Treatment of Late Infantile Neuronal Ceroid Lipofuscinosis by CNS Administration of a Serotype 2 Adeno-Associated Virus Expressing CLN2 cDNA

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ABSTRACT

Late infantile neuronal ceroid lipofuscinosis (LINCL) is an autosomal recessive, neurodegenerative lysosomal storage disease affecting the CNS and is fatal by age 8 to 12 years. A total average dose of 2.5 × 10^12 particle units of an adeno-associated virus (AAV) serotype 2 vector expressing the human CLN2 cDNA (AAV2CUhCLN2) was administered to 12 locations in the CNS of 10 children with LINCL. In addition to safety parameters, a neurological rating scale (primary variable) and three quantitative magnetic resonance imaging (MRI) parameters (secondary variables) were used to compare the rate of neurological decline for 18 months in treated subjects compared with untreated subjects. Although there were no unexpected serious adverse events that were unequivocally attributable to the AAV2CUhCLN2 vector, there were serious adverse effects, the etiology of which could not be determined under the conditions of the experiment. One subject died 49 days postsurgery after developing status epilepticus on day 14, but with no evidence of CNS inflammation. Four of the 10 subjects developed a mild, mostly transient, humoral response to the vector. Compared with control subjects, the measured rates of decline of all MRI parameters were slower, albeit the numbers were too small for statistical significance. Importantly, assessment of the neurologic rating scale, which was the primary outcome variable, demonstrated a significantly reduced rate of decline compared with control subjects. Although the trial is not matched, randomized, or blinded and lacked a contemporaneous placebo/sham control group, assessment of the primary outcome variable suggests a slowing of progression of LINCL in the treated children. On this basis, we propose that additional studies to assess the safety and efficacy of AAV-mediated gene therapy for LINCL are warranted.

INTRODUCTION

Late infantile neuronal ceroid lipofuscinosis (LINCL) is an autosomal recessive, neurodegenerative lysosomal storage disease affecting the CNS and is fatal by age 8 to 12 years. A total average dose of 2.5 × 10^12 particle units of an adeno-associated virus (AAV) serotype 2 vector expressing the human CLN2 cDNA (AAV2CUhCLN2) was administered to 12 locations in the CNS of 10 children with LINCL. In addition to safety parameters, a neurological rating scale (primary variable) and three quantitative magnetic resonance imaging (MRI) parameters (secondary variables) were used to compare the rate of neurological decline for 18 months in treated subjects compared with untreated subjects. Although there were no unexpected serious adverse events that were unequivocally attributable to the AAV2CUhCLN2 vector, there were serious adverse effects, the etiology of which could not be determined under the conditions of the experiment. One subject died 49 days postsurgery after developing status epilepticus on day 14, but with no evidence of CNS inflammation. Four of the 10 subjects developed a mild, mostly transient, humoral response to the vector. Compared with control subjects, the measured rates of decline of all MRI parameters were slower, albeit the numbers were too small for statistical significance. Importantly, assessment of the neurologic rating scale, which was the primary outcome variable, demonstrated a significantly reduced rate of decline compared with control subjects. Although the trial is not matched, randomized, or blinded and lacked a contemporaneous placebo/sham control group, assessment of the primary outcome variable suggests a slowing of progression of LINCL in the treated children. On this basis, we propose that additional studies to assess the safety and efficacy of AAV-mediated gene therapy for LINCL are warranted.

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is mutated, proteins accumulate in the lysosomes of neurons over time, leading to progressive neuronal death, likely by apoptosis (Lane et al., 1996).

LINCL typically manifests clinically at age 2 to 4 years with ataxia, myoclonus, impaired speech, and developmental regression (Boustany, 1996; Williams et al., 1999; Haltia, 2003). Seizures are often the first manifestation, but there is variability in the time of onset and the appearance of the collective symptoms. A gradual decline in visual ability follows, with blindness by age 4 to 6 years. Affected children generally become wheelchair-bound between 4 and 6 years. Toward the late stages of the disease, feeding becomes difficult, resulting in poor weight gain. Death occurs by age 8 to 12 years (Williams et al., 1999; Haltia, 2003).

