Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy

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Alpha-1 antitrypsin (AAT) deficiency is well-suited as a target for human gene transfer. We performed a phase 1, open-label, dose-escalation clinical trial of a recombinant adeno-associated virus (rAAV) vector expressing normal (M) AAT packaged into serotype 1 AAV capsids delivered by i.m. injection. Nine AAT-deficient subjects were enrolled sequentially in cohorts of 3 each at doses of 6.9×10^{12} , 2.2×10^{13} , and 6.0×10^{13} vector genome particles per patient. Four subjects receiving AAT protein augmentation discontinued therapy 28 or 56 days before vector administration. Vector administration was well tolerated, with only mild local reactions and 1 unrelated serious adverse event (bacterial epididymitis). There were no changes in hematology or clinical chemistry parameters. M-specific AAT was expressed above background in all subjects in cohorts 2 and 3 and was sustained at levels 0.1% of normal for at least 1 year in the highest dosage level cohort, despite development of neutralizing antibody and IFN- γ enzyme-linked immunosorbent responses to AAV1 capsid at day 14 in all subjects. These findings suggest that immune responses to AAV capsid that develop after i.m. injection of a serotype 1 rAAV vector expressing AAT do not completely eliminate transduced cells in this context.

AAV | CTL | immune response | pulmonary | gene therapy

Alpha-1 antitrypsin (AAT) deficiency is among the most common single gene disorders in North America and Northern Europe, with a mutant allele frequency of up to 4% (1, 2). It is inherited as a codominant disorder with variable penetrance. Normal serum AAT levels range from 20 to 53 μ M, and AAT-deficient individuals with plasma AAT levels $<11 \mu$ M are prone to spontaneous development of chronic lung disease, characterized by airway inflammation and panacinar emphysema. More than 95% of deficient alleles are of one particular proteinase inhibitor (PI) phenotype, known as PI*Z (glu342lys) (3, 4). Heterozygous carriers with 1 PI*Z allele and 1 wild-type (PI*M) allele may also have an increased risk of lung disease if they are exposed to tobacco smoke or other detrimental environmental factors (5). It is generally accepted that AAT lung disease is caused by a lack of normal function of AAT as the principal plasma antiprotease, and the only currently available treatment is protein augmentation therapy using AAT purified from normal human donor plasma (6). However, protein augmentation therapy is suboptimal because of the need for weekly i.v. infusions, high cost, and limited availability.

On the basis of these considerations, recent attempts at AAT gene therapy have focused on gene augmentation through i.m. administration of recombinant adeno-associated virus (rAAV) vectors, which are capable of long-term transduction of skeletal myofibers in mice, with sustained secretion of plasma proteins, such as AAT, erythropoietin, or factor IX (7–10). A previous phase 1 trial of i.m. rAAV–AAT based on the well-studied AAV serotype 2 showed favorable safety characteristics, but transgene expression

was only barely above background transiently in 1 of 12 subjects (11). Because of a marked efficiency advantage for vectors packaged in AAV serotype 1 capsid and injected in murine muscle, our group proceeded with preclinical development of a cross-packaged or pseudotyped rAAV–AAT vector, using the same rAAV–AAT gene cassette as in the previous clinical trial but packaged in a serotype 1 capsid (10, 12, 13).

Recent data from other clinical programs attempting to use rAAV vectors to augment plasma proteins have raised the possibility that a fundamental limitation to this approach may be posed by immune responses to the AAV capsid. In a recent trial of rAAV2–factor IX delivered by hepatic artery injection, 1 subject developed a transient rise in transaminases concurrent with a loss of transgene expression, whereas a second subject showed transient expression of factor IX without transaminase elevations. A third subject demonstrated transient elevations in transaminases, which correlated with the appearance of T cells specific for the AAV2 capsid without detectable factor IX (cells were not available from the first 2 subjects to evaluate the time course of T cell activation) (14). Although it is unclear how this observation might relate to other AAV serotype capsids and other routes of administration, preliminary results of a rAAV1–lipoprotein lipase (LPL) trial raised the possibility that such a mechanism might be generalized to i.m. delivery of different serotypes, based on the demonstration of capsid-specific T cells concurrent with a transient rise in serum creatine kinase (CK) and a reversal of a decline in serum triglycerides (an indirect measure of LPL expression) in 1 subject (15).

