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Kinetics and Passive Protection Efficacy Induced by Purified Ava Human Immunoglobulin G in Rabbits Against a Bacillus Anthracis Aerosol Challenge

Jennifer Lee Plahovinsak
Wright State University

KINETICS AND PASSIVE PROTECTION EFFICACY INDUCED BY PURIFIED AVA HUMAN IMMUNOGLOBULIN G IN RABBITS AGAINST A *Bacillus anthracis* AEROSOL CHALLENGE

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By

JENNIFER PLAHOVINSAK

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jennifer Plahovinsak ENTITLED Kinetics and Passive Protection Efficacy Induced by Purified AVA Human Immunoglobulin G in Rabbits Against a *Bacillus anthracis* Aerosol Challenge BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

_____
Robert Casillas, Ph.D.
Co-Thesis Advisor

_____
Courtney Sulentic, Ph.D.
Co-Thesis Advisor

_____
Mariana Morris, Ph.D.
Department Chair

Committee on Final Examination

_____
Courtney Sulentic, Ph.D.

_____
Robert Casillas, Ph.D.

_____
Michael Babin, Ph.D., DVM

_____
James Lucot, Ph.D.

_____
Joseph F. Thomas, Jr., Ph.D.
Dean, School of Graduate Studies

ABSTRACT

Plahovinsak, Jennifer. M.S., Department of Pharmacology and Toxicology, Wright State University, 2006. Kinetics and Passive Protection Efficacy Induced by Purified AVA Human Immunoglobulin G in Rabbits Against a *Bacillus anthracis* Aerosol Challenge.

The present study was conducted to determine the half-life, assess the toxicity, and passive protection efficacy of purified immunoglobulin G (IgG) from Anthrax Vaccine Adsorbed (AVA) vaccinated human donors. Half-life determinations were calculated from the reportable values obtained using the anti-PA ELISA assay and the Centers for Disease Control's (CDC) "ELISA for Windows" software. For toxicity evaluations animals were observed clinical for one hour post administration and for 14-days post-treatment. The protection efficacy was determined based upon the mortality results from a lethal *Bacillus anthracis* aerosol challenge. While no protection was achieved in this delayed exposure scenario, the study yielded valuable kinetics data for use in future research.

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INTRODUCTION

Background

Bacillus anthracis, the etiologic agent of anthrax is a gram-positive, rod-shaped, facultative aerobic, spore forming bacterium. *B. anthracis* causes the human disease anthrax with different clinical manifestations after introduction by gastrointestinal, cutaneous, or pulmonary routes. The bacterial incubation period varies from 12 h to 5 days depending upon the dose, but the onset may be longer following inhalational exposure (Dixon et al, 1999). Anthrax introduced by inhaling *B. anthracis* spores has the highest mortality rate. Following a low spore exposure or cessation of therapeutic intervention (e.g. antibiotics), some reports suggest a delayed disease onset of several weeks (Phipps et al, 2004). The initial clinical signs and symptoms upon inhalation of *B. anthracis* spores are non-specific and may include malaise, headache, fever, nausea, and vomiting. Initial clinical signs are followed by a sudden onset of respiratory distress with dsypnea, stridor, cyanosis, and chest pain. The onset of respiratory distress is followed by shock and death with close to 100% mortality (Inglesby et al, 2002).

Anthrax is considered a serious terrorist and military threat due to the durability of *B. anthracis* spores and the high mortality from inhalational exposure. Three genetically different strains of *B. anthracis* have been identified; Ames, Vollum, and Sterne. The bacterium commonly exists in two different states, the vegetative and the spore forms. The vegetative cell is the active state of the bacterium, whereas the spore is the dormant state in which, under a myriad of conditions, the bacterium can hibernate for decades. Vegetative cells have characteristic squared ends and form chains. In a host, spores only germinate and multiply in the presence of the required substrates (i.e. amino

acids, nucleosides, glucose, etc) (Lew 2000). The endospores are located centrally in the vegetative cell's sporangium. The spores are resistant to extreme conditions including temperature variations, radiation, and desiccation. Microscopically, the spores are resistant to gram staining and are highly refractive to light (Dixon et al, 1999).

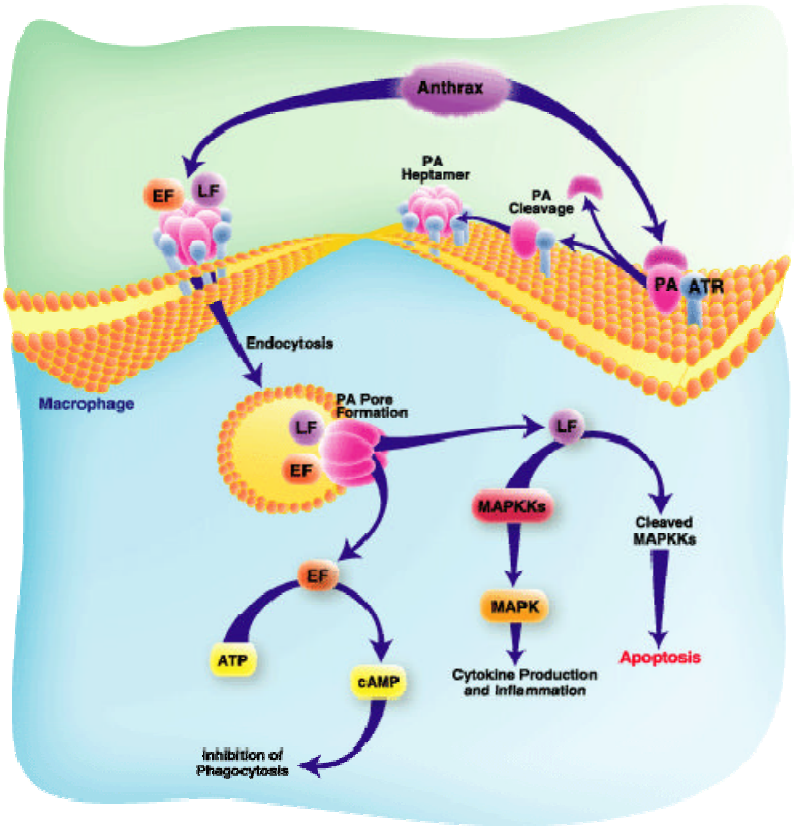
Expression of a poly-D-glutamic acid capsule and a tripartite exotoxin are essential for virulence of *B. anthracis* (Ezzel & Welkos, 1999). The Sterne strain lacks the polypeptide capsule rendering it non-virulent. The polypeptide capsule helps the bacterium evade phagocytosis by macrophages, while three secreted protein subunits form the exotoxin. The three secreted exotoxin proteins are Protective Antigen (PA), Lethal Factor (LF), and Edema Factor (EF), which combine to form two distinct toxins: lethal toxin (LT) formed by PA and LF, and Edema Toxin (ET) formed by PA and EF.

The PA subunit binds to one of two cell-surface receptors, capillary morphogenesis protein 2 (CMG2) or anthrax toxin receptor/tumor endothelial markers 8 (ATR/TEM 8). Both receptors are membrane spanning, single peptide chains with an extra cellular von Willebrand factor A (VWA) domain, and a cytoplasmic tail. The VWA domain fold, a site for dinucleotide binding, is ubiquitously located in many cell adhesion proteins and promotes protein-protein interactions (Lacy et al, 2004). These interactions promote the binding of the PA. These two receptors appear to be expressed in many different cell types (Scobie and Young, 2005).

The PA subunits are 83 kilodaltons (kDa) in size and bind to host cells via the membrane bound Anthrax Toxin Receptor. The 83 kDa PA is cleaved by furin to form a 63 kDa PA subunit. The PA subunits once cleaved, combine to form heptamers. The dissociation of the smaller fragment allows for the larger fragment, which remains

receptor bound, to self associate into a ring shape, also referred to as a prepore. The prepore results in the exposure of a binding site where up to three molecules of LF and EF compete for binding (Abrami et al, 2005). The binding of the heptameric PA to LF or EF forms LT and ET respectively, in which the LT or ET are internalized by the cells via receptor mediated phagocytosis/endocytosis. Following internalization heptameric PA forms a channel in the phagosome/endosome which allows the toxic LF and EF subunits access to the cell cytoplasm (Welkos et al, 2001). It is generally accepted that LF is a protease, which inactivates mitogen-activated protein kinase kinase 1 (MAPKK1) and MAPPK 2, which in turn inactivates the MAPK signal transduction pathway. EF is a calcium-dependent adenylate cyclase that inhibits phagocytosis (Scobie & Young, 2005).

Figure 1. *B. anthracis* Binding Pathway



http://www.biotechjournal.com/Pathways/anthrax.htm

It has been theorized that LT and ET contribute to the bacteria's ability to evade the host innate immune response, possibly by inducing apoptosis in activated macrophages, inhibiting phagocytosis, and deregulating proinflammatory cytokines (Phipps et al, 2004). These processes may allow the bacteria to multiply rapidly leading to an exponential increase in toxin production. LT and ET are likely efficacious targets for current therapeutic interventions and possible novel interference approaches.

Current Treatments

Licensed products for the prevention or treatment of anthrax include the vaccine Biothrax produced by Bioport (Lansing, Michigan; also known as Anthrax Vaccine Adsorbed, AVA) and antibiotics (described below). Limited clinical studies for Biothrax have reported higher than normal adverse reactions at local injection sites in females versus males (Joellenbeck et al, 2002). In addition to inconsistent reactions to the vaccine, the scientific basis for the recommended vaccination schedule of six subcutaneous vaccinations is unclear (Wang and Roehrl, 2005). The significant immunogen in all effective vaccines against anthrax is based upon the PA component of the anthrax toxin, although the mechanism leading to protection is unclear. While PA is the major component of the AVA, there are several other unquantified components in the vaccine preparation (McBride et al, 1998). Public skepticism also exists regarding the credibility of the limited animal data and the efficacy of the vaccine against an inhalational challenge (Ivins et al, 1998). Due to the skepticism regarding the dose regimen, efficacy, and inconsistent adverse effects of the licensed vaccine, in

combination with recent civilian attacks, an interest in novel and more refined alternative vaccines for anthrax has developed.

Currently, the recommended treatment regimen for inhalation anthrax exposure for non-vaccinated individuals centers primarily on antibiotics in combination with aggressive supportive care. Several antibiotics have been licensed for use in the treatment of anthrax including fluroquinolones, doxycycline, and penicillin/amoxicillin. Fluroquinolones (ciprofloxacin or levofloxacin) inhibit DNA replication, and ciprofloxacin has been recommended as the drug of choice by the CDC, the Working Group for Civilian Biodefense, and the Department of Defense. These recommendations are based upon *in vitro* activity against *B. anthracis* and *in vivo* efficacy demonstrated from the prophylaxis of inhaled anthrax. The recommended antibiotic treatment following possible *B. anthracis* exposure is oral ciprofloxacin (500mg) twice daily for 60 days (CDC Update: MMWR Weekly, 2001). The long duration of antibiotic therapy is due to the incubation period of the spores ranging from h to several days or weeks. However, antibiotics are effective against killing the bacteria but are not protective against the deleterious effects of the toxins. The primary mechanism for the neutralization of the toxins is toxin specific antibodies. The large amounts of toxin produced by *B. anthracis* often send the patient into shock and the disease is often fatal once symptoms are recognized (Wang and Roehrl, 2005).

The effectiveness of a biological warfare event is dependent on the susceptibility of the target population to the agent of choice. Due to the questions regarding the licensed vaccine, the long duration of antibiotic therapies, and recent civilian attacks an interest in novel and more refined treatments for anthrax has emerged. Passively

transferred antibodies provide an innovative approach. These antibodies are expected to provide immediate immunity against a biological agent regardless of the immune status of the host.

Passive Antibody Therapy

Passive antibody therapy was first reported in 1890 by Emil Behring and Shibasaburo Kitasato (Browning, 1955). Serum was collected from rabbits immunized against tetanus and the serum was injected into the abdominal cavity of mice. The treated mice survived a challenge of live virulent tetanus bacteria and exhibited no signs of infection. This demonstrated that substances present in serum following a vaccination provided adequate protection against infection, and that immunity can be passively acquired. Passively transferred antibodies induce a protective effect via several different pathways. The antibody can mark a pathogen for opsonization by binding to bacterial surfaces and initiate antibody-dependent cell-mediated cytotoxicity (ADCC). Most importantly the antibody induces recruitment of the complement pathway signaling the removal and destruction of a pathogen (Goldsby, 2001).

Passively administered antibodies to *B. anthracis* have been used *in vitro* and *in vivo*. These antibodies inhibited LT toxicity to macrophages *in vitro*, neutralized LT prior to injection in rats, and protected rats by a pre-challenge intraperitoneal (IP) treatment of a monoclonal antibody (mAb) prior to intravenous (IV) injection of LT (Koblier, 2002). Passive antibodies from varying amounts of anti-PA rabbit serum administered post-challenge in guinea pigs also demonstrated protective effects against a 25 LD_{50} Vollum spore challenge. However, 24 h after exposure the protective efficacy of

the anti-PA serum decreased. The half life of mAb was 3-4 days and the half-life of the anti-PA serum was found to be 9-10 days (Koblier, 2002). The proposed mechanism of passive antibody protection is the physical binding to anthrax toxin impeding the toxin's interaction with the host cell receptor (Little et al, 1997).

Acute lethal toxicity can be observed as an adverse effect of passive immunization. Animal efficacy studies did not predict the toxic response in humans during the development of a passive immunization with anti-meningoccal serum against *Nesseria meningitis* (meningococcus). Toxicity in humans was observed as anaphylaxis, fever, chills, dyspnea, lethargy, and serum sickness, and was not observed in the animal studies. Serum sickness, a syndrome resulting in malaise, rash, fever, and arthralgia normally occurring 7-10 days after therapy, was initiated. The formation of immune complexes from host responses to the foreign proteins was the speculated cause (Casadevall, 1994). In passively treated mice challenged with *C. neoformans*, an acute toxicity lead to death within 20-60 min after injection (Savoy, 1997). Again the toxicity appeared to be caused by the formation of immune complexes, which activate macrophages. Timing of the passive immunization in relation to the time of challenge was important with regards to toxicity. The acute lethal toxicity was most evident during the mid-stage of the infection. Toxicity was less evident during the early and late stages of infection. Mice that survived 1 h post challenge recovered fully (Savoy, 1997).

A major advantage to passive antibody therapy is that it provides a state of immediate immunity which lasts from several weeks to potentially several months. Many human IgG isotypes have half-lives that exceed 30 days, which may lead to long-lived protection for passively immunized subjects. Immunoglobulins are extremely

versatile molecules that can be tailored for use against virtually any infectious agent. One aspect that has limited the development of antibody based therapies against infectious agents is the recognition that efficacy is directly related to timing of administration. This project, will evaluate the timing of pre-exposure administration of purified human IgG and the efficacy against a lethal *B. anthracis* challenge in rabbits. The proposed method of protection relies upon purified human IgG antibodies binding to the PA exotoxin subunit, and physically inhibiting the binding of PA to the host cell's anthrax toxin receptor thus preventing the formation of LT and ET.

PURPOSE

The efficacy of a treatment against *B. anthracis* cannot be tested in humans due to ethical considerations. The results from this study will determine the clearance of the human IgG from the rabbit serum. Although the human IgG will be cleared from the rabbit at an accelerated rate, the volume of plasma required to complete the purification process cannot be obtained from a single rabbit. Post-treatment serum concentrations of anti-PA will be compared amongst groups to determine if a 20 mg/kg dose of purified AIG is sufficient to provide protection. Clinical signs of toxicity will determine whether the levels of purified human antibody administered are tolerated by the rabbit. A post treatment aerosol challenge will evaluate the efficacy of passively transferred AIG.

The mortality results will yield valuable data for future research into the use of passive antibody administration as an immediate pretreatment for *B. anthracis* exposure and eventually as a potential prophylaxis against infection. The delayed exposure scenario tested will provide the data to determine the half-life of the purified AIG material for use in future research. This half-life information will be used in future studies to determine potential as a post-exposure treatment. The half life of the material in the rabbit will provide a starting point for future research in higher species such as non-human primates.

