary antibody, goat anti-human IgG-HRP, Fc $\gamma$  specific or goat anti-human IgM-HRP, Fc $\mu$  specific antibody were added and incubated for one hour at room temperature. After another wash step, a substrate solution containing OPD (O-phenylenediamine dihydrochloride) in citrate buffer was added. After 15 minutes, 3 N HCl was added to stop the reaction and plates were read on a microplate reader at 490 nm.

#### **Human Ig $\kappa/\lambda$ quantification by ELISA**

Flat bottom microtiter plates (Nunc F96 Maxisorp) were coated overnight at 4°C with 50  $\mu\text{l}$  of goat anti-human IgG, Fc $\gamma$  specific antibody, at 1  $\mu\text{g}/\text{ml}$  in PBS. Plates were washed four times with PBS-0.1% Tween 20. Meanwhile, in a separate preparation plate, dilutions of standards (in duplicate) and unknowns were prepared in 100  $\mu\text{l}$  volume of PBS with 1 mg/ml BSA. A purified monoclonal human IgG1/ $\kappa$  or  $\lambda$  protein was used as the standard and a different IgG1/ $\kappa$  or  $\lambda$  protein serves as an internal calibrator for comparison. Diluted test samples (50  $\mu\text{l}$ ) were transferred to the wells of the assay plate and incubated for one hour at room temperature. Plates were washed as before and 50  $\mu\text{l}$  of the detecting antibody, goat anti-human kappa or lambda-HRP was added, incubated for one hour at room temperature, and developed as described above.

#### **RAW 264.7 cell line in vitro bioassay**

The presence of neutralizing (protective) antibody to anthrax toxin components in human sera, engrafted SCID mouse sera, supernatants of hybridomas or transiently transfected CHO-K1 cells were determined using an *in vitro* protection bioassay with the mouse macrophage RAW 264.7 target cell line [22]. Briefly, recombinant PA (100 ng/ml) and LF (50 ng/ml) were pre-incubated with a range of dilutions of each sample for 30 minutes at 37°C in a working volume of 100  $\mu\text{l}$  of DMEM medium supplemented with 10% fetal calf serum. This 100  $\mu\text{l}$  volume was subsequently transferred into a 96 well flat bottom tissue culture plate containing  $1 \times 10^4$  RAW 264.7 cells/well in 100  $\mu\text{l}$  of the same medium. The culture plate was incubated for 3 hours at 37°C. The lysed cells were removed by washing. The remaining cells were assayed using the CytoTox Assay96 kit (Promega Corp., WI) following the

manufacturer protocol. Briefly, the cells are lysed, an aliquot added to a substrate mix and the lactate dehydrogenase activity determined spectrophotometrically at 490 nm. This assay was used to approximate the  $\text{IC}_{50}$  of antibodies in conferring protection against lethal toxin.

#### **Binding affinity determinations**

BiaCore: Affinity constants were determined using the principal of surface plasmon resonance (SPR) with a BiaCore 3000 (BiaCore Inc.). Affinity purified goat anti-human IgG (Jackson ImmunoResearch) was conjugated to two flow cells of the CM5 chip according to manufacturer's instructions. An optimal concentration of an antibody preparation was first introduced into one of the two flowcells, and was captured by the anti-human IgG. Next, a defined concentration of antigen was introduced into both flow cells for a defined period of time, using the flow cell without antibody as a reference signal. As antigen bound to the captured antibody of interest, there was a change in the SPR signal, which was proportional to the amount of antigen bound. After a defined period of time, antigen solution was replaced with buffer, and the dissociation of the antigen from the antibody was then measured, again by the SPR signal. Curve-fitting software provided by BiaCore generated estimates of the association and dissociation rates from which affinities were calculated.

#### **Fischer rat in vivo anthrax toxin neutralization**

The *in vivo* anthrax toxin neutralization experiments were performed basically as described by Ivins [23]. Male Fisher 344 rats with jugular vein catheters weighing between 200–250 g were purchased from Charles River Laboratories (Wilmington, MA). Human anti-anthrax PA IgG monoclonal antibodies AVP-21D9, AVP-22G12, AVP-1C6, AVP-21D9.1 and AVP22G12.1 were produced from recombinant CHO cell lines adapted for growth in serum free media. The human IgG monoclonal antibodies were purified by affinity chromatography on HiTrap Protein A, dialyzed against PBS pH7.4 and filter sterilized. Rats were anaesthetized in an Isoflurane (Abbott, IL) EZ-anesthesia chamber (Euthanex Corp, PA) following manufacturers guidelines. The antibody was administered via the catheter in 0.2 ml PBS/0.1%BSA pH 7.4 and at 5 minutes, 17 hours or a week later lethal toxin (PA 20  $\mu\text{g}$  / LF 4  $\mu\text{g}$  in 0.2 ml PBS 0.1%BSA pH 7.4/ 200 g rat) was administered via the same route. Five animals were used in each test group and four animals in each control IgG (Sigma, St Louis, MO). Test and control experiments were carried out at the same time using the same batch of reconstituted PA and LF toxins (List Laboratories). Animals were monitored for discomfort and time of death versus survival, as assessed on the basis of cessation of breathing and heartbeat. Rats were maintained under anesthesia for 5 hr post exposure to lethal toxin or until death to minimize

discomfort. Rats that survived were monitored for 24 hours and then euthanized by carbon dioxide asphyxiation. All experimental protocols involving animals were reviewed and approved by the Avanir Pharmaceuticals Institutional Animal Care and Use Committee (San Diego, CA).

## Results

### Donor screening

Donors sera (X064-004b and X064-019) were screened for IgG and IgM against tetanus toxoid, PA and LF by ELISA. Figure 1 shows that both donors had significant IgG responses to tetanus toxoid and some albeit low levels of specific IgG antibody against PA and LF.