In the present study, 9 AAT-deficient (PI*ZZ) individuals were dosed sequentially in cohorts of 3, each receiving i.m. doses of 6.9×10^{12} , 2.2×10^{13} , or 6.0×10^{13} vector genome particles (vg), and immune responses were monitored by IFN- γ enzyme-linked im-

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Conflict of interest statement: T.R.F. and B.J.B. hold patents related to this research, and any monies received have been donated to the Department of Pediatrics, College of Medicine at the University of Florida. T.R.F. and B.J.B. were also founders of Applied Genetic Technologies Corporation (AGTC), the study sponsor. They do not currently hold a position with the company, and any proceeds from this study are donated to the University of Florida. T.R.F.'s laboratory receives research funding from the Alpha 1 Foundation. M.L.B. receives research funding from, holds an endowed professorship from, and is a consultant for the Alpha 1 Foundation. The University of Florida owns an interest in the study agent and also owns stock in the sponsor of the study, AGTC. The Alpha 1 Foundation is closely affiliated with AlphaNet, which owns an interest in the study agent. J.M.W. is an inventor on patents including those involving AAV1 that have been licensed to various biopharmaceutical companies.

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Table 1. Demographic and baseline characteristics of subjects

Subject	Age (y)	Gender	Weight (kg)	FEV1 (% predicted)	AAT protein augmentation therapy	Prior rAAV2 vector dose (vg)	Dose of rAAV1 vector in present trial (vg)
101	66	F	54.6	53.6	No	6.9×10^{12}	6.9×10^{12}
102	73	F	66.5	86.7	Yes	—	6.9×10^{12}
103	69	M	73.7	92.1	Yes	6.9×10^{12}	6.9×10^{12}
201	38	M	83.0	40.5	Yes	—	2.2×10^{13}
202	54	M	89.0	93.0	No	6.9×10^{13}	2.2×10^{13}
203	59	M	72.7	50.8	No	2.1×10^{13}	2.2×10^{13}
301	47	F	69.3	58.0	No	—	6.0×10^{13}
302	35	F	134.6	97.6	No	—	6.0×10^{13}
303	61	M	72.6	54.9	Yes	—	6.0×10^{13}

FEV1, forced expiratory volume in 1 s; vg, vector genome particles of rAAV2–AAT; F, female; M, male.

munospot (ELISPOT), lymphocyte proliferation, and serum antibody responses to AAV1 capsid and the AAT transgene. ELISPOT assays were performed prospectively in the last 5 subjects in the trial and retrospectively in others. All other assays were performed prospectively in all patients, as were general safety assessments, serum CK measurements, and assays for transgene expression. These studies have enabled a prospective analysis of the relationship between immune responses to capsid and sustained transgene expression, and have supported the concept that immune responses to the rAAV1–AAT vector do not completely eliminate transduced muscle cells in this context.

Results

Characteristics of the Study Population. All study subjects were enrolled at the University of Florida under a protocol approved by the Western Institutional Review Board and reviewed by an executive group of the University of Florida Institutional Review Board and the gene therapy Data and Safety Monitoring Board of the National Heart, Lung, and Blood Institute. An Investigational New Drug Application sponsored by Applied Genetic Technologies Corporation was filed with the U.S. Food and Drug Administration. Informed consent was obtained from each subject, and a decision monitoring protocol was used to ensure that subjects understood the risks of the protocol and the anticipated lack of clinical benefit from their participation.

The study population consisted of 9 subjects (5 male, 4 female) who had been diagnosed as homozygous for PI*Z with a serum AAT level $<11 \mu\text{M}$. The demographic and baseline characteristics of the subjects are presented in Table 1. Four subjects had received a serotype 2 AAV–AAT vector in a prior gene transfer study (11). Four subjects were receiving weekly AAT protein augmentation therapy, which was discontinued 28 days (subjects 102 and 103) or 56 days (subjects 201 and 303) before administration of rAAV1–AAT and resumed after the day-90 visit. Subjects were enrolled sequentially into 1 of 3 dosage level cohorts of 6.9×10^{12} , 2.2×10^{13} , or 6.0×10^{13} vg administered by i.m. injection in a total volume of 9.9 mL.

