Retroviral Gene Transfer of Human Adenosine Deaminase in Murine Hematopoietic Cells: Effect of Selectable Marker Sequences on Long-Term Expression

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Retroviral-mediated gene transfer of human adenosine deaminase (hADA) provides a model system for the development of somatic gene therapy as a therapy for diseases of bone marrow-derived cells. We have previously demonstrated that hADA can be observed in all hematopoietic lineages in a minority of mice transplanted with bone marrow cells infected with a simplified retroviral vector, ZipPGK-ADA. Here we report a majority of mice (six of eight) demonstrate expression of hADA in the peripheral blood at least 6 months after transplantation with bone marrow infected with this simplified retroviral vector, which contains no selectable marker. The failure to express hADA in two of eight mice was associated with the absence of the recombinant retroviral provirus in DNA prepared from bone marrow cells of these mice apparently due to failure to efficiently infect the reconstituting hematopoietic stem cell. In an effort to preselect bone marrow stem cells containing proviral integrations, we incorporated the selectable marker neo phosphotransferase (NEO) into a retroviral vector encoding hADA, N\textsubscript{i}$/ZipPGK-ADATKNEO, and used G418 selection of infected bone marrow cells before transplantation. In contrast to the simplified retroviral vector, hADA expression in these recipients was short lived (<8 weeks), despite the continued presence of intact provirus in DNA prepared from bone marrow of these mice. To determine whether the preselection of bone marrow using G418 was responsible for the lack of sustained hADA expression, we repeated the infection with the N\textsubscript{i}$/ZipPGK-ADATKNEO vector but omitted the G418 selection step. Again, the majority of recipient mice failed to express hADA long term, although the continued presence of provirus in DNA prepared from peripheral blood cell mononuclear cells was clearly demonstrated. Finally, we demonstrate clonal fluctuation of infected stem cells, and observe a temporal correlation between cessation of expression of hADA and the emergence of a dominant stem cell clone between 14 and 20 weeks posttransplantation in one recipient. These data suggest that inclusion of a second transcriptional unit that includes neo phosphotransferase sequences in this simplified vector is associated with decreased expression of the selectable ADA sequences.

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accomplished in these experiments, the number of mice demonstrating expression 6 months after transplantation was low (~30% to 40%). Here we report that use of the same simplified retroviral vector with a higher viral titer leads to stable expression in 75% of mice without use of any in vitro selection. In addition, we demonstrate that the introduction of a second transcriptional unit that includes the dominant selectable marker, neo phosphotransferase (encoding G418-resistance), into this vector is associated with a lack of stable expression of ADA in vivo, despite persistence of an unarranged provirus in hematopoietic cells. In one animal, progressive decrease in expression of the introduced ADA sequence is temporally related to the emergence of a dominant, presumably transcriptionally silent, clone of hematopoietic cells.

MATERIALS AND METHODS

Retroviral vectors. The ZipPGK-ADA retrovirus (hereafter referred to as PGK-ADA) (Fig 1) was constructed as previously described\(^1\) and contains the hADA cDNA expressed off the human \(X\) chromosome phosphoglycerate kinase promoter. The \(N_g\)/ZipPGK-ADATKNEO retrovirus (hereafter referred to as PGK-ADATKNEO) (Fig 2) was constructed from LTR-TKNEO, a construct in which the herpes thymidine kinase promoter (TK)-neo phosphotransferase (NEO) cassette was introduced at a Xho I site in an empty retroviral construct (\(N_g\)/Zip)\(^7\) that contains the 5’ long terminal repeat (LTR) and \(\psi\) + genome of \(N_g\) and the 3’LTR and genome of Zip retroviral vectors. The PGKprADA (PGK-ADA) cassette containing the natural ADA polyadenylation site was introduced in the antisense orientation (relative to the retroviral LTR) at an abutting BamHI site.

