Expression of Human Adenosine Deaminase in Mice After Transplantation of Genetically-Modified Bone Marrow

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A high titer retroviral vector was used to transfer a human adenosine deaminase (h-ADA) cDNA into murine bone marrow cells in vitro. The h-ADA cDNA was linked to the retroviral promoter, and the vector also contained a neomycin phosphotransferase gene as a selectable marker. Infected marrow was transplanted into syngeneic W/W' recipients, and h-ADA expression was monitored for 5.5 months. Several weeks after transplantation, h-ADA was detected in the erythrocytes of all nine recipients, eight of which expressed levels equal to the endogenous enzyme. This level of expression persisted in two of six surviving mice, while expression in three others stabilized at lower, but readily detectable, levels. Only one mouse had no detectable h-ADA after 5.5 months. Vector DNA sequences with common integration sites were found in hematopoietic and lymphoid tissues of the mice at 5.5 months, providing evidence that hematopoietic stem cells had been infected. Furthermore, all mice transplanted with marrow that had been selected in G418 before infusion had multiple vector copies per genome. While this category included the two highest h-ADA expressors, it also included the negative mouse. Thus, multiple copies of the vector were not sufficient to guarantee long-term h-ADA expression. Mice were monitored for "helper virus" infections with an assay designed to detect a wide range of replication-competent retroviruses, including those endogenous to the mouse genome. No helper virus was detected in the two highest h-ADA expressors, ruling out helper-assisted vector spread as a cause of the high h-ADA expression. These results help provide a foundation for the development of somatic gene therapy techniques to be used in the treatment of human disease.

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helper virus generation. The h-ADA cDNA is transcribed from the Moloney murine leukemia virus LTR. The neomycin phosphotransferase (neo) gene, which confers G418 resistance, is transcribed from an internal SV40 promoter. To produce ecotropic virus, cell-free supernatant from the amphotropic producer PA317/old female mice used in this study were obtained from Jackson Laboratories (Bar Harbor, ME). WBB6F/J was used in all experiments and had a titer of 2.4 x 10^9 plaque-forming units/mL by XC assay, as previously described 

Culture time for ψ2/LASN cells was minimized to avoid helper virus production, which can occur with this packaging cell/vector combination. Procedures involving recombinant viruses were performed in accordance with the National Institutes of Health (NIH) guidelines at the BL1 level of containment.

**Bone marrow infection and transplantation.** The 8- to 12-week-old female mice used in this study were obtained from Jackson Laboratories (Bar Harbor, ME). WBB6F/J +/ + mice were used as bone marrow donors. WBB6F/J W/W mice, which have an anemic phenotype due to a defect in hematopoiesis, were used as recipients. 5-Fluorouracil (150 mg/kg) was given intravenously to donors 4 days before marrow harvest. Marrow was flushed from femurs and tibias with cocultivation medium: Iscove's medium, 10% heat-inactivated (56°C for 30 minutes) FBS (Hyclone), 10% pokeweed mitogen-stimulated spleen cell conditioned medium (Terry Fox Laboratories, Vancouver, BC, Canada), Polybrene (4 μg/mL), penicillin, and streptomycin. Clumps of marrow were dispersed with 5 to 10 passages through a 10 mL pipette. For cocultivation, media was aspirated from 10 cm dishes of virus-producer cells (plated at 1 x 10^6 cells per dish, 48 hours earlier) and replaced with 10 mL of marrow suspension. Marrow from one mouse was added to each dish. The cells were maintained at 37°C in a 5% CO2 atmosphere. After 3 days, 10 mL of fresh cocultivation medium was added to each dish. Nonadherent cells were washed from each dish 1 day later. Marrow that was selected in G418 before transplantation was centrifuged at 1,200 rpm for 5 minutes and resuspended in 1/2 volume of fresh cocultivation medium containing 0.5 mg/mL G418 (active compound). The marrow was washed 2 days later and resuspended in Hanks' balanced salt solution (HBSS) for infusion into recipients. Marrow that was not G418-preselected was harvested from producer plates and transferred to 10 cm dishes for 8 hours to remove adherent fibroblasts. Nonadherent cells were then resuspended in HBSS for infusion. Recipient mice were each injected, via tail vein, with 0.4 mL of cell suspension. To decrease the morbidity occasionally associated with marrow infusion, heparin (75 units) was given, via tail vein, to each recipient 30 minutes before transplant.

