



Research paper

Development, validation and use of ELISA for antibodies to human alpha-1 antitrypsin

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ABSTRACT

Evaluation of human antibody responses to alpha-1 antitrypsin (AAT) in clinical trials and clinical practice has been limited by the lack of a validated assay. Here we describe the development and validation of an ELISA method for quantification of human and nonhuman primate antibody responses to human AAT. A reference anti-human AAT serum standard was generated using sera from a cynomolgus macaque injected with a recombinant adeno-associated virus vector expressing human AAT. The ELISA was validated for use with human serum dilutions as low as 1:10 and was able to distinguish between specific responses in cynomolgus serum and non-specific increases in apparent antibody titer in serum from subjects in a clinical trial of an AAT gene therapy vector.

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1. Introduction

Alpha-1 antitrypsin deficiency is an inherited genetic disorder caused by mutations in the *SERPINA1* gene which codes for protease inhibitor (PI) called alpha-1 antitrypsin (AAT) (Crystal et al., 1989; Silverman and Sandhaus, 2009). Normal AAT, referred to as M-type AAT or PI^M as it migrates toward the middle of an isoelectric focusing (IEF) gel, is produced in the liver, secreted into the blood, and ultimately protects the lungs from attack by neutrophil elastase. AAT deficiency is the most widely recognized abnormality of a proteinase inhibitor, affecting up to 100,000 individuals in the US, and is characterized by reduced serum levels of AAT and increased risk of developing emphysema. A glutamic acid to lysine mutation at position 342 (PI^{*Z}) results in an AAT protein that is misfolded and has reduced secretion from the liver. More than 95% of individuals with severe AAT deficiency are homozygous PI^{ZZ} and have serum AAT levels of 10 to 15% of the levels in normal PI^{MM} individuals (Ogushi et al., 1988).

A recombinant adeno-associated virus (rAAV) gene therapy vector is being developed for treatment of AAT deficiency. The vector contains an M-AAT expression cassette packaged in an AAV capsid (Chulay et al., 2011) and has been administered to AAT-deficient patients in clinical trials (Brantly et al., 2009; Flotte et al., 2011). Because the clinical effects of patient immune responses to therapeutic proteins can range from no effect to extremely harmful effects on patient health, regulatory guidance recommends assessment of immunogenicity data during development of biological products (U.S. Food and Drug Administration, 2009). However, evaluation of human antibody responses to AAT in clinical trials and clinical practice has been limited by the lack of a validated assay. We therefore developed and validated an ELISA method using a nonhuman primate reference standard serum for quantification of human antibody responses to AAT.

2. Materials and methods

2.1. Generation of anti-hAAT reference standard serum

Serum from four cynomolgus macaques (*Macaca fascicularis*) was tested to determine the neutralizing antibody titer to AAV serotype 1 (Lin et al., 2008). One animal with a titer <1:5 was given a recombinant AAV serotype 1 vector expressing human

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AAT (rAAV1-CB-hAAT) (Chulay et al., 2011) at a dose of 4×10^{12} vector genome copies (vg) per kg body weight by intramuscular (IM) injection, distributed across a total of six sites in the tibialis and gastrocnemius lateralis muscles of the lower limbs. This vector contains cDNA that expresses the M1(Val213) normal variant (Nukiwa et al., 1987) of AAT. Serum was obtained weekly from 4 to 20 weeks after vector administration and tested for levels of AAT antigen by ELISA as previously described (Chulay et al., 2011) and for antibodies to human AAT by ELISA as described below. Serum samples from weeks 8 through 16 were pooled to generate a reference standard serum master stock that was assigned an anti-hAAT titer of 1 U/mL. A vial of the master stock was diluted 1:100 with phosphate buffered saline (PBS) and aliquoted to generate a reference standard serum working stock (AGTC lot 033110) with an anti-hAAT titer of 10,000 μ U/mL.