Virus-producing cell lines. ZipPGK-ADA was introduced into \(\psi\)-2 cells\(^2\) by calcium phosphate precipitation\(^3\) and high-titer clones selected for over-expression of ADA by incubation in media containing 4 \(\mu\)mol/L xyloturanosyl arabinoside (Xyl-A) and 5 to 6 \(\mu\)mol/L deoxycoformycin (dCF) (both supplied by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) as previously described.\(^4\) The producer lines \(\psi\)-2-PGK-ADA-21 produces recombinant retrovirus at a titer of \(1 \times 10^3\) Xyl-A/dCF-resistant colony-forming units (CFU)/mL when assayed on NIH/3T3 cells. High-titer producer clones of PGK-ADATKNEO retrovirus were generated by transfection of NJZipPGK-ADATKNEO into \(N_g\)-2 cells followed by selection in G418 at 0.75 mg/mL (dry powder; GIBCO, Grand Island, NY). The clone \(\psi\)-2-PGK-ADATKNEO-91 had a titer of \(5 \times 10^5\) G418-resistant CFU/mL when assayed on NIH/3T3 cells and was used in all subsequent experiments. All \(\psi\)-2 producer cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% calf serum, penicillin 100 U/mL, and streptomycin 100 \(\mu\)g/mL (all GIBCO).

Expression of hADA in murine hematopoietic cells. The expression of transduced hADA-cDNA was analyzed as previously described.\(^1\) Briefly, cells obtained from peripheral blood, CFU-S-derived spleen colonies, spleen, thymus, or BM cells were frozen and thawed in lysis buffer (5 mmol/L K\(_2\)HPO\(_4\)/1 mmol/L EDTA). Lysate supernatants were loaded onto cellulose-acetate strips presoaked in running buffer (Supra Heme buffer; Helena Laboratories, Beaumont, TX) and ADAS isoforms were separated by electrophoresis. Enzyme activity was detected by exposing the cellulose acetate strip to a substrate containing xanthine oxidase (0.06 U/mL) and nucleoside phosphorylase (15 \(\mu\)g/mL) (both from Boehringer Mannheim, Indianapolis, IN), adenosine (2 mg/mL), dimethylthiazol diphenyltetrazolium bromide (0.5 mg/mL), and phenazine methosulphate (0.1 mg/mL) (all from Sigma, St Louis, MO) in 50 mmol/L phosphate buffer, pH 7.5, at 37°C for 12 to 15 minutes.

DNA analysis. Nucleated cells were recovered from peripheral
blood samples by lysis of red cells in Triton-X (Sigma, St Louis, MO) solution (0.32 mol/L sucrose, 10 mmol/L Tris, pH 7.5, 5 mmol/L MgCl₂, 1% Triton-X-100) and DNA was prepared from these and other cell populations by standard methods. DNA was digested with Xba I (for proviral structure) or EcoRI (for proviral integration patterns). Digested DNA was electrophoresed on a 1% agarose gel, transferred to nylon membrane (Magnagraph; Micron Separations, Inc, Westboro, MA), and hybridized to a ³²P-labeled Neo I-Hinfl fragment of hADA cDNA. Prehybridization, hybridization, and posthybridization washes were performed according to the manufacturer’s recommendations. Filters were exposed to x-ray film in the presence of calcium tungstate intensifying screens at −70°C.

**Helper virus assay.** Plasma and spleen samples were analyzed for the presence of helper virus at the time of killing, 6 months post-BMT. Peripheral blood (1.5 to 2 mL) was obtained by cardiac puncture and plasma separated by centrifugation. A single cell suspension representing one half of each spleen was prepared and the plasma and spleen cells were cocultured with monolayers of NIH/3T3 cells for 48 hours, after which the fibroblasts were incubated in medium containing Xyl-A/dCF (PGK-ADA) or G418 (PGK-ADATKNEO) for a further 12 to 14 days.

