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In Vivo Kinetics of Transduced Cells in Peripheral T Cell-Directed Gene Therapy: Role of $CD8^+$ Cells in Improved Immunological Function in an Adenosine Deaminase $(ADA)^-$SCID Patient[1]

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We previously reported successful peripheral T cell-directed gene therapy in a boy with adenosine deaminase $(ADA)^-$SCID. In the present study, to better understand the reconstitutive effect of this gene therapy on his immunological system, we investigated the in vivo kinetics and functional subsets of T cells in PBL. Apparent immunological improvements were obtained after infusion of transduced cells at more than 4×10^8 cells/kg/therapy/3 mo. Frequency of ADAcDNA-integrated cells in PBL, ADA activity in PBL and clinical improvement showed good correlation, even though $CD8^+$ cells gradually became predominant in PBL. On the basis that polyethylene glycol (PEG)-ADA was maintained at the same dosage as before gene therapy, we consider that his immunological improvement resulted from the gene therapy itself. Most $CD3^+$ cells in PBL after gene therapy expressed TCR$\alpha\beta$. Analysis of TCR repertoire based on TCR V region usage revealed no expansion of limited clones in his PBL. The T cell subset cells $CD8^+CDw60^+$ and $CD8^+CD27^+CD45RA^-$, which are reported to provide substantial help to B cells, were maintained throughout the gene therapy. Furthermore, his reconstituted peripheral T cells helped normal B cells to produce substantial IgG in vitro. Expression of both Th1- and Th2-type cytokine genes was induced in his reconstituted T cells at the same comparably high level as in normal subjects. Collectively, these results provide evidence of persistent and distinct functions of transduced cells in this patient's PBL after gene therapy. ***The Journal of Immunology,*** **1999, 163: 2256–2261.**

Over the past 8 years, a large number of protocols for gene therapy (GT)[3] have been instituted for many kinds of diseases (1). However, results with most of these have not met expectations. Stem cell-directed gene therapy, considered the best approach for congenital hemopoietic disorders, has also been tried recently, but again success has been elusive (2–4). Among the few successful examples has been GT for adenosine deaminase (ADA) deficiency, especially by a peripheral T cell-directed protocol (5–7). The first patient in the world to undergo GT has maintained her reconstituted immunological function for an unexpectedly long time. A number of problems in the peripheral T cell-directed protocol have been noted: the life of peripheral T cells is limited and $CD8^+$ population in PBL increases. The in vivo kinetics and immunological functions of transduced cells have not been reported in detail, and the precise mechanism of effective GT targeting to peripheral T cells remains to be established.

We previously reported a successful series of peripheral T cell-directed GT for a patient with ADA^-SCID (7). In the present study, we present the in vivo kinetics and functions of transduced cells in this patient's peripheral blood to better understand how peripheral T cell-directed GT has improved his immunological function in vivo.

Materials and Methods

Case report

The patient and our GT protocol for ADA deficiency have been reported in detail elsewhere (7). Briefly, a 4-yr-old boy who had shown only limited improvement after treatment with polyethylene glycol (PEG)-modified ADA was enrolled in a clinical gene therapy trial. PBMC from the patient were stimulated with anti-CD3 Ab (OKT3; Ortho, Raritan, NJ) and recombinant human IL-2 in AIM-V medium (Life Technologies, Grand Island, NY) supplemented with 5% FCS (Life Technologies) for 72 h. Transduction was performed twice during the next 48 h by exposure to a retroviral vector, LASN, that contained cDNA of human ADA gene. Cultivation was continued for a total of 7 to 11 days before infusion into the patient. No selection procedure for enrichment of gene-transduced cells was performed. Following a total of 11 infusions of transduced cells over 20 mo, a number of immunological improvements were seen, including improved isohemagglutinin titer, delayed-type hypersensitivity (DTH) skin test, serum Ig level, and specific Ab response to specific Ag.

