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Successful Peripheral T-Lymphocyte–Directed Gene Transfer for a Patient With Severe Combined Immune Deficiency Caused by Adenosine Deaminase Deficiency

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Ten patients with adenosine deaminase deficiency (ADA) have been enrolled in gene therapy clinical trials since the first patient was treated in September 1990. We describe a patient who had received periodic infusions of genetically modified autologous T lymphocytes transduced with the human ADA cDNA containing retroviral vector LASN. The percentage of peripheral blood lymphocytes carrying the transduced ADA gene has remained stable at 10% to 20% during the 12 months since the fourth infusion. ADA enzyme activity in the patient's circulating T cells, which was only marginally detected before gene transfer, increased to levels comparable to those of a heterozygous carrier individual and was associated with increased T-lymphocyte counts and improvement of the patient's immune function. The results obtained in this trial are in agreement with previously published observations and support the usefulness of T-lymphocyte–directed gene transfer in the treatment of ADA–SCID.

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Heights, IL) with [35S]dATP (Amersham Life Science) and a series of ADA-specific primers. Amplified products were sequenced through a 6% acrylamide gel (National Diagnostics, Atlanta, GA). To analyze the ADA genomic sequence, high molecular DNA was obtained from B-LCL by standard techniques. Primers and PCR conditions for amplification of ADA all exons have also been described previously. Amplified products were isolated from agarose gel and sequenced directly using the Thermal Cycler DNA sequencing kit (Circumvent; New England Biolabs Inc, Beverly, MA). ADA cDNA sequences are numbered relative to the start site of translation and genomic DNA according to Wiginton et al.

**Southern blot analysis.** High molecular weight DNA from B-LCL was digested with restriction endonuclease Rsa I, separated in 1.0% agarose gel, and transferred onto a nylon membrane (Biotrace HP; Heights, IL) with [35S]dATP (Amersham Life Science) and a series of probes randomly labeled 444-bp Rsa I-Pst I fragment from the ADA cDNA. 

**Retroviral-mediated gene transfer into patient’s peripheral T cells.** The clinical protocol used here has been described elsewhere. Briefly, peripheral T lymphocytes from the patient were obtained by apheresis (CS3000 plus, Baxter Corp, Chicago, IL), isolated by density gradient centrifugation, and then maintained in AIM-V medium (GIBCO-BRL) supplemented with 5% FCS (GIBCO-BRL), 100 mmol/L of recombinant human IL-2 (rIL-2, SHIONOGI, Osaka, Japan) and 10 ng/mL of anti-CD3 antibody (Orthoclone OKT3 Injection; Ortho, Raritan, NJ) in gas-permeable culture bags (Nipro Pretobag; Nishyo, Osaka, Japan). After 72 hours, half of the medium was removed and replaced with supernatant containing the LASN retroviral vector. 

**Identification of mutations responsible for ADA deficiency.**

To analyze mutations in our patient, we amplified full-length ADA cDNA from the patient’s EBV transformed B-LCL by RT-PCR. Sequence analysis revealed that all of the clones (66) carried a G602 to A transition resulting in replacement of the arginine residue by histidine at codon 211 (Fig 1A). The mutation eliminates a recognition site for the restriction enzyme Rsa I. We took advantage of this feature to distinguish the mutated allele from the normal allele. High molecular weight DNA extracted from the patient’s B-LCL was digested with Rsa I, blotted and hybridized to an ADA cDNA probe spanning the region from this mutation site in exon 7 to the end of exon 8, respectively. Using these primers, the amplification of DNA samples from vector-containing cells generates two bands; the larger one (250 bp) derived from the endogenous ADA gene containing intron 7 and the smaller one (174 bp) from the LASN provirus. To evaluate the frequency of transduced cells in the patient’s peripheral blood, a standard curve was prepared from a serial dilution of in vitro-transduced and G418-selected B-LCL with untransduced cells. The ratio of the amount of amplified ADA cDNA derived from the integrated vector and the amplified genomic sequence was calculated after hybridization with an ADA cDNA probe.

**Thin-layer chromatography (TLC) analysis of ADA enzyme activity.** Mononuclear cells were washed twice with phosphate-buffered saline to remove FCS and then suspended in 100 mmol/L Tris, pH 7.4 containing 1% bovine serum albumin. Cell lysates were obtained by 5 rapid freeze-thaw cycles. Cellular debris was removed by centrifugation and the lysates were stored at −80°C until used. ADA enzyme activity was assayed by the measurement of the conversion of [14C] adenosine (Amersham Life Science) to [14C] inosine and [14C] hypoxanthine followed by TLC separation of the reaction products performed as previously described. The results were expressed as nanomoles of inosine and hypoxanthine produced per min by 10^6 cells (nmol/min/10^6 cells).
lyzed exons 1 to 11 by PCR amplification of genomic DNA and direct sequencing. Sequence analyses of the amplified fragments including exon 2 showed the patient to be heteroallelic for a splice site mutation at the first position of intron 2 (G111 = A transversion) (Fig 2A). This mutation eliminates a recognition site for the restriction enzyme BspMI. BspMI digestion showed that the patient and his mother were heterozygous for this mutation, while the father showed a normal digestion pattern (Fig 2B). Reports of mutation analyses of other patients have shown that a mutation affecting a mRNA splicing mechanism may give rise to a nonfunctional or unstable mRNA.26,27 This mechanism is also supported by the fact that RsaI digestion showed that all full-length cDNA clones (48/48) from the patient’s B-LCL carried the paternal G632 to A missense mutation.