HYPOTHESIS

The administration of column purified plasma containing Immunoglobulin G (IgG) from AVA vaccinated human donors (AIG) will provide passive protection in rabbits against a lethal *B. anthracis* aerosol challenge.

SPECIFIC AIMS

1. To evaluate the pharmacokinetic parameters (i.e. half-life) of passively administered purified IgG using the Anti-PA Enzyme Linked Immunosorbent Assays (ELISA). This assay will quantify the levels of human anti-PA present in the rabbit serum at 1, 24, 48, 72, 96 h, and 7, 10, 13, 16, 19, 22, 25, and 28 days post treatment and allow for an accurate assessment of IgG clearance from the rabbit serum.

2. To evaluate the potential toxicity caused by passively administered purified human IgG using clinical observations.

3. To statistically evaluate the protective efficacy of passively administered purified IgG to a lethal challenge of *B. anthracis* spores using survival data.

EXPERIMENTAL APPROACH

Chemicals and reagents

Normal human plasma samples were received from Interstate Blood Bank (Chicago, IL). AVA human plasma samples were generously provided by Cangene Corporation (Winnipeg, Manitoba, Canada). 0.9 % physiological saline was purchased from Butler Animal Health Supply (Dublin, OH). Sterile water was purchased from Sigma, (St Louis, MO). Purified rPA for plate coating was purchased from National Institutes of Health (NIH), (Bethesda, MD), Lot number 1715AA.

ELISA wash buffer was prepared fresh and contained 1X Phosphate Buffered Saline (PBS, Sigma), with 0.1 % Tween 20 (Sigma). ELISA Diluent was prepared fresh and contained 1X PBS with 5% skim milk and 0.2 % Tween 20, pH 7.4. Master Plate Diluent was prepared fresh and contained 1X PBS with 5% skim milk and 0.5 % Tween 20, pH 7.4. Deionized water was obtained at Battelle Memorial Institute (BMI).

Quality Control Serum High was produced at BMI, lot number BMI500. (This is a high titer human serum.) Quality Control Serum Low was produced at BMI, lot number BMI502. (This is a low titer human serum.) Negative Control Serum was produced at BMI, Lot number 093K0475. (This is a negative human serum).

Naïve rabbit serum was produced at BMI, Lot number BMI012. Positive Control rabbit serum was produced at BMI, Lot number BMI023. Pooled naïve human serum was produced at BMI, Lot number BMI504. Pooled AVA human serum was produced at BMI, Lot number BMI505. Positive Control human serum was purchased from the Center for Disease Control (CDC, Atlanta, GA), Lot number ARV801.

HRPO Conjugate, ABTS Microwell Perioxidase Substrate System, and ABTS Perioxidase Stop Solution were purchased from Kirkegaard & Perry Laboratories, (Gaithersburg, MD).

Antibody Purification

Normal human plasma samples were received from Interstate Blood Bank and prescreened via ELISA (Performed at BMI, methods adapted from Quinn, et al 2002) to confirm the samples (1 L each) were negative for PA-specific IgG. Upon confirmation of anti-PA IgG negative plasma samples, all samples were pooled into a single batch. From the pooled plasma, 1 ml aliquots were removed for characterization by ELISA (to confirm the pooled material's anti-PA IgG negativity).

Similarly, the AVA human plasma samples, received from Cangene Corporation, were characterized by ELISA (Performed at BMI, methods adapted from Quinn, et al 2002) for anti-PA IgG. AVA positive plasma samples were pooled and 1 ml aliquots removed for ELISA reassessment of anti-PA IgG levels. For both the naïve and AVA plasma pools, the plasma was re-distributed into appropriately sized aliquots and shipped on dry ice to Covance Research Products (CRP), (Denver, PA) for column purification of the IgG according to CRP Standard Operating Procedures (SOP).

Passive Antibody Administration

Forty rabbits were randomly assigned (using the MREF Animal Randomization Program) to five total groups: three test groups of eight animals per group, and two control groups of eight animals per group (Table 1). Each group contained equal

numbers of male and female rabbits (four male, four female) to eliminate any potential gender differences. On Study Day 0 animals in Groups 1, 2, and 3 were administered anti-PA doses of AVA IgG (AIG) by the IP route as described in Table 1. Animals in Group 4 were IP-administered normal IgG at the same total protein dose level as the Group 3 animals. The total protein for each animal was calculated by:

$$\text{Total protein (mg/kg)} = \frac{\text{Total volume (ml)} \times \text{concentration (mg/ml)}}{\text{Weight of animal (kg)}}$$

Group 5 rabbits did not receive treatment. All study animals were weighed on Study Day -1 to calculate IP dose volumes. A non-challenge AIG 20 mg/kg group was not included in this particular study due to the limited amount of AIG material available for use.

Table 1. Group Descriptions Including Dose and Challenge Schedule.

Group (n=8)	IP Dosing Material	Dose (mg/kg)	*B. anthracis* Spore Inhalation Challenge
1	AIG	5	None
2	AIG	10	
3	AIG	20	Day 14
4	Normal IgG	Group 3 total protein dose	
5	None	None	

B. anthracis Aerosol Challenges

On Day 14 the animals in the challenge groups were randomly assigned to a challenge order using the MREF Animal Randomization Program. Animals in Groups 1

and 2 were not challenged. Challenged rabbits were placed into a plethysmography chamber in a Class III Biological Safety cabinet system and challenged with aerosolized *B. anthracis* spores (Ames Strain). Rabbits were challenged with a target dose of 200-times the mean lethal dose (LD_{50}), based upon the published rabbit aerosol LD_{50} of 1.05x 10^5 colony forming units (CFU) (Zaucha et al, 1998). The duration of the challenge was determined upon an estimated aerosol spore concentration and a cumulative minute volume gathered during the challenge. Challenges were conducted according to BMI SOP's.

Clinical Observations

Animals were observed twice daily for 14 days post-treatment (prior to challenge) for signs of protein toxicity. Potential clinical signs included, but were not limited to, loss of appetite, respiratory distress, abnormal colored urine, weakness in back legs, loss of coordination, and cool to the touch (Savoy, 1997).

Scheduled animals were challenged (on Study Day 14) with Ames strain *B. anthracis* spores as described above, and observed post challenge for an additional 14 days (twice daily) for signs of disease or mortality. However, it should be noted that experimentally infected rabbits exhibit few, if any, signs of disease prior to the day of death. Independent publications consistently report an abrupt terminal phase in lethally infected rabbits. Clinical disease is not generally apparent until the day of death, at which time rabbits become progressively lethargic and weak. Rabbits may exhibit brief periods of excitation and hyperactivity within h or min prior to death. Symptoms may include: hyperactivity or seizure denoting meningitis or encephalitis; loss of coordination,

respiratory distress, dyspnea, or forced abdominal respirations; unresponsive to touch or external stimuli; and moribundity (Zaucha et al, 1998).

Animals determined to be moribund were euthanized. Animals not challenged were euthanized on Study Day 28. Deaths or euthanasia were recorded to the nearest day.

Blood Collections

Blood samples were obtained from all study animals according to the schedule in Table 2. Blood was collected from the medial artery of the animals' ears. Approximately 2 ml of blood was collected from all groups listed in Table 2 for the anti-PA IgG ELISA. Samples were collected into a Serum Separator Tubes (Becton Dickinson, Franklin Lakes, NJ), sterile filtered (Nalgene, Rochester, NY, PES Filters, 0.2µm pore size, 13 mm diameter), cultured to confirm sterility according to BMI SOP's, aliquoted into two cryovials (VWR, West Chester, PA) each contain approximately 300 µl, and stored at -70º C until analyzed by ELISA for anti-PA IgG activity.

Table 2. Blood Collection Schedule

Group (n=8)	IP Dosing Material	ELISA[1]
1	AIG	Pre-dose Baseline (Day-1) Post dosing h:1, 24, 48, 72, and 96 Study Days:7, 10, 13, 16, 19, 22, 25, and 28
2	AIG	
3	AIG	
4	Normal IgG	

[1] Blood collection times are based specifically on each individual animal's IP treatment times.

Anti-PA Enzyme Linked Immunosorbent Assay (ELISA)

This assay, adapted from Quinn *et al*, 2001, quantifies the levels of human anti-PA present in the rabbit serum at 1, 24, 48, 72, 96 h and 7, 10, 13, 16, 19, 22, 25, and 28 days post-treatment allowing for assessment of normal human IgG or AIG clearance. The assay measures IgG antibodies against anthrax PA using an ELISA in which purified recombinant Protective Antigen (rPA) is used as the solid-phase immobilized antigen. Purified rPA for plate coating was purchased from National Institutes of Health (NIH), (Bethesda, MD) Lot number 1715AA. The secondary antibody (Anti-Human HRPO Conjugate Lot AVR423, Kirkegaard & Perry Laboratories) was used as the reporter or signal system is an enzyme-conjugated anti-gamma chain. The assay result is reported as the mean serum concentration of anti-PA specific IgG.

The primary acceptance criteria conclude whether or not the plate meets the required minimum standards. If a plate failed to meet any one of the three following criteria the plate failed and any results were disregarded.

1. The four parameter logistic log (4PL) curve was generated from the reference standard 7 point dilution must have an R^2 value of =0.9800.
2. The Quality Control samples must fall within their concentration range (average ± 3 standard deviations). Quality Control Serum High, lot number BMI500, (High titer human serum). Quality Control Serum Low, lot number BMI502, (Low titer human serum).
3. The negative control must have a mean optical density of <0.2. Negative Control Serum, Lot number 093K0475, (negative human serum).

The secondary acceptance criteria including the intra-assay and intra-dilution % coefficient of variation (CV) conclude whether or not the test sample results are acceptable.

Serum samples were aliquoted into appropriate volumes (~ 300µl), and frozen at <-70 º C until analyzed. Serum was not frozen and thawed more than 5 times with fewer freeze/thaw cycles being preferred.

ELISA plates (Immulon 2 HB, VWR, West Chester, PA) were coated with 1.0 µg/ml rPA (NIH, Lot 1715AA), covered with aluminum foil and incubated at 2-8º C overnight for at least 14 h. Coated plates were used within 7 days or discarded. Master Plate Diluent was prepared fresh and contained 1X PBS with 5% skim milk and 0.5 % Tween 20, pH 7.4. ELISA buffers were warmed to room temperature, and the rPA-coated plates were washed 3 times using an automated plate washer (Bio-Tek model ELx405, Winooski, VT). ELISA wash buffer was prepared fresh and contained 1X Phosphate Buffered Saline (PBS, Sigma), with 0.1 % Tween 20 (Sigma).

ELISA diluent buffer (100 µl/well) was added to all wells except column 12 which is reserved for quality and negative controls (Figure 1.). ELISA Diluent was prepared fresh and contained 1X PBS with 5% skim milk and 0.2 % Tween 20, pH 7.4. The serum standards were loaded into the plate wells in triplicate (columns 1-3). The quality controls and negative controls were added to column 12. The test samples were pre-diluted (typically 1:50) and loaded into the remaining wells. Samples in columns 1-11 were diluted using a two fold dilution.

The plates were incubated for 60 min at 37ºC (Nu Aire model NU-2600, Plymouth, MN). The plates were washed 3 times and 100 µl of diluted conjugate

solution was added to all plate wells, (Anti-Human HRPO Conjugate, Lot AVR423). The plates were returned to the humidified chamber to incubate for an additional 60 min. The plates were removed and washed 3 times and 100 µl of substrate was added to all the wells, (ABTS Microwell Perioxidase Substrate System, Kirkegaard & Perry Laboratories). After a final incubation in the humidified chamber for 30 min, 100 µl of stop solution was added to the plates, (ABTS Peroxidase Stop Solution, Kirkegaard & Perry Laboratories). The plates were then read within 4 h of addition of stop solution (Bio-Tek Plate Reader model ELx800). The data is then transferred to the CDC's "ELISA for Windows" analysis program and the reportable value calculated.

Table 3. Example of Test Plate Layout for ELISA Assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-HIGH
B	S2	S2	S2	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-HIGH
C	S3	S3	S3	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-LOW
D	S4	S4	S4	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-LOW
E	S5	S5	S5	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-QUAL
F	S6	S6	S6	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-QUAL
G	S7	S7	S7	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	NC
H	S8	S8	S8	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	NC

S1-S8 = Reference Standard Serum, Dilutions 1-8
TS# = Test Samples 1-4
QC-HIGH = High Concentration Serum Control
QC-LOW = Low Concentration Serum Control
QC-QUAL = Candidate Concentration Serum Control under Qualification
NC = Negative Serum Control
*Note: Columns 1-3 are reference standard samples diluted serially 2-fold down the plate, and columns 4-11 are unknown test samples diluted serially 2-fold down the plate.

ELISA Pilot Study 1

A pilot study was conducted to assess the ability of the human IgG ELISA to detect the purified AIG (Cov-060320-purIgG) in naïve human serum (093K0475), naïve rabbit serum (BMI012), anti-PA positive rabbit serum (BMI023), and PBS at a dilution series of expected concentrations. The AIG test material was spiked into the diluents at the dilution levels listed in Table 4. All dilutions in groups 1-16 were performed in triplicate. The samples were then analyzed via ELISA to determine the assay's recovery of AIG material from the samples.

Table 4. ELISA Pilot Study 1 Test Matrix

Group Number	Diluent	AVA IgG[1] Dilution Factor
1	Naïve Rabbit[2]	1:10
2		1:25
3		1:50
4		1:100
5	Anti-PA IgG Positive Rabbit[3]	1:10
6		1:25
7		1:50
8		1:100
9	Naïve Human[4]	1:10
10		1:25
11		1:50
12		1:100
13	PBS (control)	1:10
14		1:25
15		1:50
16		1:100

[1] = AIG is the test material (Lot Cov-060320-purIgG)
[2] = Lot No. BMI012
[3] = Lot No. BMI023
[4] = Lot No. 093K0475

ELISA Pilot Study 2

A second pilot study was conducted to compare the ability of the human IgG ELISA to detect the AIG (Cov-060320-purIgG), the AVA plasma (BMI505, source material for AIG), and a well characterized positive human serum (AVR801) from naïve human serum (093K0475), naïve rabbit serum (BMI012), and PBS at a dilution series of expected concentrations. The samples were prepared by spiking the test material into the diluents as listed in Table 5. All dilutions in were performed in triplicate. The samples were then analyzed via ELISA to determine the assay's recovery of AIG and AVA plasma, and positive human serum from the samples.

Table 5. ELISA Pilot Study 2 Test Matrix

Diluent	AIG[1]	AVA Plasma[2]	Anti-PA IgG positive Human Serum[3]
Naïve Rabbit[4]	1:10	1:10	1:10
	1:25	1:25	1:25
	1:50	1:50	1:50
	1:100	1:100	1:100
Naïve Human[5]	1:10	1:10	1:10
	1:25	1:25	1:25
	1:50	1:50	1:50
	1:100	1:100	1:100
PBS (control)	1:10	1:10	1:10
	1:25	1:25	1:25
	1:50	1:50	1:50
	1:100	1:100	1:100

[1] = AIG test material (Lot Cov-060320-purIgG).
[2] = Source plasma for AIG. (Pooled plasma from AVA vaccinated donors BMI-505).
[3] = Lot No. AVR801
[4] = Lot No.BMI012
[5] = Lot No. 093K0475

Statistical Analysis

Statistical evaluations were performed in StatExat 6.0, according to BMI SOPs. To determine any differences between naïve human, naïve rabbit, anti-PA positive rabbit serum, and PBS in terms of ELISA response to AIG, a regression model was fitted to the data with factors for serum type, dilution factor (AIG, AVA plasma, and anti-PA positive human serum), and the interaction. Separate models were fitted to each spiking material, slopes and intercepts were estimated for the relationship between dilution and the ELISA result for each diluent. Tests were conducted to determine if the slopes and/or intercepts for the different serum types were significantly different from each another.