### Chimeric engraft screening

The PBLs from donor X064-004b and X064-019 were engrafted into mice designated X040-042 and X040-043 respectively. After boosting, sera from engrafted mice were screened for human IgG against PA. In figure 2A the initial bleed after the first boost is plotted alongside the X064-004b donor sera. One engraft had an anti-PA IgG level that is  $9 \times$  higher than the donor sera. Moreover in figure 2B, the second bleed from the engrafted mice, a range of 8–30 fold increase in specific anti-PA IgG is observed. This increase in specific IgG over time in the engrafted mice is even more pronounced in the second engraft using cells from donor X064-019 as shown in figure 3A and 3B. The increase in specific anti-PA IgG in the second bleed is more than 500 fold relative to specific anti-PA in the donor sera.

### Immunoglobulin sequence analysis

Following fusion, hybridoma cells producing human anti-anthrax PA IgG were selected and the cDNA encoding the immunoglobulin variable regions were rescued and sequenced. The cDNA templates were used to establish stable CHO K1 cell lines producing antibodies. Four neutralizing anti-PA antibodies were discovered by this approach. The VH families were represented by the VH3, VH1 and VH4. Likewise VK 1 and VL 3 represented the VL families. Both VH and VL regions contained evidence of hyper mutation away from the germline. The Table 1 lists the antibodies isolated by this approach and the D and J regions are assigned where possible using DNAPLOT in Vbase.

### Kinetics of binding

The equilibrium dissociation constants ( $K_d$ ) for recombinant form of the antibodies were determined by BiaCore analysis. The rate constants  $k_{on}$  and  $k_{off}$  were evaluated directly from the sensogram in the BiaCore analysis and the  $K_d$  was deduced. The results are summarized in Table 2. One striking feature of all the protective antibodies isolated by the Xenerex Technology™ is the very slow

off rate, which contribute to the very high affinities  $8.21 \times 10^{-11}M$  to  $7.11 \times 10^{-10}M$ . The slow off rate may confer significant physiological advantages for toxin neutralization *in vivo*.

### In vitro lethal toxin inhibition

All the antibodies were initially selected based on binding to PA83 and secondly on inhibition of lethal toxin in a Raw 264.7 cell based *in vitro* assay. Only clones exhibiting toxin neutralization in a qualitative assay were developed further. The Raw 264.7 cell assay was adapted to compare the various antibodies for potency of toxin inhibition. In figure 4 a typical antibody dose response curve is reconstructed to provide an estimate of the  $IC_{50}$  for AVP-21D9, AVP-22G12 and AVP-1C6. Again the inhibitory potency ranking of all the selected antibodies were reflecting the same ranking observed for the binding to PA83.

### Effect of anti-anthrax PA antibodies on protection of rats from lethal toxin challenge

Figure 5 illustrates the protection profile of the three antibodies AVP-21D9, 22G12 and 1C6 in the rat model at two doses 0.5 $\times$  and 1 $\times$  molar ratios relative to toxin challenge. AVP-21D9 protected rats at 0.5 $\times$  and no deaths were observed in the 5 hr following toxin administration, likewise AVP-22G12 at 1 $\times$  also showed complete protection. However with AVP22G12 at 0.5 $\times$  the time to death was prolonged to 255 min. The administrations of lethal toxin 5 min after the infusion of 0.5 $\times$  or 1 $\times$  control human IgG resulted in time to death of 85–120 min. AVP-1C6 at 1 $\times$  conferred 80% protection and at 0.5 $\times$  were not protective.

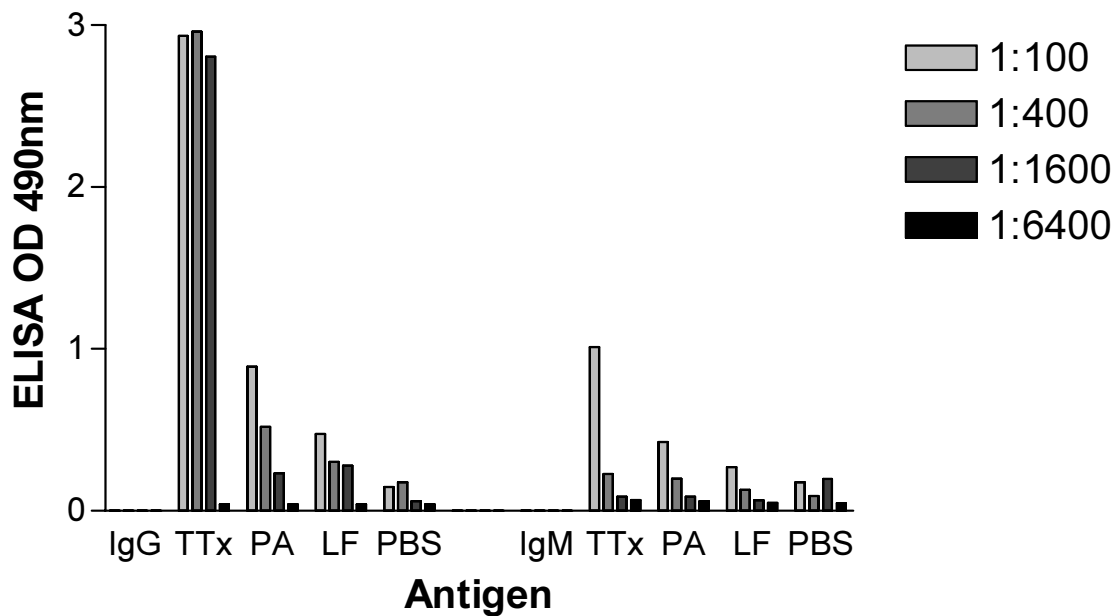
### Effect of antibody glycosylation on anti-anthrax PA antibodies on protection of rats from lethal toxin challenge

Site directed mutagenesis (N297Q) was used to remove the N-glycosylation site in the Fc region. These aglycosylated antibodies were designated as AVP-21D9.1 and AVP-22G12.1 and compared to the glycosylated counterparts in the rat toxin challenge prophylaxis model. As described earlier, antibody was intravenously administered 5 minutes prior to the lethal toxin (PA/LF) challenge. Both AVP-21D9 and AVP-21D9.1 fully protected rats against anthrax toxin with 0.5 $\times$  molar excess relative to PA toxin, whilst AVP-22G12.1 was slightly less potent than the parent molecule at 1 $\times$  as shown in figure 6.