Flow cytometric analysis

PBMC was washed with PBS containing 0.5% albumin and stained with FITC or PE-conjugated mAbs anti-CD4 (Leu-3a), anti-CD8 (Leu-2a), anti-HLA-DR, anti-CD45RA (Leu-18), anti-CD27, and anti-CDw60 for 20 min on ice. The stained cells were then washed. All mAbs were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA) except anti-CDw60, which was purchased from PharMingen (San Diego, CA). A TCR$\alpha\beta$ screening panel (Diversi-T) containing FITC-conjugated mAbs to Vα2, Vα12.1, Vβ3.1, Vβ5.3 + 5.2, Vβ5.1, Vβ6.7, Vβ8 family, Vβ12, and

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[3] Abbreviations used in this paper: GT, gene therapy; ADA, adenosine deaminase; FISH, fluorescence in situ hybridization; SAC, *Staphylococcus aureus* Cowan I; PEG, polyethylene glycol; LCL, lymphoblastoid cell line.

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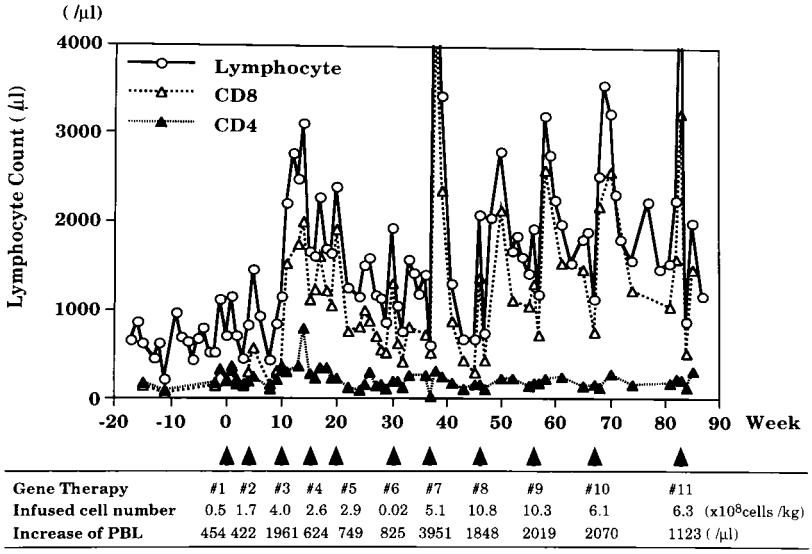

FIGURE 1. Kinetics of PBL and T cell subset during gene therapy. Open circle, open triangle, and filled triangle indicate the number of PBL, $CD8^+$ cells, and $CD4^+$ cells, respectively. A filled arrow at the *bottom* represents an infusion of transduced cells in each GT. Increase in PBL means the cell number increased during the week after each infusion.

Vβ13.1 + 13.2 was purchased from T Cell Diagnostics (Woburn, MA) for examination of TCR repertoire. Cell surface phenotype was analyzed on an Epics XL flow cytometer (Coulter, Hialeah, FL).

Purification of $CD4^+$ and $CD8^+$ cells

PBMC from the patient was reacted with MACS (Magnetic Cell Sorting) CD4 or CD8 microbeads (Miltenyi Biotec, Sunnyvale, CA) for 15 min at 4°C and washed with PBS supplemented with 1% albumin three times. They were applied to a MACS column (Miltenyi Biotec) and rinsed with PBS. After removal of the column from MACS, eluted labeled cells were collected as $CD4^+$ or $CD8^+$ cells. Purity of each cell was greater than 98% by analysis on FACScan (Becton Dickinson Immunocytometry Systems) after staining with FITC-labeled Abs.

PCR

PCR for ADAcDNA was performed using primer pairs corresponding to the first and second exons of ADA gene, which amplify ADAcDNA but not genomic ADA gene, because the first intron was too long to be amplified. Primers for β-actin were utilized as an internal control. One microgram of DNA was amplified in a final volume of 50 μl. PCR was conducted in a DNA thermal cycler (Cetus, Emeryville, CA) for 30 cycles: 1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min extension at 72°C. PCR products were then visualized by being subjected to electrophoresis on 2% agarose in 0.5× TBE buffer containing ethidium bromide.