The relationship between antibody response (measured by ELISA) and dilution of each spiking material (AIG, AVA plasma, and anti-PA positive human serum) was used to assess accuracy. This was completed by testing whether there was a significant difference between the measured ELISA and the expected values based on the estimated AIG titer and the known titer of the positive serum. This was performed separately for each serum type. The coefficient of variation (%CV) was calculated for each dilution and serum type to detect any discrepancies in pipetting precision.

RESULTS

Antibody Purification

In order to reduce the volume of material administered to the rabbits used for this study the human plasma was column purified. The purification process removed any anti-coagulants used during the plasma collection process that might interfere with the current study.

Naive IgG was purified from BMI504 (a large pool of naïve human plasma, pooled at BMI and obtained from Interstate Blood Bank) by Covance Research Products (CRP) using Protein G sepharose affinity chromatography according to CRP SOPs. The purified IgG was confirmed to be sterile and have acceptably low endotoxin levels (Cambrex, Walkersville, MD), and the total protein content (primarily IgG) was estimated to be 5.4 mg/ml by A_{280} spectrophotometric readings. BMI received 31 sealed glass vials, from CRP, containing 100 ml each of purified naïve human IgG.

Upon receipt, the product was determined to be intact and stored at approximately -20 °C. Later inspection revealed that a majority of vials stored at -20 °C freezer had developed cracks. The investigation identified that the breakage was due to the overfilling of the vials. The broken vials were thawed in sterile 5 L vessels at room temperature, and the retained IgG material was sterile-filtered and aliquoted into 100 ml bottles containing 50 ml each and stored at 2-8 °C.

Subsequently, 18 ml from bottles #5, #42, and #60 were removed and sub-aliquoted into 0.5 ml and 1.0 ml aliquots for additional characterization assays. Several 0.5 ml aliquots were sent to CRP for testing of sterility and endotoxin levels, which were determined to be acceptable. The results from the sterility, endotoxin, and total protein

concentration were provided in the certificate of analysis provided by CRP, summarized in Table 6. The results obtained by CRP for the determination of total protein were varied. The results obtained via BCA were reported as the most reliable according to CRP. The total IgG protein concentration used for the study was obtained by averaging the results from the BCA, IgG ELISA, and A_{280} spectrophotometric tests.

Table 6. Purified Naïve Human IgG Product Specifications: BMI-504-PurIgG

Description of Analysis/Test	Specification (Units)	Results (Units)	Method
Endotoxin	<1.0 EU	<0.07 EU	LAL Testing (Cambrex)
Sterility	Pass	Pass	Final Container (Taconic)
Protein IgG Concentration by ELISA	>5.0 mg/ml	8.78 mg/ml[1]	Covance SOP 46122
BCA Concentration	>5.0mg/ml	5.97 mg/ml[1]	Covance SOP 46121
PAGE-SDS Gel	Qualitative	Heavy and light chains visible	Covance SOP 46077
Concentration by A_{280nm}	>5.0 mg/ml	5.47 mg/ml[1]	Covance SOP 46069

[1] Value is an average of three samples (beginning, middle, and end vials).

The control material, once confirmed sterile with acceptable endotoxin levels, was characterized by anti-PA ELISA (BMI) to confirm the negativity. These results are summarized in Table 7.

Table 7. Anti-PA ELISA-Specific Characterization Information for Purified Naïve Human: BMI-504-PurIgG.

General Information	
Lot Number	BMI504-PurIgG
IgG Filter and Re-Pool Date	November 22, 2005
Expiration at 2 – 8°C	November 22, 2006[1]
Specific Information for Unknown Test Sample Application (n = 4)	
Average anti-PA IgG Concentration	0.000 µg/ml
Standard Deviation	0.000 µg/ml
%CV	0.00%
Acceptance Range (average conc. ±3 Std. Dev.)	N/A
Dilution for use as a Test Sample (Recommended)	1:50

[1] This date is an approximation without supporting stability studies.

The AIG test material was purified from BMI505 (a pool of human plasma from AVA vaccinated volunteers received from Cangene Corporation) by CRP using Protein G sepharose affinity chromatography. The first round of purification yielded purified total IgG (containing anti-PA IgG) and was assigned the lot number BMI505-purIgG. BMI505-purIgG was determined to have an average endotoxin level of 2.62 EU/ml (Cambrex). These levels exceeded the maximum levels required for use in animal studies (1.0 EU/ml) therefore, BMI505-purIgG required detoxification.

BMI505-purIgG material was pooled and processed by CRP using a "Detoxi-Gel" endotoxin removal column (Pierce, Rockford, IL). The final product resulting from this detoxification process was assigned the lot number BMI505-purIgG-rework. The detoxification was not successful, as BMI505-purIgG-rework was determined to have an average endotoxin level of 2.09 EU/ml (Cambrex). BMI505-purIgG-rework was pooled and processed again by CRP using a different type of detoxification matrix referred to as the "EndoTrap" column (Cambrex). The matrix utilizes a ligand technology derived from a bacteriophage to bind the endotoxin.

The resulting product, , assigned the lot number BMI506-purIgG, was determined to have an average endotoxin content of 0.076 EU/ml (Cambrex), and total protein content of approximately 4.8 mg/ml as measured by A_{280} spectrophotometric readings (CRP). The endotoxin levels were acceptably low and the total IgG concentration acceptably high for use as the test material. However, this product failed Taconic (Hudson, NY) sterility tests and filter sterilization was necessary. The final version of this product, lot number of Cov-060320-purIgG was determined to be sterile (Taconic). The results from the sterility, endotoxin, and total protein concentration were provided in the certificate of analysis provided by CRP, summarized in Table 8. Again the results obtained by CRP for the determination of total protein were varied. The total IgG protein concentration used for the study was obtained by averaging the results from the BCA, IgG ELISA, and A_{280} spectrophotometric tests.

Table 8. Purified AIG Product Specifications: Cov-060320-purIgG

Description of Analysis/Test	Specification (Units)	Results (Units)	Method
Endotoxin	<1.0 EU	<0.62 EU	LAL Testing (Cambrex)
Sterility	Pass	Pass	Final Container (Taconic)
Protein IgG Concentration by ELISA	>5.0 mg/ml	9.21 mg/ml[1]	Covance SOP 46122
BCA Concentration	>5.0mg/ml	6.36 mg/ml[1]	Covance SOP 46121
PAGE-SDS Gel	Qualitative	Heavy and light chains visible	Covance SOP 46077
Concentration by A_{280nm}	>5.0 mg/ml	5.88 mg/ml[1]	Covance SOP 46069

[1] Value is an average of three samples (beginning, middle, and end vials).

Cov-060320-purIgG was characterized by ELISA for anti-PA IgG concentration. The results used to determine the unknown anti-PA concentration for Cov-060320-purIgG were obtained by assaying the product 24 different times by a single test operator (8 independent iterations each from retention vials #1, #26, and #51). The anti-PA ELISA-specific characterization information is detailed in Table 9.

Table 9. Anti-PA ELISA-Specific Characterization Information for AIG: Cov-060320-PurIgG.

General Information	
Lot Number	Cov-060320-purIgG
Receipt Date	April 6, 2006
Expiration at 2 – 8°C	April 6, 2007[1]
Specific Information for Unknown Test Sample Application (n = 24)	
Average anti-PA IgG Concentration	688 µg/ml
Standard Deviation	23.7 µg/ml
%CV	3.44%
Acceptance Range (average conc. ±3 Std. Dev.)	N/A
Dilution for use as a Test Sample (Recommended)	1:200

[1] This date is an approximation without supporting stability studies.

Passive Antibody Administration

Animals in Groups 1-4 were administered IP treatments as described in Table 10. All treatments were given using a 16 gauge 1 in. needle attached to a 10 in. catheter. Prior to dosing, the placement of the needle was confirmed in the peritoneal cavity via aspiration with a 3 ml syringe. All doses were rounded to the nearest 0.1 ml. The dosage volumes of AIG ranged from 16.5 - 78.1 ml. The dose volumes of naïve IgG contained the same total protein dose as the animals in Group 3 (207.8 mg/kg), and ranged from 72.8 – 83.6 ml (Table 10). All doses were followed by a 3.0 ml flush of 0.9% physiological saline.

Table 10. Animal Treatment Volumes and Total Protein Dose by Group

	Animal I.D.	Group	Sex	Animal Wt (kg) 5/8/06	Treatment Dosage (anti-PA mg/kg)	Treatment Volume (ml)	Total Protein (mg/kg)
AIG	2077	1	M	2.2881	5	16.6	52.0
	2088	1	M	2.3035	5	16.7	52.0
	2090	1	M	2.3021	5	16.7	52.0
	2096	1	M	2.2756	5	16.5	52.0
	2217	1	F	2.4871	5	18.1	52.0
	2212	1	F	2.5434	5	18.5	52.0
	2206	1	F	2.4002	5	17.4	52.0
	2220	1	F	2.6891	5	19.5	52.0
	2095	2	M	2.5613	10	37.2	103.9
	2086	2	M	2.4791	10	36.0	103.9
	2080	2	M	2.3946	10	34.8	103.9
	2081	2	M	2.4058	10	35.0	103.9
	2214	2	F	2.6819	10	39.0	103.9
	2213	2	F	2.7203	10	39.5	103.9
	2221	2	F	2.5462	10	37.0	103.9
	2203	2	F	2.3746	10	34.5	103.9
	2084	3	M	2.4057	20	69.9	207.8
	2085	3	M	2.5042	20	72.8	207.8
	2076	3	M	2.3729	20	69.0	207.8
	2091	3	M	2.4106	20	70.1	207.8
	2216	3	F	2.4785	20	72.0	207.8
	2218	3	F	2.6143	20	76.0	207.8
	2207	3	F	2.6269	20	76.4	207.8
	2209	3	F	2.6857	20	78.1	207.8
Naive IgG	2078	4	M	2.5032	Group 3 total IgG	77.2	207.8
	2083	4	M	2.4783	Group 3 total IgG	76.4	207.8
	2082	4	M	2.3596	Group 3 total IgG	72.8	207.8
	2092	4	M	2.5073	Group 3 total IgG	77.3	207.8
	2205	4	F	2.5151	Group 3 total IgG	77.6	207.8
	2208	4	F	2.4923	Group 3 total IgG	76.9	207.8
	2201	4	F	2.5321	Group 3 total IgG	78.1	207.8
	2210	4	F	2.7116	Group 3 total IgG	83.6	207.8

Clinical Observations

To ensure that the passively administered material was non-toxic to the rabbits, all animals in Groups 1-4 were observed continuously for at least 1 h post IP-treatment. There were no apparent signs of toxicity observed during this period. All animals in Groups 1-4 remained normal during the twice daily observations over the 14-day period after treatment (Table 11). A single animal in Group 2 exhibited brief periods of inappetence on Study Days 7, 9, and 12 post-treatment. It can be speculated that these periods of inappetence were related to times when the animal was tranquilized for blood collections.

Table 11. Summary Table of Observed Post-Treatment Clinical Observations

Clinical Observation	Number of animals in each group that demonstrated the clinical observation at least once during the observation period				
	Group 1 (n=8)	Group 2 (n=8)	Group 3 (n=8)	Group 4 (n=8)	Group 5 (n=8)
Normal	8	8	8	8	8
Not eating	0	1	0	0	0

***B. anthracis* Aerosol Challenges**

Aerosol challenges were conducted as described in BMI SOP Numbers MREF.XIII-001 and MREF.XIII-011. A Collison 3-jet nebulizer (BGI Inc., Waltham, MA) was used to aerosolize the biological agent, *B. anthracis* (Ames strain, BMI), for the challenge. Filtered "house" air was provided to supply a continuous and regulated air source to the Collison nebulizer and for additional dilution air. As the aerosolized *B. anthracis* exited the Collison, additional humidified air was introduced through a bubbler

into the aerosol stream to dilute the aerosol and regulate humidity in the system during each exposure. Nebulizer bypass air and humidified dilution air was provided to the animal during the pre exposure periods of testing. At the initiation of each animal challenge, the bypass airflow was turned off and the air redirected to the Collison nebulizer for aerosol generation.

Supply air pressure for system operation was regulated and maintained at approximately 30 psi for all exposure challenges. The Collison nebulizer bypass airflow was regulated and controlled at approximately 7.4 L/min and the humidified dilution air at approximately 8.7 L/min using a 0 to 20 L/min mass flow controller (Sierra Instruments, Monterey, CA). Relative Humidity levels in the exposure system were maintained in the range of 52% to 87 % during exposures, and were monitored using a temperature and humidity monitor model 605-H1 (SKC, Inc., Fullerton, CA). The nebulizer flow rate was maintained at approximately 7.5 L/min by supplying a continuous and regulated air supply to the nebulizer in the range of 27 to 29 psi.

The aerosol sampling system consisted of a model 7541 impinger (Ace Glass, Inc., Vineland, NJ), and an Aerodynamic Particle Sizer® Spectrometer model 3321 (TSI, Inc., St Paul, MN). A flow validated impinger filled with approximately 20 ml of sterile water (Sigma) was used to collect a representative fraction of the challenge aerosol from the exposure system. An impinger sample was collected during aerosol challenge to determine the respirable colony forming unit (cfu) concentration delivered to the animal. A single 30 s sample was pulled from the system during each animal exposure challenge at a flow rate of 0.5 L/min. These samples were plated on trypticase soy agar (TSA) to obtain the approximate LD_{50s} administered to each animal (Table 12). Flow rate through

the impinger was maintained at approximately 6 L/min by maintaining a negative pressure of 18 inches of Hg using a 1/5 hp vacuum pump (Gast Manufacturing, Benton Harbor, MI). An Aerodynamic Particle Sizer® Spectrometer (APS) was used to measure the size distribution of the *B. anthracis* aerosol. .

Table 12. Aerosol LD_{50} Equivalents and Time to Death by Dose Group

Animal ID	Group	Dosing Material	anti-PA Dose (mg/kg)	Aerosol LD50 Equivalents	Days Till Death
2091	3	AVA IgG	20	225	2
2085	3	AVA IgG	20	349	3
2216	3	AVA IgG	20	229	3
2207	3	AVA IgG	20	190	3
2076	3	AVA IgG	20	400	4
2218	3	AVA IgG	20	306	4
2084	3	AVA IgG	20	183	4
2209	3	AVA IgG	20	188	8
2208	4	Naïve IgG	NA	377	3
2205	4	Naïve IgG	NA	268	3
2078	4	Naïve IgG	NA	220	3
2082	4	Naïve IgG	NA	339	4
2201	4	Naïve IgG	NA	295	4
2210	4	Naïve IgG	NA	220	4
2092	4	Naïve IgG	NA	191	4
2083	4	Naïve IgG	NA	333	5
2219	5	None	NA	442	3
2093	5	None	NA	345	3
2094	5	None	NA	323	3
2097	5	None	NA	297	3
2202	5	None	NA	248	3
2087	5	None	NA	228	3
2215	5	None	NA	142	4
2204	5	None	NA	179	5

Post Challenge Clinical Observations and Mortality

Adverse clinical observations on rabbits from the time of the anthrax challenge to the end of the study included: inappetence, soft stool, no stool, wheezing, sneezing, rough hair coat, lethargy, loss of coordination, abnormal posture/gait, milky lacrimations, moribundity, seizure, and death. Many rabbits showed signs of clinical illness (inappetence, lethargy and/or moribundity) leading up to their time of death or euthanasia. Inappetence was a common observation during the first few days following challenge in all groups. This inappetence could be due to the anthrax infection and/or the stress of daily activities (e.g. blood draws) in the animal rooms. Although a majority of the animals that died exhibited inappetance just prior to death, a period of inappetence was not always predictive of a fatal outcome. Other symptoms of anthrax, such as lethargy/recumbency and respiratory distress, were less commonly observed and most likely due to the rapid progression to death seen in rabbits. The post-challenge observations are detailed in Table 13.