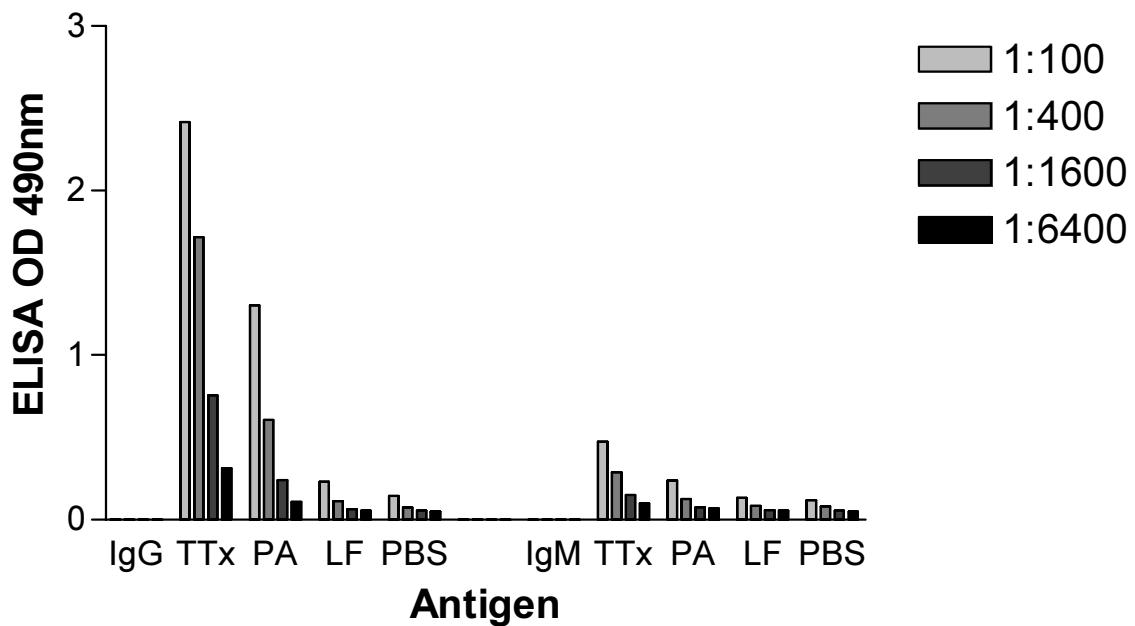
### Duration of AVP-21D9 antibody mediated protection of rats from lethal toxin challenge

To investigate the duration of the protection afforded by a fully human antibody in Fisher rats AVP-21D9 was intravenously administered 17 hours or 1 week prior to the lethal toxin (PA/LF) challenge. A single administration of AVP-21D9 at 1 $\times$  protected 100% when challenged 17 hours later. Over the extended period of time

