T Lymphocyte-Directed Gene Therapy for ADA SCID: Initial Trial Results After 4 Years

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In 1990, a clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of two children with severe combined immunodeficiency (ADA- SCID). The numberof bloodTcells normalized as did many cellular and humoral immune responses. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Although many components remain to be perfected, it is concluded here that gene therapy can be a safe and effective addition to treatment for some patients with this severe immunodeficiency disease.

The possibility of using gene transfer as a therapy for human disease has great appeal, The decision to enter clinical trials awaited the development of safe and efficient techniques of gene transfer and improved understanding of the basic pathology and biology underlying likely candidate diseases and target cells. The advent of useful retroviral vectors that permitted relatively high efficiency gene transfer and stable integration was a critical advance (1, 2), as was the demonstration that this procedure of gene transfer could be effectively and safely used in humans (3).

Severe combined immunodeficiency secondary to a genetic defect in the purine catabolic enzyme adenosinc deaminase [ADA- SCID] is characterized by defective T and B cell function and recurrent infections, often involving opportunistic pathogens. Large amounts of deoxyadenosine, an ADA substrate, are present in these patients; dcoxyadenosine is preferentially converted to the toxic compound deoxyadenosine triphosphate in T cells, disabling the immune systera (4).

Because this disease is curable by allogcneic bone marrow transplantation given without pretransplantation cytoreductive conditioning, it was initially assumed that gene therapy should be directed at the bone marrow stem cell. However, initial attempts to use stem cell gene transfer in primates resulted in only low-level, transient gene expression, insufficient for clinical use. The observation that the only donor cells detected in some patients "cured" by allogeneic bone marrow transplantation was their T cells-the others remaining ADA-deficient (5)-raised the possibility that T celldirected gene therapy also might be a useful treatment.

The introduction of enzyme replacement with ADA-containing erythrocytes (6) or with bovine ADA conjugated with polyethylene glycol (PEG-ADA) (7) has made this approach feasible. PEG-ADA has provided noncurative, life-saving treatment for ADA - SCID patients; with this treatment, most patients have experienced weight gain and decreased opportunistic infections. Full immune reconstitution has been less regularly achieved with enzyme therapy. T cell functiou as measured by in vitro mitogen responses improved in most patients, but fewer patients recovered consistcnt immune responses to specific antigens [for instance, as measured by normal delayed-type hypersensitivity (DTH) skin test reactivity] (8-10). Nearly all PEGADA-treated patients showed increased peripheral T cell counts, which provided a source of T cells for gene correction not available without enzyme therapy. Furthermore, enzyme treatment could be continued during the gene therapy trial so that the ethical dilemma of withholding or stopping a life-saving therapy to rest an unknown treatment could be avoided.

The adenosine deaminase complementary DNA (cDNA) (11) is 1.5 kh and fits within a retroviral vector. With the use of an ADA-containing retroviral vector, ADA-deficient T cell lines were transduced to express normal amounts of ADA; this rendered them normally resistant to intoxication and growth inhibition when challenged with deoxyadcnosine (12, 13). Next, studies in mice, rabbits, and nonhuman primates using T cells modified with retroviral vectors showed normal cell SUrvival and function after their reintroduction into recipient animals (14). Finally, Bordignon and colleagues (15) showed that ADA gene-corrected T cells acquired a survival advantage compared with uncorrected ADA-deficient cells when transplanted into itnFuLmodeficient, but ADA-normal BNX recipient mice.

The clinical protocol used here has been described elsewhere (16). Patients with documented ADA SCID were eligible if they did not have a human lymphocyte antigcn-niatched sibling as a potential donor for marrow transplantation and if they had been treated with PEG-ADA for at least 9 months without full immune recon-StitUtion. T cells were obtained from their blood by apheresis, induced to proliferate in Culture, transdUced with the ADA retroviral vector LASN, culture-cxpanded, and then rcinfused into the patient after 9 to 12 days (17). No selection procedure was used to enrich for gcne-transd i iced cells.

Fig. 1. Peripheral blood T cell counts since the time the diagnosis of ADA deficiency was made, dates of treatments, and the total number of cells infused for each patient. ADA level is measured in nanomoles of adenosine deaminated per minute per 1011 cells. Vertical bars indicate the dates of cell infusion, and their height represents the total number of nonselected cells in fused at each treatment. The T cell numbers represent total CD3-bearing T cells determined by standard flow cytometric analysis. (A) Patient I began gene therapy on 14 September 1990 (protocol day 0) and received a total of duplicate samples and represent EHNA-sensitive ADA enzyme activity. (13) 11 infusions. Cellular ADA enzyme level is indicated by the dashed line. ADA Patient 2 began gene therapy on 31 January 1991 (protocol day 0) and activity was determined as described (13, 25). Values shown are the mean ofreceived of a total of 12 infusions.