General Safety and Biodistribution. Each subject underwent extensive clinical and laboratory monitoring at baseline and for 1 year after study agent administration. Minor adverse events were occasionally observed, but the only such events related to the study agent were minimal to mild reactions at the injection site in 7 of 9 subjects (bruising in 6, swelling or induration in 3, redness in 2, and warmth or tenderness in 1 each) that were not considered clinically important. Only 1 serious adverse event was reported, a culture-proven *Escherichia coli* epididymitis with onset 2½ weeks after vector administration that was judged unrelated to vector administration. There were no clinically important changes in hematology, serum chemistry, or urinalysis parameters after vector administration.

Biodistribution studies demonstrated vector DNA in the blood in 2 of 3 subjects in each of the 2 lower dose cohorts and 3 of 3 subjects

in the highest dose cohort, which was maximal at day 1 and progressively decreased to become negative by day 14 or day 90 in all but 1 subject (supporting information Table S1). No vector DNA was detected in semen.

Time Course of Vector-Mediated Expression of Wild-Type (M) AAT.

Vector-mediated expression of AAT was monitored using assays of both total AAT (nephelometry) and wild-type (M) AAT (mAb-based ELISA). Total AAT levels ranged from 3.1 to 6.0 μM at day –1 and, except for subject 103 who had a transient increase of Z-type AAT to 11 μM concurrent with his bacterial epididymitis, consistent with AAT being an acute-phase reaction protein, did not change appreciably at subsequent visits (mean $\pm$ SD ratio of subsequent to baseline values, $100.8\% \pm 15.9\%$).

For the 4 subjects who had been receiving AAT protein augmentation therapy, measurement of M-type AAT levels was complicated by the reactivity of the M-specific mAb to residual infused M-AAT protein therapy. This antibody has more than 500-fold greater affinity for M-type AAT than for Z-type AAT, and background M-specific AAT levels are therefore very low in PI*ZZ individuals (mean $\pm$ SD, 10.7 ± 6.0 nM for 20 PI*ZZ individuals; upper 99% confidence interval, <25 nM). However, because weekly AAT protein augmentation therapy achieves peak serum levels $>300 \mu\text{M}$ and nadir levels $>11 \mu\text{M}$, the reactivity of the mAb resulted in measured levels of M-specific AAT at day –1 of 235 nM and 358 nM in subjects 102 and 103, who had discontinued protein augmentation therapy 28 days before vector administration, and these levels progressively decreased until day 60 and remained stable at ≈ 13 nM through day 90. Subjects 201 and 303, who had discontinued protein augmentation therapy 56 days before vector administration, had measured levels of M-specific AAT at day –1 of 33 nM and 18 nM, respectively, and these levels decreased to <10 nM at day 14, after which they increased to a maximum of 28 nM on day 90 in subject 201 and 48 nM on day 90 in subject 303 (Fig. 1). Serum M-specific AAT concentrations could not be determined after day 90 in these patients because they had resumed protein augmentation therapy.

Among the 5 subjects who were not receiving AAT protein augmentation therapy, subject 101 received the lowest vector dose and had no appreciable change in M-specific AAT levels. Among those who received the intermediate vector dose, subject 202 had an average pretreatment M-specific AAT level of 10 nM that increased to 18 nM on day 30 and slowly decreased to pretreatment levels by day 90, and subject 203 had an average pretreatment M-specific AAT level of 11 nM that increased to 21 nM on day 60 and then decreased to pretreatment levels by day 180 (Fig. 1). Among those who received the highest vector dose, subject 301 had an average pretreatment M-specific AAT level of 10 nM that increased to 43 nM on day 90 and remained above 40 nM for 1 year, and subject 302 had an average pretreatment M-specific AAT level of 8 nM that increased to 18 nM on day 45 and then seemed to decrease to

Fig. 2. Time course of IFN- γ ELISPOT responses to pools of AAV1 capsid peptides or controls. PBMC were obtained at 1 or 2 times before (pre1 and pre2) and at 14, 30, 45, 60, 75, and 90 days (D14 through D90, respectively) and 425 or 321 days (Yr1) after vector administration and stimulated with each of 3 pools (A, B, and C) of AAV1 capsid peptides (15-mers overlapping by 10 aa) or with a positive control peptide pool (CEF). Responses that were significantly increased compared with medium alone (control) are indicated by an asterisk.