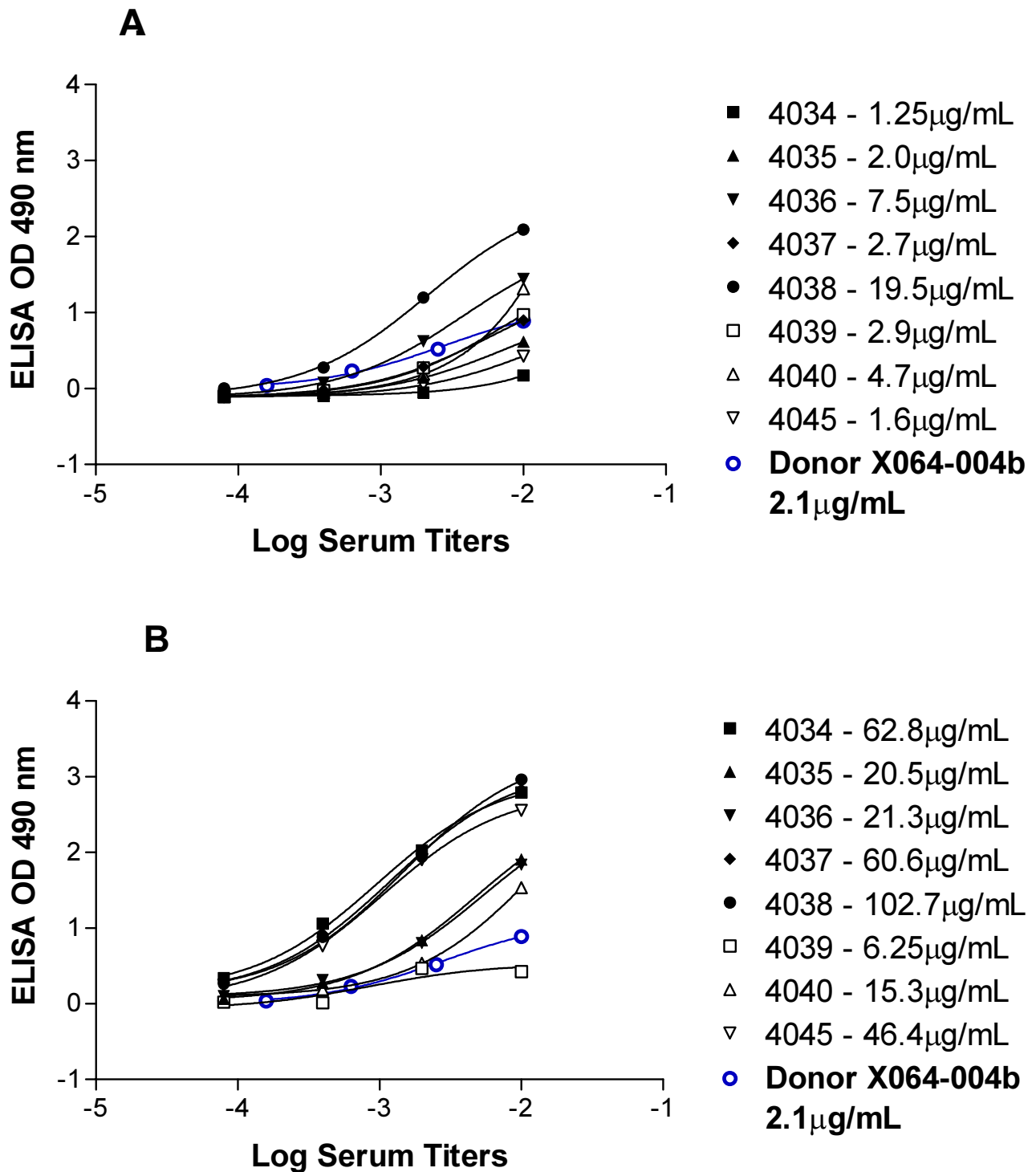
### Donor X064-004b



### Donor X064-019

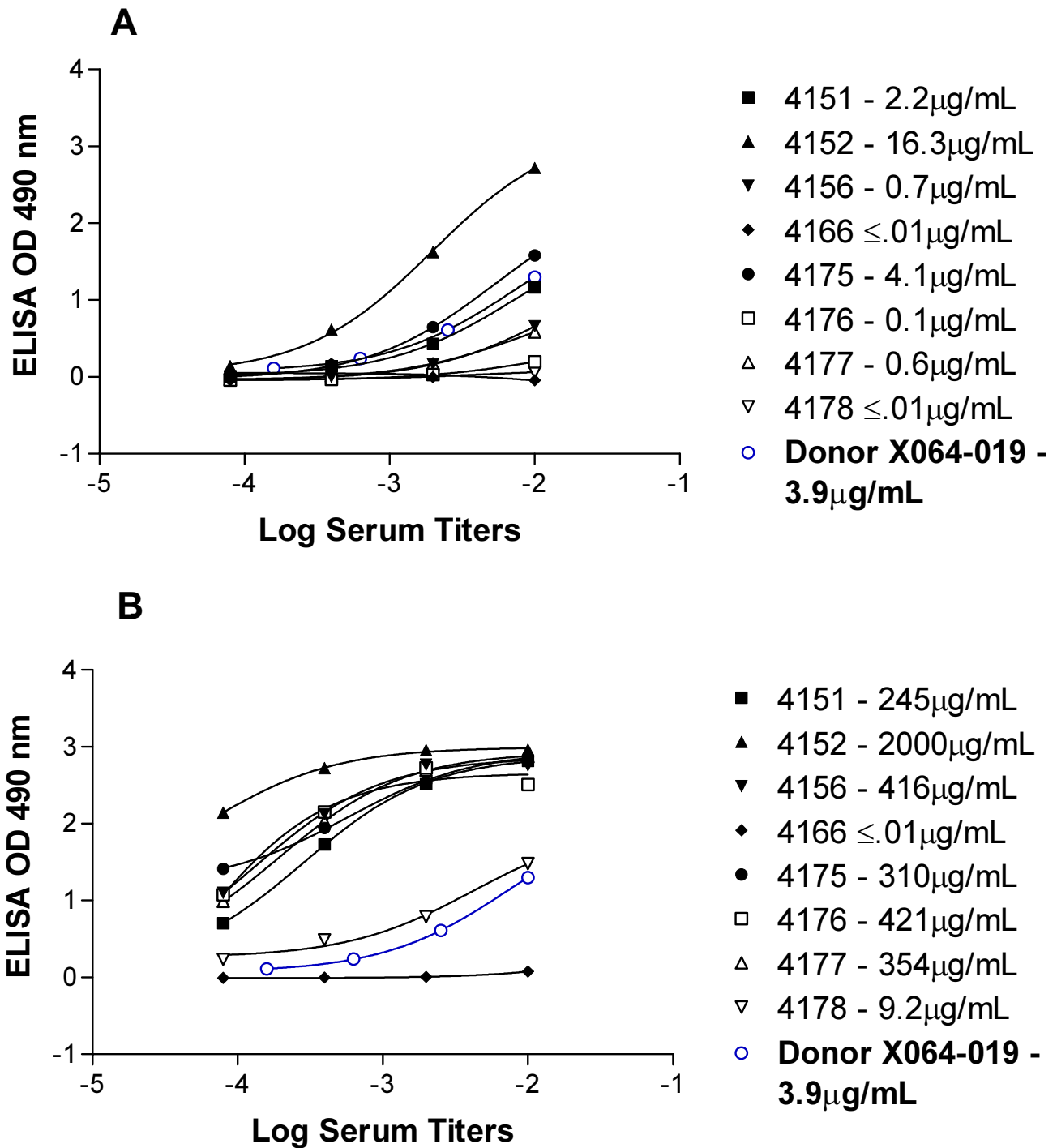


**Figure 1**  
**ELISA panels of AVA vaccinated donors** Plasma samples X064-004b and X064-019 obtained at the time of blood collection by venipuncture from anthrax-vaccinated donors were pre-screened against tetanus toxoid, PA 83 and LF in an ELISA for both IgG and IgM.



**Figure 2**  
**IgG response to PA83 in donor X064-004b engraft sera** The presence of IgG antibody to anthrax toxin PA83 components in sera of engrafted SCID mice sera were determined by ELISA after the first and second boosts. The specific levels of IgG and donor levels are shown. The IgG response from Donor X064-004b cells engrafted into SCID mice at day 15 (A) and day 30 (B).