As a strategy to treat the CNS manifestations of LINCL, we initiated a program using serotype 2 adeno-associated virus (AAV2) to transfer the normal CLN2 cDNA to the brain, exploiting the neurotrophic properties of this vector and the potential for long-term transgene expression. Preclinical studies demonstrated that direct CNS administration of AAV2_{Cdh1-CLN2}, a serotype 2 adeno-associated virus gene transfer vector expressing the human CLN2 cDNA, mediated high levels of enzymatically active TPP-I in the lysosomes of neurons of experimental animals for at least 18 months. There was no evidence of adverse effects in experimental animals (Hackett et al., 2005; Sondhi et al., 2005), and administration of the vector demonstrated suppression of the accumulation of autofluorescent material in the CNS of CLN2 knockout mice (Passini et al., 2006). On the basis of these data, the present study was designed to assess the safety of direct administration of the AAV2_{Cdh1-CLN2} vector to the CNS of children with LINCL and to develop preliminary data concerning whether this therapy slows the progression of the CNS manifestations of the disease. LINCL represents a paradigm for assessing the efficacy of therapy for rare disorders of children, in that a combination of practical and ethical concerns significantly limits clinical trial design, obviating the use of contemporaneous, matched, randomized, blinded, or placebo control subjects. With this caveat, the data demonstrate that this therapy is associated with minimal toxicity. For the children for whom data were available for >6 months posttherapy, compared with historic control subjects, the magnetic resonance imaging (MRI) assessments (the secondary outcome parameters) showed a slower but not statistically significant rate of decline. Importantly, assessment of the modified Hamburg LINCL clinical rating scale, the primary outcome variable, demonstrated that the treatment was associated with a statistically significant slower decline in neurologic status compared with control subjects. Although the number of treated children is small and the control group is not ideal to make definitive conclusions, collectively the data suggest that additional studies are warranted to assess the safety and efficacy of AAV-mediated gene therapy for the CNS manifestations of LINCL.

MATERIALS AND METHODS

Overall design

The study was carried out at the New York Presbyterian Hospital, Weill Medical College of Cornell University (New York, NY). The research protocol was reviewed and approved by the Weill Cornell Institutional Review Board, Institutional Biosafety Committee, National Institutes of Health (NIH)/General Clinical Research Center-Pediatric Scientific Advisory Committee and Data Safety Monitoring Board. At the national level, the protocol was reviewed by the NIH/Recombinant DNA Advisory Committee and an Investigational New Drug application was approved by the Center for Biologies Evaluation and Research, U.S. Food and Drug Administration (U.S. FDA, BBIND 11481). The parents of all participating children provided informed consent.

The study was designed as an 18-month follow-up subsequent to direct CNS administration of the AAV2_{Cdh1-CLN2} vector to 10 children with LINCL, 5 of whom were severely affected by the disease and 5 of whom were moderately affected (Table 1; and see Supplementary Table I at www.liebertonline.com/hum). All subjects received 1.8 to 3.2 × 10^12 particle units (average dose, 2.5 × 10^12 particle units) of the AAV2_{Cdh1-CLN2} vector. The primary outcome variable was neurologic assessment of the disease, using a modified form of the Hamburg LINCL scale (see Supplementary Table II at www.liebertonline.com/hum) (Steinfeld et al., 2002; Crystal et al., 2004). The secondary variables included CNS MRI assessment of gray matter volume as a percentage of total brain volume, ventricular volume, and cortical apparent diffusion coefficient (CADC) (Dyke et al., 2007; Worgall et al., 2007). Because of the small number of children involved and because of ethical issues regarding a neurosurgical procedure in children with a fatal neurological disease, the study did not include a formal randomized, placebo/sham, or untreated control group, and the study was not blinded (Crystal et al., 2004; Arkin et al., 2005). Data from four independent children with LINCL with two assessments separated by 1 year served as an untreated control group for comparison. For the primary variable, we also compared the rate of decline in the modified Hamburg scale in the treated subjects with the data published by Steinfeld and coworkers (2002).

Study population

The study group consisted of 10 children (6 boys and 4 girls) with LINCL. Pretherapy, the children had confirmation of CLN2 mutations, routine blood and urine studies, comprehensive neurological assessments, and quantitative MRI imaging (Crystal et al., 2004). Subjects were chosen for the gene therapy study, on the basis of specific inclusion/exclusion criteria, by an eligibility committee composed of three physicians other than the principal investigator, including a pediatric neurosurgeon, a pediatric neurologist, and a general pediatrician.

AAV2_{Cdh1-CLN2}

The gene therapy vector used in the study, AAV2_{Cdh1-CLN2}, is based on an AAV2 serotype capsid and genome. The modified genome is deleted of the AAV2 rep and cap genes, retaining only the AAV2 inverted terminal repeats flanking the expression cassette (Crystal et al., 2004; Hackett et al., 2005; Sondhi et al., 2005). The expression cassette includes the CAG promoter (consisting of the human cytomegalovirus immediate/early enhancer, the promoter, splice donor, and left-hand intron sequence from chicken β-actin, and the splice acceptor from rabbit β-globin), the human CLN2 cDNA with an optimized Kozak translational initiation signal before the start codon and a rabbit β-globin poly(A) sequence. The AAV2_{Cdh1-CLN2} vector was produced by a two-plasmid cotransfection.
procedure in the Belfer Gene Therapy Core Facility (Weill Cornell Medical College) under current Good Manufacturing Practice conditions. This included transfection into a certified 293 cell line of an expression cassette plasmid (pAAV2-CAG-hCLN2) containing the promoter and the human CLN2 cDNA expression cassette and an adenovirus/AAV2 helper plasmid (pPAK-MA2) (et al., 2005; Sondhi et al.). The genotypes of the four control subjects were as follows: two G3556C homozygotes, one G3556C/T4383C heterozygote, and one G3556C/T3016A heterozygote.