Retroviral mediated gene transfer into peripheral T cells. At the age of 4, the patient was enrolled in a clinical gene therapy trial that repeated the protocol of the first gene therapy experiment at the National Institutes of Health (NIH) in 1990.22 The patient’s peripheral mononuclear cells, obtained by apheresis, were stimulated with IL-2 (100 U/mL) and anti-CD3 antibody (OKT3; 10 ng/mL). After 72 hours of stimulation, they were transduced twice during the next 48 hours by exposure to the ADA retroviral vector LASN, expanded 20- to 50-fold in number by culturing for 6 days after the beginning of transduction, and then reinfused into the patient (see Materials and Methods). No selection procedure to enrich for gene-transduced cells was performed. Semiquantitative PCR of the cells in the first and second infusions revealed that the frequency of the vector-carrying cells ranged from 3% to 7% (data not shown).

Clinical course after gene therapy. The patient received a total of 10 infusions over the 18-month period (Fig 3). A striking increase in lymphocyte number was observed early in the trial, followed by a gradual return to the basal level. This was followed by a sustained increase after the 8th infusion (protocol day 322) and the patient’s PBL count has since remained in the normal range (PBL, 1,980/µL; CD3, 1,822/µL; CD4, 240/µL; CD8, 1,538/µL; CD19, 154/µL on protocol day 429). Progressive inversion of CD4/CD8 ratio has been observed since the 4th infusion due to an increase of the absolute CD8+ cell count. This phenomenon is thought to be the result of preferential proliferation of CD8+ cells during in vitro culture and transduction. ADA enzyme activity, nearly undetectable in the patient’s lymphocytes before gene therapy, also increased progressively after the 7th infusion (protocol day 252) and reached 27 U on protocol day 476, which is approximately comparable to that of a heterozygous carrier individual (the patient’s mother, 34.8 U). The number of transduced cells in the patient’s peripheral blood were assessed by semiquantitative PCR using PBL obtained before each infusion (Fig 4). The frequency of the genetically modified cells increased with the number of infusions of the ADA gene transduced lymphocytes and exceeded 10% of total circulating mononuclear cells just before the 5th infusion (on protocol day 126; Fig 4, lane 4). The frequency
remained normal for more than a half year without additional IVIG treatment (Fig 3 and Table 1). These results suggest that the accumulated genetically corrected T lymphocytes in the patient’s peripheral blood are associated with improvement of cellular and humoral immune responses and an increase in his circulating lymphocyte count. Although he sometimes became transiently febrile after infusions, the patient showed no serious adverse reactions to the treatments.

**DISCUSSION**

Advances in molecular biology during the past 3 decades have suggested that gene transfer could provide a new approach to the treatment of inherited diseases as well as acquired disorders such as cancer and acquired immune deficiency syndrome. The number of active gene therapy protocols has increased greatly since the first clinical gene therapy trial. ADA−SCID is one of the few early candidate disorders suitable for such interventions. Accordingly, 10 ADA−SCID patients have been enrolled in gene therapy clinical protocols that employed different strategies, retroviral vector designs, and target cell populations. The results obtained from these trials have recently been reported.

This trial of gene therapy for an ADA−SCID patient in Japan began in August 1995. Over the next 18 months he received a total of 10 infusions of cultured-expanded autologous T cells that had been transduced with the LASN retroviral vector. After an initial period of fluctuating counts, the patient’s T cells stabilized in the normal range and this has been sustained for the last half year. The frequency of integrated provirus in the patient’s peripheral blood increased to approximately 15% (0.1 to 0.2 proviral copies/cell) by the 4th infusion and has remained stable since that time. The patient’s cell associated adenosine deaminase enzyme activity has increased from barely detectable before treatment to values approaching those found in the peripheral mononuclear cells of his heterozygous carrier mother. Delayed hypersensitivity skin tests, a measure of T-cell function, have improved. Isohemagglutinin titers have also increased and his dependence on infusions of normal gammaglobulin has eased. The patient has gained 3 kg in weight during this trial. He is still receiving periodic PEG-ADA replacement and is attending public school with no more infections than his classmates.

The period of observation has simply not been sufficient to assess the full breadth or the duration of this improved clinical status and immune responsiveness. Further, additional studies will be required to reconcile the apparent dissociation between the level of T-cell ADA observed and the proportion of cells containing integrated vector at different time points. Also, the effect of withdrawal of the exogenous PEG-ADA treatment must await more complete characterization of the quality of the patient’s immune system and the repertoire of specificities represented in the transduced T-cell population.