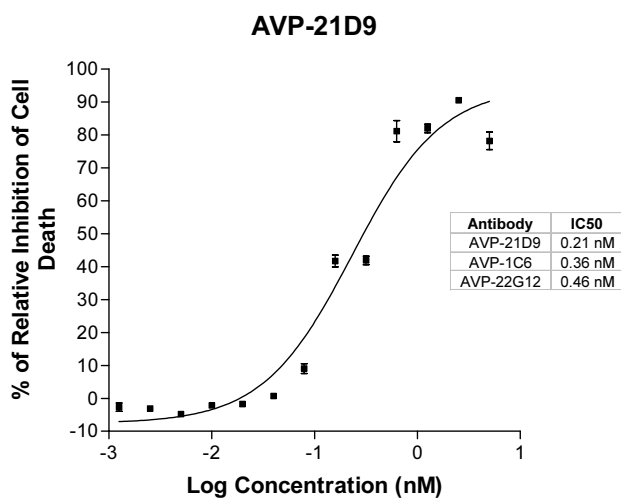
**Figure 3**  
**IgG response to PA83 in donor X064-043 engraft sera** The presence of IgG antibody to anthrax toxin PA83 components in sera of engrafted SCID mice sera were determined by ELISA after the first and second boosts. The specific levels of IgG and donor levels are shown. The IgG response from Donor X064-043 cells engrafted into SCID mice at day 15 (A) and day 30 (B).

**Table 1: Human anti-anthrax PA83 antibody classification.** The immunoglobulin sequence derived from the cDNA encoding the variable regions were used to search Ybase and the VH class, VH locus, DH and JH segments were assigned for the VH regions. Likewise VL class, VL locus and JL segments were assigned for the VL regions. Comparing the actual sequences and closest matched V family members the extent of somatic hyper mutation could be ascertained.

Designation	VH					VL			
	VH Class	VH Locus	# Mutations from germline	DH(RF)	JH	VL Class	VL Locus	# Mutations from Germline	JL
AVP-21D9	VH3	3-43	26	6-19(1)	JH4b	VK1	L12	14	JK1
AVP-1C6	VH3	3-73	8	6-13(1)	JH3b/a	VK1	L18	13	JK4
AVP-4H7	VH4	4-39	29	unknown	JH6b/a	VL3	3h	22	JL2/JL3a
AVP-22G12	VH3	3-11	20	unknown	JH5b	VL3	3r	9	JL2/JL3a

**Table 2: Human anti-anthrax PA antibody kinetic binding data.** The equilibrium dissociation constant ( $K_d$ ) for recombinant form of the antibodies was determined by BiaCore analyses. The rate constants  $k_{on}$  and  $k_{off}$  were evaluated directly from the sensogram in the BiaCore analysis and the  $K_d$  was deduced.

Antibody	Dissociation Constant ( $K_D$ ) M	Association Rate ( $k_{on}$ ) $M^{-1}S^{-1}$	Dissociation Rate ( $k_{off}$ ) $S^{-1}$
AVP-21D9	$8.21 \times 10^{-11}$	$1.80 \times 10^5$	$1.48 \times 10^{-5}$
AVP-1C6	$7.11 \times 10^{-10}$	$1.85 \times 10^5$	$1.31 \times 10^{-4}$
AVP-4H7	$1.41 \times 10^{-10}$	$1.74 \times 10^5$	$2.45 \times 10^{-5}$
AVP-22G12	$5.12 \times 10^{-10}$	$1.01 \times 10^5$	$5.17 \times 10^{-5}$

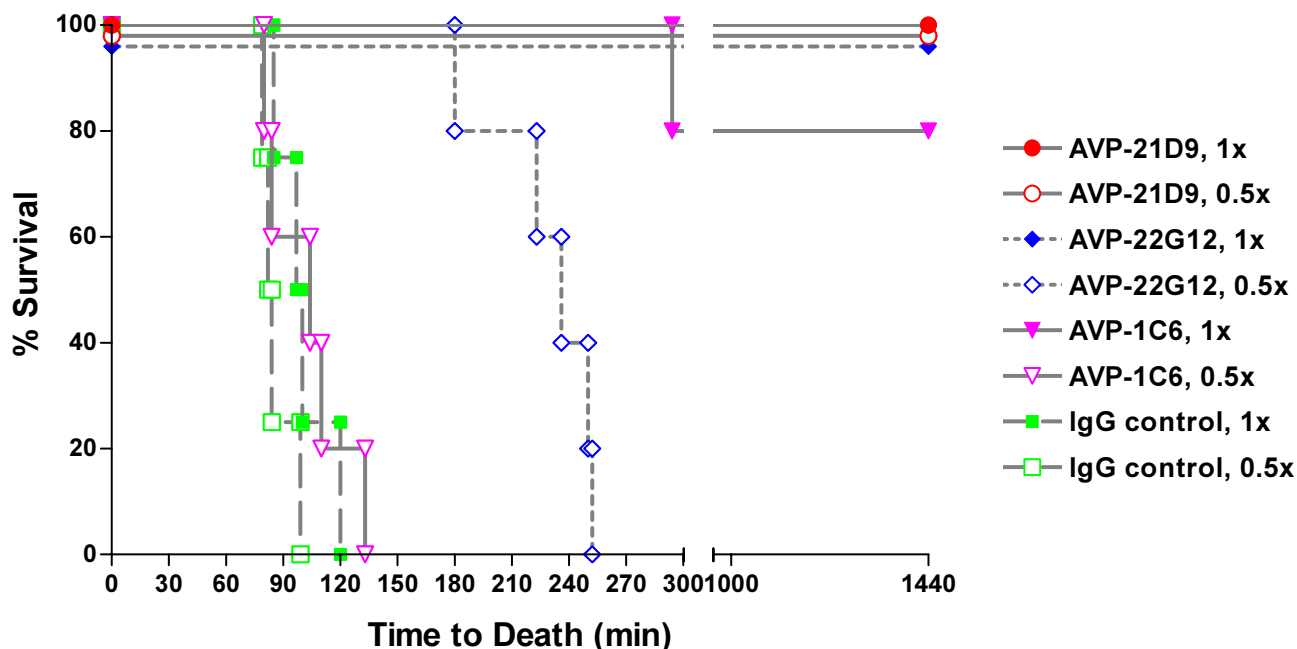


**Figure 4**  
**Determination of AVP-21D9 IC<sub>50</sub> using RAW 264.7**  
**264.7 cell based assay** 1.2 nM PA and 0.56 nM LF in a 96 well assay on confluent RAW 264.7 264.7 cells cause 100% cell lysis. The AVP-21D9 was assessed at various concentrations for the ability to inhibit the lethal toxin. From the dose response curve an IC<sub>50</sub> values was estimated. AVP-22G12 and AVP-1C6 IC<sub>50</sub> determinations were carried out likewise.

administration of AVP-21D9 at  $10 \times$  dose showed 80% protection. Almost all the control animals died within 120 min, one outlier had delayed time of death to 230 minutes (Figure 7).