The clinical histories and ADA gene murations of each patient have been reported (18, 19). Patient I presented with infection at 2 days of age and had recurrent infections and very poor growth until 26 months of age, when the diagnosis of ADA deficiency was established and she was started on PEG-ADA [30 U per kilogram of body weight per week (30 U/kg/week)]. Treatment with PEG-ADA enzyme for approximately 2 years had resulted in significant, but incomplete, benefit. With PEG-ADA she gained weight, had fewer infections, and transiently developed a normal peripheral blood T cell count (Fig. IA), and her T cetls had acquired the ability to respond to mitogens in vitro. However, significant immune deficiency persisted, including recurrence of her T lymphopcnia (Fig. IA), DTH skin test ancrgy (Table 1), depressed in vitro i1mMine reactivity to specific antigens such as tetanus toxoid, failure to generate normal cytotoxic T cells to viral antigens or allogeneic cells, defective immunoglobulin production and absent or weak antibody responses to several vaccine antigens, and borderline isohelflagglUtinin titers (Table 0. At 4 years of age, she was enrolled in this trial.

The course of disease in patient 2 (who was 9 years old when enrolled in the trial) was milder than that seen in classic SCID (19), She had her first serious infection at age 3, and septic arthritis at age 5; the diagnosis was finally established at age 6 when significant lymphopcnia with ADA deficiency was confirmed. This patient had an excellent initial improvement in peripheral T cell numbers after the start of PEGADA therapy (30 U/kg/week) at age 5, but lymphopenia recurred in the third and fourth years of enzyme treatment (Fig. 113). During the year before gene therapy, repeatcd evaluation of her immune system showed persisting immunodeficiency, but less severe than that in patient 1. Despite 4 years of enzyme treatment, DTH skin test rcactivity was absent (Table I), cytotoxic T cells to viral antigens and allogeneic cells were deficient, and isohemagglutinins were barely detectable. However, illustrating the variability seen in the responses of patient 2 over time, blood lymphocytes that were cryopreserved from the day the clinical trial began and tested later showed normal cytotoxic activity to allogencic cells.

Within 5 to 6 months of beginning gene therapy, the peripheral blood T cell counts for patient I (Fig. IA) rapidly increased in number and stabilized in the normal range and have remained normal since that time (20). ADA enzyme activity, nearly undetectable in her blood lymphocytes initially, progressively increased in concentration during the first 2 years of treatment to reach a level roughly half the concentration found in heterozygous carriers (expressim, only one intact ADA atlele) and has remained at that level since (Fig. IA). Thus, both the reconstituted number of peripheral blood T cells and the elevated T cell ADA enzyme concentration have persisted since the patient's last treatment, indicating that peripheral T cells can have an unexpectedly long life-span and that gene expression from the retroviral vector has not been silenced over this period.

Table 1. DTH skin test reactivity and isohemagglutinin titers in sera of each patient at various times during the treatment protocol. Skin tests were applied as Multitest (Pasteur Meneux, Lyon, France) and scored according to the manufacturer's instructions 48 to 72 hours after being placed. Seven antigens were placed on the dates indicated, although only five were technically satisfactory on day 1252 for patient 1 and on day 1118 for patient 2. Isohemagglutinin titers were determined by standard blood bank techniques (34). Ninety five percent of normal children over the age of 2 years will have a titer of ~:1: 16 and 82% will have a titer *LE-1:* 32 (35). IND, not done. For the DTH skin tests, positive tests were elicited; T, tetanus toxoid; D, diphtheria toxoid; C, *Candida albicans; P,* Proteus antigen; S, streptococcal antigen; OT, old tuberculin.

Patient 2, who had variable immune rcactivity before enrollment, responded to the institution of lymphocyte infusions, with her peripheral T cell count rapidly increasing to levels in the high normal range (Fig [B), Beginning with infusion 5, which inClUdcd protocol modifications to partially deplete CD8 cells from the initially cultured cell population (21), her T cell Count fell into the mid-normal range, where it persisted throughout the treatment period and for a year after the last cell infusion. In contrast to those in patient 1, ADA enzyme levels in the circulating T cells of patient 2 did not rise significantly above the small amounts seen before gene therapy treatment (-1.5 nmol/10' cells per minute).

The differences in final lymphocyte ADA concentration are consistent with the levels of gene transfer reached in these patients. For several months in the second protocol year during which cell infusions were not given, LASN vector sequences detected by polymerase chain reaction (PCR) maintained a stable frequency in the peripheral

blood of patient I at a level greater than the PCRpositive control standard containing the equivalent of 0.3 vector copics/cell (Fig. 2). By contrast, although vector-contaiming cells were also stably detected throughout a similar period in patient 2, their level reached only a value equivalent to 0.1 to 1.0% of her circulating cells carrying the inserted ADA vector.

The principal contributor to the differencc in the final frequency of LASN vector-modified T cells in patients I and 2 was the low gene transfer efficiency in the cells of patient 2; this was consistently only a tenth or less of what was routinely achieved in the cells from patient 1. Despite the gross differences in the final proportion of vectorcontaining cells reached in these two patients, both CD4 and CD8 T cell populations from each have remained consistently positive for integrated vector sequences since the first infusion through protocol day 1480 for patient I and through protocol day 1198 for patient 2 (Fig. 2).