Semiquantitative PCR was performed as described previously (7). Briefly, primer pairs were designed to amplify the sequence between exon 7 and exon 8, which generated two bands of DNA samples from vector-containing cells by PCR, the large one derived from the endogenous ADA gene containing intron 7 and the smaller one 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-lymphoblastoid cell lines (LCL) with untransduced cells. The ratio of the amount of amplified ADAcDNA derived from the integrated vector and that of the amplified genomic sequence was calculated after hybridization with an ADAcDNA probe. We have confirmed that this technique could provide quantitative results in samples with a transduction frequency below 50% (7).

To investigate the expression of each cytokine gene, RT-PCR was performed as described elsewhere (8). Briefly, cDNAs were generated from RNA templates in a 15-μl reaction mixture using a First-Strand cDNA synthesis kit (Pharmacia P-L Biochemicals, Piscataway, NJ). Two microliters of cDNA was amplified in a final volume of 50 μl using specific primers for IL-2, IL-4, IL-5, and IFN-γ. Primers for β-actin were utilized as an internal control. PCR was conducted for 25 cycles: 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. PCR products were then visualized by being subjected to electrophoresis on 2% agarose.

Two color fluorescence in situ hybridization

Details of the method of two color fluorescence in situ hybridization (FISH) on interphase nuclei of the PBL have been described elsewhere (9). Briefly, the clone pADA21l containing 1.1-kb human full ADA-cDNA and a 15-kb lambda-genomic ADA clone isolated from the EMBL3 SP6/T7 Lambda Library were used as probes. The cDNA and genomic probes were labeled with digoxigenin-11-dUTP and biotin-16-dUTP, respectively, by nick translation. A probe mixture (cDNA and genomic probes) and DNA of interphase cells on glass slides were denatured. The slides were hybridized at 37°C overnight in a 25-μl mixture containing the denatured probes, human COT-1 DNA, and 25% dextran sulfate, and washed at room temperature. The slides were then incubated at 37°C for 1 h in a 100-μl solution containing FITC avidin and anti-digoxigenin-rhodamin and washed. Interphase nuclei were counterstained with diamidino phenylindole, diazabicyclooctane, and 90% glycerol. Hybridized signals were observed under a fluorescence microscope with dual band pass filters. The sensitivity of the cDNA probe in this FISH system for the transgene has been estimated to be 85% (9).

ADA activity

ADA activity was measured as described previously (7). Briefly, PBMC were washed twice with PBS to remove FCS and then suspended in 100 mmol/l Tris (pH 7.4) containing 1% BSA. Cell lysates were obtained by five rapid freeze-thaw cycles. Cellular debris was removed by centrifugation, and the lysates were stored at −80°C until use. ADA enzyme activity

Table I. *$CD4^+$ and $CD8^+$ cells in infused cells and PBL*

		GT 7	GT 8	GT 9	GT 10	GT 11
Number of infused cells ($\times 10^7$ cells/kg)	CD4	2.7	3.5	4.7	1.2	0.6
	CD8	49.1	104.0	99.8	60.7	62.3
Increase of cell number[a] (per μl)	CD4	181	122	54	126	173
	CD8	3763	1579	1867	1828	940

[a] Increase of cell number is the difference between PBL count before and 1 wk after GT.

Table II. *T cell subset in PBL*

	GT 7		GT 8		GT 9		GT 10		GT 11	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
$CD8^+HLA\text{-}DR^+/CD8^+$ (%)	8.9	23.9	15.5	44.3	31.2	39.4	22.5	49.7	14.7	34.5
$CD8^+CD45RA^+/CD8^+$ (%)	ND[a]	ND	87.4	37.1	92.3	55.3	77.3	34.9	94.8	62.1

[a] ND, not done.

was assayed by measurement of the conversion of [^{14}C]adenosine (Amersham Life Science, Arlington Heights, IL) to [^{14}C]inosine and [^{14}C]hypoxanthine followed by TLC separation of the reaction products. The results were expressed as nanomoles of inosine and hypoxanthine produced per min by 10^8 cells (nmol/min/10^8 cells).