2.2. Human serum samples

Part of the assay validation studies used sera obtained from six subjects with AAT deficiency. These sera were from individuals with PI ZZ as determined by IEF gel analysis (Constans et al., 1980; Brantly et al., 1991) for allelic discrimination, and had serum AAT concentrations ranging from 2.9 to 4.7 μ M as determined by nephelometry (Brantly et al., 1991). Sera were also obtained from nine individuals before and 14 days and 1, 3 and 12 months after administration of rAAV1-CB-hAAT in a Phase 2 clinical trial, in which the vector was administered by multiple IM injections at a single time point in three groups of three subjects each at one of three dosage levels (6×10^{11} , 1.9×10^{12} or 6×10^{12}) vector genome particles per kg body weight (Flotte et al., 2011).

2.3. Development of anti-hAAT ELISA

For initial evaluation of sera from a macaque injected with rAAV1-CB-hAAT, we used an ELISA method in which a serum (AGTC lot TH020609), collected from an immunosuppressed (IS) macaque after IM injection of rAAV1-CB-hAAT (4×10^{12} vg/kg) in a separate preclinical study, was used to generate the standard curve. A weak antibody-response to human AAT was observed despite immunosuppression with mycophenolate mofetil and sirolimus (GJ Ye, unpublished observations). Microtiter plates (Apogent, MaxiSorp, Thermo Scientific, NY) were coated with 1 μ g per well of hAAT (Athens Research & Technology, Athens, Georgia) in 100 μ L of 0.2 M sodium carbonate-bicarbonate buffer (pH 9.4) overnight at 4 °C, washed with PBS containing 0.05% Tween 20 (PBS-T) and then blocked with blocking buffer (1% non-fat milk in PBS-T) for 1 h at room temperature. Serial dilutions of serum from the IS macaque, test samples and a positive control (dilution from a separate aliquot of the IS macaque serum) were added to the plate and incubated at room temperature for 2 h. Plates were washed with PBS-T and secondary antibody [HRP-labeled goat anti-monkey IgG (Fitzerald Industries International, North Acton, MA)] diluted 1:5000 in blocking buffer was added. After incubation for 1 h at room temperature plates were washed with PBS-T and (3,3',5,5'-Tetramethylbenzidine) TMB substrate (Sigma, St. Louis, MO) was added followed by stop solution after 5 min. Absorbance (OD₄₅₀) was measured using

a μ Quant plate reader (BioTek, Winooski, VT) and data analyzed using Gen5 data analysis software.

After initial evaluation of the new macaque sera and creation of the macaque reference standard serum, the ELISA was performed as above, except that the secondary antibody was HRP-labeled goat anti-human IgG (Chemicon/Millipore, Billerica, MA), the dilution buffer was 0.1% bovine serum albumin (BSA) in PBS-T, serial 4-fold dilutions of the macaque reference standard serum from 100 to 0.098 μ U/mL were used to generate a standard curve, and a 1:2000 dilution of the IS macaque serum was used as a positive control.

2.4. Validation of anti-hAAT ELISA

In a pre-validation study, the anti-hAAT macaque reference standard serum was added to six PI ZZ test sera at a final test serum dilution of 1:10 in blocking buffer and final reference standard concentrations of 1.56, 3.125, 20 or 50 μ U/mL. Because of poor recovery at the two lower spike concentrations, assay validation was performed using a final test serum dilution of 1:20 and final reference standard concentrations of 5, 10, 20, 40 or 80 μ U/mL.

Parameters tested during assay validation were precision (repeatability and reproducibility), accuracy, lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), selectivity, stability, linearity and range. All samples were tested in duplicates. Repeatability was evaluated by performing independent measurements of control sera containing three different levels of anti-hAAT antibody known to be within the linear range of the assay. Accuracy was assessed by determining the mean value of these three controls and comparing them to their known theoretical values. Reproducibility was evaluated by having two analysts perform six assays over time, with reagents changed midway through the testing. The LLOQ and ULOQ were established based on the lowest and highest concentrations of the reference standard in the standard curve that could be accurately measured in six independent assays with acceptable precision. Selectivity was evaluated by testing six PI ZZ serum samples neat and after being spiked with the control antibody at LLOQ and a middle and high concentration. Stability of the reference standard diluted to a nominal concentration of 30 μ U/mL was evaluated under three conditions: room temperature for up to 4 h, refrigerated temperature (4 °C) for up to 3 days and freeze/thaw over a period of 3 days.