Table 13. Post Challenge Clinical Observations

Study Day		15		16		17		18		19		20		21		22	
Animal ID	Group	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
2084	3	N	N	N	N	NE	N	D									
2085	3	N	N	N	N	NE	D										
2076	3	N	N	N	N	NE	L	D									
2091	3	N	N	L, U, M, E													
2216	3	N	N	N	N	NE, S, E											
2218	3	N	N	NE,L	NE	NE	NE,L	NE,L, D									
2207	3	N	N	N	N	NE	D										
2209	3	N	N	N	N	N	N	N	NE,L	NE	NE	NE	NE,NS	NE	NE,NS	NE,L,D	
2078	4	N	N	N	NE, L	D											
2083	4	N	N	N	N	NE	SS	N, SN	NE, RD	D							
2082	4	N	N	N	N	NE	NE, L	D									
2092	4	N	N	SS	N	NE, L	SS, NE, L, RD	D									
2205	4	N	N	N	N	NE	D										
2208	4	N	N	L	NE, L	D											
2201	4	N	N	NE	NE	NE	N	D									
2210	4	N	N	NE	NE	NE, W	NE, RD	D									
2097	5	N	N	N	NE	D											
2087	5	N	N	N	L, NE, ML, RHC, AP	D											
2094	5	N	N	N	N	D											
2093	5	N	N	N	NE	D											
2202	5	N	N	NE	NE	D											
2219	5	N	N	NE	NE	NE, L, U, AP, D											
2215	5	N	N	NE, L	NE,L	NE, L	NE, NS, L										
2204	5	N	N	N	N	N	N	NE	NE,L	NE,L,D							

AP=Abnormal Posture D=Dead E=Euthanized L=lethargic M=Moribund ML=Milky Lacrimations N=Normal NE=Not Eating NS=No Stool
RD=Respiratory Distress RHC= Rough Hair Coat S=Seizure SS=Soft Stool SN= Sneezing U=Uncoordinated W=Wheezing

Figure 2 shows the cumulative percent of totals dead by days post-challenge. Groups 4 and 5 reached 100% mortality by Study Days 4 and 5 post-challenge, respectively. Group 3 reached 100% mortality by 8 days post-challenge.

Figure 2. Percent of Total Dead versus Days Post Challenge

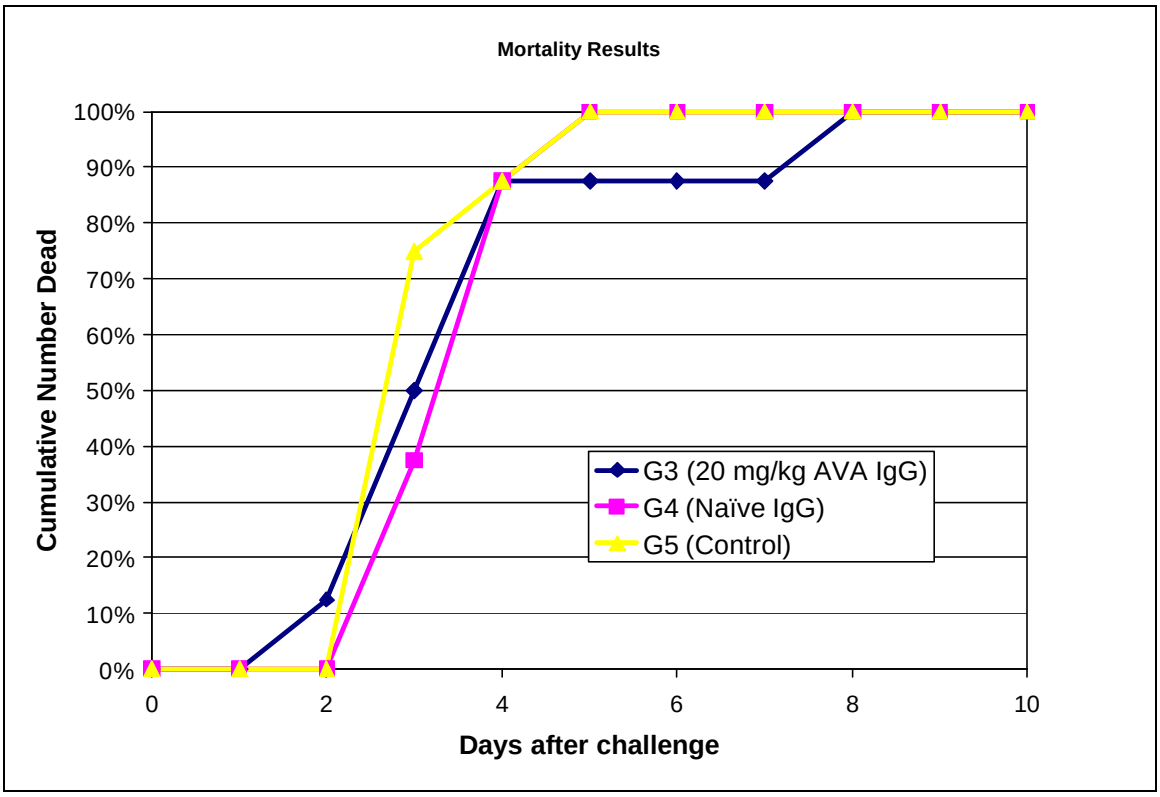


Table 14 shows the survivability and time to death statistics for Groups 3-5. The average to time death and standard deviations are included for each group.

Table 14. Survivability and Time to Death for Challenge Groups

Animal ID	Sex	Group	Ames LD 50	Challenge Date and Time	Survival Status	Date and Time of Death	Days to Death
2076	F	3	400	5/23/06 13:29	Died	5/27/06 6:20	3.7
2084	F	3	183	5/23/06 13:15	Died	5/27/06 6:20	3.7
2085	M	3	349	5/23/06 12:08	Died	5/26/06 13:50	3.1
2091	M	3	225	5/23/06 10:41	Died	5/25/06 9:27	1.9
2207	F	3	190	5/23/06 11:44	Died	5/26/06 13:50	3.1
2209	F	3	188	5/23/06 10:54	Died	5/31/06 8:15	7.9
2216	M	3	229	5/23/06 11:34	Died	5/26/06 7:30	2.8
2218	M	3	306	5/23/06 11:56	Died	5/27/06 8:20	3.8
				Survival	0/8	Average	3.8
						Stnd Dev	1.8
2078	F	4	220	5/23/06 11:08	Died	5/26/06 6:50	2.8
2082	F	4	339	5/23/06 12:55	Died	5/27/06 6:20	3.7
2083	M	4	333	5/23/06 13:40	Died	5/28/06 6:32	4.7
2092	M	4	191	5/23/06 9:45	Died	5/27/06 6:20	3.9
2201	F	4	295	5/23/06 12:31	Died	5/27/06 6:20	3.7
2205	F	4	268	5/23/06 10:12	Died	5/26/06 13:50	3.2
2208	M	4	377	5/23/06 12:20	Died	5/26/06 6:50	2.8
2210	M	4	220	5/23/06 9:56	Died	5/27/06 6:20	3.8
				Survival	0/8	Average	3.6
						Stnd Dev	0.6
2087	F	5	228	5/23/06 9:10	Died	5/26/06 6:50	2.9
2093	F	5	345	5/23/06 13:53	Died	5/26/06 6:50	2.7
2094	M	5	323	5/23/06 13:09	Died	5/26/06 6:50	2.7
2097	M	5	297	5/23/06 12:44	Died	5/26/06 6:50	2.8
2202	F	5	248	5/23/06 10:26	Died	5/26/06 6:50	2.8
2204	F	5	179	5/23/06 11:20	Died	5/28/06 8:32	4.9
2215	M	5	142	5/23/06 9:32	Died	5/27/06 6:20	3.9
2219	M	5	442	5/23/06 9:23	Died	5/26/06 7:40	2.9
				Survival	0/8	Average	3.2
						Stnd Dev	0.8

ELISA Pilot Study 1

This pilot study was conducted to determine the ability of the human IgG ELISA to recover the purified AIG (Cov-060320-purIgG) in naïve human serum (093K0475), naïve rabbit serum (BMI012), positive rabbit serum (BMI023), and PBS for a series of expected concentrations. It was unclear how accurately the human anti-PA ELISA would detect the AIG material from positive rabbit serum collected for this project. This pilot study compared the assay's recovery rate from the four different test diluents.

Table 15 contains the baseline ELISA results for the individual materials used in this pilot study. The ELISA test results are presented in Table 16 and Figure 4. The actual ELISA values listed represent the mean from the 3 independent results. The expected concentrations are based upon the AIG material having a concentration of 705 µg/ml.

Table 15. ELISA Results on Individual Test Materials

Test Material	Mean Anti-PA ELISA Concentration (µg/ml)[1]
AIG	705
Diluents	
Naïve Rabbit	0
anti-PA IgG Positive Rabbit	27
Naïve Human	0
PBS	0

[1] Results are from anti-PA ELISA using anti-human conjugate

Table 16. ELISA Pilot Study 1 Results for the Test Samples

			Mean Anti-PA ELISA Concentration (µg/ml)		
Group	**Diluent**	**AIG[1] Dilution Factor**	**Actual**	**Expected[5]**	**% Recovery**
1	Naïve Rabbit[2]	10	43	71	61 %
2		25	23	28	82 %
3		50	14	14	100 %
4		100	5.4	7	77 %
5	Anti-PA IgG Positive Rabbit[3]	10	103	98	105 %
6		25	48	55	87 %
7		50	37	41	90 %
8		100	33	34	97 %
9	Naïve Human[4]	10	55	71	77 %
10		25	20	28	71 %
11		50	10	14	71 %
12		100	4.9	7	70 %
13	PBS (control)	10	50	71	70 %
14		25	18	28	64 %
15		50	9.0	14	64 %
16		100	4.4	7	63 %

[1] AIG is the test material (Lot Cov-060320-purIgG)
[2] Lot No. BMI012
[3] Lot No. BMI023
[4] Lot No. 093K0475
[5] Expected values is based upon the AIG mean anti-PA ELISA concentration of 705 µg/ml

Figure 3 clearly shows that the ELISA assay detects both the purified human AIG and the rabbit IgG from the known positive rabbit serum (BMI023). The results from the assays show a slight decrease in recovery from the expected values. However, this is not unlikely as the test material AIG has not been as extensively characterized as the positive control serum BMI 023.

Figure 3. ELISA Pilot Study 1 Results with Lines for Expected Concentration.

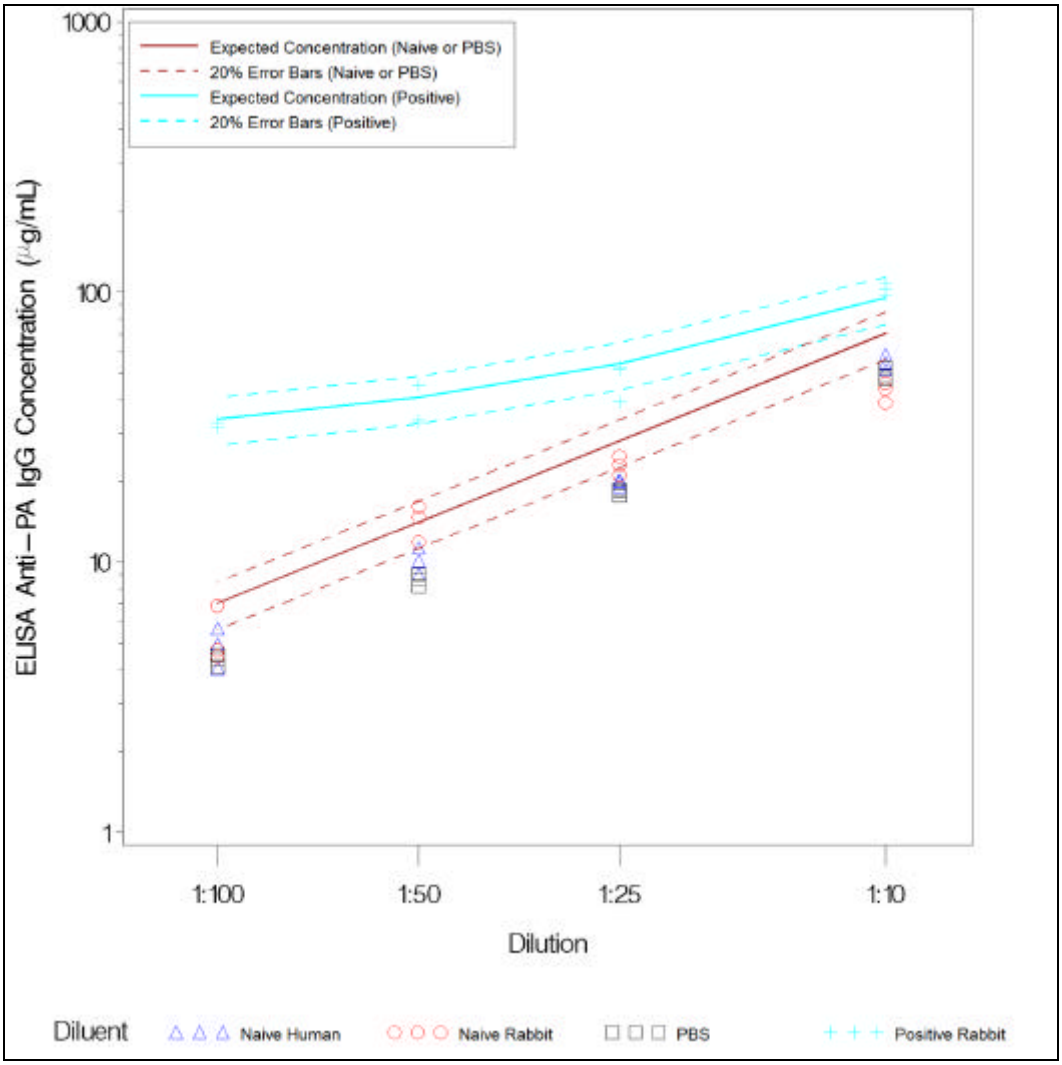


Figure 4 shows the ELISA data plotted against the expected titers. The expected titers were calculated from the dilutions used and the estimated titers of the purified AIG test material and the known positive rabbit serum (BMI023). The solid lines are regression lines for each diluent and the dotted line represents the 45 degree line.

Figure 4. ELISA Pilot Study 1 Values Plotted Versus Expected Concentrations.

