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ARTICLE

Successful Reconstitution of Immunity in ADA-SCID by Stem Cell Gene Therapy Following Cessation of PEG-ADA and Use of Mild Preconditioning

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Gene therapy is a promising treatment option for monogenic diseases, but success has been seen in only a handful of studies thus far. We now document successful reconstitution of immune function in a child with the adenosine deaminase (ADA)-deficient form of severe combined immunodeficiency (SCID) following hematopoietic stem cell (HSC) gene therapy. An ADA-SCID child who showed a poor response to PEG-ADA enzyme replacement was enrolled into the clinical study. Following cessation of enzyme replacement therapy, autologous CD34$^+$ HSCs were transduced with an ADA-expressing gammaretroviral vector. Gene-modified cells were reinfused following one dose of preconditioning chemotherapy. Two years after the procedure, immunological and biochemical correction has been maintained with progressive increase in lymphocyte numbers, reinitiation of thymopoiesis, and systemic detoxification of ADA metabolites. Sustained vector marking with detection of polyclonal vector integration sites in multiple cell lineages and detection of ADA activity in red blood cells suggests transduction of early hematopoietic progenitors. No serious side effects were seen either as a result of the conditioning procedure or due to retroviral insertion. Gene therapy is an effective treatment option for the treatment of ADA-SCID.

Key Words: gene therapy, SCID, retroviral vector, adenosine deaminase

INTRODUCTION

Deficiency of the enzyme adenosine deaminase (ADA) leads to abnormal T, B, and NK cell development, resulting in severe combined immunodeficiency (SCID). Affected children suffer from severe, recurrent infection and failure to thrive and the condition is fatal in the first year of life if untreated. Hematopoietic stem cell transplantation (HSCT) from an HLA-matched donor offers good immunological and biochemical correction with survival >85%, but in the mismatched donor setting, the outcome is significantly worse [1]. PEG-ADA enzyme replacement therapy is used when a matched donor is unavailable but although overall survival is good [2], immune reconstitution is variable and prolonged treatment results in significant lymphopenia [3], variable defects in T cell function including decreased thymic output [4], and susceptibility to viral infection [5].

Initial retroviral vector-mediated gene therapy trials for ADA-SCID demonstrated efficient transduction of hematopoietic progenitors [6,7] and long-lived expression of the ADA gene in transduced T lymphocytes [8,9], but immune reconstitution was poor and most probably compromised by the concomitant use of PEG-ADA, which abrogated the survival and growth advantage of gene-modified cells [10]. Success of gene therapy for ADA-SCID has more recently been shown in patients who did not commence PEG-ADA and who received a nonmyeloablative chemotherapy regimen [11]. It is likely that the lack of

Abbreviations used: ADA, adenosine deaminase; dATP, deoxyadenosine; HSCT, hematopoietic stem cell transplantation; SCID, severe combined immunodeficiency; LTR, long terminal repeat; PEG-ADA, polyethylene glycol-conjugated bovine ADA.

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TABLE 1: Immunological and metabolic values pre- and post-gene therapy

	12 months pre-GT	1 month pre-GT (prior to cessation of PEG-ADA)	26 months post-GT (or latest follow-up)	Normal values
ALC (cells $\times 10^9$/L)	1170	360	720	1100–5900
CD3 (cells $\times 10^9$/L)	420	100	600	700–4200
CD4 (cells $\times 10^9$/L)	180	60	300	300–200
CD8 (cells $\times 10^9$/L)	370	30	300	300–1800
CD19 (cells $\times 10^9$/L)	230	140	60	200–1600
%CD27$^+$/CD45R0$^-$ (of T cells)	17.5	7.8	39	n/a
CD4$^+$ TRECs (/10^6 cells)	nd	0	1314	n/a
CD8$^+$ TRECs (/10^6 cells)	nd	0	966	n/a
PHA proliferation (cpm)	173,085 (dpm)	52,278	47,865	>12,500
CD3 proliferation (cpm)	nd	nd	3245	>2000
IgA (g/L)	0.44	0.30	0.11	0.4–2.0
IgM (g/L)	0.23	0.15	0.11	0.5–2.0
dATP (μmol/L)	38	10	70	Undetectable

nd, not done; n/a, not available; GT, gene therapy.

PEG-ADA restores a survival advantage to transduced lymphocytes and that chemotherapy allows increased engraftment of gene-transduced progenitors. We established a protocol whereby PEG-ADA treatment was stopped in poor responders and patients received mild chemotherapy prior to the return of gene-transduced autologous HSCs. We now report reconstitution of cellular immunity, reinitiation of thymopoiesis, and metabolic correction for over 2 years in one patient enrolled into this protocol.