Because immunoassay calibration curves are inherently non-linear and non-linear curve fitting algorithms are used to describe the data, five independent curves were analyzed for linearity of results within the dynamic range of the assay, i.e. between LLOQ and ULOQ. Calibration curves were established using a four-parameter curve fitting method with at least six points in the middle portion of the curve.

Acceptance criteria for accuracy was that the mean value be within $\pm 20\%$ of the expected value. Acceptance criteria for repeatability and reproducibility were that the coefficient of variation (CV) be less than 20%.

2.5. Specificity test of anti-AAT responses in clinical trial

Each clinical trial test serum sample was thawed at room temperature and kept on ice. An aliquot of the anti-AAT

macaque reference standard serum was thawed and diluted with blocking buffer to a final concentration of 100 $\mu\text{U/mL}$. An aliquot of purified hAAT (Athens Research and Technology) at 1 mg/mL was used to prepare a series of dilutions at 200, 40 and 8 $\mu\text{g/mL}$ in blocking buffer. In a 1.5 mL microcentrifuge tube, 40 μL of blocking buffer, 5 μL anti-AAT reference standard (100 $\mu\text{U/mL}$) or clinical trial test sample, and 5 μL of dilutions of hAAT protein (200, 40, 8 and 0 $\mu\text{g/mL}$) were added (total volume 50 μL). The reaction mixtures were incubated at 37 $^{\circ}\text{C}$ for 1 h, then diluted 1:20 by adding 950 μL of blocking buffer to each reaction, and triplicate 100 μL aliquots of each sample was added to appropriate wells of ELISA plates that had been coated with 1 μg of purified hAAT in 100 μL of blocking buffer, after which the ELISA analysis was performed according to the method detailed in Section 2.3.

3. Results

3.1. Generation and evaluation of macaque anti-hAAT serum

Serum from a cynomolgus macaque injected with a recombinant AAV vector expressing human AAT was obtained weekly from 4 to 20 weeks after vector administration. Low levels of hAAT antigen were detected at 4 weeks (84 nM) and 6 weeks (8.3 nM) after vector administration but hAAT antigen was not detected in serum obtained at week 8 or thereafter.

Antibodies to human AAT rose progressively from week 4 to week 9 and then reached a plateau through week 13, followed by a gradual decline in antibody titer over the next several weeks (Fig. 1). Sera from week 8 through week 16 were pooled, assigned an anti-hAAT titer of 1 U/mL, and used as the reference standard macaque anti-hAAT serum for development of a clinically applicable anti-AAT ELISA.

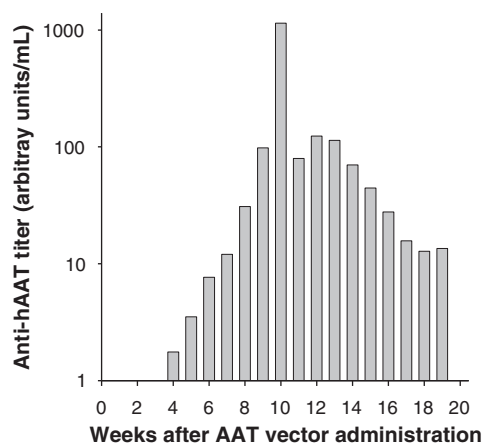


Fig. 1. Development of anti-human AAT antibodies in an immune competent cynomolgus macaque. Serum from a cynomolgus macaque injected with rAAV1-CB-hAAT was collected weekly from 4 to 20 weeks after vector administration and the anti-hAAT antibody concentration was measured by ELISA. A standard curve was generated with anti-hAAT serum from an immunosuppressed macaque after IM injection of the same vector, with the serum from the immunosuppressed macaque assigned an arbitrary titer of 1 U/mL. The titer in the immune competent macaque sera ranged from 1 to 1000 U/mL.

3.2. Development of an anti-hAAT ELISA

Serial dilutions of the reference standard macaque anti-hAAT serum were tested by ELISA to generate a standard curve that was fit to a 4-parameter equation and reliably achieved a correlation coefficient >0.99 (Fig. 2). Anti-hAAT concentrations in test sera were calculated by comparison with the reference standard curve.

During assay development, a separate dilution of the reference standard macaque serum was used as a positive control. The calculated serum anti-hAAT concentration in the positive control ranged from 87.4% to 94.4% (mean $\pm$ SD = $90.4\% \pm 3.64\%$, $n=3$) of the expected value.