In vitro IgG production

A total of 1×10^5 peripheral B cells from a normal individual was cultured with the same number of peripheral T cells from the patient or a normal individual in round-bottom microtiter plates. Cells were stimulated with PWM or *Staphylococcus aureus* Cowan I (SAC) to produce IgG in triplicate. Culture supernatants were harvested after 6 days for determination of IgG secretion by an IgG-specific ELISA.

Results

PBL count after infusion of transduced cells

The dose of transduced cells for infusion was first increased stepwise to check the safety of the GT procedure (Fig. 1). PBL count increased to a peak level within 1 wk after each infusion and decreased gradually to near the pre-GT level over the next several weeks. PBL count just before each GT increased constantly from the 7th infusion on, suggesting an accumulation of transduced cells. Maintenance of the patient's PBL count at a high level seemed to require infusion of more than 4×10^8 cells/kg/therapy/3 mo. The change in $CD8^+$ cell count was parallel to that in total PBL count, whereas $CD4^+$ cell count remained low over the entire course. These findings indicate that most of the transduced cells were of the $CD8^+$ population.

In vivo kinetics of $CD4^+$ and $CD8^+$ cells in PBL

The number of $CD4^+$ and $CD8^+$ cells in both infused cells and patient's PBL were examined after the 7th GT. The number of infused $CD4^+$ cells was quite small, because more than 90% of cells were $CD8^+$ cells after stimulation with anti-CD3 Ab for a gene transduction and subsequent in vitro culture with IL-2 (Table I). This may be the major reason why the increase in $CD4^+$ cell number in PBL after each infusion was so small compared with that in $CD8^+$ cell number.

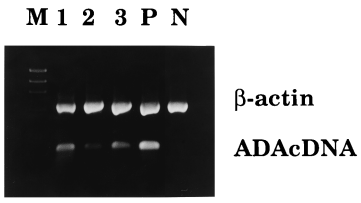

FIGURE 2. Integration of transduced ADAcDNA in peripheral T cell subset. Vector-derived ADAcDNA and β-actin gene were amplified from DNA sample purified from each type of cell just before the 11th GT and detected after electrophoresis on agarose gel. P, N, and M indicate positive control (LASN-transduced LCL which contained human ADAcDNA), negative control (nontransduced LCL), and DNA marker, respectively. *Lanes 1* to *3* represent PCR products from total PBL, $CD4^+$, and $CD8^+$ cells, respectively.

The kinetics of two $CD8^+$ T cell subsets in PBL, one the $CD8^+HLA\text{-}DR^+$ cells representing activated cells and the other $CD8^+CD45RA^+$ cells, most of which were considered to be naive cells, were also investigated (Table II). Ratios of $CD8^+HLA\text{-}DR^+$ cells vs total $CD8^+$ cells in PBL increased consistently after each infusion, suggesting that most infused cells were activated cells. In contrast, $CD8^+CD45RA^+$ cells decreased after each infusion, but increased to become the major population before the next infusion, while both absolute and relative numbers of $CD8^+HLA\text{-}DR^+$ cells decreased. These results suggest that most of the activated $CD8^+$ cells had a short life span and that only a small but distinct portion survived in vivo for a longer time. Such long-lived cells might be accumulated in vivo after a series of infusions. This may explain why a large number of transduced cells were required to maintain PBL count at a higher level.

These data raised the question of whether $CD4^+$ cells were activated less efficiently than $CD8^+$ cells during transduction, leading to failure in integration of ADAcDNA into $CD4^+$ cells, and whether most ADAcDNA-integrated $CD8^+$ cells were eliminated shortly after each infusion. To address these, we next investigated the integration of ADAcDNA in both purified $CD4^+$ and $CD8^+$ cells.