Fig. 2. PCR evaluation of the frequency of LASN vector-positive cells in the blood of patients 1 and 2 at various protocol days. (A) Cells from patient 1 for protocol days (D) 304 to 591 (see Fig. 1 A). PCR analysis was performed as described (26) in an ethidium-stained gel. (113) Cells from patient 2 for protocol days (D) 333 to 501 (see Fig. 13). PCR products were probed with 3 2 P-labeled neo gene as described (26). (C) Purified CD4' and CD8' cell subpopulations from patient 1 (D1480) and patient 2 (D1 198) prepared by separation of peripheral blood mononuclear cells (PBMCs) by fluorescence- activated cell sorting (FACS). The purity of the separated T cell subpopulations from which DNA was extracted exceeded 98%, as confirmed by FACS analysis. Direct PCR with [31 P]deoxycytosine triphosphate was performed as described (27). Standards (STD) were prepared from DNA obtained from cell mixtures of a known proportion of LASN-transduced cells containing a single vector insert mixed with vector-negative cells. C, vector-negative control cells.

To more accurately measure the proportion of vector-containing cells in patient 1, we performed quantitative Southern (DNA) hybridization analysis for vector sequence on DNA isolated from her peripheral blood T cells at different days during the course of this protocol. On protocol days 816 and 1252, which represent samples taken 109 and 545 days after the last treatment, the vector concentration was at the level of approximately one vector copy per cell (Fig. 3). Longitudinal studies of samples obtained rhroUghOUt the study show that this large amount of integrated vector was reached by infusion 8 (D707) and that it has remained in this range since that time (22).

The use of a restriction encionuclease that cuts only once within the vector sequence does not give detectable bands (Fig. 3), indicating that the population of blood T cells at these dates is not oligoclorial with respect to integrated vector. Vector-dcrived mRNA was readily detected by reverse transcription (RT)-PCR at these same tunes (Fig. 3), confirming that vector expression persisted and was correlated with the presence of ADA enzyme activity in her circularing T cells.

To evaluate the effect of gene therapy on the immune function of these two patients in addition to its beneficial effect on T cell numbers, we per-formed a panel of immunologic studies both before, and at various times after, treatment. DTH skin test reactivity to common environmental and vaccine antigens tests the overall competence of the cellular imrrrune system because a response depends on the full complement of cellular functions, not just cell proliferation or secretion of a single cytokine (Table I). Patient I was anergic before our protocol treatment despite nearly 2 years of PEG-ADA treatment. Eight months after the initiation of gene therapy (protocol day 251), she had a brisk DTH response to a single intradcrinal skin test with tetanus toxoid. By protocol day 455, DTH responses to five of seven antigens were present, and this increased responsiveness has persisted, through day 1252.

Fig. 3. Quantitative Southern hybridization analysis of DNA prepared from the blood mononuclear cells of patient I on protocol days (D) 816 and 1252 (28). DNA digested with Sst I should yield a single restriction fragment of 3.1 kb containing both the vector neo and ADA genes. Eco RI cuts only once within the vector sequence, and therefore a detectable band would indicate that a predominant clone with a single unique vector integration site was present in that blood sample. None was detected. Polyadenylated mRNA was extracted from the patient cells on days 0, 816, and 1252 and analyzed for vector message by RT-PCR (29). The primer locations used are indicated as short solid lines above the vector diagram. SV, SV40 early promoter; (A), polyadenylation site; 11, extended retrovirus packaging signal. Hatched regions indicate protein coding regions.

Before the protocol, patient 2 had no positive DTH skin test (Table 1). At protoc0l clay 501, five positive DTH skin tests were elicited, and this increased DTH reactivity had persisted when she was last tested on day 1118. She also acquired palpable lymph nodes and visible tonsils during the period of protocol treatment.

To corroborate the improved itrununе function indicated by these DTH tests, we evaluated the capacity of peripheral T cells from our patients to produce interleukin-2 (IL-2) or to kill antigenic target cells in vitro. In several patients treated with PEGADA, in vitro T cell proliferative responses to mitogcns may normalize,

whereas responses to specific antigens are less improved (710). During PEG-ADA treatment before gene therapy, T cells from patient I produced IL-2 in response to stimulation with the mitogen phytohcmagglutinin (PHA) (Fig. 4A) but were unable to produce IL-2 in response to stimulation with influenza A virus or tetanus toxoid, despite repeated irnmunization with these antigens. Over the first months of gene therapy, IL-2 production improved and became normal after I year (Fig. 4A). Again before gene therapy, patient I's T cells failed to show significant cytolytic reactivity against either allogeneic cells or influenza A-infected target cells, Almost mirroring the steady increase in IL-2 production, she acquired normal in vitro cytolytic T cell responses to these antigens, reaching normal values in her second year of treatment. (Fig, 4B).

Fig. 4. Evaluation of the in vitro cellular immune responses of blood T cells from patients 1 and 2 at various times before and during the gene therapy trial. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (A) Production of T-2 by cultured cells from patient 1 after stimulation with the mitogen PHA and with the specific antigens tetanus toxoid and influenza A virus as described (30). T-2 was quantitated by bioassay measuring the proliferation of the IL-2-dependent T cell line CTLL at a 1: 2 dilution of the lymphocyte culture supernatant. The fine dashed line indicates the patient's T cell count for reference. Solid triangles along the base line indicate the dates of cell infusion. (113) In vitro killing of a ~5'Crdabeled, influenza A-infected autoiogous B cell line and a ~,'Cr-labelecl allogeneic target B cell line by blood Tcells from patient 1 as described (31), Lysis (as percent specific isotope release during a 6-hour incubation of effector and target cells at a ratio of 60:1) was measured after in vitro pre-stimulation for 7 days. Solid triangles along the base line indicate the dates of cell infusion. (C) In vitro killing of a "'Cr-labeled, influenza A-infected autologous B cell and a 51 Cr-labeled allogeneic target B cell line by blood T cells from patient 2 as described above.