**ADA assay.** Blood was collected at intervals from tail veins, and red blood cells (RBCs) were analyzed for h-ADA. RBCs were lysed 1:1 with water, and the stroma was removed by toluene extraction. ADA assay was assayed in other tissues after maceration and sonication on ice. ADA activities were measured by a spectrophotometric assay, and mouse and human ADA were distinguished by starch gel electrophoresis. RBC samples that contained h-ADA at levels of greater than 20% of the endogenous mouse ADA were readily detected by the standard starch gel method (Fig 2A and B). To identify low levels of h-ADA in mouse tissues, h-ADA combining protein (ADCP) was added to mouse tissues. ADCP retarded the migration of h-ADA and permitted identification of low levels of h-ADA in mouse tissues (Fig 2C). ADCP was derived from cultured skin fibroblasts obtained from patients with ADA deficiency. Test samples were incubated with ADCP for 30 minutes before starch gel analysis. Control mouse tissues were mixed with human T-cell ADA and ADCP to determine the limit of detection for h-ADA. We estimated that addition of ADCP enabled detection of...
0.001 μmol/l of h-ADA activity per lane, which is about 1% of the level of endogenous mouse RBC ADA. Human ADA activity levels in mouse tissues were estimated from starch gels by comparison with human ADA standards of known activity prepared from human T cells.

**Assessment of erythroid reconstitution.** Erythroid reconstitution was assessed 3 months after transplantation. Blood was diluted 1:10 in phosphate-buffered saline (PBS) containing heparin and analyzed for RBC number and size on a Sysmex E-2500 blood analyzer (Baxter Scientific, Chicago, IL). Donor mice had a normal erythrocyte profile, with an RBC count of 11.6 cells per picoliter and a mean cellular volume of 50 fl. Recipients had a macrocytic anemia with an RBC count of 5.2 cells per picoliter and a mean cellular volume of 75 fl. Successful reconstitution with donor marrow has been shown to result in a cure of the recipient's anemia.\(^2\) We considered transplanted animals to be reconstituted if the RBC number was greater than 10 cells per picoliter and if the macrocytosis was eliminated.

**Helper virus assay.** Blood from recipient mice was assayed for endogenous and exogenous helper virus at 2 and 3 months post-transplantation, respectively, by using sensitive vector rescue assays. Several cell lines that were sensitive to different murine helper viruses, and that contained a replication-defective retrovirus vector carrying the neo gene (N2\(^{2+}\)), were used as recipients for test virus. Endogenous (ecotropic, amphotropic, and xenotropic) viruses were assayed on Mus dunni fibroblasts (Dunnii/N2). Exogenous viruses were assayed on NIH 3T3 fibroblasts (NIH 3T3/N2). In one case, Balb/c 3T3/N2 and HeLa/N2 cells were also used to extend the assay range to include B-tropic and additional xenotropic viruses, respectively. Preliminary experiments showed that addition of an appropriate helper virus to these vector-containing cells resulted in rescue of the neo-virus and the appearance of this virus at high titer in medium exposed to the cells.