Integration and expression of ADAcDNA in PBL

The integration of ADAcDNA in $CD4^+$ and $CD8^+$ cells purified from the patient's PBL just before the 11th GT was examined (Fig. 2). ADAcDNA was apparently integrated in both $CD4^+$ and $CD8^+$ cells, but integration in $CD4^+$ cells was less than that in $CD8^+$ cells. These data indicate that both $CD8^+$ and $CD4^+$ cells carrying ADAcDNA were maintained for at least several months in the patient's PBL, although the increase in $CD4^+$ cells was less satisfactory.

ADA activity in the patient's PBL and frequency of ADAcDNA-integrated cells in PBL were investigated by semiquantitative PCR and FISH during the GT series. As shown in Fig.

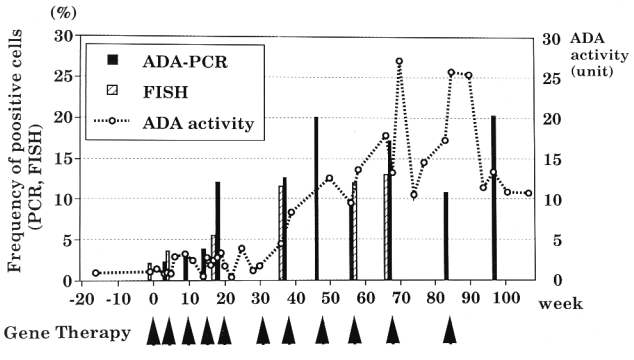

FIGURE 3. Integration and expression of transduced ADAcDNA in PBL. An open circle indicates ADA activity in PBL. A filled or a hatched bar represents the frequency of transduced cells in PBL detected by semiquantitative PCR or FISH.

Table III. *TCR repertoire*

	Pre-GT	Pre-5	Pre-8	Pre-11
TCR-$\alpha\beta^+$CD3$^+$ (%)	42.4	84.0	73.0	74.8
TCR-$\gamma\delta^+$CD3$^+$ (%)	8.3	3.1	3.2	1.8
TCR-Vα2 (%)	1.2	2.5	1.9	1.9
TCR-Vα12.1 (%)	ND[a]	ND	3.7	2.6
TCR-Vβ3.1 (%)	ND	ND	1.7	1.9
TCR-Vβ5.3 + 5.2 (%)	1.3	2.8	2.4	3.4
TCR-Vβ5.1 (%)	1.3	1.3	0.8	0.5
TCR-Vβ6.7 (%)	1.5	2.6	0.9	0.5
TCR-Vβ8 (%)	1.9	2.2	0.8	1.0
TCR-Vβ12 (%)	0.7	1.6	0.8	0.8
TCR-Vβ13.1 + 13.2 (%)	ND	ND	0.5	0.7

[a] ND, not done.

3, ADA activity in PBL progressively increased in parallel with the frequency of integrated cells in PBL. Significantly, ADA activity in PBL markedly increased to a level greater than 10 units, in other words almost one-tenth the level in normal individuals, after the frequency of transduced cells constituted more than 10% of PBL. This result suggests that the integrated ADAcDNA was correctly transcribed and promoted a definite function in vivo.

4) Peripheral TCR repertoire

Most of the increased CD3$^+$ cells in the patient's PBL after GT expressed TCR$\alpha\beta$, even though TCR$\gamma\delta^+$ cells were slightly decreased during that time (Table III). Analysis of TCR repertoire based on TCR V region usage revealed that no limited clones were expanded in his PBL. These results suggest that the transduction procedure did not promote preferentially the growth of T cells bearing specific TCR V regions. On this basis, the increased CD8$^+$ cells in his PBL may be considered to have contained functionally diverse populations, as seen in normal individuals.

Functional subset of peripheral CD8$^+$ T cells

Human CD8$^+$ T cells are functionally heterogeneous and reported to contain a distinct population that possesses helper activity for B cells (10–12). We then analyzed T cell subset expressing CD8$^+$CDw60$^+$ or CD8$^+$CD27$^+$CD45RA$^-$, both of which have been reported to provide substantial help to B cells (13, 14). CD8$^+$CDw60$^+$ cells were maintained in his PBL throughout the GT, whereas the CD8$^+$CD27$^+$CD45RA$^-$ cell population decreased (Table IV). However, this did not mean a decrease in the number of CD8$^+$CD27$^+$CD45RA$^-$ cells in his peripheral blood, because the total number of peripheral CD8$^+$ cells after GT was almost ten times than that before. In contrast, CD8$^+$CD27$^-$CD45RA$^+$ cells were significantly increased after GT. This population has been reported to include cytotoxic effector T cells (14).