The results of these cytolytic assays for patient 2 are shown in Fig. 4C. Tests done 120 days before the beginning of gene therapy also showed impaired responses. However, cells that were obtained at the time of the first gene therapy infusion, cryopreserved, and subsequently tested some nionths later showed a normal cytolytic response to allogeneic cells. After a year on gene therapy, cytolytic T cell activity against influenza also became normal.

To evaluate the effects of our treatment on Immoral immune function in these patients, we measured antibody responses to several antigens. Despite their PEG-ADA treatment, both patients I and 2 had only low or borderline titers of isoheinagglutinins on repeated testing before gene therapy. Each patient showed significant clevations in the levels of these antibodies within 90 to H 5 days of beginning treatment with gene-modified cells (Table 1). lsohcmagglutinins are antibodies that react with group A and B red blood cell antigens and Occur spontaneously as a result of environ mental exposure to cross-reacting antigens. Isohernagglutinin responses are, therefore, less dependent on the timing of previous immunizations than are responses to contmon vaccine antigens. After gene therapy, each patient also had improvement in antiboJy responses to vaccines to *Hemophilits influenz,ae* B (HIB) and tetanus toxoid (Fig. 5). With enzyme therapy alone, peripheral lymphocytes from each patient were unable to produce irnmun0gl0bUIin M (IgM) in vitro after stimulation with pokeweed mitogcn (PWM), but made robust responses after a year on the gene therapy protocol (Fig. 5A). Immunoglobulin production to PWM depends on T cells; these results ftirther confirm the reconstitution of T cell function associated with gene therapy.

The effects of this treatment on the clinical well-being of these patients is more difficult to quantitate. Patient 1, who had been kept in relative isolation in her home for her first 4 years, was enrolled in public kindergarten after I year on the protocol and has missed no more school because of infectious disease than her classmates or siblings. She has grown normally in height and weight and is considered to be normal by her parents. Patient 2 was regularly attending public school while receiving PEG-ADA treatmentalone and has continued to do well clinically. Chronic sinusitis and headaches, which had been a recurring problem for several years, cleared completely a few months after initiation of the protocol.

This trial of retroviral-mediated gene transfer shows that the survival of reinfused transduced peripheral blood T cells is prolonged in vivo; the erroneous assumption that T cells Would not have Such long-term survival was often cited as a potential problem with this treatment strategy. Patient I has had a normal total peripheral T cell count since the last cell infusion, and the proportion of her circulating T cells carrying vector DNA has remained stable over that period. Further, expression of the ADA transgene under the influence of the retroviral long terminal repeat (LTR) promoter has persisted for a long period in vivo withOut Obvious extinction. There have been swings in the level of ADA enzyme in her peripheral lymphocytes throughout the period of observation, but the level of blood ADA enzyme activity at 4 years (protocol day 1480) is equivalent to that found immediately after the last cell infusion 2 years earlier (Fig. 1A). Although the data have not yet been completely analyzed, blood obtained after 5 years showed continuation of this trend with, again, a normal T lymphocyte count and an equivalent ADA level.

The rnechanism by which Our treatment aided immune reconstitution in patient 2 is less clear. The responses of patient 2 to some in vitro immunologic tests were variable before beginning our treatment protocol, ranging from little or no detectable response to nearly normal responses on the blood sample from the day gene therapy began. This patient produced a normal antibody response to iniritunization with bacteriophagc ~pX 174 about a year before beginning gene therapy (8). Although we have shown several examples of depressed cellular and humoral inimurie responses that strongly improved after gene therapy, this highly variable immune reactivity while patient 2 was on PEG-ADA therapy alone complicates interpretation of the contribution of our therapy. There was a temporal relation between initiation of gene therapy and a normalized peripheral T cell Count, improved DTH, appearance of tonsils and palpable lymph nodes, normalized isohemagglutinin response, and improved PWM response, as well as other factors. In view of the relatively low level of ADA gene transfer achieved in this patient, the potential contribution of the infusions of the culturc-activated T cells to the patient's response must also be considered. Perhaps ex vivo T cell activation somehow bypassed a differentiation block that PEG-ADA alone was unable to relieve. Despite the low final percentage gene transfer achieved, a I % level of ADA gene-corrected cells could represent 10' to 10" ADA-expressing T cells distributed throughout the body that could readily contribute to immune improvement.