For vector rescue assay, 10 μL fresh mouse blood was mixed with 4 μL culture medium containing 4 μg/mL Polybrene (Polysciences, Inc., Warrington, PA) and 4 μg/mL Polybrene (Sigma Chemical Co., St Louis, MO), and the mixture was added to 6 cm dishes. The cells were passaged every 3 to 4 days for 2 weeks to allow infectivity. One day later, the blood cells were washed from the dish and the adherent cells were trypsinized and split 1:10 into 6 cm dishes. The cells were passaged every 3 to 4 days to allow potential helper virus to spread. Medium was harvested after a 16-hour exposure to confluent dishes of cells, filtered (0.45 μm pore size), and 1 mL was assayed for the ability to transfer G418 resistance (0.75 to 1.0 mg/mL active compound) to homologous cells (Dunnii, NIH 3T3, Balb/c 3T3, or HeLa). Negative controls included vector-containing cells that had never been exposed to mouse blood, but which were passaged for 2 weeks. Positive controls included vector-containing cells infected with Moloney murine leukemia (ecotropic, N2; pMLV- K),\(^3\) 1504A (amphotropic, N2; pMLV-K),\(^3\) or NZB virus (xenotropic; NZB-JU-6).\(^3\)

**DNA analysis.** Tissue samples were collected from bone marrow, spleen, thymus, lymph node (axillary, cervical, mesenteric) and liver. DNA was prepared from each tissue and analyzed for the presence of the LASN provirus by Southern blotting.\(^2\) DNA samples were digested with *XbaI* to assess proviral integrity and *HindIII* to assess proviral integration patterns.

**RESULTS**

**Experimental design.** For transfer and expression of h-ADA, we used the retroviral vector LASN (Fig 1) for two reasons. The LTR promoter/enhancer had previously been shown to drive the h-ADA cDNA as well as or better than other promoters (CMV and SV40) in cultured human hematopoietic cells.\(^4\) Second, this vector produced very high titer virus in the ecotropic packaging line v\(^2\). After cocultivation, marrow used in most of the animals was preselected in G418. Preselection acts to destroy those stem cells that remain uninfected, thereby enriching for infected stem cells and boosting the average number of vector inserts per genome in reconstituted animals.

In preliminary studies involving transplantation of DBA mice, we found that after radiation treatment, 19 of 20 DBA mice developed life-long infections with an endogenous ecotropic virus (data not shown). Previous work has shown that 30% of DBA mice spontaneously activate an endogenous, ecotropic, N-tropic retrovirus as they age.\(^5\) Furthermore, radiation treatment has been shown to activate ecotropic and xenotropic retroviruses in vivo in a dose-dependent manner.\(^6\) Therefore, to avoid unknown effects of the helper viruses on the experimental results, we used *W/W\(^v\)* mice as bone marrow recipients since radiation is not required to achieve engraftment in these animals.\(^8\) *W/W\(^v\)* mice have a stem cell defect and can be competitively reconstituted by wild type marrow in the absence of radiation treatment. Fj hybrid resistance is enhanced in the absence of irradiation,\(^3\) thus, we used syngeneic WBB6F, mice as donors instead of parental C57Bl to avoid the possibility of graft rejection due to hybrid resistance.

**Persistent h-ADA expression in transplanted mice.** Six mice were transplanted with G418-preselected marrow, each receiving the marrow from eight donors. Human ADA expression was monitored by starch gel analysis of RBC lysates at intervals after transplantation (Table 1, Fig 2). At time points up to 1 month, all six expressed h-ADA at levels approximating that of the murine enzyme. At 5.5 months, two mice (P1 and P2) continued to express the same high level, and two mice (P3 and P4) showed a decrease in h-ADA expression. In addition to standard analysis by starch gel electrophoresis (Fig 2A and B), mice P3 and P4 were analyzed by a more sensitive assay using human ADA-combining protein, which showed that only one mouse (P4) had undetectable h-ADA levels (less than 1% of endogenous ADA).

Three mice were transplanted with marrow that had not been G418-selected. Each received the marrow from two donors. Again, appreciable h-ADA levels were noted at the early time point, 3 weeks post-transplant (Table 1). Two mice, NP1 and NP3, expressed levels approximating that of the endogenous enzyme; ie, similar to the mice that received G418-preselected marrow. One mouse, NP2, exhibited a somewhat lower level. With time, h-ADA levels in all three mice stabilized at low, but readily detectable, levels (Table 1, Fig 2C).