**RESULTS**

**Generation of virus-producing cell lines.** Previously, we have reported that ~33% of mice transplanted with BM cells infected with the PGK-ADA producer clone ψ-2 PGK-ADA-120.3 (titer, 2 × 10⁶ Xyl-A/dCF CFU/mL) expressed hADA 6 months after transplantation. Because a high titer of retrovirus in the supernatant of retroviral producer lines is important for infection of HSC, we screened multiple new producer cell clones of PGK-ADA retrovirus. One producer clone (ψ-2 PGK-ADA-21) of 60 clones screened had a titer fivefold higher (1 × 10⁶ Xyl-A/dCF CFU/mL) than the producer cell line used in our previously reported experiments and was used in the experiments reported here. Selection with Xyl-A/dCF depends on expression of ADA above endogenous levels and viral titer based on this selection consistently is lower than titers based on G418. Because several investigators have shown that selection of BM cells in G418 following infection with retroviral constructs that contain the neo phosphotransferase (NEO) gene leads to effective elimination of non-NEO containing colony-forming cells, we also established retroviral producer cell lines of the construct N₂/ZipPGK-ADATKNEO (Fig 2). Retroviral vectors containing the TKNEO cassette have been useful for the long-term and stable expression of G418 resistance in BM. Screening of multiple G418-resistant producer clones yielded one (ψ-2 PGK-ADATKNEO-91) with a titer of 5 × 10⁵ G418 CFU/mL and this producer cell line was used in subsequent BM experiments.

**Infection of BM cells and expression of hADA in vivo.** Infection of murine BM cells following prestimulation with multiple growth factors uniformly yielded successful gene transfer into HSC. In contrast with our previous results, the use of a higher titer PGK-ADA virus was associated with expression of hADA in the majority of transplant-recipient mice 6 months (180 days) following transplantation (Fig 3). The level of expression of the transferred ADA gene is variable (estimated to be 5% to 25% of endogenous murine ADA expression), but easily detectable in peripheral blood samples in six of eight mice 6 months following transplant (animals 1 through 3 and 6 through 8, Fig 3). These mice represent two representative experiments performed concurrently with the experiments reported below. In addition, comparison of expression at 4 months and 6 months demonstrated that one animal (lane 8) was expressing hADA at 6 months, although not apparently expressing human enzyme at 4 months. Such fluctuations in stem cell clonal dominance have been previously reported using retroviral integration as a marker for stem cell tracking at

**Fig 3.** Long-term expression of hADA in peripheral blood of mice transplanted with BM cells infected with the PGK-ADA virus. In situ enzyme analysis of peripheral blood samples of eight separate mice at 4 and 6 months posttransplantation. The location of human and murine ADA enzymes and murine hemoglobin (Hgb) are as noted. The positive lane represents a fibroblast cell line expressing both human and murine ADA. The number of the lane denotes the identity of individual mice at each time point.
the DNA level.\textsuperscript{16} The level of expression of the introduced hADA cDNA compared with endogenous murine ADA is in agreement with our previous experience using this vector.\textsuperscript{16,17} In addition, also as previously noted, animals that did not express hADA were shown to not contain the retroviral provirus by Southern blot analysis of DNA obtained from the spleen and BM (not shown). Southern blot analysis of DNA obtained from hADA(+) animals showed the provirus to be unarranged and estimated at 0.1 to 0.3 genome copies (not shown). This result implies that reconstituting HSC were not efficiently infected in all animals, but that the presence of the PGKADA provirus is uniformly associated with expression of the transferred ADA cDNA. No helper virus was detected in the serum or by coculture of spleen cells with NIH/3T3 cells from these mice. Rare NIH/3T3 colonies survived the Xyl-A/dCF selection following attempted helper rescue and these were analyzed for the expression of hADA by the method described above. None of these expressed hADA when analyzed by ADA isoenzyme assay, demonstrating the lack of helper virus in vivo.