Fig. 5. Humoral immune function of patients 1 and 2 before (solid bars) and after (hatched bars) gene therapy. (A) IgIVI production by the patient's peripheral blood mononuclear cells in cultures stimulated with the T cell-dependent polyclonal activator PWM performed as described (32). "Before" sai , iples were from Di Follow-up cultures were at D500 (patient 1) and D560 (patient 2). In each case, the patient's cells stimulated with the T cell-independent B cell stimulant EBV (33) produced normal amounts of gIVI (not shown), indicating intact B cell function before and after gene therapy, as expected. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (113) Serum antibody response to *Hemophilus influenzae* B. Patient 1 had failed to respond to two immunizations while on PEG-ADA alone [Di shown]. Her response at protocol D591 is shown, after immunization. Patient 2 had some HIB-specific antibodies present before therapy [D(- 122)], whose amounts increased without additional immunization during the protocol (D560). (C) Serum tetanus antibody. Patient 1 had negligible response to five separate tetanus immunizations before gene therapy [D(-48) shown] but responded briskly at D731, 24 days after reimmunization. Serum titers for patient 2 are shown for D(-9), 140 days after immunization while on PEG-ADA alone, and after receiving gene therapy 132 days after a booster tetanus immunization.

Since the beginning of the trial, the dose of PEG-ADA enzyme given to each of our patients has been decreased by more than half, (patient 1, 14 U/kg/week; patient 2, 10 U/kg/ week), during which time their immune function has improved. By contrast, worsened immune function has been seen in other ADASCID patients when their dose of enzyme has been similarly reduced (10, 23). We do not want to expose these patients to the potential risk of recurrent immunodeficiency by completely stopping PEG-ADA enzyme treatment until we have better information about the qualityand duration of the immune improvement achieved by this first-generation gene therapy trial. The role of continued exogenous enzyme treatment will be clarified here or in companion studies attempting stem cell gene correction (24).

The safety of rctroviral-mccliatcd gene transfer has been a central concern. At least in the short and intermediate terin, no problems have appeared in any clinical trial using these vectors. In the longer term, the theoretical potential for retroviral vectors to cause insertional mutagenesis remains the primary concern. To date, there has been no indication that malignancy associated with this process will be a complication of retroviral-mccliated gene transfer.

Our trial here has demonstrated the potcntial efficacy Of using gene-corrected antologous cells for treatment of children with ADA SCID. Eleven children with this disease have been enrolled in various gene therapy protocols, each using different strategies and retroviral vector designs and focusing on different target Cell Populations. The experience gained from these approaches should provide guidance for gene therapy as a treatment for this disorder as well as for a larger array of inherited and acquired diseases.

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17. Peripheral T cells from the patients were collected by apheresis, isolated by density gradient centrifugation, washed extensively, and then cultured in 24well culture plates in medium supplemented with 100 to 1000 lUlml of recombinant IL-2 and 10 ng/ml of Ol to stimulate T cell proliferation. After 24 hours, half the medium was removed and replaced with supernatant containing the LASN retroviral vector supplemented with IL-2 and protamine (10 fLg/ ml) to give an initial multiplicity of infection of 1. The LASN vector contains the human ADA cDNA under the transcriptional control of the promoter-enhancer in the retrovlral LTR and a neomycin phosphotransferase gene (neo) controlled by air internal SV40 promoter F. A. Hock, A. D. Miller, W. R. A. Osborne, *Blood 74,* 876 (1989)] LASN was packaged with PA31 7 amphotropic retrovirus packaging cells (2). The LASN vector preparation, manufactured under good manufacturing practices by Genetic Therapy, Gaithersburg, MD, had a titer of 1 X 10`1 to 3 X 105. The cells were returned to the incubator and the transduction process repeated, with the addition of fresh retroviral supernatant and Ill twice daily for a total of three to five additions of vector. The cultured cells were transferred to gas-permeable culture bags at the conclusion of the transduction process, The proliferating T cell cultures were observed daily, split, and fed as necessary with periodic samples tested for viability and microbial contamination. Gene transfer efficiency was variable from treatment to treatment and patient to patient, ranging from 1 to 10% for patient 1 and 0. 1 to I % for patient 2. On days 9 to 12, the cultured cells were washed extensively with saline containing 0.5% human albumin and were then infused into the patient over a period of about 1 hour. During the 9 to 12 days of culture, the cell populations had expanded 17- to 135-fold. Preliminary studies testing the T cell receptor 0 gene repRJoire showed that T cell cultures remained polyclonal for at least 3 weeks under these culture conditions. The culture period used in the clinical trial was held to half this time period to ensure a polyclonal T cell repertoire in the infused cell population.
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20. The protocol was reviewed and approved by the Clinical Research Subpanels of the NCI and NHLBl, the NCI Cancer Treatment and Evaluation Program (CTEP), the NIH Biosafety Committee, the Human Gene Therapy Subcommittee, the Recombinant DNA Advisory Committee, the Director of NIH, and the U.S. Food and Drug Administration, Informed consent was obtained from the parents of each patient.