We therefore tested whether his peripheral T cells, in which CD8$^+$ cells were predominant, contained helper activity for mitogen-induced B cell differentiation. Results showed that peripheral T cells were able to help normal B cells to produce a substantial amount of IgG in vitro (Fig. 4). We also investigated cytokine gene expressions in his PBL. Expression of both Th1- and Th2-type cytokine genes was induced at a comparably high level, as in normal subjects (Fig. 5). These results provided further evidence of the distinct functions of his peripheral T cells.

Table IV. *Functional subset of T cells*

	Pre-GT	Pre-GT 9	Pre-GT 11
CD8$^+$CDw60$^+$/CD8$^+$ (%)	31.3	34.6	22.8
CD8$^+$CD27$^+$CD45RA$^-$/CD8$^+$ (%)	18.3	3.4	4.1
CD8$^+$CD27$^-$CD45RA$^+$/CD8$^+$ (%)	16.4	64.8	70.8

Discussion

We successfully performed peripheral T cell-directed GT for a boy with ADA$^-$SCID and report here the in vivo kinetics of transduced cells. Several animal studies on in vivo kinetics of transferred T cells have been described. Cell transfer to athymic nude mice demonstrated the inherent ability of CD8$^+$ T cells to survive long term and to self-renew (15). In normal euthymic mice, in which the thymus likely continue to export T cells to the periphery, donor-derived mature CD8$^+$ T cells in PBL declined in number with time, and their half-life was estimated to be 34 days. Transfer of an equal number of CD4$^+$ and CD8$^+$ T cells suggested that the ultimate size of the T cell pool was apparently determined by the combined sum of CD4$^+$ and CD8$^+$ T cells. In our SCID patient, peripheral T cells declined after each infusion of transduced cells as in normal euthymic mice. On the basis that nonintegrated T cells likely had a short life span and the peripheral T cell pool was not full, however, cells integrating ADAcDNA in our SCID patient should have had a growth advantage and survived long term, as in athymic nude mice. Although we have no direct evidence for the

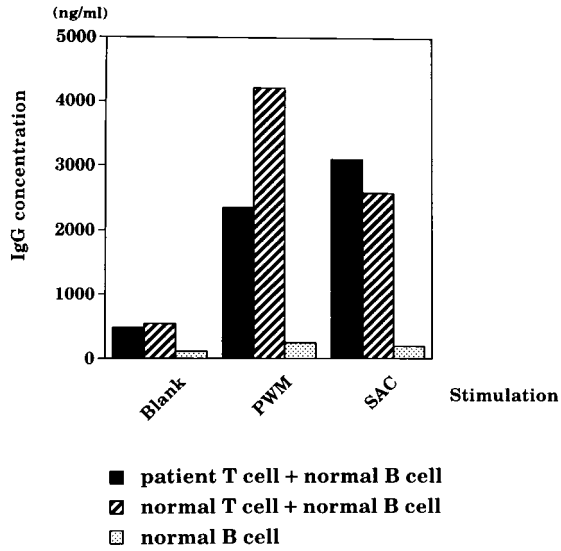

FIGURE 4. Helper function of patient's T cells in in vitro IgG production. IgG production from normal B cells with combination of normal or patient's T cells was measured by IgG-specific ELISA assay after 6 days' culture with stimulation of PWM or SAC.

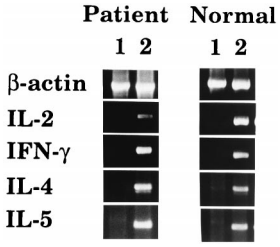

FIGURE 5. Cytokine gene expression in PBL. Expression of each cytokine gene was analyzed by RT-PCR using RNA samples from patient's PBL just before the 11th GT. *Lane 1* and *2* indicate RT-PCR products from PBL without and with stimulation by PHA, respectively.

selective survival of transduced cells, it will become clearer on long-term follow-up study in this patient after the discontinuation of gene therapy.