RESULTS

Patient Characteristics

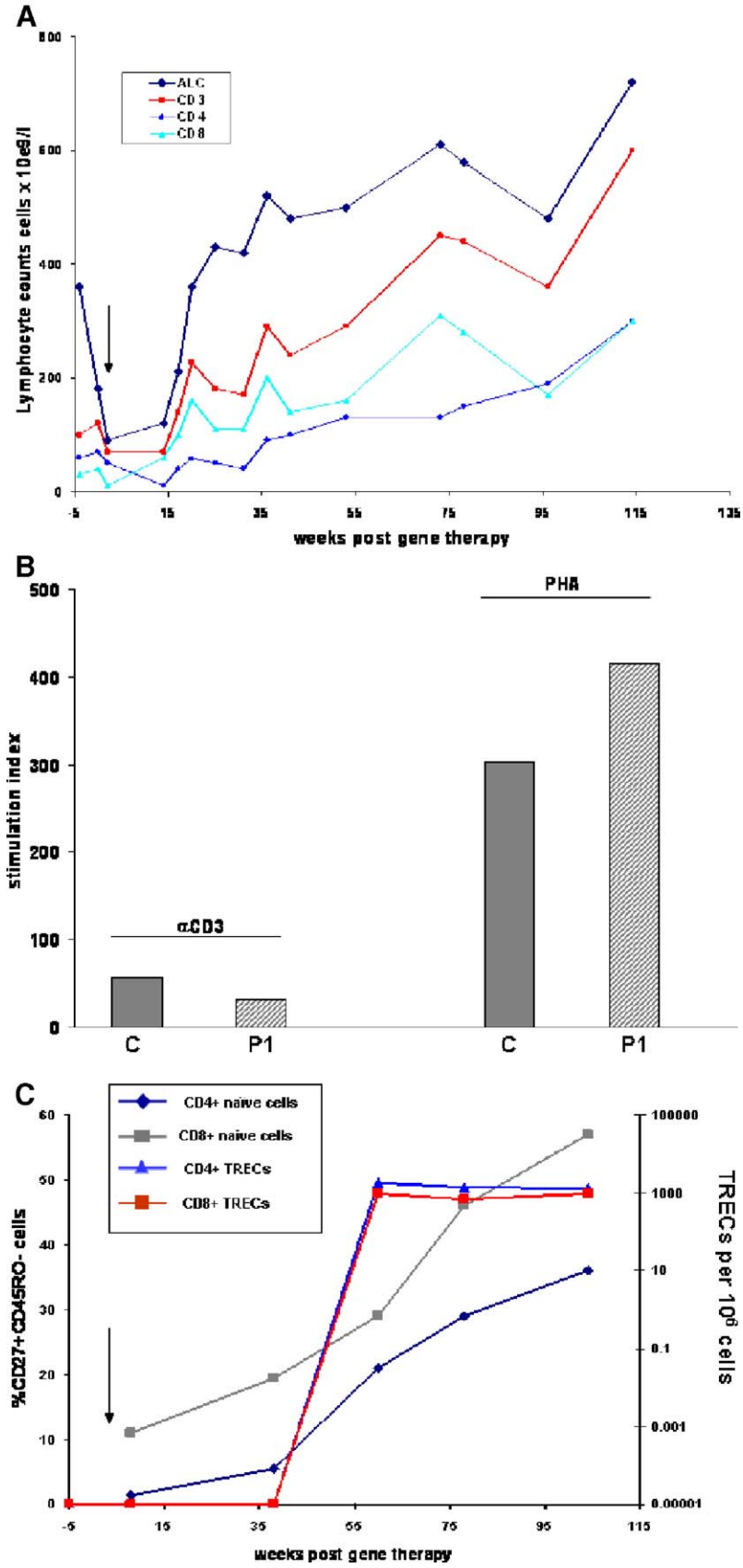
P1 was diagnosed at 2 months of age and, due to the unavailability of a matched donor, started on PEG-ADA. Despite effective metabolic correction, good clinical status, and no infective problems, immune recovery was poor, with significant T cell lymphopenia, poor immunoglobulin production, and compromised thymic function as evidenced by the disappearance of naïve T cells and no detectable T cell receptor excision circles (TRECs). At 3.2 years of age and 3 years after starting PEG-ADA, he was enrolled into the gene therapy study. The protocol was approved by the UK Gene Therapy Advisory Committee, the Medicines Control Agency (now Medicine and Healthcare Products Regulatory Agency), and the local Institutional Research Ethics Committee. Criteria for entry into the study were confirmation of ADA-SCID by identification of the genetic defect or biochemical analysis (in this case, both a genetic defect (Q3X) and biochemical abnormality (presenting dATP of 1531 μmol/L) were confirmed), lack of an HLA-matched related/unrelated or cord blood donor, and failure of PEG-ADA therapy (as defined by a lymphocyte count of $<1.5 \times 10^9$ cells/L and hypogammaglobulinemia requiring Ig replacement). Written informed consent was obtained from the parents following discussion of alternative treatment options.