21. Beginning with culture 9 for patient 1 and culture 5 for patient 2, the patients' lymphocyte populations obtained by apheresis were fractionated by adherence to flasks coated with CD8 monoclonal antibodies (Applied Immune Sciences) following the manufacturer's instructions. This protocol modification for CD8 depletion was introduced because both patients were developing a progressively inverted CD4CD8 ratio. This effect was apparently the result of preferential growth of CD8 I cells during the last 4 to 5 days of culture and the subsequent persistence of these infused CD8 I cells in the circulation. Corisequently, each subsequent apheresis sampled the recently increased number of CD81 cells, and thus the skewing of the ratio of CD4 to CD8 cells became compounded with each additional treatment. By partially depleting the apheresis sample of CD81 cells by an immuncaffinity selection process, the later treatments for each patient consisted of cells with a more balanced phenotype. The perturbation in normal CD4-CD8 cell proportions did not have detectable untoward effects for either patient.
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25. The ADA enzyme activity assay was performed in duplicate as described (13). Positive control cells were obtained from healthy normal donors and had a mean of 82 U (normal range, 66 to 102 U). Duplicate samples were run in the presence of the ADA enzyme inhibitor EHNA (30 VLM). Specific ADA activity was calculated as total adenosine dearninating activity minus EHNA-resistant activity. EHNA-resistant activity represents metabolic activity of a nonspecific aminchydrolase present in human cells.
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28. Southern hybridization analysis for LASIN vector consisted of the following: 10 tLg of DNA was digested with Ssf I and hybridized with a 728-bp Nco I fragment from LASN corresponding to the SV40 promoter and neo gene, DNA from K562-LASN cells served as a positive control.
29. RT-PGR analysis for LASIN vector transcripts was as follows: 3 tLg of polyadenylated RNA was treated with deoxyribonuclease and reverse-transcribed. The cDNA (0.3 Rg) was amplified with LASN vector specific primers in a 30-cycle PCIR reaction. The oligonucleotides 5'-CAGCCTCTGCAGGGCAGAAC-3'(corresponding to the 3' end of the ADA gene in LASN) and 5'-GCCCAGTCATAGCCGAATAG-3' (complementary to 5'end of the nee gene in FASN) were used as primers. After eiectrophoresis and blotting, the sequences were hybridized with a 527-ble probe corresponding to the entire length of the predicted PCR product.
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36. The authors acknowledge superb technical and nursing support by C. Able, K. Snitzer, A. Boole, M. Brown, F. May, R. Gutieriez, M. Yu, H. Goetzman, C. Warnebo, B. Sink, and L. Top. In addition, we thank R. Sorensen for providing the initial patient blood samples, Applied Immune Sciences for CELLector T-1 50 flasks, and Genetic Therapy for clinical-grade LASN vector. Finally, we also thank our earlier collaborators D. B. Kohn, E. Gilboa, P. W. Kantoff, M. A. Eglitis, R. Moen, K. Cometta, A. Gillio, R. J. O'Reilly, and C. Bordignon for their contributions that helped prepare the way for this clinical trial.

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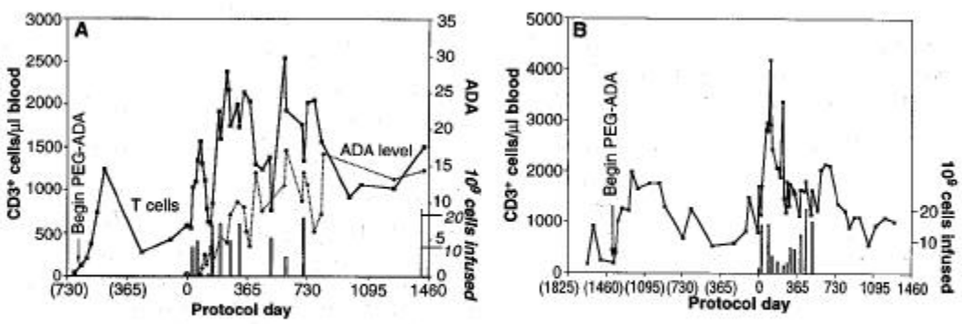

Fig. 1. Peripheral blood T cell counts since the time the diagnosis of ADA deficiency was made, dates of treatments, and the total number of cells infused for each patient. ADA level is measured in nanomoles of adenosine deaminated per minute per 10^8 cells. Vertical bars indicate the dates of cell infusion, and their height represents the total number of nonselected cells infused at each treatment. The T cell numbers represent total CD3-bearing T cells determined by standard flow cytometric analysis. (**A**) Patient 1 began gene therapy on 14 September 1990 (protocol day 0) and received a total of 11 infusions. Cellular ADA enzyme level is indicated by the dashed line. ADA activity was determined as described (*13, 25*). Values shown are the mean of duplicate samples and represent EHNA-sensitive ADA enzyme activity. (**B**) Patient 2 began gene therapy on 31 January 1991 (protocol day 0) and received of a total of 12 infusions.