In preliminary assay qualification studies, test sera from six AAT deficient humans (PI ZZ) were tested neat and after spiking with known amounts of the reference standard macaque serum. At a test serum dilution of 1:5 there was a non-specific background signal that was higher than the signal at the lowest point on the reference standard curve. At a dilution of 1:10 the background signal was generally just below the lowest point on the reference standard curve. In addition, the calculated recovery of anti-hAAT antibody in the spiked samples was lower in PI ZZ serum diluted 1:5 than in PI ZZ serum diluted 1:10. This suggested that the AAT antigen in PI ZZ serum was interacting with the anti-hAAT antibody in the reference standard macaque serum. This was tested by adding known amounts of purified AAT to various dilutions of the reference standard macaque serum, which demonstrated that excess AAT antigen in a test serum will inhibit the ability of the anti-hAAT antibody to react with AAT antigen on the ELISA plate (see Section 3.4 and Fig. 5 below). These results confirmed the antigen specificity of the reference standard macaque serum. During assay validation,

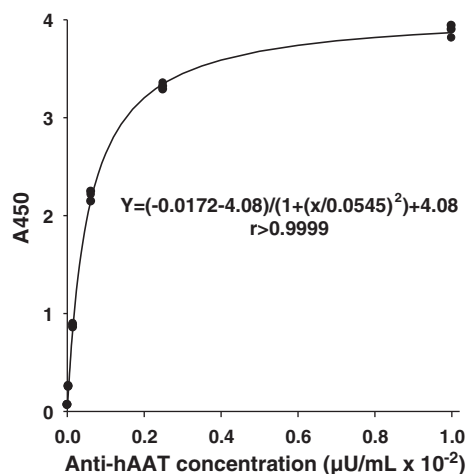


Fig. 2. A standard curve generated using a reference serum obtained by pooling the Week 8 through Week 16 sera from an immune competent cynomolgus macaque. This reference standard serum was assigned a titer of 1 U/mL and serial 4-fold dilutions, from 100 $\mu\text{U/mL}$ to 0.098 $\mu\text{U/mL}$, were added in triplicate to wells coated with hAAT. Antibody binding was detected using HRP-labeled goat anti-monkey IgG as described in the Material and methods section. Four parameter curve fitting ($Y = (A - D) / (1 + (X/C)^B) + D$) of the average A_{450} for the six antibody concentrations was used to describe the standard curve.

a final test serum dilution of 1:20 was used for evaluation of selectivity.

3.3. Validation of an anti-hAAT ELISA

3.3.1. Accuracy and repeatability

Using the reference standard macaque anti-hAAT serum diluted in BSA buffer to nominal concentrations of 3.5 $\mu\text{U/mL}$, 20 $\mu\text{U/mL}$ and 50 $\mu\text{U/mL}$, the mean $\pm$ SD observed values were 3.81 ± 0.14 $\mu\text{U/mL}$ (108.8% of predicted), 17.75 ± 1.95 $\mu\text{U/mL}$ (88.8% of predicted) and 44.1 ± 1.93 $\mu\text{U/mL}$ (88.1% of predicted). The CV at these three concentrations was 3.8%, 11.0% and 4.4%, respectively.

3.3.2. Reproducibility

When the assay was performed six times by two analysts over 3 days, inter-assay precision (CV) at 1.56 $\mu\text{U/mL}$ (the LLOQ), 20 $\mu\text{U/mL}$ and 50 $\mu\text{U/mL}$ was 2.0%, 11.0% and 6.2%, respectively – well within the acceptance criterion of 20%. Different analysts, different assay dates and different assay reagents had no significant impact on the results.

3.3.3. Lower limit of quantification

Using the reference standard macaque anti-hAAT serum diluted in BSA buffer, the LLOQ was determined to be 1.56 $\mu\text{U/mL}$. Accuracy at this concentration was 93.0% of predicted with a CV of 2.0%. At the next lower concentration (0.78 $\mu\text{U/mL}$) accuracy and repeatability were inconsistent.