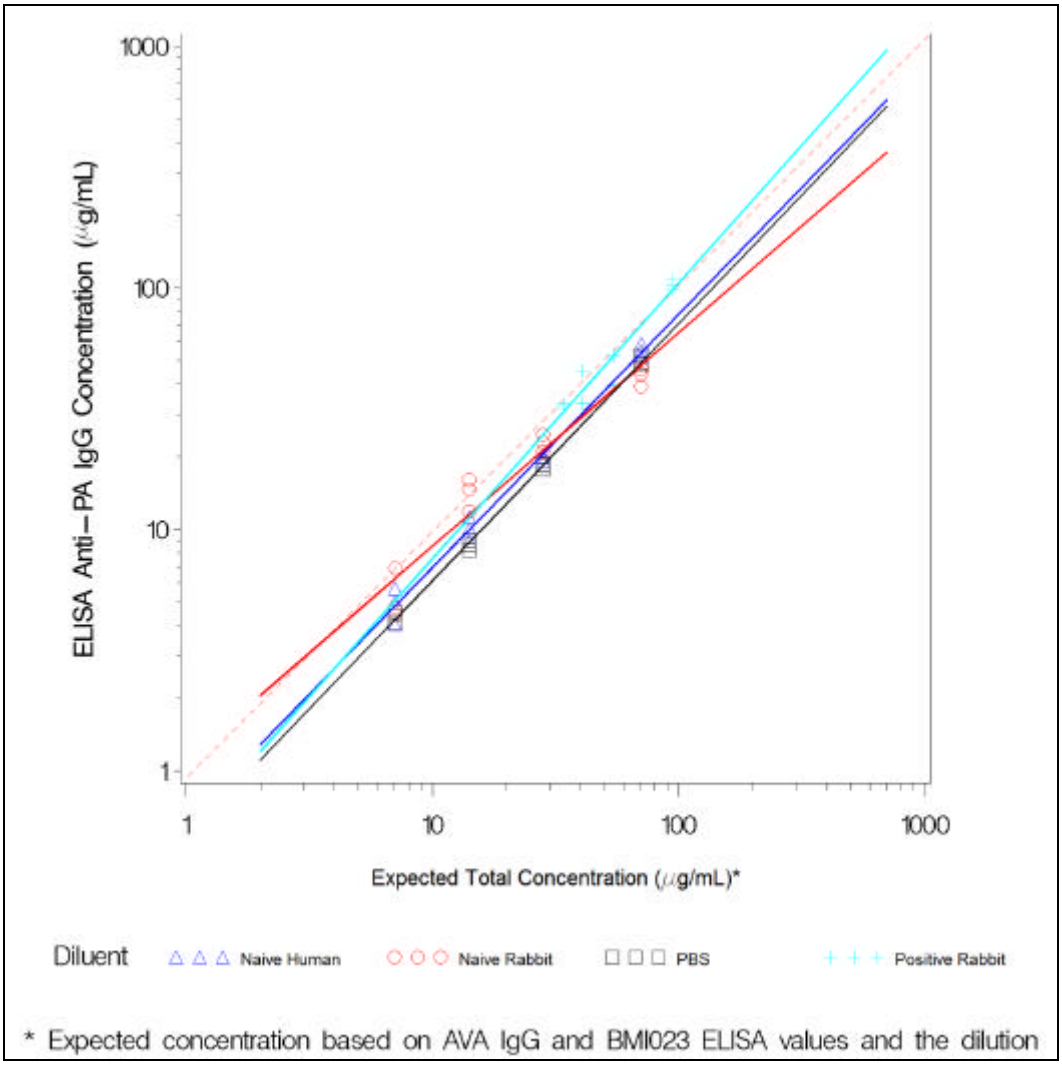


Table 17 contains the slope estimates from the analysis of covariance models fitted to the logarithms of the ELISA data. The models included a continuous covariate for the logarithm of the expected titer, a factor for diluent and the interaction. The table also contains the p-value for testing whether the slope estimate was significantly different from one. To maintain an overall p value at the 0.05 level across the four diluents, p-values were considered significant if they were less than the Bonferroni adjusted level of

0.0125 (if p=0.05/4 (4 represents the number of different diluents tested)). No slopes were significantly different from one. Thus, the hypotheses of dilutional linearity for the ELISA assays were not rejected for any of the diluents. The intercepts from the ELISA model for the naïve human (p-value=0.0023) and PBS (p-value<0.0001) diluents were significantly less than zero. This indicates a slight under-recovery for these diluents.

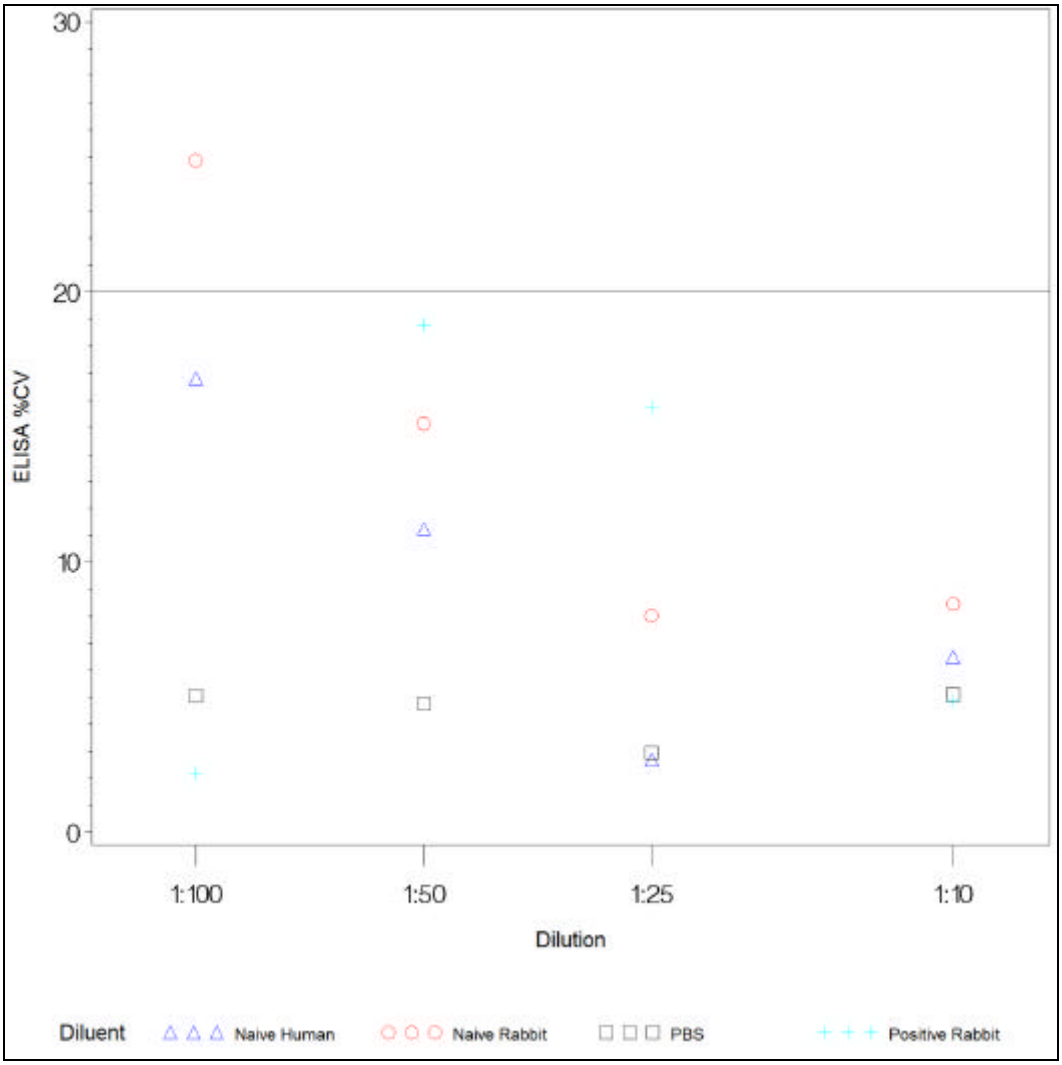
Table 17. Slope Estimates from ELISA Models with P-values for Testing If Slope is Significantly Different From One.

Diluent	ELISA	
	Slope Estimate	P-value*
Naïve Human	1.05	0.3018
Naïve Rabbit	0.88	0.0139
PBS	1.06	0.1756
Positive Rabbit	1.14	0.1709

* No slopes significant at the Bonferroni adjusted 0.0125 (=0.05/4) level.

Figure 5 shows the coefficient of variation (CV) from the ELISA data for each diluent and dilution. The variability in the data appears to be greater for the 1:100 dilutions which is not unexpected as these values were near (and sometimes less than) the ELISA Limit of Quantification (LOQ) of 2.5 µg/ml. CVs for the other dilutions were less than 20 percent. These plots provide evidence that the different diluents utilized in this pilot study do not adversely affect the precision of the human IgG ELISA assay. The ELISA results confirm that there were no statistically significant differences for AIG between any of the naïve diluents and the results for positive rabbit serum were slightly elevated when compared to the naïve diluents.

Figure 5. Coefficients of Variation for Each Diluent and Dilution for ELISA.



Pilot Study 2 Results

The under recovery of the positive AIG from the negative diluents was a concern since the majority of the samples used in determining the half-life of the AIG material were collected before the rabbit's immune response would be detectable (less than 7 days). This pilot study would determine any statistical significant difference in the human IgG ELISA's ability to recover positive test materials (AIG, the AVA source plasma, and a known positive human serum) from naïve diluents (naïve human serum, naïve rabbit serum, and PBS) at a dilution series of expected concentrations.

The test results are presented in Tables 18-20 and Figures 6-8. All actual values in the tables are the mean result of the three test samples results, while the figures plot the individual results.

Table 18. ELISA Pilot Study 2 Results for AIG in Each Diluent

		Anti-PA ELISA Concentration (µg/ml)		
Diluent	**Dilution Factor**	**Actual**	**Expected[1]**	**% Recovery**
Naïve Rabbit	10	60	69	88%
	25	23	28	82%
	50	11	14	77%
	100	5.7	6.9	83%
Naïve Human	10	59	69	86%
	25	26	28	94%
	50	13	14	95%
	100	5.9	6.9	85%
PBS (control)	10	57	69	83%
	25	25	28	91%
	50	11	14	81%
	100	4.6	6.9	68%

[1] Expected ELISA values are based on AIG having an anti-PA level of 688 µg/ml.

Table 19. ELISA Pilot Study 2 Results for AVA Plasma in Each Diluent

		Anti-PA ELISA Concentration (µg/ml)		
Diluent	**Dilution Factor**	**Actual**	**Expected[1]**	**% Recovery**
Naïve Rabbit	10	69	90	77%
	25	24	36	67%
	50	15	18	83%
	100	7.1	9.0	79%
Naïve Human	10	78	90	87%
	25	25	36	69%
	50	15	18	83%
	100	7.5	9.0	83%
PBS (control)	10	68	90	76%
	25	25	36	69%
	50	12	18	67%
	100	6.3	9.0	70%

[1] Expected ELISA values are based on AVA Plasma having an anti-PA level of 901 µg/ml.

Table 20. ELISA Pilot Study 2 Results for Anti-PA IgG positive Human Serum in Each Diluent

		Anti-PA ELISA Concentration (µg/ml)		
Diluent	**Dilution Factor**	**Actual**	**Expected[1]**	**% Recovery**
Naïve Rabbit	10	10.5	10.9	96%
	25	4.3	4.4	98%
	50	2.6	2.2	118%
	100	1.0	1.1	92%
Naïve Human	10	11.0	10.9	101%
	25	4.6	4.4	105%
	50	3.0	2.2	136%
	100	1.7	1.1	160%
PBS (control)	10	9.5	10.9	87%
	25	3.7	4.4	84%
	50	1.7	2.2	80%
	100	0.9	1.1	82%

[1] Expected ELISA values are based on anti-PA IgG positive Human Serum having an anti-PA level of 109 µg/ml.

Figures 6-8 clearly show that the ELISA assay does detect the positive materials (AIG, AVA Plasma, and positive human serum) in all three of the diluents. Plots of the ELISA data show that most of the results are within 20-30 percent of the expected value for all dilutions and spiking materials. The AVA plasma had the lowest recovery in all diluents, yet the recovery was relatively consistent across diluents. The recovery of the positive materials from PBS was lower than in the naïve rabbit and naïve human serum. This is consistent with the lower recoveries observed in the initial pilot study.

Figure 6. ELISA Pilot Study 2 Results for AIG with Lines for Expected Concentration.

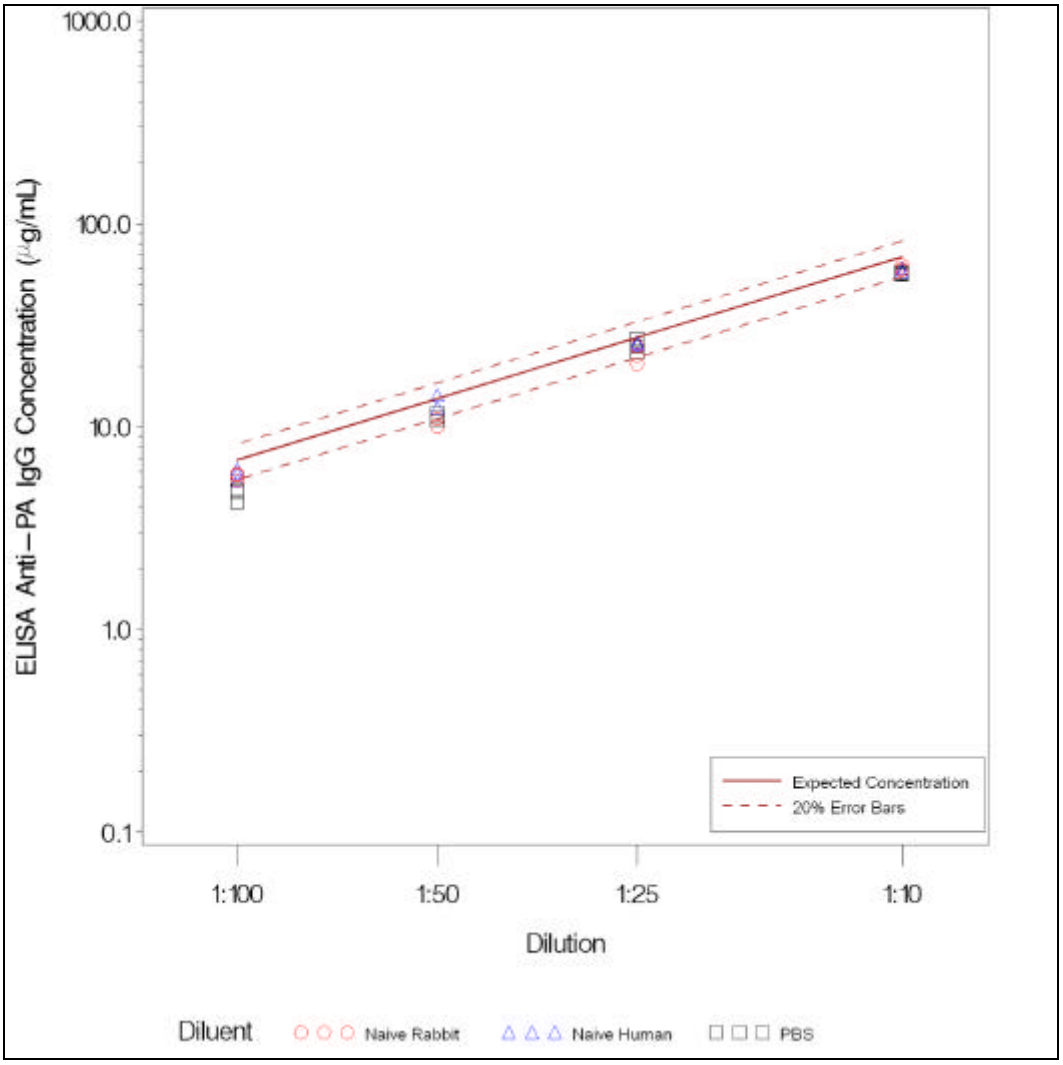


Figure 7. ELISA Pilot Study 2 Results for AVA Plasma with Lines for Expected Concentration.