Because 25\% of the mice transplanted with BM cells infected with PGK-ADA retrovirus did not contain provirus integrations, we introduced a dominant selectable marker, Neo, expressed off the herpes virus TK promoter into a PGK-ADA retrovirus (Fig 2). Initial experiments analyzed the optimal infection and selection protocol with the vector using day 12 CFU-S-derived spleen colonies to assay efficiency of retroviral infection. In a representative experiment, 6\% of isolated spleen colonies were transduced following 48 hours of cocultivation (no prestimulation), while 21\% were positive after 48 hours of cocultivation and 24 hours of selection (in 1.2 mg/mL G418), and, optimally, 53\% of CFU-S were transduced following 48 hours of cocultivation and 48 hours of selection in G418. Selection in higher concentrations of G418 was associated with excessive CFU-S toxicity and too few stem cells to allow efficient reconstitution of transplanted mice. Even at the 1.2 mg/mL concentration of G418, only 6\% of BM cells survived the infection and selection manipulations (range, 1\% to 10\% in eight experiments) and expression of the transferred gene was transient. Ten of 13 mice (77\%) expressed detectable hADA at the first analysis posttransplantation (14 days). However, from the total of 13 mice transplanted after in vitro selection with G418, no recipient expressed hADA longer than 56 days posttransplantation. The majority of mice in these experiments (7 of 13) demonstrated no detectable hADA expression beyond 42 days posttransplantation (range for group, 19 to 56 days).

In an effort to more closely compare the results of experiments using the PGK-ADA and the PGK-ADATKNEO vectors, we used the identical infection protocol for gene transfer with the PGK-ADATKNEO virus as described above for the simplified PGK-ADA virus (48 hours of prestimulation, 48 hours of cocultivation, no selection in G418). As seen in Fig 4A, the expression of hADA (in relation to endogenous murine ADA) was uniformly lower (judged to be <5\% of endogenous) in peripheral blood of

Fig 4. Expression (A) and proviral structure (B) in peripheral blood cells from mice transplanted with BM cells infected with PGK-ADATKNEO retrovirus. (A) In situ enzyme analysis of peripheral blood samples as in Fig 3. Below lanes are the weeks posttransplantation of the blood samples: lane 1 (+) represents the location of endogenous murine ADA. Lane 1 (-) represents uninfected control, lane 2 (+) represents a single-copy positive control. As seen by the location of the endogenous band, the proviral structure in lane 2 (+) is artifactually running slightly fast. Both (A) and (B) represent serial blood samples of a single mouse.
these animals compared with expression directed by the simplified PGK-ADA vector (compare with Fig 3). Following the expression of hADA in the peripheral blood of individual mice, three patterns of expression were noted: (1) lack of expression in the peripheral blood from the initial analysis despite the presence of intact provirus in the DNA of peripheral blood mononuclear cells by Southern blot analysis (8 of 15); (2) sustained but low level expression (1 of 14); and (3) transient expression lasting from 10 to 14 weeks associated with persistence of the provirus by Southern analysis after cessation of expression (4 of 14) (Figs 4 and 5). As seen in Fig 4A, a representative example of the last pattern, the level of expression of hADA is low, but detectable in serial bleeds from one mouse until 20 weeks posttransplant (Fig 4A, lane 6). Simultaneous Southern blot analysis of Xba I-restricted DNA from peripheral blood cells obtained with this sample (Fig 4B) shows the presence of an unarranged 2.7-kb ADA-hybridizing provirus even at 20 weeks (Fig 4B, lane 7). Compared with the single-copy control (Fig 4B, lane 2), we estimate that ~25% to 50% of these cells contain an intact provirus and that the relative number of cells derived from transduced stem and progenitor cells was stable over the 4 to 20 weeks examined.

The systematic analysis of hADA expression in serial peripheral blood samples obtained from individual mice along with simultaneous Southern blot analysis of DNA from these same samples led to the observation that decreasing expression of the transferred gene was temporally related to the emergence of a dominant, presumably transcriptionally silent, stem cell clone in one recipient. As seen in Fig 5A, expression of hADA in this mouse was easily seen from 4 to 10 weeks posttransplantation (Fig 5A, lanes 1 through 3). Southern blot analysis of DNA obtained from these same blood samples was analyzed for integration patterns by cutting with EcoRI and hybridization with 32P-labeled hADA cDNA (Fig 5B). Cross-hybridization with the endogenous murine ADA gene yields a slow migrating band (at arrowhead), while DNA from a transfected NIH/3T3 cell line yields a single integration band (at arrow) consistent with a single-copy integration. DNA from blood samples at 4 to 10 weeks posttransplant showed a smear at 4 weeks (consistent with multiple integrations into more differentiated progenitors and precursor cells), major integration bands (at a and b) and the emergence of an additional clone of cells (represented by integration band c) at 10 weeks (Fig 5B, lane 5). No expression of hADA is seen after 14 weeks (lane 4, Fig 5A). Simultaneously, the dominant clone represented by integration c (lanes 6 and 7, Fig 5B) becomes the only peripheral blood cells containing the integrated provirus by DNA analysis. These data