3.3.4. Upper limit of quantification

Using the reference standard macaque anti-hAAT serum diluted in BSA buffer, the ULOQ was determined to be 50 $\mu\text{U/mL}$. Accuracy at this concentration was 89.4% of predicted with a CV of 6.2%. At the next higher concentration (100 $\mu\text{U/mL}$) the CV was 8.3% but accuracy was only 79.8% of predicted, which is below the acceptance criterion of 80%.

3.3.5. Linearity and range

The dynamic range for the assay was determined to be 0.78 $\mu\text{U/mL}$ to 100 $\mu\text{U/mL}$. Five standard curves were evaluated over this range and the slope for each was 1.0 with an intercept between -0.02 and $+0.02$ and an $r^2 > 0.99$ for all five curves. One of the five standard curves is shown in Fig. 3A.

3.3.6. Selectivity

The assay validation parameters above were determined using the reference standard macaque serum diluted in BSA buffer. Selectivity was determined using six independent human PI ZZ serum samples spiked with five levels of the antibody standard (5, 10, 20, 40 and 80 $\mu\text{U/mL}$). Average recoveries ranged from $23.8 \pm 5.0\%$ of predicted with the 5 $\mu\text{U/mL}$ spike to $9.4 \pm 0.86\%$ of predicted with the 80 $\mu\text{U/mL}$ spike (Table 1). The CV for the six samples was 21.1% with the 5 $\mu\text{U/mL}$ spike and $<20\%$ for the other conditions.

Although the recovery from all spiked samples was low, there was a linear relationship between the amount of anti-hAAT antibody added and the antibody level determined by ELISA (Fig. 3B).

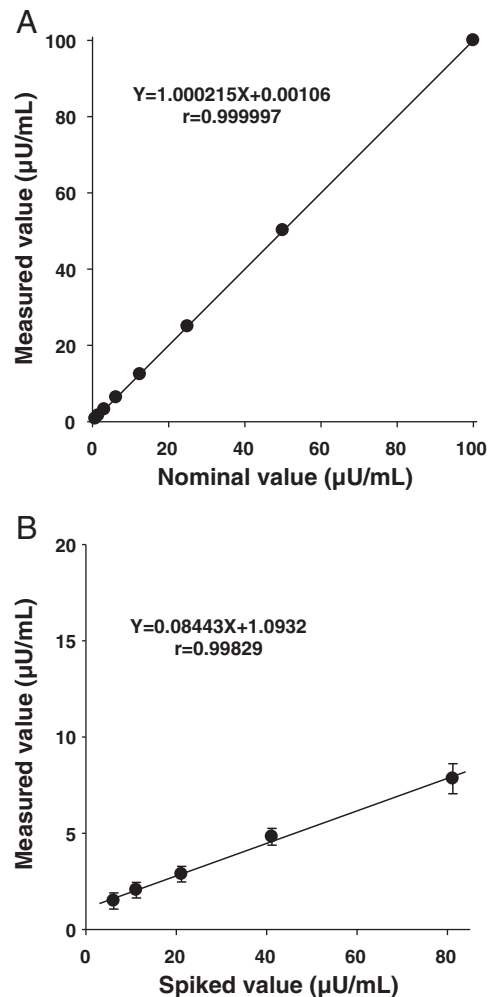


Fig. 3. Linearity of anti-hAAT ELISA results. A. Samples of a reference standard macaque serum were diluted in BSA buffer to achieve a range of nominal antibody concentrations from 0.78 $\mu\text{U/mL}$ to 100 $\mu\text{U/mL}$. Mean values are plotted. The measured antibody concentrations were 5.25% lower than the nominal concentration for the 0.78 $\mu\text{U/mL}$ dilution and were within $\pm 2\%$ of the nominal concentration for all other dilutions. B. Sera from six PI ZZ subjects were spiked with the reference standard macaque serum to achieve a final anti-hAAT antibody concentration ranging from 5 $\mu\text{U/mL}$ to 80 $\mu\text{U/mL}$. The mean $\pm$ SD measured antibody concentrations are plotted as a function of the spike concentration. The measured antibody concentration as a percent of the spiked concentration ranged from $13.5 \pm 1.6\%$ at the lowest spike concentration to $9.4 \pm 0.86\%$ at the highest spike concentration.