## Discussion

The engraftment of human peripheral blood lymphocytes into severe combined immunodeficient (SCID) mice in order to reconstitute a functional human immune system has been reported previously [24]. However the subsequent rescue and immortalization of specific B-cells has had mixed results. In this study we recruited donors that had been actively immunized with the current licensed anthrax vaccine (AVA). Despite vaccination the serum levels of anti-PA83 specific IgG and IgM were relatively low (2-3  $\mu$ g/ml) in comparison to the anti-tetanus responses in both donors. We utilized the SCID-HuPBL platform to demonstrate that we could selectively direct the response by immunization of the chimeric animals. Immunization of the chimeric mice with recombinant PA83 resulted in a significant increase in specific IgG in some of the engrafted mice, in one case as high as 2 mg/ml (mouse 4152 figure 3A). In comparing the 1<sup>st</sup> bleeds (figure 2A & 3A) with the 2<sup>nd</sup> bleeds (figure 2B & 3B) for both sets of chimeric mice, it is clear that a specific response was selectively enhanced in the animals upon boosting with anti-



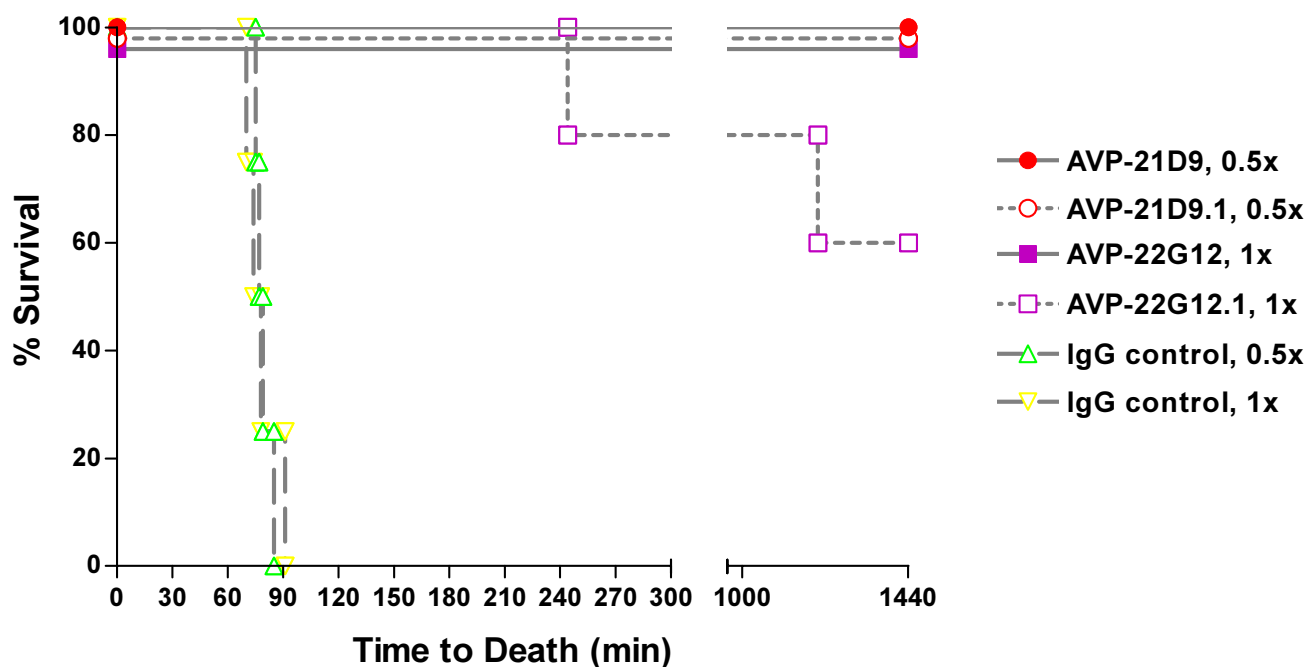
**Figure 5**

**Protection of rats from a lethal toxin challenge five minutes after administration of antibody** Male Fisher 344 rats with jugular vein catheters weighing between 200–250 g were administered human anti-anthrax PA IgG monoclonal antibodies AVP-21D9, AVP-22G12, AVP-1C6, or the control human IgG in 0.2 ml PBS 0.1% BSA pH 7.4, 5 minutes later lethal toxin (PA 20  $\mu$ g + LF 4  $\mu$ g/200 g rat in 0.2 ml PBS 0.1% BSA pH 7.4) was administered. The dose of antibody was 0.25 and 0.12 nmols/rat corresponding to 1 $\times$  and 0.5 $\times$  molar equivalent to the lethal toxin. Five animals were used in each test group and four animals in each control. Test and control experiments were carried out at the same time using the same batch of reconstituted PA and LF toxins. Animals were monitored for discomfort and time of death, as assessed on the basis of cessation of breathing and heartbeat. Rats were maintained under anaesthesia for 5 hr post exposure to lethal toxin or until death to minimize discomfort.

gen (see figure 3A & 3B). However not all the chimeric mice demonstrated such a robust response, hence it is not a stochastic process. We speculate that in mice that responded well to antigen challenge, we have recalled the human memory B cell response and recruited specific human helper T-cells. The specific recall leads to proliferation of antigen specific plasma cells. The antibody producing cells in the chimeric mice were recovered from the spleen and peritoneal washes in sufficient numbers to permit fusion with a standard mouse myeloma P3X63Ag8.653 [21] to form hybridomas. Others [25,26] and we have noted that the formation of mouse/human hybridomas using a murine fusion partner with human derived plasma cells results in unstable hybrids, usually these are difficult to clone, expand and isolate. We circumnavigate this problem by rescuing the transcripts encoded by mRNA from a small cluster of cells and generating stable recombinant CHO cell lines and testing these for the activity. Hence the fusion with P3X63Ag8.653 with the

human cells results in hybrids of antibody-producing cell, which permits identification of positive wells for specific IgG production and the rescue of immunoglobulin transcripts.