Clinical Course

PEG-ADA was stopped 1 month prior to treatment. We isolated autologous CD34$^+$ cells from bone marrow and performed transduction of cells as detailed under Materials and Methods. Transduction efficiency was 25–30% by flow-cytometric analysis of intracellular ADA expression and PCR analysis for the ADA transgene in cultured hematopoietic progenitor cell colonies (data not shown). On day −2 the patient received one dose of Melphalan (140 mg/m^2) prior to the return of transduced cells. The total number of CD34$^+$ADA$^+$ cells at reinfusion was ~1.4×10^6/kg. The patient experienced Melphalan-induced cytopenias and was transfusion independent after 3 weeks.

The patient was known to be EBV positive prior to initiation of gene therapy, with a viral load of 4100 copies/ml whole blood, although there were no clinical signs of EBV infection. At day +28, an increase in EBV viral load (4568 copies/ml whole blood at day +24 to >800,000 copies/ml at day +40) was followed by a transient expansion of a CD8$^+$ TCR Vβ1 clone to $>2900 \times 10^9$ cells/L with rapid resolution of EBV viremia (data not shown). These cells did not contain the ADA

FIG. 1. Immune reconstitution following gene therapy. (A) T lymphocyte recovery from time of stopping PEG-ADA at week −4 to week +115. The ALC, total CD3, CD4, and CD8 are shown. The early expansion of T cells in response to EBV infection is not shown for the sake of clarity. Arrow indicates timing of gene therapy. (B) Proliferative responses to CD3 stimulation and PHA at >70 weeks following gene therapy, P1 compared to control (C). The stimulation index for proliferation in response to each mitogen is shown. (C) Increase in thymic function following gene therapy. CD4$^+$ and CD8$^+$ naïve T cells (%CD27$^+$CD45RO$^-$) increase in number post-gene therapy. TRECs in both CD4$^+$ and CD8$^+$ cells were detected for the first time at 60 weeks following gene therapy. Arrow indicates timing of gene therapy.



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transgene (determined by qPCR on the sorted cell population), indicating that a preexisting EBV-specific memory T cell population generated while on PEG-ADA had expanded in response to viral reactivation. As this coincided with the re-emergence of B cells after Melphalan-induced lymphopenia, we also treated the patient with a monoclonal antibody against the B-cell-specific surface receptor CD20 (Rituximab—four doses at 375 mg/m^2 at weekly intervals).

Immune Reconstitution Post-gene Therapy

Following gene therapy, there was a progressive rise in total lymphocyte numbers (Table 1 and Fig. 1A). In comparison to values at cessation of PEG-ADA, total T cells rose from 100×10^9 to 600×10^9 cells/L, with an increase in $CD4^+$ counts from 60×10^9 to 300×10^9 cells/L and in $CD8^+$ lymphocytes from 30×10^9 to 300×10^9 cells/L. T cell recovery was accompanied by the acquisition of normal proliferative responses to CD3 stimulation and phytohemagglutinin (PHA) (Fig. 1B). B lymphocyte counts recovered after Rituximab therapy to 60×10^9 cells/L, coinciding with a steady increase in endogenous production of IgM and IgA, although levels remained below the normal range (data not shown). NK lymphocyte counts also recovered and were 40×10^9 cells/L at 96 weeks posttherapy (data not shown). P1 remains on immunoglobulin replacement but has discontinued prophylactic antibiotics and is thriving, free of infection, and attending normal school.

Prior to gene therapy there had been a progressive decrease in $CD27^+CD45RO^-$ T cell populations and we were unable to detect TRECs in separated $CD4^+$ and $CD8^+$ cells, indicating severe compromise of thymic function. Following gene therapy, there was a progressive re-emergence of $CD27^+CD45RO^-$ T cells (Fig. 1C). We detected TREC activity in both $CD4^+$ and $CD8^+$ separated populations at ~1 year post-gene therapy (Fig. 1C) and it has been sustained, indicating successful reinitiation of thymopoiesis.

Diversity of the T Cell Repertoire Following Gene Therapy

Flow-cytometric analysis of T cells for TCR Vβ expression showed usage of all families and demonstrated a polyclonal T cell repertoire equivalent to that of age-matched controls (Fig. 2A). We saw transient expansion of a TCR Vβ1 clone at the time of EBV expansion as described above but this expansion resolved soon after. Analysis of TCR Vβ complementarity-determining region 3 (CDR3) fragment lengths after PCR amplification (TCR spectratypes) showed progression from oligoclonality within individual Vβ families to polyclonality with time and reflects the evolution of a complex and diverse repertoire (Fig. 2B—three representative families shown, and see supplemental data). Longitudinal analysis using both of the above techniques has not shown any evidence of persistent clonal expansion or pathological proliferation.