3.3.7. Stability

Within the range of conditions tested storage at room temperature for up to 4 h or at $2-8^\circ\text{C}$ for up to 2 days, or freezing and thawing one or two times, had minimal effects on the assay results (Fig. 4). The mean $\pm$ SD values as a percent of the values obtained before exposure to the stability-testing condition were $92.3 \pm 11.8\%$ and $86.3 \pm 4.1\%$ after storage at room temperature for 2 or 4 h, respectively, $81.3 \pm 8.7\%$ and $83.3 \pm 9.4\%$ after storage at $2-8^\circ\text{C}$ for 1 or 2 days, respectively, and $87.7 \pm 18/2\%$ and $78.2 \pm 2.2\%$ after one or two freeze and thaw cycles, respectively.

Table 1

Selectivity of the anti-hAAT ELISA. Six independent human PI ZZ serum samples were spiked with five levels of the antibody standard, from 5 $\mu\text{U/mL}$ to 80 $\mu\text{U/mL}$. The theoretical value is the measured value in serum with no AAT added plus the concentration of antibody added in the spike. Percent recovery is the ratio of the measured value to the theoretical value.

Antibody added ($\mu\text{U/mL}$)	Measured value ($\mu\text{U/mL}$)						Theoretical value ($\mu\text{U/mL}$)					Recovery (%)				
	0	5	10	20	40	80	5	10	20	40	80	5	10	20	40	80
Serum 1	0.53	1.08	1.64	2.56	4.84	6.78	5.53	10.53	20.53	40.53	80.53	19.5	15.6	12.5	11.9	8.8
Serum 2	0.54	0.99	1.49	2.24	4.12	7.16	5.54	10.54	20.54	40.54	80.54	17.9	14.1	10.9	10.2	8.9
Serum 3	2.03	1.75	2.19	3.11	4.62	7.61	7.03	12.03	22.03	42.03	82.03	24.9	18.2	14.1	11.0	9.3
Serum 4	0.89	1.41	2.06	2.86	5.44	8.67	5.89	10.89	20.89	40.89	80.89	23.9	18.9	13.7	13.3	10.7
Serum 5	1.55	2.11	2.55	3.15	4.88	8.02	6.55	11.55	21.55	41.55	81.55	32.2	22.1	14.6	11.7	9.8
Serum 6	1.41	1.56	2.32	3.30	4.99	8.66	6.41	11.41	21.41	41.41	81.41	24.3	20.3	15.4	12.1	10.6
Mean												23.80	18.21	13.54	11.38	9.41
SD												5.01	2.95	1.62	0.80	0.86
CV (%)												21.1	16.2	12.0	6.99	9.13

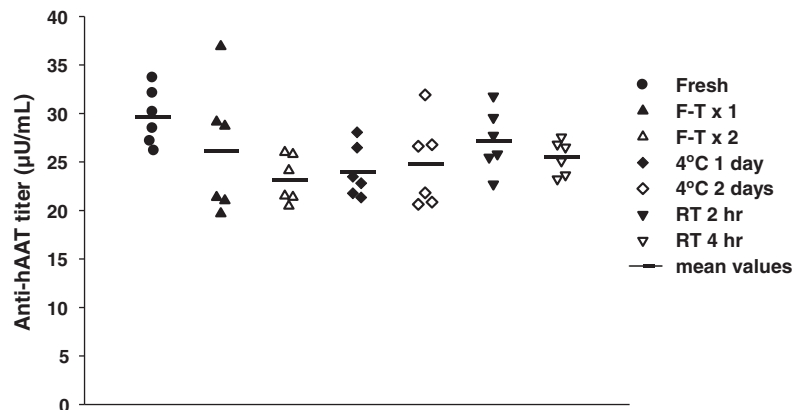


Fig. 4. Stability of anti-hAAT antibodies. Aliquots of the macaque reference standard serum were diluted to approximately 30 $\mu\text{U/mL}$ and tested fresh, after storage at room temperature (RT) for 2 or 4 h, after storage at 2–8 °C for 1 or 2 days, or after 1 or 2 cycles of freezing and thawing (FT). Individual values for six replicate measurements for each condition are plotted, with mean values indicated by horizontal lines. The mean value for each condition as a percent of the value obtained before exposure to the stability-testing condition ranged from 78.2% after two FT cycles to 92.3% after storage at room temperature for 2 h.