![Diagram](image_url)
suggest that the lack of expression of ADA after 10 weeks in this recipient is due to the replacement of cells expressing ADA with progeny of a transduced but transcriptionally silent reconstituting stem cell.

The results shown in Figs 4 and 5 were derived from representative mice from four independent experiments performed concurrently with the experiments using PGK-ADA virus and shown in Fig 3. As seen in Table 1, a summary of the six experiments reported here, 1 of 15 mice transplanted with BM infected with the PGK-ADATK-NEO virus demonstrated stable, but very low level, expression of ADA after 4 months posttransplantation. None of an additional 13 mice transplanted with BM infected with this same virus and selected in vitro with G418 demonstrated detectable ADA expression longer than 56 days posttransplantation. In contrast, six of eight mice transplanted with the simplified PGK-ADA virus demonstrated easily detectable (estimated at 5% to 25% endogenous) ADA following full hematopoietic reconstitution.

**DISCUSSION**

Efficient infection of reconstituting stem cells and stable expression of the introduced sequences in the progeny of these cells is essential to the successful application of gene transfer methods to the correction of human diseases of BM-derived cells. The ability of retrovirus vectors to infect the HSC is dependent on a number of factors including duration of cocultivation, the titer of the packaging cell line, and the cycling status of the target cell population, because cellular division is crucial for the integration of the incoming virus. We and others have demonstrated stable integration of retroviral vectors and expression of transferred genomes for up to 6 months posttransplantation. However, long-term expression has not been seen in all transplanted mice. Loss of expression of transferred genes has been attributed to the emergence of cells derived from uninfected stem cells. There is increasing evidence that hematopoietic recovery after BMT initially involves numerous hematopoietic progenitor and precursor cells, each contributing clonal progeny to the blood cellular elements. With time, the cells of some of these clones undergo terminal differentiation and death, so called 'clonal extinction,' and hematopoiesis becomes oligoclonal.

In an attempt to increase the proportion of recipients demonstrating long-term expression of ADA, we used a protocol that included an in vitro selection step, with the aim of transplanting only those cells in which the provirus had been integrated. The presence of a selectable marker has been successfully exploited by other groups, who have reported infection efficiencies of CFU-C and CFU-S approaching 100% in the transduced gene activity was observed in 70% of mice transplanted in these experiments, which confirms data reported by Bender et al using a retroviral vector containing Neo' and the β-globin gene. In their report the level of expression of the transferred β-globin gene was quite low, 0.24% to 1.5% of endogenous mouse globin mRNA. In the experiments reported here using G418 selection, expression of the second gene, ADA cDNA, was also low and not sustained long term in most recipients. The failure to achieve long-term enzyme activity was undoubtedly multifactorial, but previously accepted explanations of low infection efficiencies of HSC and 'clonal extinction' are not applicable in this case. Simultaneous documentation of lack of expression of the transferred ADA cDNA and intact provirus up to 5 months (150 days) posttransplantation confirmed infection of a reconstituting stem cell. We and others have previously reported the lack of expression of retroviral vectors in primary hematopoietic cells in the presence of unarranged proviral integrations. However, in these experiments no expression of the transferred genetic sequences was initially seen in vivo. A similar observation was reported by Bowtell et al in which the proportion of G418-resistant colonies derived from mice transplanted with cells infected with a retrovirus containing Neo' did not correlate with the proviral content.