Metabolic Correction Following Gene Therapy

Biochemical detoxification in the absence of exogenous PEG-ADA has been well maintained with deoxyadenosine triphosphate (dATP) levels consistently <100 μmol/L (dATP at diagnosis 1531 μmol/L), levels that compare favorably to those found in patients successfully treated by allogeneic HSCT (average dATP ~100 μmol/L) (Fig. 3). Red blood cell (RBC) activity in P1 at diagnosis was undetectable as a result of his mutation, which results in complete loss of ADA activity. During PEG-ADA therapy, RBC ADA activity remained undetectable since PEG-ADA acts extracellularly and does not cross the cell membrane. Following gene therapy we saw an initial sharp rise in RBC ADA activity, which is likely explained by exogenous blood transfusion during cytopenia and possibly the differentiation of transduced short-term erythroid progenitors. However, from 1 year posttherapy onward, when the effect of RBC transfusions would no longer be detectable, we have observed low but sustained RBC ADA activity at 3–5 nmol/mg Hb/h, which is ~10% of normal activity (normal range 40–60 nmol/mg Hb/h) (Fig. 3). This would suggest gene transduction of long-lived erythroid progenitors and continued generation of ADA-expressing erythrocytes.

Proviral Copy Number and Lineage-Specific Integration Analysis

Quantitative PCR analysis for the transgene in flow-sorted cell populations showed that proviral copy number in T and NK cells was >0.5 copies per cell from 16 weeks post-gene therapy onward. This may indicate the coincident continued persistence of nontransduced cells that had been generated while the patient was on PEG-ADA or the generation of new nontransduced cells presumably as a result of systemic detoxification (Fig. 4A). In comparison, the proportion of circulating B cells containing transgene was ~10% (copy number 0.1), whereas marking in myeloid cells gradually declined to ~0.1% over the period of observation. We also isolated and expanded single T cell clones. Analysis of five independent transduced clones demonstrated that the copy number within each individual clone was 1 (data not shown).

Analysis of proviral integration sites by linker-mediated PCR demonstrated the presence of polyclonal integration sites in all sorted cell populations at successive time points (Fig. 4B). These data suggest effective correction of multiple progenitors. Sequencing of individual integrants is ongoing.

DISCUSSION

Gene therapy for correction of monogenic hematopoietic disorders is now being realized by a number of clinical