3.3.8. Positive control

Each assay included an aliquot of the IS macaque serum as a positive control. In nine assays the mean value for the positive control serum was 15,500 $\mu\text{U/mL}$ with a CV of 4.02%. Based on these results, a positive control acceptance range was established to be between 12,900 $\mu\text{U/mL}$ and 18,600 $\mu\text{U/mL}$.

3.4. Evaluation of serum samples from a clinical trial

Serum was obtained before and after administration of a recombinant AAV vector expressing AAT in a Phase 2 clinical trial (Flotte et al., 2011) and tested for antibodies to hAAT

using the validated ELISA described above. The LLOQ for the ELISA is 1.56 $\mu\text{U/mL}$ and each serum was diluted 1:10 before testing; thus the LLOQ for the assay as performed for patient samples was 15.6 $\mu\text{U/mL}$.

At baseline all subjects had very low values (<20 $\mu\text{U/mL}$, range <LLOQ to 18.8 $\mu\text{U/mL}$). Four subjects (302, 304, 305 and 308) had an increase to more than 3 times the LLOQ (46.8 $\mu\text{U/mL}$) at one or more time points after administration of study drug (Table 2).

For the four subjects who had an increase to more than 3 times the LLOQ at one or more time points after administration of study drug, the specificity of the reactions was evaluated in

Table 2

Changes in serum anti-hAAT ELISA values after administration of rAAV1-CB-hAAT in a clinical trial (Flotte et al., 2011). Results are based on comparison with a reference standard macaque anti-hAAT serum assigned a value of 1 U/mL and are presented as $\mu\text{U/mL}$. Serum was obtained at a screening visit and at visits 14 days and 1, 3 and 12 months after vector administration.

Subject	301	302	303	304	305	306	307	308	401
Screening	16.6	17.7	<LLOQ	<LLOQ	18.4	<LLOQ	<LLOQ	<LLOQ	18.8
Day 14	<LLOQ	21.1	<LLOQ	<LLOQ	18.5	<LLOQ	<LLOQ	<LLOQ	19.1
Month 1	<LLOQ	50.7	17.0	37.5	20.2	<LLOQ	<LLOQ	<LLOQ	24.2
Month 3	16.2	265.8	<LLOQ	140.8	30.7	<LLOQ	<LLOQ	<LLOQ	21.3
Month 12	<LLOQ	153.2	<LLOQ	46.5	49.4	20.2	41.3	213.8	<LLOQ

LLOQ = limit of quantification (15.6 $\mu\text{U/mL}$).

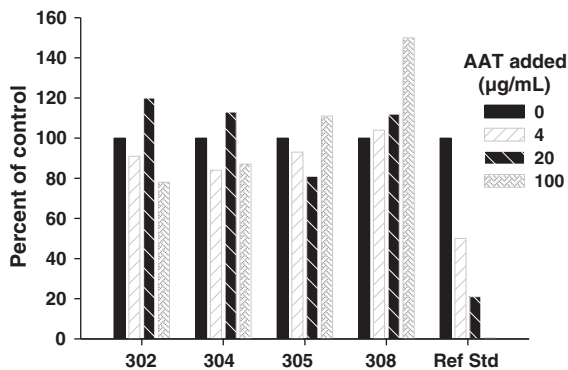


Fig. 5. Specificity of anti-hAAT responses. Purified AAT was added to achieve a final antigen concentration of 4, 20 or 100 µg/mL in a serum sample with the highest anti-hAAT ELISA titer from clinical trial subjects 302, 304, 305 and 308 or to the reference standard macaque serum (Ref Std) diluted to a nominal concentration of 100 µJ/mL before determining the anti-hAAT ELISA titer. Reactivity in the anti-hAAT ELISA assay was blocked in a dose-dependent manner by excess antigen added to the reference standard but not in serum from the clinical trial subjects.

the serum sample with the highest anti-hAAT ELISA titer, by adding serial dilutions of purified AAT to each serum before determining the anti-hAAT ELISA titer. Reactivity in the anti-hAAT ELISA assay was blocked in a dose-dependent manner by addition of a range of amounts of purified AAT to the reference standard macaque serum diluted to a nominal concentration of 100 µJ/mL, but reactivity in the anti-AAT ELISA assay was not blocked by addition of the same amounts of purified AAT to the sera from subjects 302, 304, 305 and 308 (Fig. 5). This indicates that the increase in anti-AAT values in these subjects was not antigen-specific and not directed against AAT.