Serial analyses of mice demonstrating unsustained expression of ADA after transplantation with cells exposed to PGK-ADATKNEO virus confirmed our previous observations, namely that cessation of expression was not necessarily due to reconstitution with progeny of uninfected HSC. One explanation for these observations is that the progeny of some infected stem cells are unable to express the transduced gene, while the immediate progeny of infected progenitor and precursor cells are permissive for expression of these same sequences. Alternatively, transcriptional shut off could occur in some cells during reconstitution of the recipient by transduced stem cells.

Hematopoiesis after BMT has been characterized using retroviral integration patterns by fluctuations in clonal populations followed by the emergence of a stable clone. Our data confirm these observations at the DNA level of analysis and contribute new expression data showing the temporal relationship between the loss of expression of the introduced genome and the emergence of a dominant and transcriptionally silent stem cell clone. There are several possible explanations for this observation. It is possible that integration into the genome of a primitive cell occurs at a site that is transcriptionally active only in immature cells. As the cell differentiates, transcription at that site may cease and the retroviral genes may concomitantly become transcriptionally silent. Secondly, the presence of more than one gene within the provirus may have a deleterious effect on their expression. Bowtell et al have also observed that retrovirus vectors carrying two genes are poorly transcribed in vivo, even in the absence of selection and despite the presence of provirus in the host hematopoietic cells. Our data are very similar and remarkable for the difference in expression of the transferred hADA when vectors containing the neo phosphotransferase gene are compared with the simplified vector containing only the hADA cDNA.

**Table 1. Long-Term Expression of ADA Recipient Mice**

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<th>Vector</th>
<th>Days Post-BMT</th>
<th>hADA Mice/Total Survivors</th>
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<tr>
<td></td>
<td>&lt; 30</td>
<td>31-60</td>
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<tr>
<td>PGK-ADATKNEO</td>
<td>7/15</td>
<td>4/14</td>
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<tr>
<td>PGK-ADA</td>
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and no other dominant selectable marker, in which 75% of mice exhibit easily detectable levels (estimated at 5% to 25% of endogenous) hADA 6 months after transplant. In addition to the inclusion of a dominant selection cassette (TKNeo), the PGK-ADATKNEO vector described here differs from the simplified vector in two other ways: (1) the 5' region contains an additional 1 kb of GAG sequence (so called GAG + ) that has been shown to improve the recombinant titer; and (2) the ADA cDNA is expressed in the reverse orientation with respect to the 5' LTR. Therefore, the difference in expression of the transferred hADA observed in animals transplanted with BM infected with these two viral vectors may be due to any one of these changes and further experiments will be required to determine the exact mechanism(s) involved.

Previous work by our laboratory and by Wilson et al reported the successful use of retroviral vectors containing the ADA cDNA and no additional selectable markers for the transduction of reconstituting murine HSC. These experiments demonstrated high levels of hADA (in some mice the level of hADA was higher than murine ADA) in all hematopoietic lineages of normal irradiated recipients after full hematopoietic reconstitution. The additional data reported here (Fig 3) demonstrate the importance of recombinant viral titer on the transduction of HSC. In addition, Moore et al reported the presence of hADA by immunoblotting techniques in 30% of mice 18 to 31 weeks posttransplantation with BM infected with a vector containing ADA and no dominant selectable marker. In contrast, work by Osborne et al and Kaleko et al demonstrated that a minority (one of three) of W/W mice expressed low levels of hADA (<5% of endogenous) following transplantation of normal congenic BM following infection with vectors containing the neo phosphotransferase gene.

Progress in the application of retroviral vectors for somatic gene therapy will be dependent on a more thorough understanding of the molecular nature of loss of expression of introduced genes in progeny of HSC. Resulting changes in vectors might include altering the position of the genes in relation to regulatory sequences or other gene sequences, the inclusion of enhancer sequences, or the deletion of inhibitory elements. In addition, further understanding of the biology of HSC will be needed, especially in larger animal species, to improve the efficiency of gene transfer into reconstituting HSC. This understanding has already lead to the development of improved infection protocols with retrovirus vectors containing only single genes with no need for selection in vitro or in vivo. The use of purified populations of HSC, stem cell-specific growth factors, and optimization of the microenvironment during infection and/or selection protocols may prove useful in the future.

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