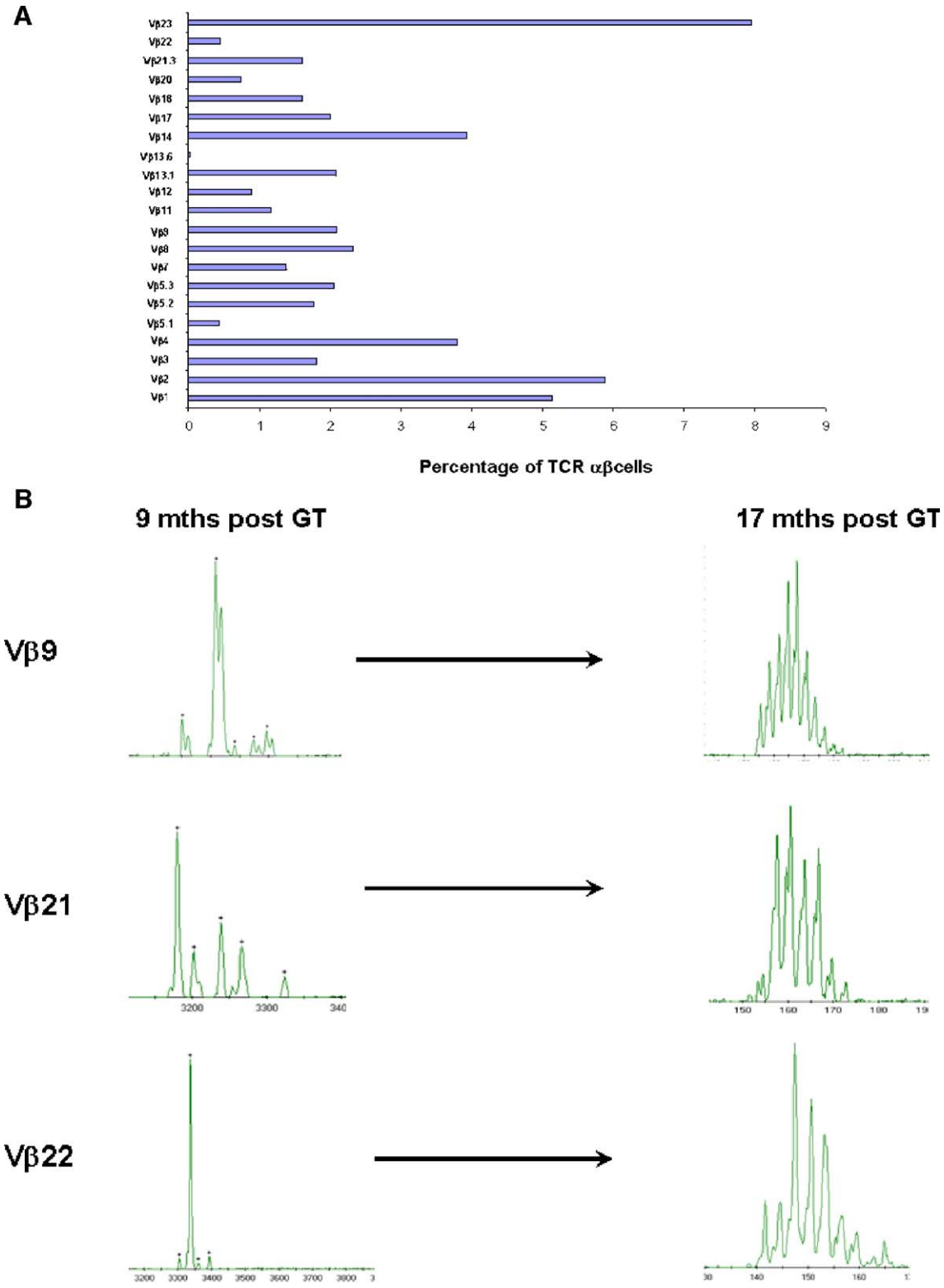

FIG. 2. Diversity of the T cell repertoire following gene therapy. (A) Normal range of TCR Vβ assessed by flow cytometry at 70 weeks post-gene therapy demonstrates the diversity of the T cell population (normal ranges for the TCR Vβ families are available as supplemental data). (B) Evolution of TCR spectratypes following gene therapy. There is progressive complexity and polyclonality of TCR Vβ families from 9 to 17 months posttreatment in $CD4^+$ selected cells. Three representative Vβ families are shown.

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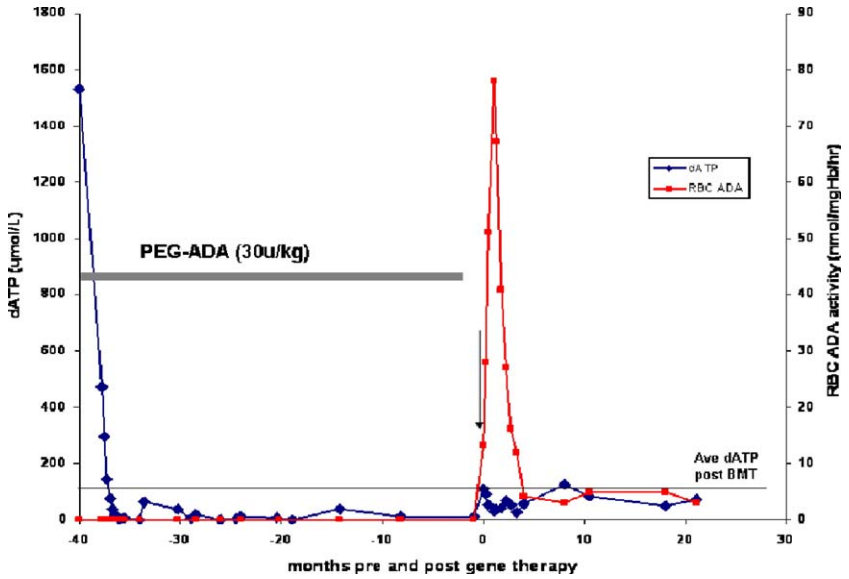

FIG. 3. Biochemical detoxification and increase in red cell ADA activity following gene therapy. Detoxification of dATP while on PEG-ADA and following gene therapy (blue). The thin gray line indicates the average dATP level post-successful allogeneic HSCT in a cohort of 11 ADA-SCID patients transplanted at our institution (average level 103 μmol/L). RBC ADA activity is undetectable prior to gene therapy and increases immediately after gene therapy and is sustained (red). Arrow indicates timing of gene therapy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

trials [11–13]. This report, the first in which PEG-ADA has been electively withdrawn, demonstrates that gene therapy alone can correct immunological and biochemical defects in ADA-SCID. We believe that the cessation of PEG-ADA and use of chemotherapy are necessary pre requisites for successful engraftment and proliferation of gene-modified cells. This is supported by data from Aiuti *et al.*, who have shown that withdrawal of PEG-ADA from patients in early gene therapy trials led to an increase in the proportion of transduced T cells [10]. The use of conditioning has not been experimentally tested but a recent ADA clinical trial in which PEG-ADA was withdrawn but without administration of chemotherapy has shown only limited immunological recovery in two patients [14].

Importantly, in our study immune recovery was superior to that achieved while on PEG-ADA. In particular naïve T cell numbers have improved considerably and are associated with normal mitogenic responses and TCR diversification. The sustained increase in naïve T cells and TREC activity suggests that there has been reinitiation of thymic function. Sustained multilineage engraftment of transduced cells and detection of prolonged RBC ADA activity suggest transduction of long-term repopulating HSC and would predict sustained immunological and metabolic recovery, the extent of which can be determined only by continued clinical monitoring. It is also interesting to note that significant numbers of nontransduced T and NK cells have remained for over 2 years, suggesting that effective systemic detoxification provided by transduced populations allows rescue of nontransduced cells. Recovery of humoral immunity has been limited despite low-level gene marking in B cells. It is clear that B cells in ADA-SCID are not intrinsically abnormal since there is reconstitution of humoral immunity in a large number of patients treated by PEG-ADA alone. Thus the humoral deficit in our patient may relate to insufficient T cell help and reestablishment of lymphoid architecture at this time point but may resolve with progressive T cell recovery. The use of Rituximab may have also had an adverse effect on B cell recovery.