Cheever et al. (16) reported that cultured T cells could proliferate in vivo in response to stimulation by Ag, distribute widely, and survive long term to provide effector function and immunologic memory. Tough et al. (17) reported that long-lived T cells have multiple phenotypes and contain a mixture of memory cells, naive cells, and memory cells masquerading as naive cells. In humans, chromosome marker studies on irradiated patients indicate that $CD45RA^{low}$ cells divide more frequently than $CD45RA^{high}$ cells (18). However, the turnover of $CD45RA^{low}$ cells is quite slow, because most can remain in interphase for months. All these previous reports suggest the relatively long-term survival of transferred T cells and are consistent with our observation that PBL count just before each GT had constantly increased since the 7th infusion, and that most of these were $CD45RA^+$ cells including not only naive but also memory cells.

The median survival time of transferred cells in adoptive T cell therapy is reported to be proportionally increased, as the dose of effector T cells is increased (16). Our results indicate that at least 4×10^8 cells/kg/therapy/3 mo were required to increase and maintain our patient's PBL count. In two patients receiving GT at the National Institutes of Health (5), both were infused a substantial number of transduced cells, but only the first case has shown an apparent clinical improvement. Satisfactory GT is therefore additionally dependent on other factors, including differences in gene mutation, while the number of transduced cells may be an essential factor in peripheral T cell-directed GT.

In serial infusion, $CD8^+$ cells gradually increase to become a major population of the patient's PBL. This imbalance in T cell subset may be a characteristic problem in peripheral T cell-directed GT, on the basis of the fact that the major regulatory T cells are considered to be in the $CD4^+$ population. In our patient, however, the frequency of transduced cells in PBL, ADA activity in PBL, and clinical improvement correlated well, even though $CD8^+$ cells were predominant. Moreover, because the patient continued to receive the same dose of PEG-ADA during GT as before, his clinical improvement should have resulted from the GT itself.

To better understand the reconstitutive effect of GT on our patient's immunological system, we investigated the repertoire and functional subset of T cells in his peripheral blood. Analysis of TCR repertoire in PBL after GT showed no limit in TCR V region usage, after $CD8^+$ cells became predominant in his PBL. $CD8^+$ cells in his PBL should therefore have contained functionally diverse populations as in normal individuals. Recent reports have shown increasing evidence of the existence of regulatory $CD8^+$ cells, even though the main regulatory T cells should be in $CD4^+$ population. Rieber et al. (13) reported that the $CD8^+CDw60^+$ subset provided substantial help to B lymphocytes. T cells with this phenotype were maintained in our patient throughout the GT. Hamann et al. (14) also reported that $CD8^+CD27^+CD45RA^-$ cells had helper activity. $CD8^+$ cells with this phenotype were also maintained in number, although the relative number of these cells in his PBL decreased after GT. It is thus conceivable that these two $CD8^+$ T cell subsets may have played a role in the improvement of his immunological function. On the other hand, $CD8^+CD27^-CD45RA^+$ cells, which are considered to be CTL-type effector cells, significantly increased after the GT. These may also play a role in host defense against some pathogens. In addition, on the basis that low numbers of highly purified $CD4^+$ cells, but not $CD8^+$ cells, were successfully engrafted in the spleen of congenic SCID mice (19), a small but distinct population of $CD4^+$ cells with transduced ADAcDNA likely had a longer life span and were involved in his immunological function.

Presently, peripheral T cell-directed GT is the most effective method available in GT for ADA^-SCID. Its fundamental drawback is its limited duration of effect. Technical improvements and modifications such as increased transduction efficiency with new vectors and increased $CD4^+$ population during transduction procedures may improve efficiency. These approaches will improve the efficacy of this type of GT in providing an immediate but relatively short-term effect, but the final goal of GT for hemopoietic disorders must remain stem cell-directed GT.

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