The diagnosis of LINCL for this subject was based on quantitative assessment of TPP-I deficiency in white blood cells and the presence of a single known mutation for LINCL in the genome.

**Table 1. Demographics of Study Population**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at time of therapy</th>
<th>Race/ethnicity</th>
<th>Sex</th>
<th>Residence</th>
<th>CLN2 mutations</th>
<th>LINCL severity</th>
<th>Other medical conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-01</td>
<td>8.6</td>
<td>C M USA</td>
<td></td>
<td></td>
<td>G3556C/T3016A</td>
<td>Severe</td>
<td>Prior otitis media</td>
</tr>
<tr>
<td>BD-02†</td>
<td>10.0</td>
<td>C M USA</td>
<td></td>
<td></td>
<td>G3556C/G3085Ab</td>
<td>Severe</td>
<td>Hypospadias, prior septic arthritis hip</td>
</tr>
<tr>
<td>BD-03†</td>
<td>6.9</td>
<td>C M USA</td>
<td></td>
<td></td>
<td>G3556C/G3085Ab</td>
<td>Severe</td>
<td>Otis media</td>
</tr>
<tr>
<td>BD-04</td>
<td>8.1</td>
<td>C F England</td>
<td></td>
<td></td>
<td>G3556C</td>
<td>Severe</td>
<td>Drug allergy</td>
</tr>
<tr>
<td>BD-05</td>
<td>4.5</td>
<td>C F Germany</td>
<td></td>
<td></td>
<td>G3670T homozygote</td>
<td>Severe</td>
<td>—</td>
</tr>
<tr>
<td>BD-06</td>
<td>5.4</td>
<td>C M USA</td>
<td></td>
<td></td>
<td>C3670T/unknown</td>
<td>Moderate</td>
<td>—</td>
</tr>
<tr>
<td>BD-07†</td>
<td>4.5</td>
<td>C M Australia</td>
<td></td>
<td></td>
<td>G3556C homozygote</td>
<td>Moderate</td>
<td>Atopic dermatitis, food allergies</td>
</tr>
<tr>
<td>BD-08</td>
<td>3.6</td>
<td>C F England</td>
<td></td>
<td></td>
<td>G3556C</td>
<td>Moderate</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>BD-09</td>
<td>5.3</td>
<td>C M Germany</td>
<td></td>
<td></td>
<td>C3670T homozygote</td>
<td>Moderate</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>BD-10†</td>
<td>3.4</td>
<td>C F Australia</td>
<td></td>
<td></td>
<td>G3556C homozygote</td>
<td>Moderate</td>
<td>Atopic dermatitis, food allergies</td>
</tr>
</tbody>
</table>

Abbreviations: CLN2, ceroid lipofuscinosis, neuronal 2; LINCL, late infantile neuronal ceroid lipofuscinosis; TPP-I, tripeptidylpeptidase I.

* Listed in order of the initial evaluation; BD, Batten disease; and * and † are used to identify sibling pairs.

C, Caucasian; M, male; F, female.

Mutations are named by location in the gene (Sleat et al., 1999) with the wild-type nucleotide before and mutant nucleotide after. G3556C is a splice junction mutation also referred to as IVS5-1G > C or c.509-1G > C. The C3670T mutation is a premature stop codon also called R208X or c.622C > T. The genotypes of the four control subjects were as follows: two G3556C/C3670T homozygotes, one G3556C homozygote, and one G3556C/T4383C homozygote.

LINCL severity based on the modified Hamburg LINCL scale; see Materials and Methods and Supplementary Table II (at www.liebertonline.com/hum).

Medical problems unrelated to the known clinical manifestations of LINCL (Boustany, 1996; Williams et al., 1999).

Premature stop codon at amino acid 104.

Missense (Arg>Gln) mutation at amino acid 127.

Lamotrigine.

The diagnosis of LINCL for this subject was based on quantitative assessment of TPP-I deficiency in white blood cells and the presence of a single known mutation for LINCL in the genome.

Vector administration and follow-up assessment

The children were prepared for anesthesia and surgery in a standard fashion. The BrainLAB system for image-guided surgery (BrainLAB, Westchester, IL) was used to map vector administration loci on the skull, based on a preoperative MRI scan with sentinels on the head. For standardization, the coronal sutures and bony landmarks of the cranium were used to draw a line in the sagittal plane on the MRI. The burr holes were produced at the mapped locations and the dura was cut, permitting the brain to be directly visualized. At the completion of the six burr holes, 150-μm-diameter flexible glass catheters (Polymer Technologies, Phoenix, AZ) were used to administer the vector. A 20-gauge spinal needle was placed on the surface of the brain orthogonal to the skull to act as a guide for the insertion of the catheters 2 cm into the predetermined locations in the brain. Intravenous mannitol (typically 1.0 g/kg) was given as needed throughout the period of vector administration to minimize brain edema.