Our report adds significantly to the evidence for efficacious gene therapy in ADA-SCID [11]. In one previously published study, two patients for whom PEG-ADA was unavailable for socioeconomic reasons were treated with gene therapy following a nonmyeloablative conditioning procedure using oral Busulphan 4 mg/kg (2 mg/kg/day on 2 successive days). Transduction conditions were similar for both studies, although several other features differ. First, we have preferred to use Melphalan because it can be administered as a single intravenous dose and because of our extensive experience with this agent in both our allograft (at 140 mg/m^2 in combination with other agents) and our autograft (at 200 mg/m^2 alone) HSCT program [15]. For future protocols in which the *ex vivo* culture of cells is intended to be short (for example using lentiviral vectors), the use of a single dose may have distinct advantages in terms of timing

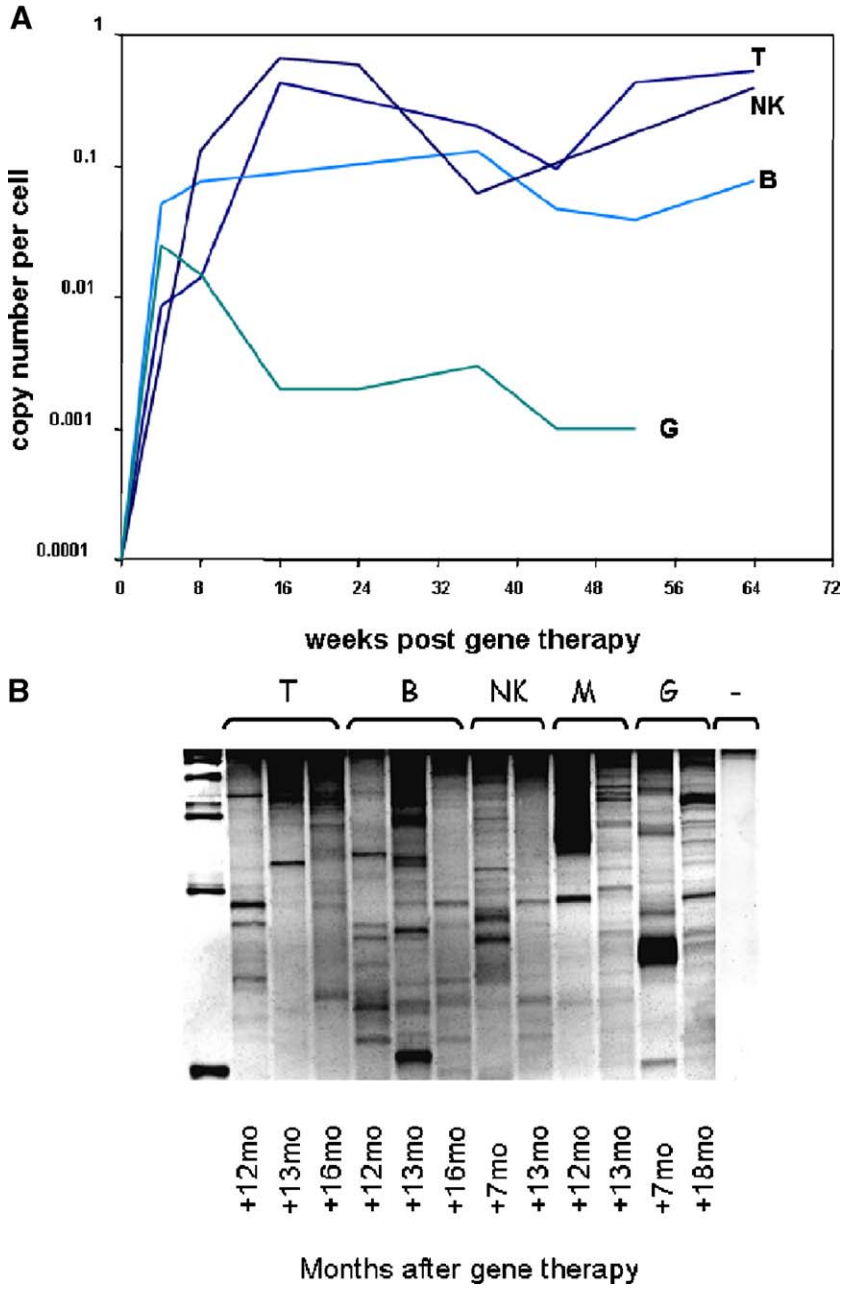

FIG. 4. Lineage-specific transgene copy number and site-specific vector integration analysis. (A) Quantitative PCR analysis for transgene in sorted cell populations. T cell, NK cell, B cell, and granulocyte (G) numbers are shown. Copy number of ~0.5 is seen for T and NK cells and low but detectable sustained levels are seen for B cells and granulocytes. (B) Vector integration analysis performed on T, B, NK, monocyte (M), and granulocyte (G) purified populations at specific time points after gene therapy. T cells show a polyclonal pattern with few specific expansions. Multiple integration sites are seen in all other lineages, suggesting transduction of early progenitors. —, negative control lane, which consists of the same analysis on untransduced cells.