Tissue samples were assayed for ADA activity 5.5 months after transplantation. h-ADA activity was detected in the bone marrow samples (Fig 3), but the levels did not correlate well with those found in RBCs. Mouse P2 had the highest level of h-ADA in the marrow, accounting for about 10% of the total ADA. Mice P1 and P4 exhibited 1% to 2% h-ADA expression in the marrow, while h-ADA was not detected in marrow from mouse P3 or the mice that received nonselected marrow. No h-ADA activity was detected in spleen (Fig 3),
thymus, or lymph node (Table 2) from any animal. Liver was also examined as a negative control, since donor bone marrow cells are not expected to reconstitute the liver, and all samples were negative for h-ADA. Thus, h-ADA was detected in RBCs and bone marrow, but not in other hematopoietic or lymphoid tissues, including spleen, thymus, or lymph node. It should be noted, however, that tissues in which h-ADA was not detected express mouse ADA at levels at least 30-fold higher than RBCs, which may have prevented detection of h-ADA expression at levels found in RBCs (Table 2).

Efficient erythroid reconstitution. The extent of reconstitution with donor erythroid cells was assessed at 3 months post-transplant (Table 3). We were concerned that the decrease in RBC h-ADA expression noted in mice P3, P4, and NP1-3 may have resulted from poor or transient engraftment with donor marrow, but this was not the case. Six of the seven mice that were analyzed were found to be nearly completely or completely reconstituted with donor erythroid cells. That is, their hemodynamic profiles, including RBC number and mean cellular volume, closely matched that of the donors rather than the recipients. Blood from one mouse, NP3, was found to have a low RBC number, but only moderate macrocytosis. This animal was considered to be partially engrafted, and the anemia was attributed to a nonspecific illness.

Efficient vector transfer. A second possible explanation for the decrease in RBC h-ADA expression seen in some
animals after 1 month, and for the lack of h-ADA expression in spleen, thymus and lymph node, was that the recipient animals were transplanted with donor stem cells that had not been infected with the LASN vector. At 5.5 months post-transplant, animals were sacrificed and genomic DNA was prepared from bone marrow, spleen, thymus, lymph node, and liver. The samples were digested with Xba I, which cuts in each LTR (Fig 1), and analyzed by Southern blotting using a neo gene probe (Fig 4A through C). Two genomic controls were included on each blot; NIH 3T3/N2 has a single copy of the N2 vector per genome and $2/LASN provides a marker for LASN vector size. Genomic samples from a WBB6F1 +/+ donor animal provided additional negative controls.

Despite variations in their levels of h-ADA expression, all four mice that had received G418-preselected marrow exhibited the same DNA hybridization pattern (Fig 4A and B). Dense bands of appropriate size were seen in the bone marrow, spleen, and thymus lanes (except mouse P4, for which no thymic tissue was found). These bands were clearly denser than the single copy control showing that, on average, there were several vector copies per genome. Fainter bands were seen in each lymph node lane. This may be attributed to incomplete reconstitution of the lymph node compartment due to the omission of radiation treatment. No bands were seen in the liver lanes or any of the lanes representing tissues from the negative control animal.

Similar bands of appropriate size were seen in the bone marrow, spleen, and thymus lanes from animals that had received nonselected marrow (Fig 4C). However, the band intensities were fainter, indicating an average genomic copy number significantly less than 1.

To examine the pattern of vector insertions, the genomic DNAs were digested with HindIII, which cuts once in the vector just upstream from the neo gene (Fig 1) and at random sites in flanking genomic DNA. The DNA was analyzed by Southern blotting, using a Neo probe. The NIH 3T3/N2 Xba I digest is again included to indicate single copy band intensity. All four mice that received G418-preselected marrow exhibited multiple insertions (Fig 4D). For each of the three tissues examined—bone marrow, spleen, and thymus—the insertion patterns were similar, suggesting that the same stem cell(s) reconstituted all of these tissues. Mouse P1 was the exception, where it appears that an additional stem cell(s) repopulated the thymus. Bands representing single insertion sites have intensities similar to the single copy control band (NIH 3T3/N2), indicating that the spleen, thymus, and bone marrow were predominantly repopulated with infected cells that derive from one to a few infected stem cells. No bands were visible in the bone marrow lanes from the mice that had received nonselected marrow. However, it is likely that these lanes also represent a pattern of multiple bands, in that digestion with Xba I produced a readily visible single band (Fig 4C).