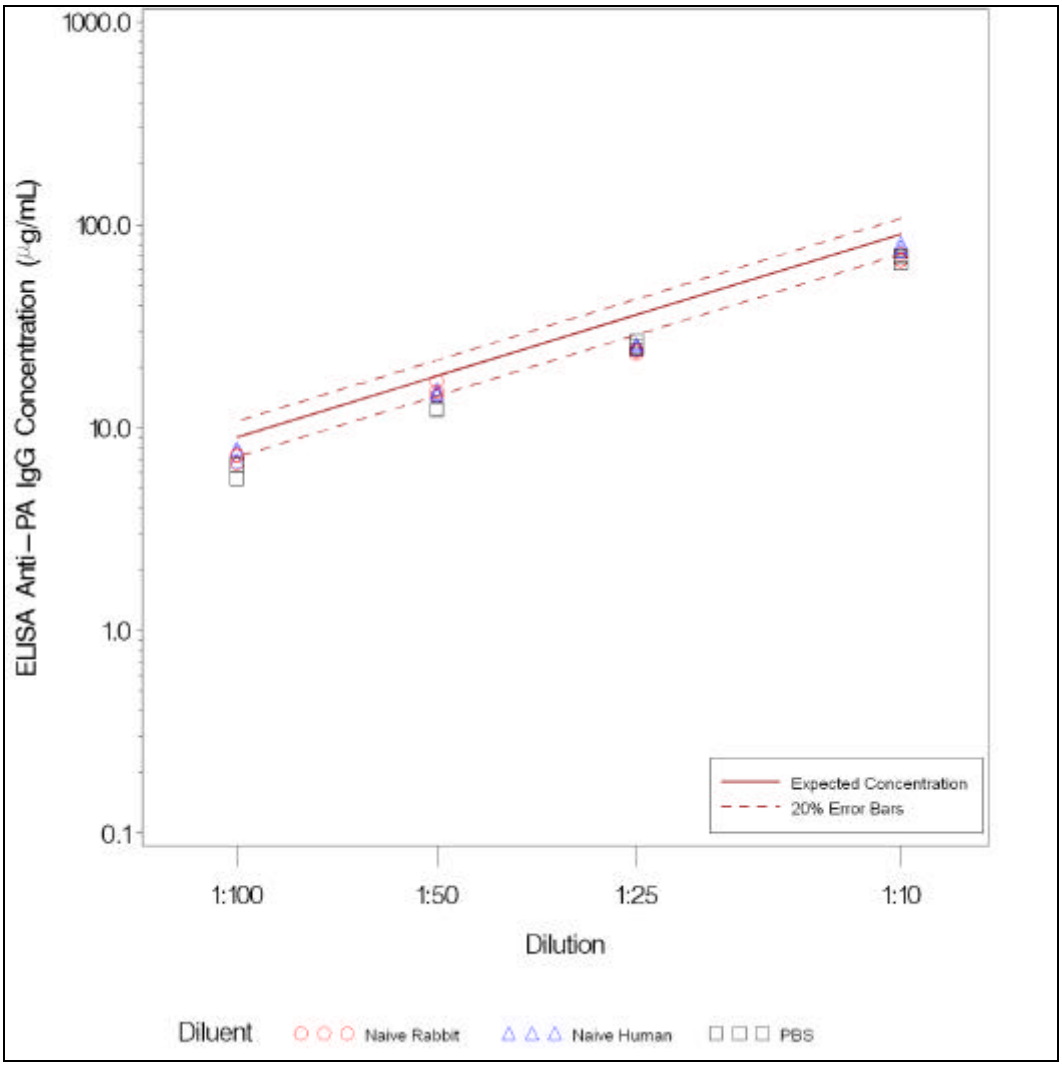
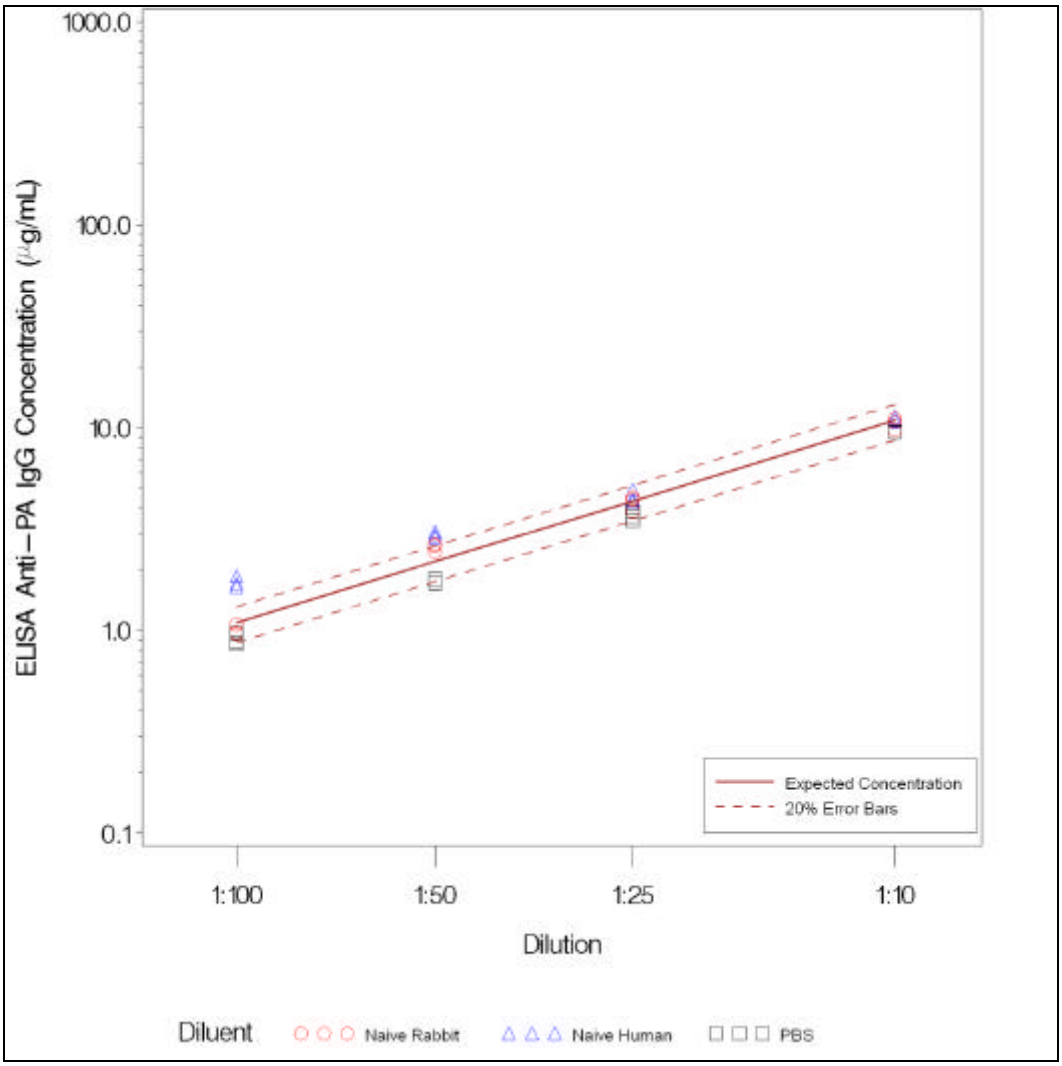


Figure 8. ELISA Pilot Study 2 Results for Anti-PA IgG positive Human Serum with Lines for Expected Concentration.



Figures 9-11 show the ELISA data plotted against the expected titers. The expected titers were based on the dilutions used and the estimated titers of the positive serum (AIG, AVA Plasma and positive human serum). The solid lines in the plots are the regression lines fitted to the data and the dotted line in each plot is the 45 degree line.

Figure 9. ELISA Pilot Study 2 Values for AIG Plotted Versus Expected Concentrations.

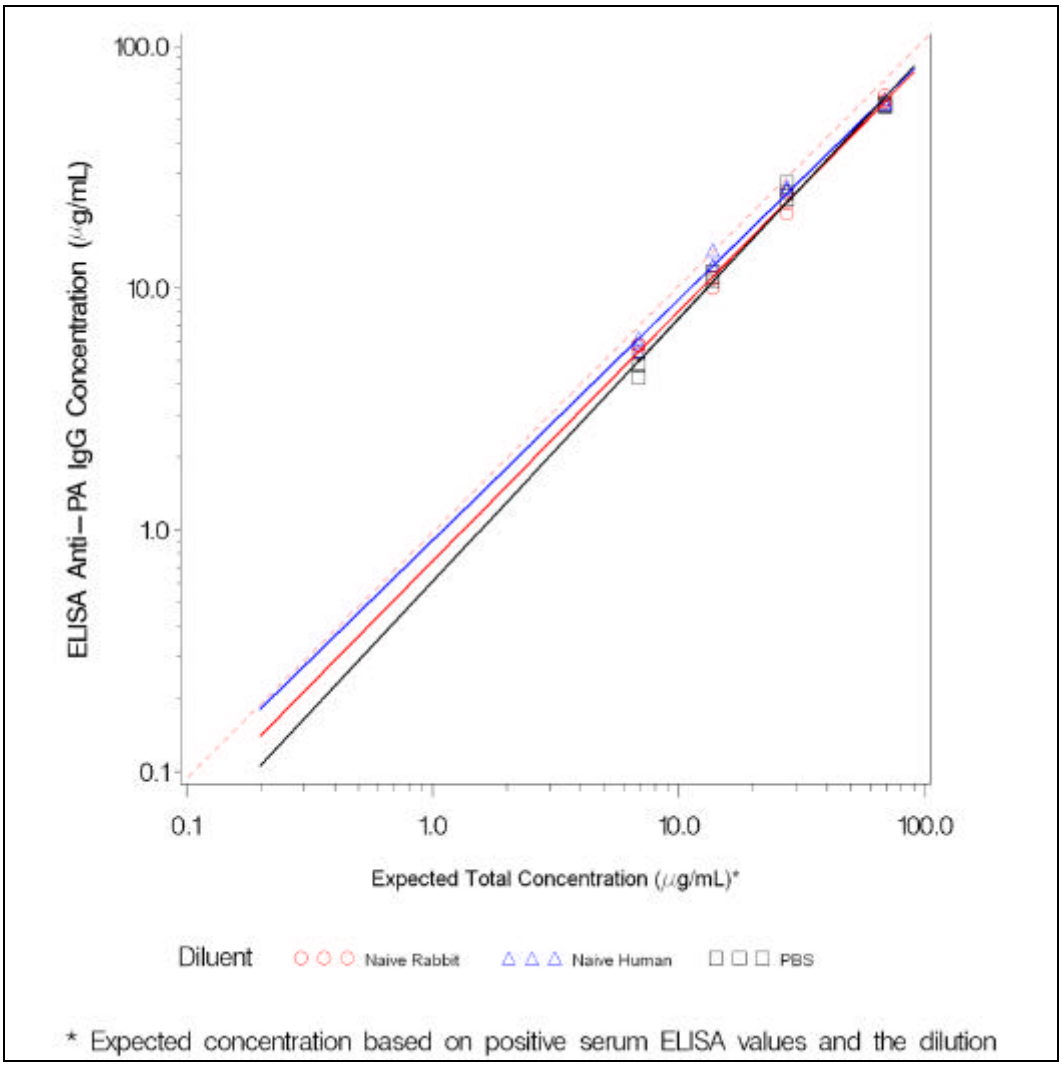


Figure 10. ELISA Pilot Study 2 Values for AVA Plasma Plotted Versus Expected Concentrations.

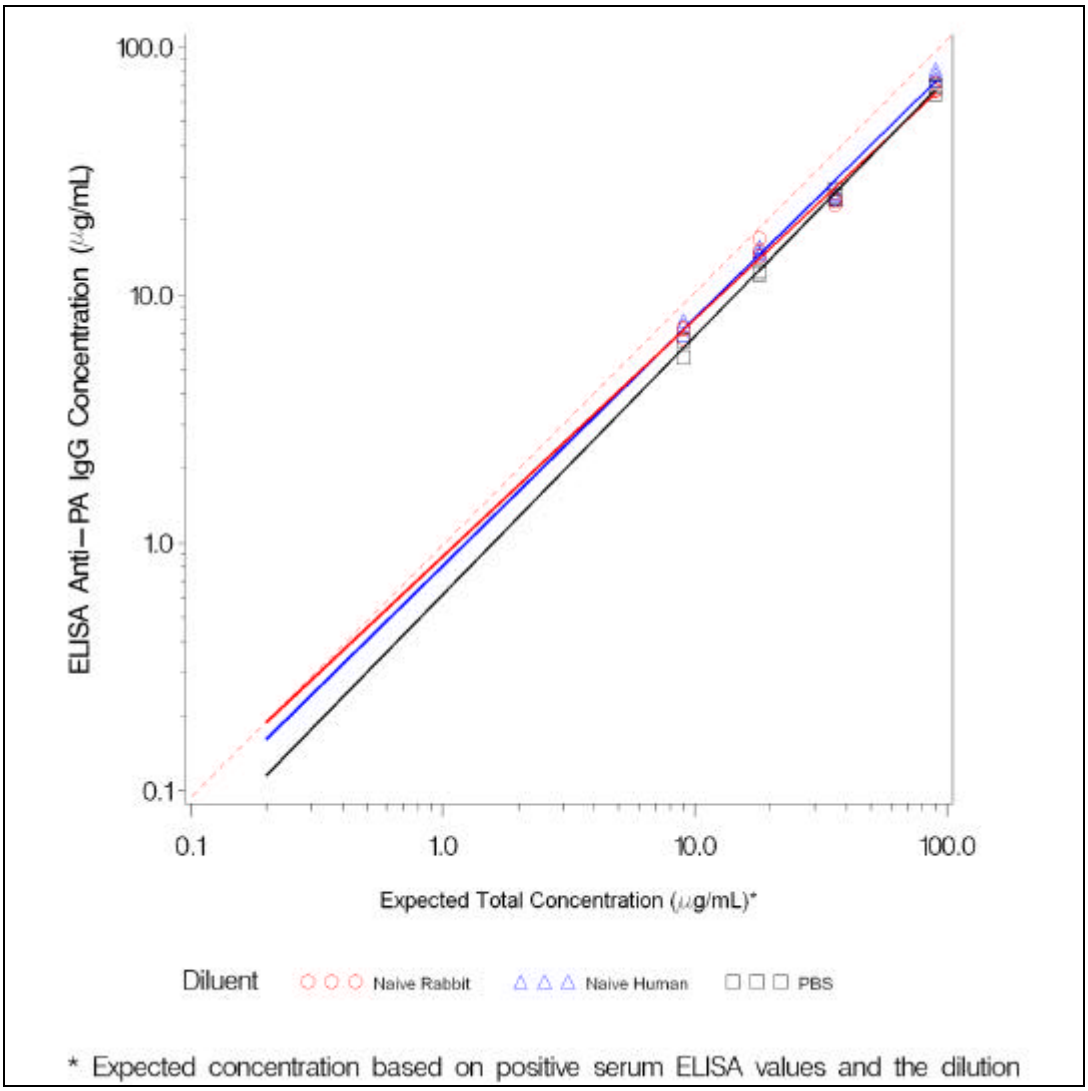


Figure 11. ELISA Pilot Study 2 Values for Anti-PA IgG Positive Human Serum Plotted Versus Expected Concentrations.