between cell harvest and reinfusion. We have also stopped PEG-ADA in an elective manner and thus the findings in our study may, if repeated in further patients, have major implications on the management strategy for ADA patients. We have utilized a vector that is designed in principle to maximize expression of ADA in hematopoietic cells. This is important not only for successful growth and survival of immune cells, but also for enhancement of the effects of systemic detoxification in other tissues that are metabolically compromised by ADA

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deficiency. For example, expression of the transgene is regulated by the spleen focus-forming virus (SFFV) LTR, which is known in experimental models to perform well in hematopoietic cells [16], and is enhanced by the inclusion of the woodchuck hepatitis posttranscriptional regulatory element (WPRE). The viral particles are also pseudotyped with the GALV envelope, which may have advantages for transduction of HSC populations and which we have used successfully for gene therapy of SCID-X1 [13]. In contrast, the vector used in the Italian study is a Moloney-based gammaretroviral vector encoding the hADA gene together with the gene for neomycin resistance and pseudotyped with an amphotropic envelope. T cell recovery in our patient was similar to that observed in the first patient treated in the Italian study but was substantially better than that observed in the second patient in that study. It is interesting to note that the second patient in the study of Aiuti *et al.* received the lowest number of gene-transduced cells. At present it is not yet possible to make direct comparisons between the performance of each vector, although there are potential advantages of that used in our study.

The time window within which HSC gene therapy will be optimally effective for diseases such as ADA-SCID is likely to be finite and restricted by thymic potential [17]. The patient treated in this study achieved substantial but incomplete recovery despite metabolic detoxification, suggesting that this potential was already limited. The influence of PEG-ADA is uncertain, but recent reports suggest that prolonged enzyme replacement results in lymphopenia with diminished proliferative responses together with decreased thymic function [3,4]. Thus prolonged use not only may result in significant clinical infection as reported [5], but also may compromise the corrective capacity of other treatment modalities. More importantly, this study shows that immune recovery after intrinsic gene modification of hematopoietic progenitors can surpass that mediated by PEG-ADA alone.

The entry of patients into this study, as for all gene therapy trials, is a careful balance of possible benefit against the risks of the procedure. In the case of ADA-SCID, for which PEG-ADA offers a treatment option in addition to HSCT, this adds a further compounding factor. Our inclusion criteria at the time of entry of P1 required failure of PEG-ADA and the unavailability of a matched family or unrelated donor (MUD). Given the problems in immune and thymic function associated with long-term PEG-ADA therapy, entry into the study at the first signs of decline in immune function appears justified. The entry criteria into our protocol have been revised in 2006 and gene therapy is now considered before a matched unrelated donor transplant. This arises from the ~60% survival rate for ADA-SCID MUD HSCT in the European and IBMTR (International Bone Marrow Transplant Registry) databases together with the safety profile of ADA gene therapy thus far. Failure of gene therapy would also not compromise a further allogeneic HSCT and therefore undertaking gene therapy with its low short-term morbidity profile as the initial procedure seems appropriate.

Recent concern over the use of retroviral vectors due to their oncogenic potential [18,19] has been highlighted by the development of T cell leukemias in one SCID-X1 gene therapy trial [20]. Similar concerns have been raised over the use of the WPRE, which in *in utero* murine gene transfer studies using lentiviral vectors has been implicated in hepatic tumor development [21,22]. However, we have used a mutated form of the WPRE (including a 5-bp deletion in the X protein promoter and a mutated X protein start codon) that has not been associated with tumors in the same model in the context of HIV-1-based vectors [23]. Although follow-up in our study is relatively short, no adverse events related to LTR-mediated mutagenesis or WPRE-mediated toxicity have been observed (analysis of vector integration sites is ongoing). Monitoring of patients treated for over 10 years in early ADA gene therapy trials, some of whom have significant levels of transgene marking, has also not shown any evidence of clonal lymphoproliferation [8].

Our report shows that successful gene therapy is possible after withdrawal of PEG-ADA supplementation. More importantly, the quality of immunological reconstitution may be superior and associated with sustained expression of ADA in multiple cell lineages. Careful evaluation in a larger number of patients is warranted to determine the long-term efficacy of gene therapy.

MATERIALS AND METHODS

Retroviral vector construction, production, and testing. The gammaretroviral vector (SFada/W) contained human ADA cDNA transcribed from the SFFV LTR and also contained a mutated WPRE (containing mutations in both the promoter and the initiation codon) to increase intracellular ADA expression (gift from Dr. Thomas Hope). Vector genomes were packaged in PG13 cells. Clinical-grade vector supernatant was manufactured by EUFETS (Idar-Oberstein, Germany). The viral titer was 5×10^5 transducing units per milliliter.