**Helper virus does not contribute to h-ADA expression.** Previous work in our laboratory showed that WBB6F1 mice do not spontaneously become infected with ecotropic, N-, or B-tropic retroviruses (data not shown). However, they do activate a replication-competent xenotropic retrovirus that spreads well on Mus dunii fibroblasts, poorly on HeLa cells, and not at all on NIH 3T3 or Balb/c 3T3 cells. The mice in this study were assayed for endogenous and exogenous (packaging line-generated) helper virus infection with a vector rescue assay on Mus dunii/N2 fibroblasts and NIH 3T3/N2 fibroblasts, respectively (Table 1). None of the mice assayed showed any evidence of ecotropic or amphotropic replication-competent retroviral infections, as the N2 vector was never rescued from NIH 3T3/N2 cells. However, despite our avoidance of radiation treatment, three mice (P3, P4, and NP1) were infected with a xenotropic retrovirus. That is, blood from these mice rescued the N2 virus from Mus dunii/N2 cells. No xenotropic virus was detected in the other mice. It is noteworthy that mice P1 and P2, which had the highest level of h-ADA expression, showed no evidence of helper virus infection.

<table>
<thead>
<tr>
<th>Table 2. Specific Activity of ADA in Mouse Tissues</th>
<th>Mouse ADA (μmol/h/mg protein)</th>
<th>Human ADA (μmol/h/mg protein)</th>
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<tbody>
<tr>
<td>RBC</td>
<td>0.09 ± 0.02</td>
<td>&lt;0.001-0.1</td>
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<tr>
<td>Bone marrow</td>
<td>3.0 ± 0.8</td>
<td>&lt;0.06-0.4</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.0 ± 0.5</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Thymus</td>
<td>13.7 ± 3.2</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Lymph node</td>
<td>4.6 ± 2.1</td>
<td>&lt;0.09</td>
</tr>
<tr>
<td>Liver</td>
<td>0.86 ± 0.14</td>
<td>&lt;0.01</td>
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<tr>
<th>Table 3. Reconstitution of W/W+ Recipients With +/+ Donor Marrow</th>
<th>RBC Parameters</th>
<th>Mouse</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>RBC No.</td>
<td>MCV</td>
</tr>
<tr>
<td></td>
<td>(cells/PL)</td>
<td>(FL)</td>
</tr>
<tr>
<td>+/+ donor</td>
<td>11.6</td>
<td>50</td>
</tr>
<tr>
<td>W/W+ recipient</td>
<td>5.2</td>
<td>75</td>
</tr>
<tr>
<td>1:1 mix of donor and recipient blood (70% donor cells)</td>
<td>8.3</td>
<td>57</td>
</tr>
<tr>
<td>P1</td>
<td>10.7</td>
<td>51</td>
</tr>
<tr>
<td>P2</td>
<td>11.1</td>
<td>51</td>
</tr>
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<td>P3</td>
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<tr>
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</tr>
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<tr>
<td>NP3</td>
<td>5.6</td>
<td>59</td>
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Erythroid reconstitution was assayed 3 months post-transplant. Blood volume was analyzed for RBC number and mean RBC volume. W/W+ blood is characterized by a macrocytic anemia, which is "cured" by reconstitution with donor marrow. P1 through P4 and NP1 through NP3 are the same mouse designations used in Table 1. All mice were considered reconstituted except NP3, which was considered partially reconstituted and anemic.
DISCUSSION