No particular heavy chain family or light chains dominated the human anti-PA response. In all but two cases we could assign D<sub>H</sub> segments usage. The array of J<sub>H</sub> and J<sub>L</sub> segments observed in the panel suggest that the approach is capturing the diversity present in the natural response to anthrax PA via vaccination with AVA. Another striking feature of the antibodies is the exceptional high affinity for the target antigen and the very slow off-rates. We have seen similar high affinities and slow off rates for anti-tetanus toxoid antibodies derived from engrafted SCID-HuPBL mice boosted with antigen (data not shown). This may be a general feature of the protective anti-bacterial toxin response in humans.



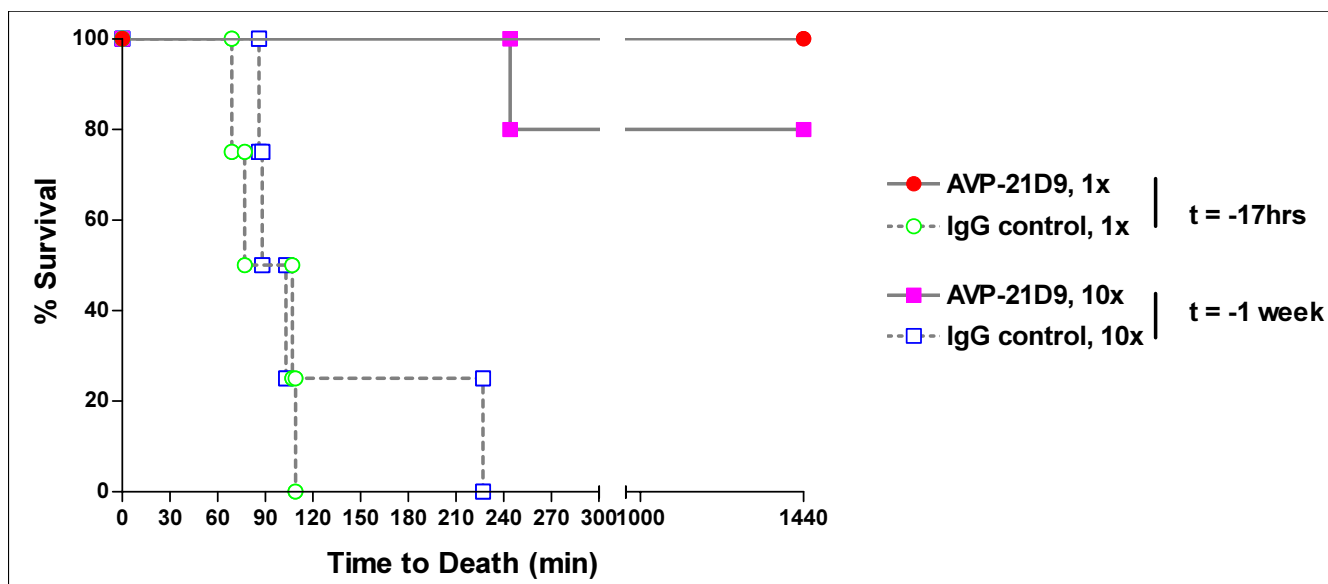
**Figure 6**

**Protection of rats from a lethal toxin challenge by aglycosylated antibody** Male Fisher 344 rats with jugular vein catheters weighing between 200–250 g were administered human anti-anthrax PA IgG monoclonal antibodies AVP-21D9, AVP-22G12, the aglycosylated forms AVP-21D9.1 and AVP-22G12.1 or the control human IgG in 0.2 ml PBS 0.1% BSA pH 7.4, 5 minutes later lethal toxin (PA 20  $\mu$ g / LF 4  $\mu$ g in 0.2 ml/200 g rat PBS 0.1% BSA pH 7.4) was administered. The dose of antibody was 0.25 and 0.12 nmols/rat corresponding to 1 $\times$  and 0.5 $\times$  molar equivalent to the lethal toxin. Five animals were used in each test group and four animals in each control. Test and control experiments were carried out at the same time using the same batch of reconstituted PA and LF toxins. Animals were monitored for discomfort and time of death, as assessed on the basis of cessation of breathing and heartbeat. Rats were maintained under anaesthesia for 5 hr post exposure to lethal toxin or until death to minimize discomfort.

Currently in the event of an inadvertent *Bacillus anthracis* spore exposure two preventative measures can be taken. If the risk can be assessed well in advance, vaccination can be employed. In the event of near term or immediate post exposure antibiotic such as Cipro may be effective. Anthrax Vaccine Adsorbed (AVA) is the only licensed human anthrax vaccine in the United States. The vaccine is known to contain a mixture of cell products including PA, LF and EF, however the exact amounts are unknown [27]. The immunization schedule consists of three subcutaneous injections at 0, 2 and 4 weeks and booster vaccination at 6, 12 and 18 months and it is suggested that annual boost may be required to maintain immunity. Mass vaccination in the event of anthrax spore release is an unlikely scenario. First, the time taken for effectiveness of such vaccination based on AVA or various rPA molecules in development may be too short, weeks as opposed to minutes. The utilization of antibiotic can inhibit bacterial growth and spread, and may prevent some of the

symptoms, but the administration needs to be timely and preferably as a prophylactic, even as such, the toxins released during the early stages of an infection may impair the immune system to cause lasting damage. Ideally, a combination of approaches that inhibit anthrax bacteria and toxins is desirable.