Each individual received a total dose of 1.8–3.2 × 1012 particle units of AAV2CUhCLN2, equally divided among 12 cortical locations delivered through 6 burr holes (2 locations at 2 depths through each hole), 3 burr holes per hemisphere. The exact locations of the administration of the vector were determined on a case-by-case basis, but were generally in the same regions. The vector was administered at a rate of 2.0 μl/min to each of the six sites in parallel by a microperfusion pump (Hamilton, Reno, NV). After the specified dose was administered over a period of 75 min to the six sites, the catheters were left in place for 5 min to ensure tissue penetration. The catheters...
were then withdrawn approximately half-way from the bottom of the catheter tract to the brain surface, and the remaining 50% of the dose was administered, in parallel, to each of the six sites as described above.

After vector administration, the surgical wounds were closed by standard techniques. A postoperative fluid-attenuated inversion recovery (FLAIR) MRI was performed within the first 48 hr after the surgical procedure to assess for bleeding or other possible perioperative adverse events. Each child was monitored postoperatively in a recovery room or intensive care unit, and once stable, was transferred to the Children’s Clinical Research Center unit. The children were discharged from the hospital at the discretion of the attending neurosurgeon, usually 7 days after vector administration. All families were asked to remain in the proximity of the hospital until the evaluation on day 14.

Subjects were assessed at Weill Cornell on days 7 and 14, and at months 1, 6, 12, and 18 after treatment (see Supplementary Table I). At months 2 and 3, they were also assessed for adverse effects at the office of the child’s personal physician. Details of the assessments are described in Supplementary Tables III, IV, and V (at www.liebertonline.com/hum). The children continue to be monitored for general status once yearly by telephone contact as mandated by the U.S. FDA 15-year annual follow-up requirement for all gene transfer studies (U.S. Food and Drug Administration, 2003).

Safety and efficacy parameters

Each individual underwent two baseline evaluations to determine eligibility and to establish baseline values for safety and efficacy parameters; these two baseline evaluations are referred to as the “screening” studies (to determine eligibility) and the “pretherapy” studies (to obtain current laboratory studies just before the surgical procedure and vector administration). The safety parameters included a general assessment (history, physical examination, vital signs, height, and weight), blood and urine analyses, human immunodeficiency virus test, vector-related tests (serum anti-AAV antibody levels), electrocardiogram, neurologic assessment, chest X-ray, and ophthalmologic examination (split lamp examination and direct ophthalmoscopy). After the death of subject BD-04, 49 days posttherapy (see below), 24-hr continuous electroencephalogram (EEG) and levels of anticonvulsant medication levels pretherapy and on day 14 were added to the protocol as per U.S. FDA recommendations. A 24-hr, 16-channel video EEG was performed 4 days before treatment and 14 days after treatment in the six subsequent subjects. In addition to the qualitative assessments of the EEG background, analysis of sleep patterns, spike and wave discharge frequency, and duration was carried out. Quantitative spike analysis was performed during wakefulness for 1 hr of each video EEG study by two readers blinded as to treatment status. The efficacy parameters used to develop estimates of the impact of therapy over time included (1) the primary variable, the modified Hamburg LINCL clinical rating scale (Steinfeld et al., 2002; Crystal et al., 2004); and (2) the secondary variables, all based on quantitative assessment of CNS MRI scans (Dyke et al., 2007; Worgall et al., 2007).

Primary variable. The primary efficacy variable for the study was the modified Hamburg LINCL clinical rating scale (see Supplementary Table II). This scale, originally developed by Steinfeld and coworkers (2002), was modified to eliminate the ophthalmologic variable, because the therapy was directed only at the CNS manifestations of the disease (Crystal et al., 2004). The modified Hamburg scale assesses motor function, seizure activity, and language skills. Each of these individual scores is ranked from 0 to 3 and then the individual scores are added to provide a total rating, with 0 being the most severe and 9 the mildest.

Secondary variables. The secondary efficacy parameters included quantitative CNS MRI assessment at various intervals pre- and posttreatment (see Supplementary Tables I and IV). Three imaging parameters were chosen on the basis of data from untreated children demonstrating a correlation with the progressive deterioration associated with LINCL (Worgall et al., 2007). All imaging data were acquired on a 3.0-T MRI system (GE Medical Systems, Milwaukee, WI). Imaging included T1-weighted, T2-weighted, and fluid-attenuated inversion recovery sequences. To assess gray matter volume as a percentage of total brain volume, a spoiled gradient recalled pulse sequence was employed. Ventricular volume assessments were performed on T1-weighted data sets. The CADC was measured with a spin-echo diffusion-weighted echo-planar pulse sequence implemented over the entire brain, and assessed for the cortex specifically (Dyke et al., 2007; Worgall et al., 2007). Details of the MRI assessments are described in Supplementary Table IV.