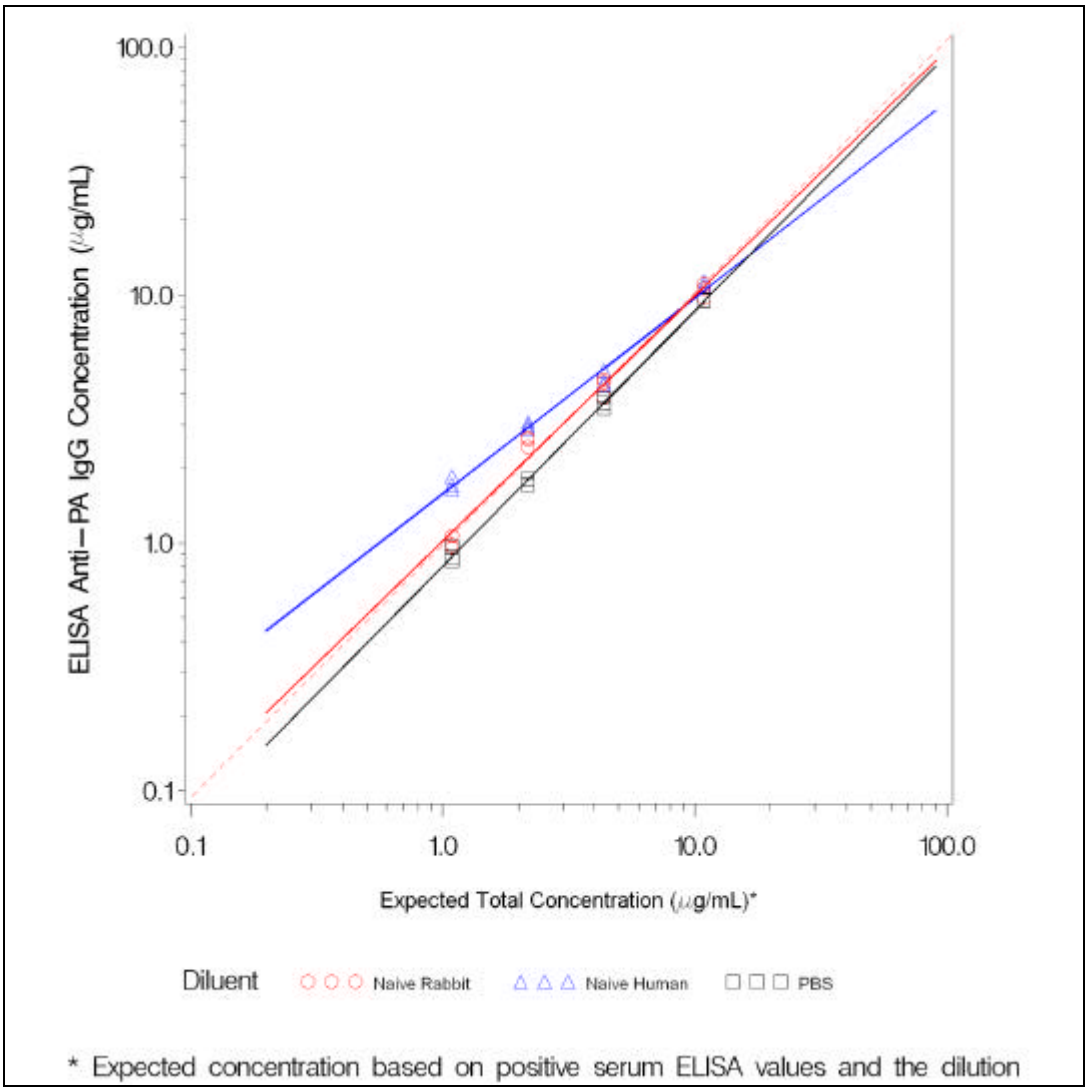


Table 21 contains the slope and intercept estimates from the analysis of covariance models fitted to the logarithms of the ELISA data for each spiking material. The models included a continuous covariate for the logarithm of the expected titer, a factor for diluent and the interaction. The table also contains the p-values for testing whether the slope estimate was significantly different from one and whether the intercept

estimate was significantly different from zero for each diluent. To maintain an overall 0.05 level across the three diluents, p-values were considered significant if they were less than the Bonferroni adjusted level of 0.0167 (0.05/ 3).

The slopes from the ELISA models were significantly different from one and intercepts were significantly different from zero for AIG diluted into PBS and anti-PA IgG positive human serum diluted into naïve human serum. In addition, the intercepts from the ELISA model using anti-PA IgG positive human serum diluted in naïve human serum (p-value<0.0001) was significantly greater than zero and diluted in PBS (p-value<0.0001) was significantly less than zero. These results may be due to values that are less than the LOQ for the assay. For AVA Plasma diluted into PBS the intercept (p-value<0.0001) was significantly less than zero. The intercept was significantly less than zero for AIG diluted into naïve rabbit serum (p-value=0.0018) and PBS (p-value<0.0001). It appears that these results are indicative of a slight under-recovery in PBS and in naïve rabbit serum for AIG.

Table 21. Slope and Intercept Estimates from ELISA Models With P-values for Testing if Slope was Significantly Different from One and Intercept was Significantly Different from Zero

Positive Serum	Diluent	Slope		Intercept	
		Estimate	P-value	Estimate	P-value
AIG	Naïve Human	1.00	0.9440	-0.04	0.2474
	Naïve Rabbit	1.03	0.2306	-0.13	0.0018*
	PBS	1.09	0.0028*	-0.21	<.0001*
AVA Plasma	Naïve Human	1.00	0.9620	-0.09	0.0470
	Naïve Rabbit	0.96	0.1525	-0.05	0.2380
	PBS	1.04	0.1828	-0.21	<.0001*
Anti-PA IgG positive Human Serum	Naïve Human	0.79	<.0001*	0.20	<.0001*
	Naïve Rabbit	0.99	0.7494	0.01	0.7044
	PBS	1.03	0.2913	-0.09	<.0001*

* Significant at the Bonferroni adjusted 0.0167 (=0.05/3) level.

Figures 12-14 show the coefficient of variation (CV) from the ELISA data for each positive serum, diluent and dilution. For the ELISA data all CVs were less than 20 percent. These plots provide evidence that the different diluents do not adversely affect the precision of the ELISA assay.

Figure 12. Coefficients of Variation for AIG ELISA Values from Each Diluent and Dilution.

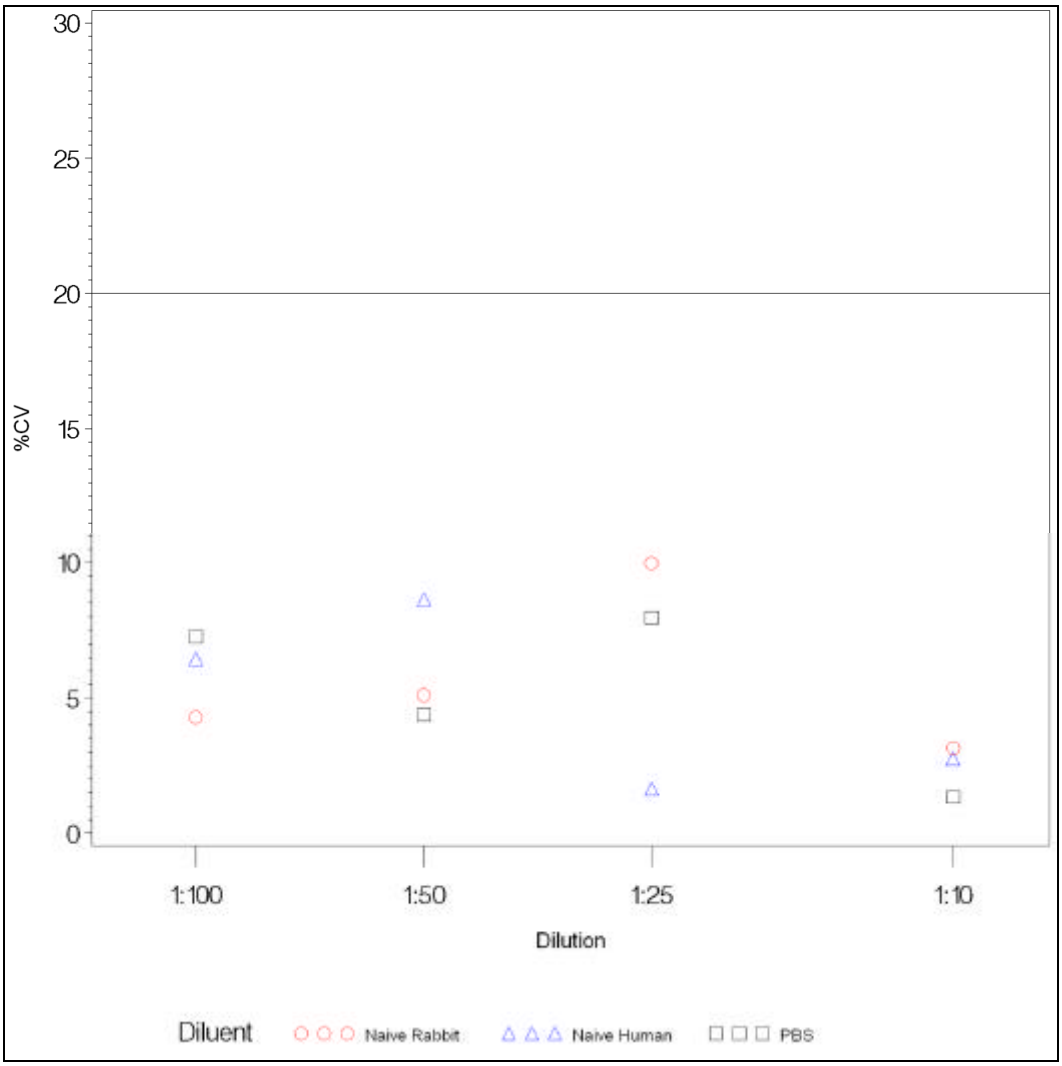


Figure 13. Coefficients of Variation for AVA Plasma ELISA Values from Each Diluent and Dilution.

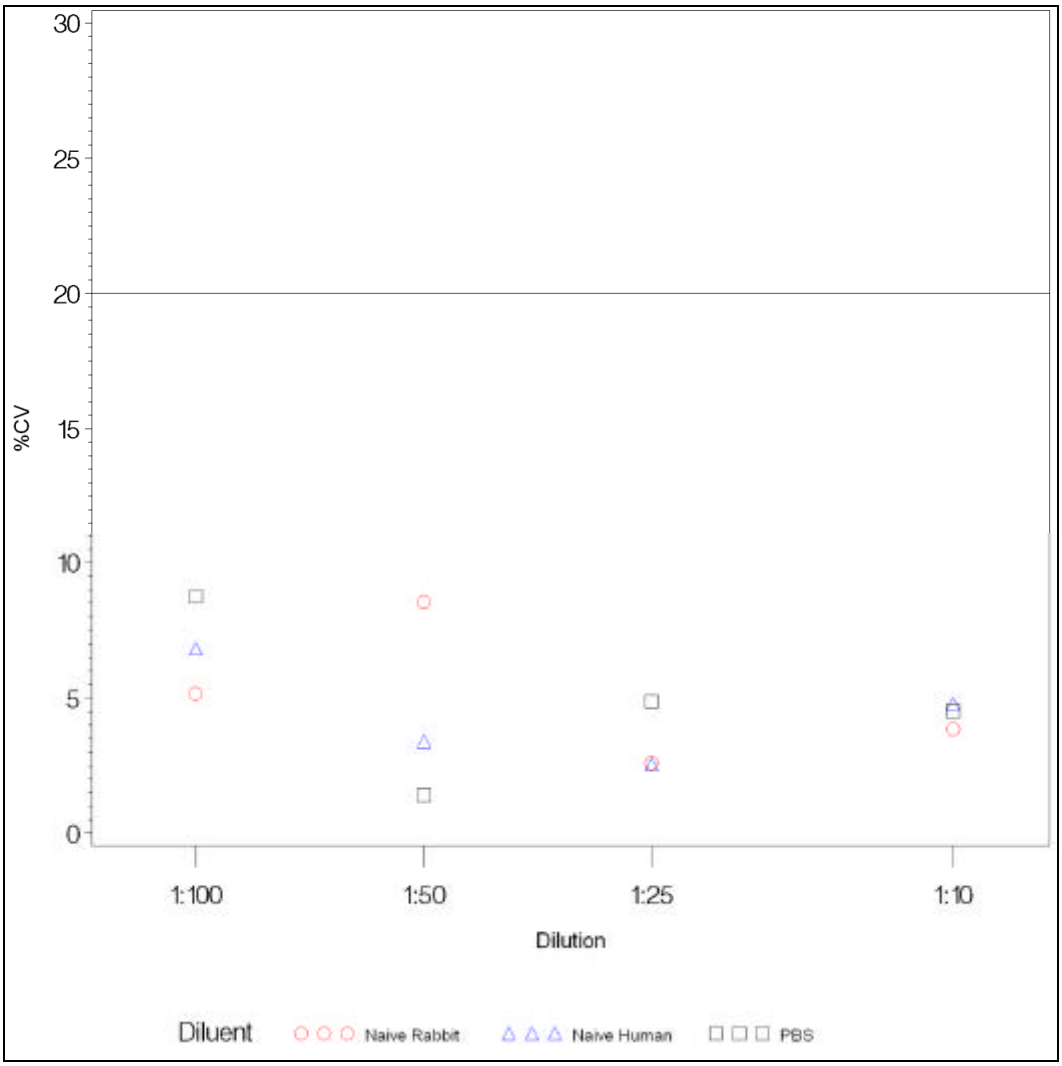
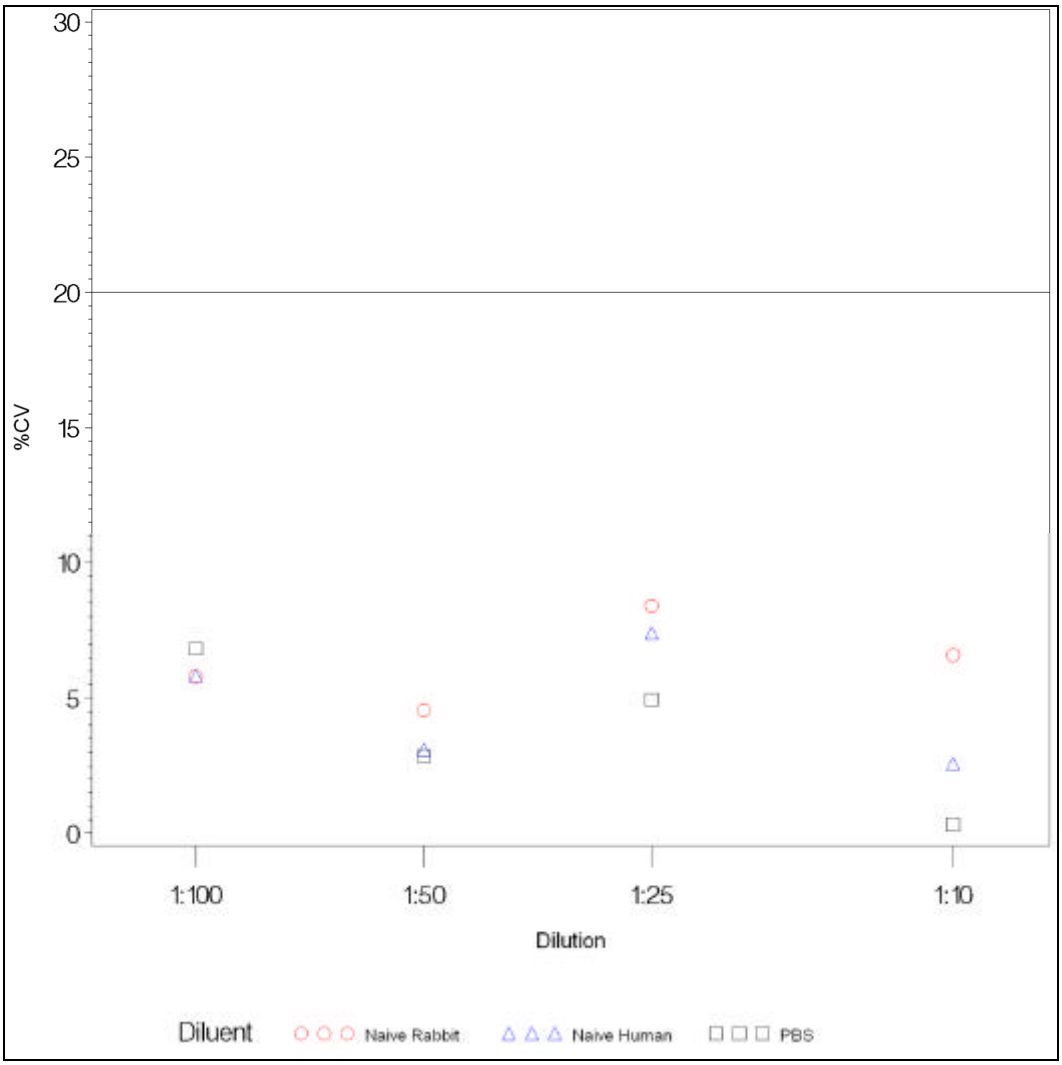


Figure 14. Coefficients of Variation for Anti-PA IgG Positive Human Serum ELISA Values from Each Diluent and Dilution.



The ELISA data for the second pilot study is less consistent than data from the first pilot study, but overall there does not appear to be a clear issue caused by spiking AIG, AVA plasma, or positive human serum into naïve rabbit serum. Spiking serum into PBS lead to lower than expected results indicating an under recovery. Based on the results of these pilot studies spiking positive material into PBS versus serum (human or

rabbit), one would expect the serum-spike material recovery to be higher. This conclusion is supported by the data in this pilot study.