Human antibodies are safe and well tolerated for a range of therapeutic indications and are logical choices for an immediate anthrax therapeutic or prophylactic in humans. Passive protection against anthrax toxins in rats and anthrax infection in guinea pigs has been demonstrated for murine monoclonal antibodies [28,29] and polyclonal antibodies [17], respectively. Recently a human monoclonal antibody against PA has demonstrated efficacy in protecting rats challenged with lethal toxin [20]. The antibody was fully protective at 0.3 nmol/250 g rat. It is expected that exceptionally high affinity human monoclonal antibodies against the anthrax toxin



**Figure 7**

**Protection of rats from a lethal toxin challenge 17 hours and 1 week after administration of antibody** Male Fisher 344 rats with jugular vein catheters weighing between 200–250 g were administered human anti-anthrax PA IgG monoclonal antibodies AVP-21D9 or the control human IgG in 0.2 ml PBS 0.1% BSA pH 7.4, 17 hours or 1 week later lethal toxin (PA 20  $\mu$ g + LF 4  $\mu$ g/200 g rat in 0.2 ml/ PBS 0.1% BSA pH 7.4) was administered. The dose of antibody was 0.25 and 2.5 nmols/rat corresponding to 1 $\times$  and 10 $\times$  molar equivalent to the lethal toxin respectively. Five animals were used in each test group and four animals in each control. Test and control experiments were carried out at the same time using the same batch of reconstituted PA and LF toxins. Animals were monitored for discomfort and time of death, as assessed on the basis of cessation of breathing and heartbeat. Rats were maintained under anaesthesia for 5 hr post exposure to lethal toxin or until death to minimize discomfort.

should have a therapeutic potential to treat anthrax exposure in humans. In this study we compare 3 human antibodies that neutralize anthrax lethal toxin *in vitro* and *in vivo* rat toxin challenge model. The most potent inhibitor of the anthrax toxin AVP-21D9 protected rats with as little as 0.5 $\times$  antibody to toxin *in vivo*, this corresponds to 0.12 nmols/200–250 g rat (figures 5 & 6). The potency ranking observed in the *in vitro* assay was matched in the rat *in vivo* protection assay. Removing the carbohydrates associated with the constant domains of the IgG did not reduce the potency of the AVP-21D9 antibody. AVP-22G12 was also potent at inhibiting the toxin *in vivo* at 1 $\times$ , but not as potent as AVP-21D9 at the 0.5 $\times$  dose. Removal of the glycosylation site in AVP-22G12 did impact on its potency suggesting that although the effector functions are not required, in the absence of the carbohydrates the overall structure of the antibody is impacted to reduce its efficacy to 80% survival at the designated 5 hour time point, which dropped to 60% due to an additional death at 12 hours. At the lower dose of AVP-22G12 the time to death was delayed significantly. AVP-1C6 at 1 $\times$  was only 80% protective and failed to protect or delay time to death at

the lower dose. The *in vivo* potency trend observed AVP21D9 > AVP-22G12 > AVP-1C6, is similar to the potency *in vitro* and correlates well with affinity of antibody to PA. Moreover dosing rats with AVP-21D9 at 1 $\times$  or 10 $\times$  and challenging with lethal toxin the next day hours or a week later with lethal toxin respectively were also protective as shown in figure 7.

High affinity human monoclonal PA neutralizing antibodies may provide immediate neutralization of the anthrax toxins. In this investigation we have accessed the human IgG response to the PA83 component of AVA and isolated a panel of high affinity potent PA neutralizing monoclonal antibodies. These antibodies were selected on the criteria of binding to PA83 (the form of the anthrax toxin released by the bacteria prior to cell bound furin processing) and inhibition of lethal toxin. This technology will be particularly useful for the generation of fully human monoclonal antibodies against various infectious disease targets from vaccinated or naturally exposed yet protected individuals. Moreover it may be possible to access self-antibodies from autoimmune individuals.

## Conclusions

We have successfully engrafted human PBL's from anthrax-vaccinated donors into SCID/bg mice and demonstrated that a specific recall response can be selectively enhanced by immunization with PA83. Moreover, we have shown that the cells producing the antibodies can be isolated, the transcript mRNA encoding the desired antibodies can be readily recovered and stable recombinant cell lines producing human monoclonal antibodies can be generated. The human monoclonal antibodies generated are of very high affinity for PA83 and neutralize lethal toxin in an *in vitro* cell based assay. Vaccination with Anthrax Vaccine Adsorbed can induce the production of a range of protective antibodies. Here we show that most of the human anti-anthrax toxin antibodies selected by the Xenerex technology™ are potent inhibitors of the lethal toxin *in vivo*. The three parental antibodies and the two aglycosylated forms described may be therapeutically useful against anthrax infection and in the passive protection of high risk individuals. In particular the two most potent anthrax toxin-neutralizing antibody AVP-21D9 and AVP-22G12 were completely effective at a dose corresponding to 0.12 nmols/rat and 0.25 nmols/rat respectively.

## Competing interests

None declared.

## Authors' contributions

IJ carried out cell engrafting immunization and cell recovery/fusion. RS-H carried out Ig cloning, expression and coordinated rat studies. FW carried out vector construction, IgG engineering and purification. SMS was responsible for fusion/cell culture and antibody production. RN carried out PBL preparation, antibody expression, *in vitro* assay and *in vivo* studies. Paul Ruther was responsible for donor/mice sera screening, IgG quantification, affinity determinations. DM and AA were responsible for SCID mouse facility and carried out the rat studies. PRM<sup>†</sup> was Project initiator. ASK Project leader. All authors read and approved the final manuscript

<sup>†</sup>Phillip R. Morrow deceased.

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