Four gene therapy clinical trials including 10 ADA−SCID patients have been performed since the first trial in 1990. Although these trials provided much data that suggested how future gene therapy might be improved by changing retroviral vector design, transduction methods and target cell populations, we found it difficult to compare the efficacy of these various trials because of differences inherent within these basic strate-
gies. Our trial has been performed following the identical protocol and vector preparations and autologous T lymphocyte isolation procedures that were used in the NIH trial. From this perspective, our trial provides an additional opportunity to evaluate the effectiveness of peripheral T lymphocyte-directed gene therapy for ADA-SCID patients. Interestingly, the clinical course of our patient is quite similar to that observed in patient 1 in the NIH trial. Both trials have shown high gene transfer efficiency, remarkable increase of the ADA enzyme activity and eventual improvement of immune function. In contrast, patient 2 in the NIH trial experienced a low gene transfer efficiency and no significant increase in the ADA enzyme activity even though she exhibited some increase in immunological function. Although the factors leading to this difference have not yet been completely identified, a striking difference in the transduction efficiency of peripheral T cells between the three patients may be relevant. Transduction efficiencies before infusion were 3% to 7% for the present case, 1% to 10% for patient 1 and 0.1% to 1% for patient 2 in the NIH trial. An abbreviated proliferative capacity of patient 2 in the NIH trial was also observed. In addition, a contribution of the development of an immune response to the neomycin resistance gene must be considered since the existence of dominant selectable markers of nonhuman origin may result in unwanted immune reactivity that could eliminate or functionally impair transgene-expressing cells.31

The severity of the underlying ADA gene defects could also affect gene transfer. In addition to the mutation analysis reported here, specific ADA gene defects have also been reported for the two NIH patients.20 These three cases can be classified by the severity of their clinical presentation. Both the present case and patient 1 in the NIH trial are of the “delayed onset” type, have splice site mutation defects and have achieved significant levels of “gene-corrected” circulating cells. However, the NIH patient 2 carries compound missense mutations and has manifested low transduction efficiency despite her less severe “late onset” type of presentation at age 5. Although there are insufficient numbers of treated patients to draw firm conclusions at this point, it does appear thus far that the responses of patients with “more severe” gene defects and clinical presentations are at least as responsive as cases with “milder ADA defects.”

It should be noted that the ADA gene transduced T lymphocytes possess a selective advantage over the nontransduced cells due to the latter’s high intracellular concentration of deoxyadenosine.32,33 In the ADA-SCID newborn trial using gene-corrected CD34+ cells obtained from the patient’s umbilical cord blood,16 LASN vector was detected in the peripheral blood T cells of these patients at a stable frequency of approximately 0.01% during the first 18 months of observation. Then, after a 50% reduction in their weekly dose of PEG-ADA, the proportion of
GENETIC CORRECTION OF ADA−SCID

Table 1. Isohemagglutinin Titer, DTH Skin Test Reactivity, and Ig Levels Before and After Gene Therapy

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<td>Isohemagglutinin titer</td>
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<td>IgA</td>
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<td>IgM</td>
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<td>DTH skin test (mm)</td>
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Table 1. Isohemagglutinin titer and DTH skin tests were tested using standard protocols before gene therapy (before) and at 11 months after the beginning of gene therapy (after) while on PEG-ADA. The patient serum Ig levels were measured just before the Ig replacement on protocol day – 60 (before) and 478 (after). The patient received the last Ig replacement at protocol day 348.

*The patient had been immunized with BCG at 5 months of age.

ADA vector-containing T cells in the blood increased to approximately 10% in each case (D.B. Kohn, personal communication, September 1995). In the present case, the dosage schedule of PEG-ADA enzyme has remained constant since the beginning of the trial (18 U/kg/wk on the protocol day 431), during which time the patient’s immune function has substantially improved. It might be expected that the proportion of the transduced cells in the patient’s PBL will increase as the PEG-ADA dosage is decreased.

To date, three clinical trials have been performed to assess the possibility of treating ADA−SCID patients by correcting hematopoietic progenitor cells.15−17 The results obtained from these trials suggest that cord blood provides a stem cell population more suitable for efficient retroviral-mediated gene transfer than does bone marrow. Taken with the observations made in the NIH trial, our results strongly suggest that the effectiveness of T lymphocyte-directed gene transfer is a viable addition to the treatment programs that should be considered for ADA−SCID patients. After additional courses of treatment and continued observation to determine the breadth and durability of these positive responses, we hope to reduce or eliminate exogenous ADA enzyme supplementation in this patient. Improvements in vector design to permit higher levels of ADA expression and innovative strategies that provide greater efficiency of stem cell gene transduction may make gene therapy the treatment of choice for ADA−SCID patients.

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