Anti-AAV2 neutralizing antibody titers

Anti-AAV2 neutralizing antibody titers were assessed in serum before and at various times after therapy by mixing serial 2-fold dilutions of serum, starting at a 1:10 dilution, with 2 × 10^5 particle units of AAV2LacZ (identical to the AAV2CuCLN2 vector, with β-galactosidase substituted for the CLN2 cDNA) (Hackett et al., 2005) for 30 min at 37°C. The mixture was then used to infect 293 ORF6 cells in a 96-well plate (Brough et al., 1996). After growth for 48 hr, a cell lysate was made and β-galactosidase activity was determined. The reciprocal dilution required for 50% inhibition of infection was interpolated. In all assays, a positive control serum sample from a normal human was included and the assay was accepted only if the reciprocal titer for the control serum was 200 ± 50.

Data analysis and statistical considerations

Individuals (other than the principal and coprincipal investigators) collected, tabulated, and verified the clinical parameters, and adverse effects were tabulated on the basis of standard methods (Crystal et al., 2004). Seizure activity (by EEG) before and after therapy was compared for n = 6 subjects by unpaired t test. For assessment of the primary and secondary parameters, the change of each parameter over time from pre- to posttherapy was compared with observations obtained from four untreated children (one was moderate and three severe on the modified Hamburg LINCL rating scale at first visit) with LINCL for whom data were available from two visits separated by approximately 1 year. None of these children were enrolled in the gene therapy protocol, but their genotypes were comparable to those of the enrolled subjects. For the primary variable, the modified Hamburg scale, the data from the four untreated
children monitored for 1 year was supplemented with data published by Steinfeld and coworkers (2002) representing an average based on 16 subjects. For the treated children with >6 months of follow-up, the change in each parameter over time posttherapy was compared with the control data. To reduce uncertainty in baseline values for the treated subjects, because all pretherapy data for each subject were obtained within 2.3 ± 0.4 months, when more than one data point was available pretherapy, the pretherapy data points were averaged and considered to be “zero” time. The rates of change for both primary and secondary parameters for the treated and untreated subjects were compared by nonparametric Mann–Whitney test.

**RESULTS**

**Study population**

Subjects were from the United States (n = 4), England (n = 2), Australia (n = 2), and Germany (n = 2). Their ages at the time of vector administration ranged from 4.5 to 10.0 years (7.6 ± 0.7 years; median, 8.1 years) for the severe group (BD-01 to BD-05) and from 3.4 to 5.4 years (4.4 ± 0.3 years; median, 4.5 years) for the moderate group (BD-06 to BD-10; see Table 1). There were two sibling pairs, one pair in the moderate group and one pair in the severe group. The CLN2 mutations of the 10 subjects included 5 different mutations in 19 of the 20 alleles; no mutation was detected in 1 allele of subject BD-06. The sibling pairs each had the same mutations. The most frequent mutation, the splice mutation G3556C, was present in 11 of 19 (58%) alleles; 4 of the subjects were homozygous for this mutation. The second most frequent mutation, C3670T, which results in a premature stop codon at amino acid 208, was present in 5 of 19 (26%) alleles; 2 subjects were homozygous for this mutation. Both mutations lead to undetectable TPP-I activity. Comparison of genotype with the clinical progression of LINCL has been reported to be relatively homogeneous, uninfluenced by genotype (Sleat et al., 1999; Worgall et al., 2007).

Of the 10 subjects who were treated, 1 died during the study period, 8 completed the study through 6 months, and 7 completed the study through the 18-month follow-up period (see Supplementary Tables III and IV for details regarding reasons for missed predetermined data points).

**Safety of administration of AAV2CUhCLN2**

MRI scans performed within 48 hr postsurgery demonstrated no evidence of hematoma or other surgery-related adverse effects. Of the 154 adverse events reported posttherapy, 60 were ranked as “serious” and 94 as “nonserious” (Table 2). In no instance was it possible to directly attribute a serious or nonserious adverse event to the vector per se versus the anesthesia/surgery/administration procedure or the natural history of the disease. However, because it was not possible to prove that these events were not related to the vector, most adverse events were recorded as probably or possibly related to the vector. Thirteen of the 60 serious adverse events (22%) and 52 of the 94 nonserious events (55%) occurred within 2 weeks of administration of the vector and were most likely related to the surgical procedure. The most common of the serious adverse events were seizures, and, to a much lesser extent, myoclonus or anemia.

One serious event resulted in withdrawal of a subject from the study. BD-04, an 8-year-old girl with severe LINCL, had an uneventful postoperative course, and was discharged from the hospital on day 7. On day 14 after vector administration, she developed status epilepticus. Although the seizures could be controlled by pentobarbital coma, the subject could not thereafter regain consciousness without reverting to status epilepticus. Her family eventually decided to decline further treatment and she died 49 days after vector administration. There was no evidence of CNS inflammation in the cerebral spinal fluid 15 days after vector administration (i.e., normal cell counts, protein, and glucose, and no detectable bacteria or viruses), or in CNS by FLAIR MRI on days 1, 21, and 44 after vector administration. Because seizures are a part of the natural history of LINCL, it is unclear to what extent this event was related to the underlying disease, the surgical procedure, or the experimental drug itself. In response to this event, with the advice of the U.S. FDA, the study design was modified for all subsequent study subjects (n = 6) to include EEG monitoring and assessment of the levels of antiseizure medications pretherapy and 14 days after therapy. No subjects had electrographic seizures pre- or posttherapy while being monitored. The pretherapy EEGs showed generalized moderate- to high-amplitude spike and wave discharges with maximum amplitude occipitally. The background was characterized by diffuse 3–to 5-Hz rhythmic activity and features of normal sleep patterns were generally preserved. This EEG background did not change significantly posttherapy. The mean spike frequency over 1 hr in six subjects before gene therapy was 56.6 ± 15.5 (SD) and the mean spike frequency 2 weeks after gene therapy was 38.8 ± 16.7 (SD), which was significantly decreased compared with pretherapy (paired samples t test, p < 0.007). Thus, although it is possible the seizures starting on day 14 in subject BD-04 were linked to the vector, assessment of seizure activity on day 14 in the subsequent six treated subjects showed no increased propensity toward seizure activity.