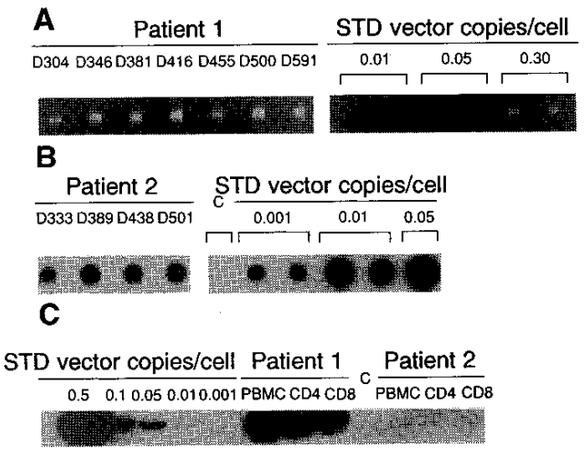

Fig. 2. PCR evaluation of the frequency of LASN vector–positive cells in the blood of patients 1 and 2 at various protocol days. (**A**) Cells from patient 1 for protocol days (D) 304 to 591 (see Fig. 1A). PCR analysis was performed as described (*26*) in an ethidium-stained gel. (**B**) Cells from patient 2 for protocol days (D) 333 to 501 (see Fig. 1B). PCR products were probed with ^{32}P-labeled *neo* gene as described (*26*). (**C**) Purified $CD4^+$ and $CD8^+$ cell subpopulations from patient 1 (D1480) and patient 2 (D1198) prepared by separation of peripheral blood mononuclear cells (PBMCs) by fluorescence-activated cell sorting (FACS). The purity of the separated T cell subpopulations from which DNA was extracted exceeded 98%, as confirmed by FACS analysis. Direct PCR with [^{32}P]deoxycytosine triphosphate was performed as described (*27*). Standards (STD) were prepared from DNA obtained from cell mixtures of a known proportion of LASN-transduced cells containing a single vector insert mixed with vector-negative cells. C, vector-negative control cells.

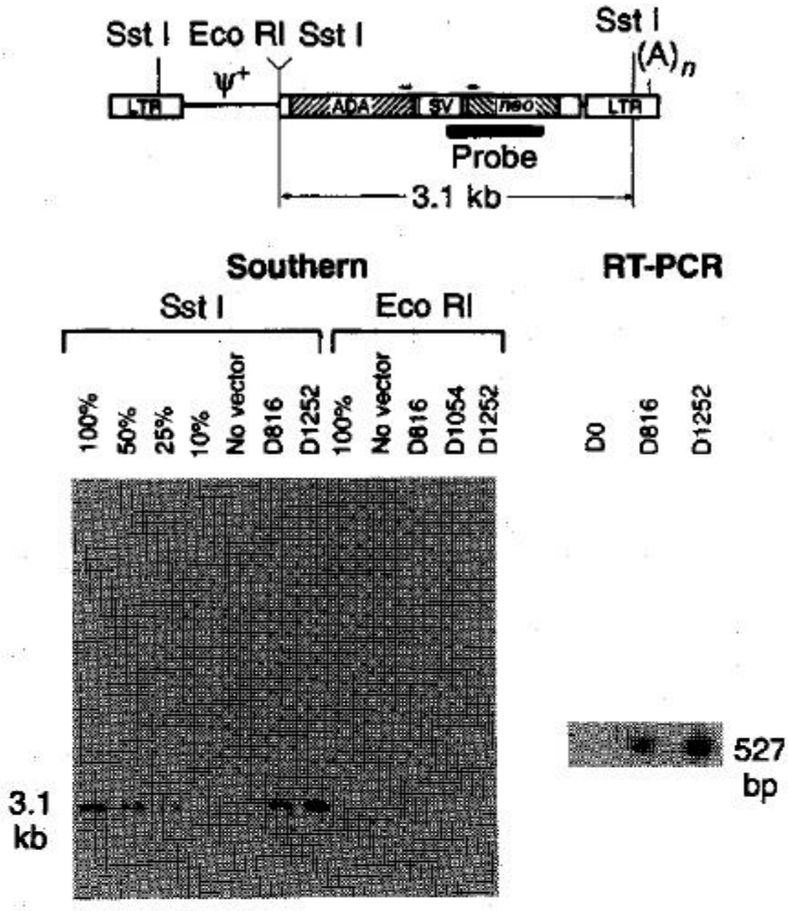

Fig. 3. Quantitative Southern hybridization analysis of DNA prepared from the blood mononuclear cells of patient 1 on protocol days (D) 816 and 1252 (*28*). DNA digested with Sst I should yield a single restriction fragment of 3.1 kb containing both the vector *neo* and ADA genes. Eco RI cuts only once within the vector sequence, and therefore a detectable band would indicate that a predominant clone with a single unique vector integration site was present in that blood sample. None was detected. Polyadenylated mRNA was extracted from the patient cells on days 0, 816, and 1252 and analyzed for vector message by RT-PCR (*29*). The primer locations used are indicated as short solid lines above the vector diagram. SV, SV40 early promoter; $(A)_n$, polyadenylation site; Ψ, extended retrovirus packaging signal. Hatched regions indicate protein coding regions.