The gene transfer protocol used in this study resulted in persistent expression of h-ADA in circulating erythrocytes of transplanted mice. At time points up to 1 month after transplantation, eight of nine mice tested expressed h-ADA at levels equal to endogenous mouse ADA levels. h-ADA was still detectable in five of the six mice that survived to 5.5 months and, in two animals, remained about equal to endogenous mouse ADA levels. Based on levels of ADA achieved in patients treated by infusion of irradiated RBCs as a source of ADA, about 25% of the normal level of ADA in blood would be sufficient for treatment. Thus, even without preselection, the h-ADA levels obtained approached this level. G418 preselection boosted h-ADA expression in two mice into this putative therapeutic range.

LASN vector DNA was present in bone marrow, spleen, thymus, and to a lesser extent, lymph node tissue from all six mice that survived to 5.5 months after transplantations (Fig 4). Gene transfer was fairly uniform within each group of mice. Both nonpreselected mice had approximately 1/4 vector copy per bone marrow genome, whereas all four preselected mice had approximately 3 to 5 copies per bone marrow genome. The presence of vector DNA and the presence of common viral integration sites in the marrow, spleen, and
thymus of each preselected animal at 5.5 months after transplantation indicates that the vector was transferred to pluripotent hematopoietic stem cells. Furthermore, the finding that the band intensities of the integrants approximated that of the single copy standard (NIH 3T3/N2, Fig 4D) indicates that both myeloid and lymphoid tissues (with the exception of the single copy standard (NIH 3T3/N2, Fig 4D) indicates that both myeloid and lymphoid tissues (with the exception of lymph node) were almost completely reconstituted by the infected donor stem cells. These results contrast with several previous reports in which poor stem cell infection resulted in the eventual loss of the vector sequences, accounting for diminished expression with time.\textsuperscript{1,2,6}

The DNA data also allow an estimate of the number of G418-preselected stem cells that actually reconstituted each transplant recipient. Comparison of Figs 4A and B with D reveals that the number of unique vector insertions (HindIII digest) is roughly equivalent to the average number of vector copies per genome (XbaI digest). This suggests that each recipient was reconstituted by one to a few stem cells. Previous reports involving gene transfer have also noted reconstitution by low numbers of genetically-modified stem cells.\textsuperscript{2,3,35}

We found that h-ADA expression in erythrocytes was relatively uniform at times up to 1 month after transplantation, but that expression in some animals decreased at later times. As noted above, this decrease in h-ADA expression was not due to loss of vector sequences. This suggests that infected erythroid progenitors, which contribute to RBC expression at the early time points, are readily infectable and actively express the LASN vector. In contrast, infected stem cells, whose progeny contribute to RBC expression at the later time points, may suppress vector expression. Mouse P4 provides an example in which no h-ADA expression was detected at 5.5 months, despite the presence of at least seven vector integrations in the reconstituting cells. These data emphasize the need to examine vector expression after long-term animal reconstitution, rather than rely on short-term assays, to evaluate the potential use of vectors for gene therapy.

We detected h-ADA only in RBCs and bone marrow despite the presence of vector DNA in spleen, thymus, and, to a lesser extent, lymph node. The use of h-ADA combining protein with starch gel electrophoresis provided a sensitive assay for the presence of h-ADA in mouse tissues; we estimate the limit of detection of h-ADA to be 1% of mouse RBC ADA and 2% of mouse ADA in the other tissues tested (Table 2). This suggests that in the differentiated leukocytes, the LTR promoter was repressed. However, the high levels of endogenous ADA in spleen, lymph node, and especially thymus do alter the limit of detection for h-ADA (Table 2) and could obscure low-level h-ADA expression. The pattern of h-ADA expression found here contrasts with a previous h-ADA gene transfer report by Belmont et al.,\textsuperscript{1} where h-ADA expression was found in spleen and, to a lesser extent, in lymph node, thymus, and bone marrow in two mice sacrificed at 12 weeks post-transplantation. Their vector construct differed from ours, but in both cases, the h-ADA cDNA was transcribed from a Moloney murine leukemia virus LTR. The explanation for differences in tissue specificity remains unknown.