There appeared to be recovery issues with spiking AVR801 (positive human serum) in any of the negative matrices at 1:50 and 1:100. This dilution of AVR801 (109.4 µg/ml) resulted in an expected concentration of 2.2 and 1.1 µg/ml, (below the ELISA's LOQ of 2.5 µg/ml). The other recoveries of AVR801 at lower dilutions are excellent. The recoveries of the AIG and AVA plasma are slightly lower than expected. However, they have not had the same level of characterization in the anti-PA ELISA to determine their expected concentrations. It is not unexpected that these recoveries are lower. The recoveries are consistent across matrices suggesting that any diluents' effects are negligible, however it appears that PBS consistently produced a lower recovery than human or rabbit serum.

Anti-PA ELISA and KINETIC RESULTS

Table 22 shows the median ELISA anti-PA IgG concentration (µg/ml) for all groups at the listed time points. All data points below the assay's LOQ are listed as < 2.5. Group 4 (Naïve IgG) was undetectable at all time points as expected. Groups 1-3 showed an increase in anti-PA concentration for most animals at the 1 h time point and an increase in anti-PA concentration for all treatment animals at 24 h. This change in anti-PA concentration indicated a proper placement of the catheter during antibody administration. The 24 h time point also represents the mean peak for Groups 1-3.

The animal ELISA kinetic data shows that ample protection against a lethal *B. anthracis* should be present at 24 h post treatment for Groups 2 and 3 (10 and 20 AIG

mg/kg, respectively). This is based on historical data showing that a titer of 60-65 µg/ml will provide nearly 95% protection against a lethal aerosol challenge in NZW Rabbits. It is also probable that some protection will be afforded at 7 days post treatment for Group 3 dose levels.

Table 22. Median ELISA anti-PA IgG Concentrations for all Samples

		Sample Time (h post IP administration)													
		-1	1 hr	24 hr	48 hr	72 hr	96 hr	Day 7	Day 10	Day 13	Day 16	Day 19	Day 22	Day 25	Day 28
	Animal	**Median ELISA anti-PA IgG (µg/ml)[1]**													
Group 1 (5 mg/kg anti-PA IgG)	2077	<2.5	<2.5	41	43	38	29	27	21	12	9	6	5	3	<2.5
	2088	<2.5	<2.5	58	40	39	34	17	11	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
	2090	<2.5	5	79	57	46	35	16	4	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
	2096	<2.5	14	75	49	45	29	15	6	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
	2217	<2.5	<2.5	48	47	41	39	18	19	6	4	<2.5	<2.5	<2.5	<2.5
	2212	<2.5	<2.5	30	43	41	35	22	9	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
	2206	<2.5	7	58	58	45	31	15	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
	2220	<2.5	<2.5	29	51	53	40	30	25	6	<2.5	<2.5	<2.5	<2.5	<2.5
Group 2 (10 mg/kg anti-PA IgG)	2095	<2.5	8	148	95	79	71	43	33	12	5	<2.5	<2.5	<2.5	<2.5
	2086	<2.5	4	134	98	111	57	53	32	10	<2.5	<2.5	<2.5	<2.5	<2.5
	2080	<2.5	<2.5	12	8	9	7	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
	2081	<2.5	4	9	90	103	66	55	35	10	<2.5	<2.5	<2.5	<2.5	<2.5
	2214	<2.5	55	207	128	113	100	78	53	25	15	10	6	<2.5	<2.5
	2213	<2.5	7	187	111	90	75	56	43	13	4	<2.5	<2.5	<2.5	<2.5
	2221	<2.5	18	168	104	81	76	48	29	13	6	<2.5	<2.5	<2.5	<2.5
	2203	<2.5	4	186	95	77	67	36	13	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
Group 3 (20 mg/kg anti-PA IgG)	2084	<2.5	13	272	236	173	154	111	65	34	5	DEAD			
	2085	<2.5	18	402	182	144	132	105	81	42	9	DEAD			
	2076	<2.5	9	410	211	155	178	94	45	3	0	DEAD			
	2091	<2.5	7	444	186	143	133	67	19	<2.5	<2.5	DEAD			
	2216	<2.5	23	553	241	193	151	90	62	5	<2.5	DEAD			
	2218	<2.5	4	217	181	171	98	120	125	53	44	DEAD			
	2207	<2.5	36	391	238	200	147	132	138	57	6	DEAD			
	2209	<2.5	15	404	277	207	174	141	109	49	20	<2.5	181	DEAD	
Group 4 (Control)	2078	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	DEAD			
	2083	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	DEAD			
	2082	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	DEAD			
	2092	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	DEAD			
	2205	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	DEAD			
	2208	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	DEAD			
	2201	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	DEAD			
	2210	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	DEAD			

[1] < 2.5 represents a value below the ELISA LOQ of 2.5 µg/ml.

Table 23 shows the means for groups 1-4 and the respective standard deviations.

Table 23. Means and Standard Deviations for all Dose Groups

		Day -1	1 hr	24 hr	48 hr	72 hr	96 hr	Study Day 7	Study Day 10	Study Day 13	Study Day 16	Study Day 19	Study Day 22	Study Day 25	Study Day 28
Group 1 (5 mg/kg AIG)	Mean	< LOQ	9	52	49	44	34	20	14	8	7	6	5	3	< LOQ
	S.D.	< LOQ	5	19	6	5	4	6	8	4	4	< LOQ	< LOQ	< LOQ	< LOQ
Group 2 (10 mg/kg AIG)	Mean	< LOQ	14	131	91	76	65	53	34	14	7	10	6	< LOQ	< LOQ
	S.D.	< LOQ	19	78	36	30	26	13	12	6	5	< LOQ	< LOQ	< LOQ	< LOQ
Group 3 (20 mg/kg AIG)	Mean	< LOQ	16	386	219	170	146	107	81	35	14	< LOQ	181	< LOQ	< LOQ
	S.D.	< LOQ	10	103	35	29	25	24	41	22	16	< LOQ	< LOQ	< LOQ	< LOQ
Group 4 (Naive)	Mean	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
	S.D.	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

Table 24 lists the Natural Log (Ln) [Median ELISA anti-PA IgG (µg/ml)] for Groups 1-3 at the respective time points for all positive response groups and the kinetic evaluation of these groups including slopes, R^2, and half-life ($t_{½}$). The kinetics evaluations represent Study Days 0-14 (prior to challenge). The mean $t_{½}$ for groups 1-3 is 4.267 days with a standard deviation of 0.06. These results illustrate that $t_{½}$ is independent of dose for 1st order kinetics as expected.

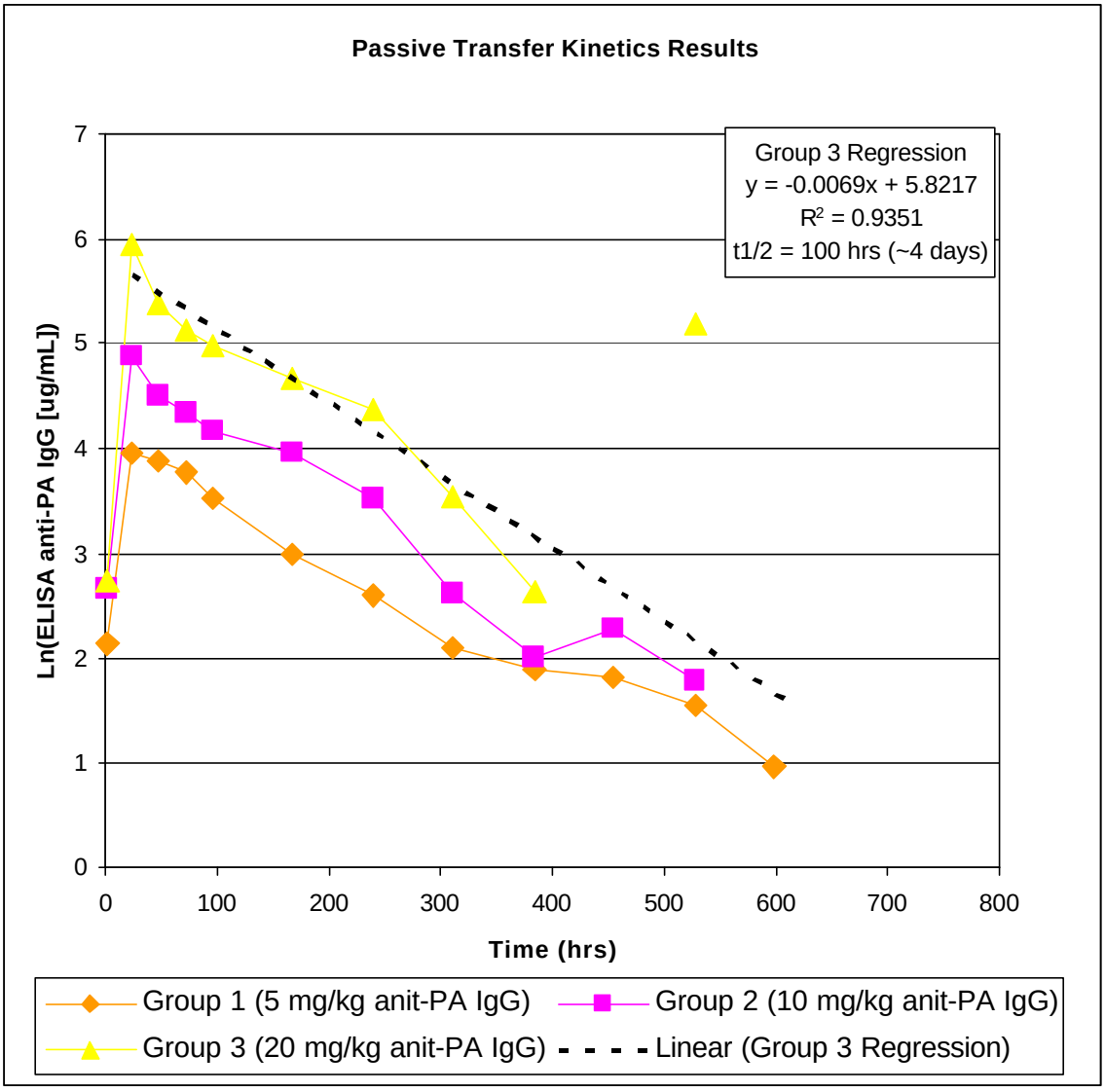
Figure 15 shows the mean values from Table 19 plotted versus time for all AIG dose groups.

Table 24. Kinetic Determinations for AIG Dose Groups

Ln [Median ELISA anti-PA IgG (µg/ml)]									Kinetics Evaluation of results			
	1 hr	24 hr	48 hr	72 hr	96 hr	Study Day 7	Study Day 10	Study Day 13	Slope	R^2	$t_{½}$ [1] (hr)	$t_{½}$ [1] (d)
Group 1 (5 mg/kg AIG)	2.1	4.0	3.9	3.8	3.5	3.0	2.6	2.1	-0.0067	0.994	104	4.3
Group 2 (10mg/kg AIG)	2.7	4.9	4.5	4.3	4.2	4.0	3.5	2.6	-0.0068	0.952	103	4.3
Group 3 (20mg/kg AIG)	2.8	6.0	5.4	5.1	5.0	4.7	4.4	3.5	-0.0069	0.935	100	4.2

[1] Half-life = Ln^2 /Slope R^2

Figure 15. Mean anti-PA Concentrations Versus Time for all AIG Dose Groups



DISCUSSION

The efficacy of a treatment, such as passive immunization, cannot be tested in humans due to ethical considerations. New treatments are tested in at least one appropriate animal species prior to submission to the Food and Drug Administration (FDA). The rabbit model was chosen for this project since lesions observed following an inhalation anthrax challenge are similar to those of inhalation anthrax found in humans and rhesus macques (Zaucha et al., 1998). The rabbit model in this study consisted of an aerosol *B. anthracis* challenge with an average 272 LD_{50} (Ames strain). The objectives of this study were to 1) evaluate the kinetics of passively transferred human immunoglobulin G (IgG) AIG via Anti-PA ELISA, to; 2) investigate the potential lethal toxicity, of the passively administered test and control materials; and 3) determine the efficacy against a delayed lethal aerosol challenge of *B. anthracis* (Ames strain).

All animals receiving IP treatments were observed continuously for one h post administration and twice daily for signs of toxicity. All animals appeared normal during the one h post AIG or naïve IgG administration. There were no significant changes in appearance, behavior, or responsiveness. No clinical signs consistent with toxicity were observed during the 14 days post-treatment. A single animal exhibited a brief decrease in appetite which correlates closely with the periodical sedation for blood draws.

Adverse clinical observations post-challenge were consistent with a *B. anthracis* infection in the rabbit model. The clinical symptoms observed for this study included: not eating, soft stool, no stool, wheezing, sneezing, abnormal posture or gait, lethargy, loss of coordination, milky lacrimations, morbundity, and seizure. All animals were observed to be abnormal during the 24-h period proceeding death or euthanasia. There

was no protection efficacy afforded by the passively transferred antibodies in the delayed exposure scenario used for this study. All animals in Groups 3, 4, and 5 were found dead or euthanized by Study Day 8 post-challenge. There was no statistically significant increase in time to death between groups. The average time to death for Groups 3, 4, and 5 were 3.8, 3.6, and 3.2 respectively as shown in Table 12 (pp. 37).

Prior to testing the rabbit sera on this study (via anti-PA ELISA), two pilot studies were conducted to evaluate the assay's ability to detect the human IgG in rabbit sera. Several well characterized BMI reagents were used for these pilot studies. The first pilot study evaluated the recovery of the purified AIG material spiked into naïve rabbit serum, anti-PA IgG positive rabbit serum, naïve human serum, and PBS. The calculations for percent recovery were performed using the expected and observed anti-PA concentration (µg/ml). The results presented in Table 15 (pg 38) clearly show that the ELISA assay detects the purified AIG from the tested diluents. The slight decreases observed in recovery were not unlikely due to the limited characterization of the AIG test material. PBS was the least reliable of the diluents.

The CV from the ELISA data shows an increase in variability for the 1:100 dilutions. These values were near and sometimes less than the assay's LOQ which explains the increase in variability. The ELISA results confirm that there were no statistically significant differences for AIG between any of the naïve diluents and the results for AIG in positive rabbit serum were slightly elevated. Additionally, the data demonstrates the dilution linearity and was close to expected levels (within normal variance for analysis methods).

Pilot study 2 was conducted to further assess the ability of the anti-PA ELISA to detect the human IgG. In this study three positive materials were compared to determine any differences between the purified AIG, the source plasma for the purified AIG (BMI505), and a well characterized known positive human serum. The results presented in Tables 16-18 (pp. 44-45) were less consistent than the results in the initial pilot study. The mean recoveries across dilution factors and diluents were similar for the AIG and AVA plasma (BMI505) demonstrating that the purification process had negligible affects on the anti-PA IgG within the material. Overall the second pilot study supported the conclusion from the first pilot study that the ELISA assay (when processed with human reagents) has the ability to consistently detect the human anti-PA in both naïve and positive rabbit sera.

The ELISA assays were performed on the rabbit sera samples following the passive administration of the test and control articles at 1, 24, 48, 72, 96 h, 7, 10, 13, 16, 19, 22, 25, and 28 days. In order to accurately determine the half-life ($t_{½}$) of the AIG material the $t_{½}$ for each dilution (groups 1-3) was averaged. The $t_{½}$ of the material was calculated to be 4.3 days with a standard deviation of 0.06. The peak serum concentration was observed at 24 h (+/-15 min) post-administration for all test groups. The serum concentration for group 4 never exceeded the assay's LOQ, indicating no anti-PA activity present in the naïve human IgG.

While the test material afforded no protection against the lethal *B. anthracis* aerosol challenge, the study achieved its primary goal. There is an accurate assessment of the half-life of the AIG material. This allows for future work to focus on the window of opportunity for prophylaxis and treatment against a lethal inhalation challenge. Future

work can assess the protection efficacy of passively administered antibodies against an inhalation challenge when serum is at peak concentrations (24 h post-treatment). Furthermore, future work could investigate passively transferred IgG as a treatment post challenge. The knowledge that the AIG material causes no lethal toxicity will allow for this work to move forward without concerns of adverse effects on the study animals.

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