Subject BD-06 died 704 days after vector administration. Although beyond the 18-month follow-up period for the primary and secondary variables, the death was identified in the post-study yearly follow-up call (see Table 2). This subject was not included in the efficacy analysis, as the subject did not return for visits subsequent to 1 month posttherapy (see Supplementary Table III, footnote 8). For both subjects who died, the families did not agree to an autopsy, and thus brain tissue was not available for verification of gene transfer by DNA/RNA-based methods or by detection of TPP-I protein.

**Anti-AAV antibodies**

Before gene transfer, no subjects had detectable serum anti-AAV2 neutralizing antibodies (Fig. 1). Four of the 10 subjects (3 severe and 1 moderate) developed a mild humoral immune response to the AAV2 capsid after CNS administration of the AAV2CUhCLN2 vector. In no subject did the anti-AAV neutralizing titer rise to >270. For two of the four subjects who developed detectable anti-AAV2 antibodies, the response was within 1 month, and for the other two, it was delayed, and not observed until 6 months posttherapy. For three of the four who developed anti-AAV2 antibodies, the titers returned to baseline by 18 months posttherapy, remaining mildly elevated in only
one subject (BD-03). Thus, CNS administration of the AAV2CUhCLN2 vector to this population results in only a mild, mostly transient systemic antivector humoral immune response.

**Disease progression assessed by CNS imaging**

The rates of change for all treated subjects were calculated from the raw data (see Supplementary Table IVB) and the means and standard errors were determined. For all three of the MRI parameters, the measured rates of decline of the treated subjects were slower, albeit the numbers were too small for statistical significance. For the gray matter volume as a percentage of total brain volume, for the six treated children for whom data were available for >6 months, there was a trend, although not significant, toward reduction in the mean rate of change ($-2.6 \pm 0.7\%$/year) compared with that of the four untreated children ($-2.84 \pm 1.3\%$/year; Fig. 2A and [see Supplementary Table IVA and B]; $p = 0.8$ by Mann–Whitney test). Likewise, the mean rate of change of the ventricular volume of the seven treated children for whom data were available ($14.0 \pm 4.1$ cm$^3$/year) showed a trend toward reduction in the mean rate of change over time compared with that of the four untreated children ($17.2 \pm 4.7$ cm$^3$/year; Fig. 2B and [see Supplementary Table IVA and B]), although this did not achieve statistical sig-
nificance (p = 0.5 by Mann–Whitney test). Consistent with these data, the CADC for the eight treated children with data available for 6 months showed a mean rate of change \[0.034 \pm 0.015 \text{ mm}^2 \text{ (10}^{-3})/\text{sec \cdot year}\] that trended toward being slower than for the untreated control subjects \[0.083 \pm 0.023 \text{ mm}^2 \text{ (10}^{-3})/\text{sec \cdot year}\] (Fig. 2C [and see Supplementary Table IVA and B]), although this difference was not statistically significant (p = 0.1 by Mann–Whitney test).

Disease progression assessed by the primary variable

At baseline, all study subjects had neurological abnormalities, with the extent of the abnormalities dictating the severity category (moderate or severe) of the disease (see Supplementary Table V at www.liebertonline.com/hum). Typically, there was decreased strength in the upper and lower extremities, increased muscle stretch reflexes, an upward plantar response, and other motor sys-

FIG. 1. Anti-AAV2 neutralizing titers after gene transfer with AAV2_CuCLN2. Serum samples were taken at intervals before and after administration of the vector. The anti-AAV2 neutralizing titer was assessed by determining the dilution of serum required to inhibit in vitro gene transfer by 50%. Open symbols represent the pretherapy values; solid symbols represent posttherapy values.