Our data are consistent with the hypothesis that we have transferred the LASN vector into pluripotent stem cells that reconstitute the transplanted animals, but that after differentiation, the vector is only active in erythroid progeny of the stem cell. An alternative hypothesis is that the vector remains transcriptionally silent in all progeny of the infected stem cells, but that we have also infected a self-renewing or long-lived erythocyte progenitor in which the vector is actively transcribed. The existence of such progenitors has been postulated based on the finding that under certain conditions, W/W\textsuperscript{v} animals are primarily repopulated with donor erythroid cells, but not donor leukocytes, for 20 weeks after transplantation.\textsuperscript{26} This was clearly not the case for the animals reported here, because DNA analysis of thymus, which is primarily a lymphoid organ, shows efficient repopulation with vector-infected donor cells. Thus, while it is formally possible that an erythroid-restricted stem cell is responsible for the generation of erythrocytes that express h-ADA, we find no evidence to support such a conclusion from our DNA analysis. In addition, mouse RBCs have a lifespan of less than 2 months,\textsuperscript{29} so h-ADA expression seen at 5.5 months is not due to persistence of RBCs that expressed h-ADA at the earliest time points.

One objective of this study was to rule out any contribution of replication-competent helper virus to vector spread in vivo. Helper virus, presumably generated by the vector packaging cells, was detected in all mice in a previous h-ADA gene transfer study.\textsuperscript{3} However, we found that even in the absence of packaging line-generated helper virus, the mice used in gene transfer protocols may produce replication-competent retroviruses. These viruses are endogenous to each mouse strain and their activation is enhanced by radiation treatment.\textsuperscript{3,10} Their detection is dependent on the choice of an appropriate assay. For example, the B-tropic viruses that infect B-type mice, such as C57B1, are not detectable by using N-tropic NIH 3T3 cells in a vector rescue assay.

We opted to omit radiation treatment to minimize the risk of endogenous virus activation. Despite this precaution, a xenotropic retrovirus was cultured from the blood of three animals. It is not known if this xenotropic virus can infect and spread in the cells of the WBB6F\textsubscript{v} mice. However, the absence of any helper virus infection in those mice expressing the most h-ADA clearly indicates that helper virus is not necessary for the success of murine gene therapy experiments.

One problem with this and other gene transfer protocols is that large amounts of marrow are required for the transplantation procedure. We used eight donors per recipient with G418 preselection to insure reconstitution with infected marrow. This was necessary to determine the role of vector expression in the reconstituted animals. But the large marrow requirement renders this approach impractical for clinical use. In the absence of preselection, the marrow requirement was substantially lower, approaching a clinically relevant amount. It is possible that the amount of donor marrow could have been further reduced without sacrificing reconstitution. It is also clear that large numbers of stem cells were lost during cocultivation. These large losses may have been due in part to stem cell adherence to the fibroblast...
monolayer. Methods of retroviral transfer that preserve stem cell viability will provide a significant advance.

Three of nine animals died during the course of the study. This is not indicative of insufficient marrow transplantation; these mice were not irradiated and should live normal lifespans with or without marrow engraftment. The deaths seem to be inherent to the W/W' genotype. In our experience, a minor subset of these mice are frail and self-mutilating. These animals fail to thrive and die even in the absence of experimental manipulation.

In conclusion, the results of this study show improved transfer of h-ADA into mice, both with respect to the extent of gene transfer and the percentage of mice that continued to express h-ADA long-term. From mouse to mouse, h-ADA expression was still variable and was not detected in spleen, thymus, or lymph node, despite the presence of vector sequences. Further work will be directed at minimizing the marrow losses presently associated with cocultivation, developing vectors that effect uniform, high-level gene expression in all infected cells, and adapting gene transfer protocols to large animal models.

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