Cell purification and transduction. Bone marrow $CD34^+$ cells (CliniMACS; Miltenyi Biotec) were cultured in Lifecell X-Fold cell culture containers (Nexell, Irvine, CA, USA), at a concentration of 0.5×10^6 cells/ml in serum-free X-Vivo 10 medium (Biowhittaker) supplemented with 1% human serum albumin (Baxter, UK) and cytokines (SCF 300 ng/ml, TPO 100 ng/ml, IL-3 20 ng/ml, and Flt3-L 300 ng/ml; R&D Systems) for 40 h at 37°C in 5% CO_2 (preactivation). Lifecell X-Fold cell culture containers were precoated with the CH296 human fragment of fibronectin (25 μg/ml) (donated by Takara Bio, Inc., Japan). SFada/W retroviral supernatant was added on three occasions over a 56-h period. Cells were then harvested, washed three times, and infused into the patients.

Flow cytometry. Whole blood was labeled with combinations of monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP), cychrome (Cy), or tricolor (TC). Isotype-matched fluorochrome-conjugated antibodies served as controls (BD Biosciences: CD3 FITC, CD45RO FITC,

CD27 FITC, CD19 PE, CD27 PE, CD4 PE, CD8 PE, CD45 PerCP, CD4 PerCP, CD8 Cy, CD45RO Cy, CD16+CD56 FITC PE Simultest. Caltag: IgM FITC, IgD FITC, CD19 TC). The resulting three-color cell staining was analyzed with an Epics XL flow cytometer (Beckman Coulter, High Wycombe, UK). Cells were sorted using a Beckman Coulter Epics Altra with Expo32 software.

Intracellular staining for ADA expression was performed according to the protocol of Otsu *et al.* [24]. Briefly, cells were fixed and permeabilized using an Intrastain fixation and permeabilization kit (DAKO, Glostrup, Denmark). Cells were then stained with a mouse anti-ADA antibody (gift from M. Hershfield, Duke University) or an isotype control. This was followed by staining with a biotinylated anti-IgG1 antibody (PharMingen, San Diego, CA, USA) and a streptavidin–phycoerythrin tertiary antibody (PharMingen) before analysis.

Immunoglobulin superfamily antigen receptor repertoires. Direct immunofluorescence was performed using fluorochrome-conjugated antibodies, PE- and PerCP-labeled TCR β, TCR δ, and TCR Vβ family antibodies, obtained from BD, Beckman Coulter, and Serotec. For TCR Vβ analysis samples were analyzed on a FACSCalibur flow cytometer. TCR Vβ expression is represented as a percentage of CD3-positive cells for each family.

CDR3 TCR spectratyping was performed as previously described [25].

T cell functionality. PBMCs (5×10^5) were seeded into a 96-well plate in RPMI containing 5% human AB serum. Mitogens (CD3 or PHA) were added to the wells in a final volume of 200 μl. A minimum of three replicates were performed on each sample, with a positive control and unstimulated control. Three days later, samples were pulsed with 1 μCi/ml tritiated thymidine for 6 h and the cells were harvested and the thymidine incorporation was measured on a scintillation counter. The stimulation index (maximal counts per minute (cpm)/background cpm) is presented.

TREC analysis. $CD4^+$ and $CD8^+$ TREC analysis was carried out as previously described [15].

Integration site analysis. Twenty to fifty nanograms of patient DNA from FACS-sorted cells was subjected to restriction digestion with the enzyme *Tsp*905I. Digested DNA was used as a template for a 100-cycle linear PCR amplification (94°C for 1 min, 65°C for 30 s, 72°C for 45 s) with a 5′ biotin-labeled SFFV LTR primer (5′-TGGCCCAACGTTAGCTATTTTCATGTA-3′). Magnetic streptavidin C1 beads (Dynal Biotech) were used to isolate the 5′-biotinylated single strands. Second-strand synthesis was performed by Klenow polymerase (New England Biolabs) primed by an asymmetric degenerate linker created by annealing the oligos 5′-TAGGGCACTA-TAGGGCACGCGTGNNNNNN-3′ and 5′-CACGCGTGCCCTATAG TGCCCTA-3′. The new double strands served as a template for exponential PCR amplification (94°C for 1 min, 65°C for 1 min, 72°C for 1 min; 31 cycles) using primers forward, 5′-TAGGGCACTATAGGGCACGC-3′, and reverse, 5′-TGGCCCAACGTTAGCTATTTTCATGTA-3′. A nested PCR was then performed (94°C for 1 min, 60°C for 1 min, 72°C for 1 min; 31 cycles) with primers forward, 5′-CTATAGGGCACGCGTG-3′, and reverse, 5′-CCTTGATCTGAACTTCTCTATTCTTGGTTTG-3′. The PCR products were analyzed by electrophoresis on the Spreadex high-resolution system (Elchrom Scientific).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2006.06.007.

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