4. Discussion

AAT protein purified from human plasma has been administered to many thousands of patients without any apparent immune response, although the data supporting this conclusion are quite limited. The package inserts for three augmentation products (Prolastin, Aralast and Zemaira) state that none of the patients in clinical trials developed detectable antibodies to AAT, but these trials enrolled small numbers of patients and the methods used to try and detect anti-AAT antibodies are not described in either the package inserts or publications stating that antibodies to AAT were not detected (Wewers et al., 1987; Hubbard and Crystal, 1988; Schmidt et al., 1988; Stoller et al., 2002; Stocks et al., 2010).

In the current study we generated primate immune sera that were used as a positive control and as a reagent to generate the standard curve in order to quantify antibody responses. After IM administration of an AAV vector expressing human AAT, human AAT protein was detected in the serum of the macaque at very low levels for only a short period of time but was clearly sufficient to generate large amounts of anti-hAAT antibodies. The low and transient levels of serum hAAT did not allow us to characterize the expressed protein, but in previous studies in which the same vector was injected IM in C57BL/6 mice the muscle-expressed human AAT protein was functionally equivalent to AAT purified from human plasma, as determined by its ability to be cleaved by and form a complex

with human neutrophil elastase and to inhibit neutrophil elastase activity *in vitro* (Lu et al., 2006). Muscle-expressed human AAT also has a similar appearance on immunoblots of SDS-PAGE gels, suggesting that post-translation modifications of AAT in muscle and liver are similar (Song et al., 1998). However, it is possible that there could be subtle differences in post-translation modifications of AAT secreted from human muscle which could affect immunogenicity of the expressed protein, and it was therefore important to develop and validate an assay that could be used to evaluate this possibility.

One of the important issues in the development of an assay to detect immune responses to biological proteins is development of an appropriate positive control. FDA guidance recommends that if an antihuman immunoglobulin reagent will be used to detect patient antibodies, the positive control and quality control samples should be detectable by that reagent (e.g., primate immune sera, humanized monoclonal) (U.S. Food and Drug Administration, 2009). In previous clinical trials of rAAV vectors expressing AAT (Brantly et al., 2006; Brantly et al., 2009), potential development of antibodies to AAT after vector administration was evaluated using serum from a baboon injected with rAAV2-CB-hAAT (Song et al., 2002) as a positive control. However, this reagent was available in only limited amounts, and it was therefore not possible to conduct assay validation studies.

Components in the matrix other than antibodies can interfere with assay results. Of greatest concern is the presence in the matrix of product or its endogenous counterpart. Specifically, if large quantities of product-related material are present in sera/plasma, that material can prevent detection of antibodies in the test format (U.S. Food and Drug Administration, 2009). Partial inhibition of antibody detection by AAT in test sera was indeed observed for our assay. When serial dilutions of the reference standard macaque serum were added to BSA buffer, there was a linear dose-response and excellent recovery (>88% recovery at antibody levels between 1.56 µJ/mL and 50 µJ/mL). However, when serial dilutions of the reference standard macaque serum were added to human serum from individuals with AAT deficiency (PI ZZ), recovery was poor due to competition between AAT antigen in the test serum and AAT antigen on the ELISA plate. Despite the poor overall recovery, a linear dose-response was observed between the amount of antibody added and the measured antibody titer (Fig. 3B), indicating that the assay is able to detect low levels of antibody even in the presence of the concentrations of AAT (2.9 to 4.7 µM = 150 to 244 µg/mL) found in the PI ZZ sera evaluated.

The ability of exogenous AAT to specifically inhibit anti-AAT antibodies was also useful in evaluating the apparent small increase in anti-hAAT titer in four of nine subjects in a clinical trial of a rAAV vector expressing AAT. The failure of purified AAT to block ELISA reactivity in the clinical trial subject sera (Fig. 5) indicates that the increase in anti-AAT values in these subjects was not antigen-specific and not directed against AAT.

The availability of a validated assay will facilitate evaluation of immune responses to AAT in clinical trials and clinical practice.

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