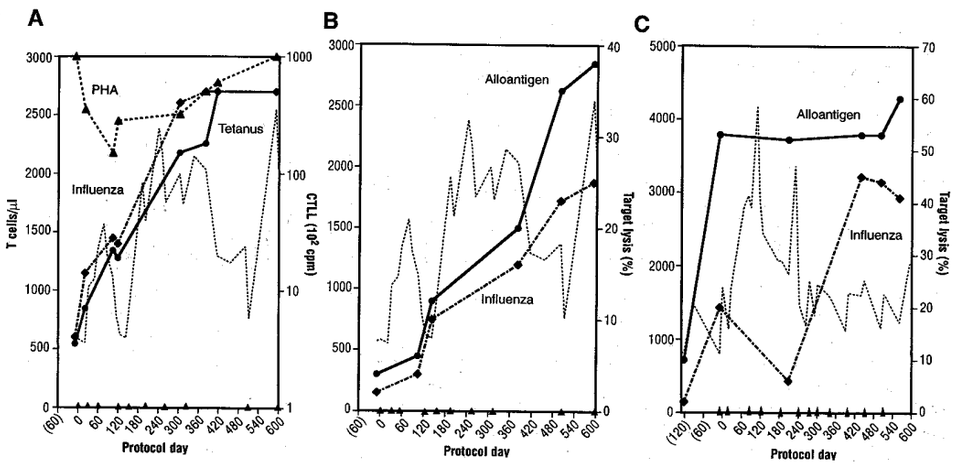

Fig. 4. Evaluation of the in vitro cellular immune responses of blood T cells from patients 1 and 2 at various times before and during the gene therapy trial. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (**A**) Production of IL-2 by cultured cells from patient 1 after stimulation with the mitogen PHA and with the specific antigens tetanus toxoid and influenza A virus as described (*30*). IL-2 was quantitated by bioassay measuring the proliferation of the IL-2–dependent T cell line CTLL at a 1:2 dilution of the lymphocyte culture supernatant. The fine dashed line indicates the patient's T cell count for reference. Solid triangles along the base line indicate the dates of cell infusion. (**B**) In vitro killing of a ^{51}Cr-labeled, influenza A–infected autologous B cell line and a ^{51}Cr-labeled allogeneic target B cell line by blood T cells from patient 1 as described (*31*). Lysis (as percent specific isotope release during a 6-hour incubation of effector and target cells at a ratio of 60:1) was measured after in vitro pre-stimulation for 7 days. Solid triangles along the base line indicate the dates of cell infusion. (**C**) In vitro killing of a ^{51}Cr-labeled, influenza A–infected autologous B cell and a ^{51}Cr-labeled allogeneic target B cell line by blood T cells from patient 2 as described above.

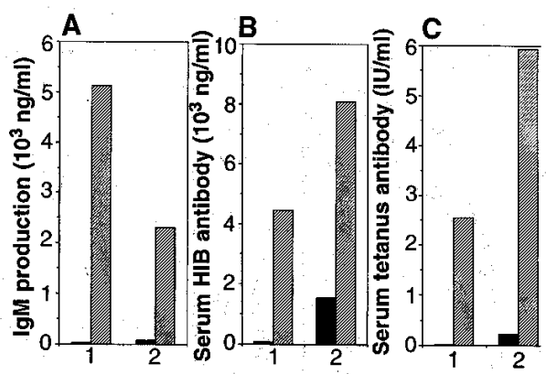

Fig. 5. Humoral immune function of patients 1 and 2 before (solid bars) and after (hatched bars) gene therapy. (**A**) IgM production by the patient's peripheral blood mononuclear cells in cultures stimulated with the T cell–dependent polyclonal activator PWM performed as described (*32*). "Before" samples were from D(−9). Follow-up cultures were at D500 (patient 1) and D560 (patient 2). In each case, the patient's cells stimulated with the T cell–independent B cell stimulant EBV (*33*) produced normal amounts of IgM (not shown), indicating intact B cell function before and after gene therapy, as expected. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (**B**) Serum antibody response to *Hemophilus influenzae* B. Patient 1 had failed to respond to two immunizations while on PEG-ADA alone [D(−9) shown]. Her response at protocol D591 is shown, after immunization. Patient 2 had some HIB-specific antibodies present before therapy [D(−122)], whose amounts increased without additional immunization during the protocol (D560). (**C**) Serum tetanus antibody. Patient 1 had negligible response to five separate tetanus immunizations before gene therapy [D(−48) shown] but responded briskly at D731, 24 days after re-immunization. Serum titers for patient 2 are shown for D(−9), 140 days after immunization while on PEG-ADA alone, and after receiving gene therapy (D592), 32 days after a booster tetanus immunization.

Table 1. DTH skin test reactivity and isohemagglutinin titers in sera of each patient at various times during the treatment protocol. Skin tests were applied as Multitest (Pasteur Merieux, Lyon, France) and scored according to the manufacturer's instructions 48 to 72 hours after being placed. Seven antigens were placed on the dates indicated, although only five were technically satisfactory on day 1252 for patient 1 and on day 1118 for patient 2. Isohemagglutinin titers were determined by standard blood bank techniques (*34*). Ninety five percent of normal children over the age of 2 years will have a titer of ≥1:16 and 82% will have a titer ≥1:32 (*35*). ND, not done. For the DTH skin tests, positive tests were elicited; T, tetanus toxoid; D, diphtheria toxoid; C, *Candida albicans*; P, Proteus antigen; S, streptococcal antigen; OT, old tuberculin.

Protocol day	Isohemagglutinins	DTH skin tests
	Patient 1	
−9	16	None (0/7)
115	256	ND
251	128	ND
314	32	T, D, C
455	32	T, D, C, S, P
510	64	ND
707	32	ND
1252	ND	D, C, P
	Patient 2	
−122	4	None (0/7)
−9	4	ND
90	256	ND
186	128	ND
291	128	ND
501	128	T, D, C, S, OT
676	64	ND
957	16	ND
1118	ND	T, D, S, P