FIG. 2. Quantitative assessment of CNS MRI imaging. MRI parameters were assessed on the screening visit, the day before treatment, and 6 and 18 months after treatment. Details regarding the data points available for analysis may be found in Supplementary Table IVA (see www.liebertonline.com/hum). Data are presented as the change in the parameter relative to mean baseline values. Raw data are provided in Supplementary Table IVB (see www.liebertonline.com/hum). If more than one baseline value was available and was obtained within 4 months of treatment, the average of the baseline values was used. Four untreated LINCL subjects, for whom two assessments done ~1 year apart were available, acted as control subjects. (A) Mean rate of change in gray matter volume (expressed as a percentage of total brain volume); (B) mean rate of change in ventricular volume; (C) mean rate of change in cortical apparent diffusion coefficient. Rates of change for the treated subjects were compared with the mean rate of change for the combined control group for each of the MRI parameters by Mann–Whitney test. CADC, cortical apparent diffusion coefficient.
tem abnormalities, including gait abnormalities, myoclonus, tremors, and swallowing dysfunction. These features were more pronounced in the subjects with severe disease.

The modified Hamburg LINCL clinical rating scale was the primary variable used to assess effects of the treatment on the slowing of the progression of the disease (see Supplementary Table III). The modified Hamburg rating was assessed both as a function of age (Fig. 3A) and as a function of time after treatment (Fig. 3B). To assess the impact of treatment (Fig. 4), the mean rate of change in modified Hamburg scale was compared with that in a combined control group consisting of (1) four untreated control subjects who were monitored for 1 year without therapy and (2) the historical data from the Steinfeld and coworkers study (2002), employing only the parameters used in the modified Hamburg scale, giving a mean rate of $-1.8 \pm 0.48$/year (standard error). The decline per year for the eight treated subjects for whom data were available for $>6$ months was significantly slower in comparison with the decline for the combined control groups This was the case when the slopes of decline of the treated children were calculated using just the pre- and 12-month data points (to match the times of assessment with that of the four untreated control subjects; mean rate of change, $0.0 \pm 0.30$/year; $p < 0.01$ by Mann–Whitney test) or when the slopes of decline were calculated using all the data from the pretherapy visit through the 18-month observation points (mean rate of change, $-0.33 \pm 0.29$/year; $p < 0.05$ by Mann–Whitney test).

**DISCUSSION**

Gene transfer with adeno-associated viral vectors represents a logical approach to treat the CNS manifestations of the neu-

![A](image1)

**FIG. 3.** Progression of LINCL in the 10 treated subjects as a function of time as determined by the modified Hamburg LINCL scale, the primary outcome variable. (A) Data pre- and posttherapy, shown as a function of the age of each subject. Symbols for each subject are identical to those in Fig. 1. The gray shaded area represents the 5th to 95th percentile area of untreated children with LINCL from Germany and Switzerland published by Steinfeld and coworkers (2002), modified to eliminate the ophthalmologic parameter (Crystal et al., 2004; Steinfeld et al., 2002). * represents the death of subject BD-04. The dashed line represents the median. Open symbols represent the pretherapy values and solid symbols represent posttherapy values. (B) Data pre- and posttherapy, assessed as a function of time before and after vector administration. The data are the same as in (A), but shifted to time 0 as the time of administration. The time of treatment is indicated by the vertical dashed line. Symbols for each subject are identical to those in (A).

rological lysosomal storage disorders (Skorupa et al., 1999; Bosch et al., 2000; Frisella et al., 2001; Sondhi et al., 2001, 2005; Janson et al., 2002; Haskell et al., 2003; Cressant et al., 2004; Passini et al., 2005; Griffey et al., 2006). On the basis of extensive supporting animal data (Hackett et al., 2005; Sondhi et al., 2005; Passini et al., 2006), the present study establishes the feasibility of treating LINCL by AAV2-mediated gene transfer. A strategy was developed to administer the AAV2CUhCLN2 vector through 6 burr holes to 12 sites in the cortex to a total of 10 children with LINCL, 5 with severe and 5 with moderate disease. Serious adverse events were observed, but all were anticipated and none could be definitively linked to the gene transfer vector per se. Although the control groups were not matched, randomized, placebo/sham or blinded, and the numbers are small, assessment of the decline in the modified Hamburg LINCL clinical rating scale (the primary variable) in the treated subjects monitored for $>6$ months suggests that the therapy is associated with a slowing of disease progression. With the caveats that the small numbers of subjects and control subjects preclude more in-depth analyses of the effects of genotype, concurrent medications, severity, and other parameters on the outcome, the data support the hypothesis that AAV-mediated CNS gene transfer can slow the rate of progression of the CNS manifestations of the disease, and support the concept that additional studies using AAV-mediated CNS gene transfer to treat the CNS manifestations of LINCL are warranted.

**Safety**

Preclinical toxicology studies in experimental animals demonstrated no safety issues regarding the use of the AAV2C-
Challenges for successful gene therapy for LINCL

With rare diseases such as LINCL, the design of clinical studies is challenged by the paucity of data on the natural history of the disease and the logistical and ethical difficulty of designing a controlled study. In this context, assessment of new therapeutics in rare, childhood genetic disorders such as the LINCL is a dilemma. Like most CNS disorders, there are no biomarkers that can be used to assess therapy in the brain. Because the therapy requires direct CNS administration by a multihour neurosurgical procedure, it is challenging to design a contemporaneous, matched, randomized, blinded or placebo controlled study. In the absence of such a control group, rigorous statistical assessment of the outcome data in the present study is not possible. The data are further confounded by factors such as differences among individuals regarding genotype, standard of medical care, and other undefined factors. Thus, it was only possible to gain insight into the suggestive effect of therapy on progression of the disease. With the caveats that the control group was limited to four untreated subjects assessed twice at an interval of 1 year plus the published data of Steinfield and coworkers (2002), assessment of the change of clinical rating scale in the treated group suggests, but does not prove, that CNS administration of the AAV2CUhCLN2 vector might provide some therapeutic benefit.