Fig. 3. Evaluation of capsid-specific CD4⁺ and CD8⁺ T cell responses in subjects 301 and 302. (A) PBMC isolated at postinoculation day 45 were stimulated for 6 h with peptides derived from AAV-1 capsid (15-mers overlapping by 10 aa, bottom row) or were unstimulated (top row). Frequencies shown represent viable responding memory CD3⁺CD4⁺CD8⁺ T cells within the respective quadrant gate after having gated out naïve cells (CCR7⁺CD45RO⁻). (B) Bar chart representing the functionality of AAV-specific CD4⁺ T cells. (C) PBMC isolated at postinoculation day 81 were stimulated for 6 h with peptides derived from AAV-1 capsid (15-mers overlapping by 10 aa, bottom row) or were unstimulated (top row). Frequencies shown represent viable responding memory CD3⁺CD8⁺CD4⁺ T cells within the respective quadrant gate after having gated out naïve cells (CCR7⁺CD45RO⁻). (D) Bar chart representing the functionality of AAV-specific CD8⁺ T cells. Each bar represents the proportion of the total response positive or negative for the respective functions shown in the key below the x axis. Only bars with positive responses above the 0.005% threshold are shown.

points were available for subject 301 (at day 425) and subject 303 (at day 321) and were also positive. The magnitude of the response to any individual pool was generally less than 200 spot-forming cells (SFC) per 10^6 peripheral blood mononuclear cells (PBMC) for 4 subjects but was higher in subject 302 (e.g., the response to pool 3 was 330 SFC/ 10^6 PBMC at day 45, and the response to pool 2 was 428 SFC/ 10^6 PBMC at day 90).

We further investigated by polychromatic flow cytometry the phenotype and functionality of the capsid-specific T cells from 2 subjects. PBMC from day 45 of subject 301 and day 81 of subject 302 were stimulated for 6 h with the AAV1 peptide library and assessed simultaneously for 6 CD8⁺ T cell functions, including degranulation (CD107 α and perforin), production of cytokines (IFN- γ , TNF- α , and IL-2), and chemokine (MIP-1 α) and cell-surface markers (CD3, CD4, CD8, CD27, CD45RO, CD57, and CCR7) to determine whether the capsid-specific T cells were CD4⁺ or CD8⁺ cells and the functional profile of the responding T cells. Subject 301 had a response to AAV1 capsid mediated by CD4⁺ cells that predominantly expressed TNF- α and IL-2 (Fig. 3 A and B) and expressed markers indicative of central memory phenotype (CCR7⁺CD27⁺CD45RO⁺). Subject 302 had a response to AAV1 capsid mediated by CD8⁺ T cell that generally seemed effector-like in functionality, with expression of perforin, MIP-1 α , CD107 α , and TNF- γ in various combinations, with relatively little IL-2 and IFN- γ production (Fig. 3 C and D). All *ex vivo* and cultured ELISPOT assays using AAT peptides were negative at all time points tested.

T cell responses were also evaluated in all subjects using a lymphocyte proliferation assay performed at baseline, day 14, and day 90. For the AAV antigen, subject 202 had a stimulation index (SI) <1 at baseline and day 14 and an SI of 7.0 at day 90. In subject 301 the SI was 3.3, 0.7, and 2.6 at baseline and days 14 and 90,

respectively. All other subjects had negative responses (SI <2.5) to both AAV and AAT antigens at all time points tested.

Serum CK values were also assessed over time (Fig. 4). Only 5 of the 36 values measured were above the reference range (>170 U/L). Of these, 3 were baseline values, and the other 2 were from 1 of the subjects (102) who had had a high baseline value. None of the values was greater than 1.5 times the upper limit of normal, and none of the subjects for whom an effector T cell response was demonstrated by day 14 had an elevated CK value after vector administration.

Discussion

In this first-in-human phase 1 study, we have observed reproducible expression of wild-type M-AAT protein from a cross-packaged rAAV1-AAT vector after i.m. administration that was sustained at subtherapeutic levels for at least 1 year in 2 subjects and at least 90 days in the third subject in the 6.0×10^{13} vg dosage level cohort, despite effector T cell responses that developed within the first 14 days after vector delivery and persisted 90 days

Fig. 4. Serum CK levels before and after vector administration.

subject with inclusion and exclusion criteria was confirmed. (For more details refer to [SI Text](#).)