It is of interest that although there was evidence of statistically significant slowing of the decline of the disease as assessed by the primary variable, the clinical rating scale, the quantitative MRI parameters suggested, but did not prove, a difference compared with the control subjects. Because there are no biochemical markers for the disease, imaging represents a rational choice for secondary parameters to complement the clinical rating scales (Ramirez-Montealegates and Pearce, 2007; Worgall et al., 2007). However, in diseases such as cerebral palsy and Parkinson’s disease, and in our studies of LINCL, the correlation between clinical and neuroimaging results based on the current methods is variable (Bohnen et al., 2007; Worgall et al., 2007; Korzeniewski et al., 2008). In this regard, it would be useful for the design of future therapeutic studies to develop less variable high-resolution MRI or other imaging parameters that are sensitive to the disease process.

Successful gene therapy for the CNS manifestations of LINCL requires vector-driven expression of TPP-I in the CNS that is of a sufficient level, distribution, and duration to treat 49 days after vector administration. An extensive review was inconclusive as to the cause, that is, whether the onset of the seizure activity was related to the progression of the disease, the surgical procedure, or the vector itself. The lack of evidence of inflammation in the cerebrospinal fluid and by MRI argued against the vector as a cause. Of note, the LINCL-affected, but untreated younger sibling of that patient died at approximately the same age 2 years later. Seizures are a cardinal feature of LINCL and intractable seizures and status epilepticus are a known feature of advanced LINCL (Boustany, 1996). Assessment of 24-hr continuous EEG at 14 days posttherapy (the timing of the onset of the seizures in the subject who died) in the six subsequent subjects who underwent treatment showed a decrease in subclinical seizure activity after therapy compared with the same subjects pretherapy.
the disease. The requirement for long-term expression comes from the knowledge that the accumulation of the lysosomal storage defect is progressive. The range of required therapeutic levels of expression can be estimated from the observation that affected children with ~5% of normal TPP-I activity have a late-onset variant of the disease and that heterozygous carriers are normal (Sleat et al., 1999). These requirements were met in the present study by using doses of the AAV2/3-herCLN2 vector that, scaled from doses used in mice and monkeys, should provide long-term expression in the human CNS of physiological levels of TPP-I protein, at least in the general proximity of the sites of vector administration. The major challenge for successful therapy of LINCL is the requirement for wide distribution of the therapy within the brain, based on the fact that LINCL affects the brain diffusely (Boustany, 1996; Williams et al., 1999; Haltia, 2003). On the basis of safety constraints limiting the volume that could be administered per unit time, the limits on the concentration of the vector to avoid aggregation, and the number of burr holes, we designed the vector administration to be through six burr holes with vector deposition at two depths per burr hole. With these physical limitations, it is likely that the goal of spreading the vector over as wide a volume of brain as possible will likely come from vector design, that is, by developing new gene transfer vectors that distribute more widely through the CNS.

Although the data in the present study cannot prove we achieved the levels, duration, and distribution of TPP-I expression in the treated children to provide a true therapeutic benefit, we suggest that the data provide a rationale for developing a second-generation clinical trial of AAV gene transfer in individuals with LINCL. On the basis of studies in rodents, non-humans primates, and cats, it is likely that the greatest impact may be obtained from the use of serotypes of AAV such as 1, 5, 8, 9, or rh.10, all of which provide much higher levels and a much wider distribution of transgene expression in the brain than the AAV2-based vector used in the present study (Vite et al., 2003; Bunnell et al., 2005; Broekman et al., 2006; Cearley and Wolfe, 2006; Passini et al., 2006; Cabrera-Salazar et al., 2007; Sondhi et al., 2007a). On the basis of the suggestive evidence of an impact on disease progression obtained in the present study with an AAV2 serotype-based vector, these second-generation AAV vectors are now prime candidates for the development of a new clinical trial for the treatment of the CNS manifestations of LINCL. Further, on the basis of LINCL knockout mouse data published by Cabrera-Salazar and coworkers (2007), using an AAV1-based vector, and our similar data with AAVrh.10-based vector (Sondhi et al., 2007b), an enhanced therapeutic outcome will likely be achieved by treating the disease as early as possible. For a second-generation clinical trial of AAV gene transfer we would also propose the use of a newly developed clinical severity scale, which correlated better with disease progression in individuals with LINCL (Worgall et al., 2007).

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**AUTHOR DISCLOSURE STATEMENT**

No competing financial interests exist.

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