Injection Procedure. The deltoid muscle of the nondominant arm (left arm for right-handed individuals and vice versa) was chosen as the site of injection. Before vector injection, a baseline computed tomography scan of the chest and upper extremity was performed to assess baseline morphology and rule out underlying abnormalities of the upper extremity. Doppler ultrasound was performed over the potential injection site immediately before and during the injection to avoid vascular structures with observable flow. The needle was also directly visualized and the outflow of vector visualized with Doppler ultrasound in real time. The dose was administered in a constant 9.9-mL volume, regardless of vector dose, using lactated Ringers solution to dilute the vector to the proper concentration. At the time of the injection, 9 separate injections of 1.1 mL each were injected slowly over 1 min each.

Biodistribution Assays. Blood samples (and semen when available) were obtained at screening and on days 1 (blood only), 3, 14, and 90. DNA was extracted, and vector sequences were detected using Taqman real-time PCR techniques with a previously published primer/probe set directed against a vector-specific CB promoter sequence (26, 27). All samples were tested in duplicate, with a third sample containing a positive spike-in control. The technique has a sensitivity of 100 copies per microgram of input DNA.

Measurement of Total and Wild-Type (M-Specific) AAT. Total AAT was measured using a clinically approved nephelometry method, an automated assay in which polyclonal rabbit anti-AAT antibody (Siemens Healthcare Diagnostics) is mixed with serum and the resulting antigen-antibody complexes are detected by light scattering in a nephelometer (Dade-Behring Nephelometer II; Siemens). M-specific AAT was measured by ELISA, monoclonal antibody was developed in a standard fashion by injecting mice with highly purified PI M1M1 AAT, isolating hybrid clones and identifying clones that made antibody to M but not Z AAT using a dot blot. The M-specific ELISA is a standard double sandwich assay, primary antibody is monoclonal to M AAT, and secondary is rabbit antihuman AAT (Dako). Assays were run on a SPECTRAmax (Molecular Devices) plate reader using a highly purified PI M1M1 AAT (Athens Research). The assay was validated for accuracy, reproducibility, selectivity, lowest limit of quantification, and stability ([Fig. S1](#)). Dilutions of serum are added to wells coated with this mAb at 167 ng/mL and antigen-antibody complexes detected using a polyclonal rabbit antihuman AAT at 20 ng/mL followed by a peroxidase-conjugated goat antirabbit Ig, with O-phenylenediamine added for colorimetric analysis. The assay is validated for accuracy, repeatability, and precision using a broad range of concentrations and is also validated for serum stability at various temperatures ranging from 24 °C to

–80 °C. The lower limit of quantification for this assay is 8.8 pM, with selectivity and reproducibility at the lower limit of detection >90%.

Antibody Responses. Serum was obtained at screening and on days 14 and 90 for measurement of neutralizing antibodies to AAV and antibodies to AAT and AAV by ELISA. Neutralizing antibodies were measured as previously described (28). Anti-AAV2 and anti-AAT antibodies were assayed by ELISA as previously described (11). In the case of the anti-AAT antibody assay, the positive control serum was taken from a baboon immunized against human AAT in a prior study (27).

Cellular Immune Responses. Blood was collected and PBMC separated at screening and on days 14 and 90 for measurement of lymphocyte proliferative responses to AAV and AAT antigens as previously described (26, 29). For more details refer to [SI Text](#).

In Vitro Stimulation and Staining of Cells and Polychromatic Flow Cytometry. Costimulatory antibodies (anti-CD28 and CD49d, 1 μ g/mL; BD Biosciences), monensin (GolgiStop, 0.7 μ l/mL; BD Biosciences), brefeldin A (10 μ g/mL; Sigma-Aldrich), and anti-CD107a-PE-Cy5 were added and the cells aliquoted at 1 mL to a tube containing 2 μ g of AAV1 peptide libraries. A positive control (*Staphylococcus enterotoxin B*, 1 μ g/mL; Sigma-Aldrich) and unstimulated negative control were included in each assay. After fixation, cells were washed twice in perm buffer, stained with antibodies against intracellular markers, and then washed, fixed with PBS containing 1% paraformaldehyde, and stored at 4 °C until analysis (performed within 24 h). Analysis was performed using FlowJo software (TreeStar) as previously described (31).

Long-Term Follow-Up. Upon completion of this study, each subject will be followed annually for an additional 4 years (5 years total) and questioned about the development of any delayed malignant, neurologic, immunologic, hematologic, or other adverse events.

Statistical Analysis. All ELISPOT data were compared using an unequal variance two-tailed Student's *t* test. Differences were considered statistically significant if *P* < 0.05.

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