EXPRESSION OF HUMAN ADENOSINE DEAMINASE IN NONHUMAN PRIMATES AFTER RETROVIRUS-MEDIATED GENE TRANSFER

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The development of retroviral vectors capable of efficient insertion of genes into mammalian hematopoietic cells (1–7) has generated interest in gene therapy as a potential approach for the treatment of lethal genetic disease (8, 9). The cloning of a cDNA for the human adenosine deaminase enzyme (h-ADA) (10–12) has made the ADA deficient form of severe combined immunodeficiency (SCID) a potential candidate for such therapy. We recently described (13) a retroviral vector called SAX that contains the h-ADA cDNA (Fig. 1). Using this vector, the h-ADA gene was transferred into ADA-deficient human T cells that had been previously immortalized with human T cell leukemia virus (HTLV-1). After gene transfer, the T cells produced the ADA enzyme from the new gene at levels similar to those seen in normal cells. This level of expression was sufficient to make the treated cells resistant to levels of deoxyadenosine that are toxic to untreated ADA-deficient T cells in vitro (13). Therefore, the SAX retroviral vector appears to function efficiently in T cells cultured in vitro from ADA-deficient patients.

Several groups have attempted to establish in vivo models of h-ADA gene transfer in the mouse. Considerable success has been achieved in transferring the h-ADA gene into murine hematopoietic cells (14), and recently expression...
of this gene has been observed in spleen foci (15), but not yet in the fully reconstituted animal. To test the feasibility of a bone marrow transplant/gene transfer approach in a large animal model more analogous to man, we have developed a primate autologous transplantation model for the study of retrovirus-mediated ADA gene transfer into bone marrow cells. This report provides evidence for a low level of transfer and expression of the h-ADA gene in circulating hematopoietic cells in cynomolgus monkeys for short periods of time.

Materials and Methods

Animals and Cell Lines. The animals used in these studies were cynomolgus macaque monkeys obtained from Hazelton Research Animals. NIH 3T3 cells were kindly provided by Dr. Sandra Ruscetti (NCI, NIH) and the PG-4 cell line (used as a helper virus indicator for S"L" assays) by Dr. Robert Bassin (NCI, NIH).

Vector Construction. The construction of the retroviral vector SAX (Fig. 1) and the generation of the virus producer cell line S3A have been described previously (13). Briefly, SAX was made by inserting simian virus 40 (SV40)-promoted human ADA cDNA into the previously described (5, 6, 16) parental vector, N2. A fusion gene was created between the SV40 promoter and the ADA structural gene by placing the 400 bp Kpn I–Hind III fragment containing the enhancer and promoter elements of the SV40 early region immediately upstream of a 1,500 bp sequence containing the full-length ADA cDNA (10) (Eco R1–Acc I fragment of clone ADA 211).

Bone Marrow Processing and Transplantation. Marrow (~40–60 ml) was harvested from the long bones of the animal before irradiation. Mononuclear cells were isolated by 3% gelatin sedimentation and Ficoll/Hypaque gradient separation. These cells were then incubated with vector-producing cells or vector-containing supernatants at 37°C in the presence of 8 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, WI) as described in the text. The cynomolgus primates received a dose of 1,000 rad total body irradiation (60Co at 10 rad/min) immediately followed by infusion of their autologous bone marrow infected with the SAX vector. This dose of irradiation produces permanent aplasia in unreconstituted animals in this primate model.

Hematopoietic Cell Culture. The CFU-C assay for myeloid progenitors has been described (17, 18). The assay medium was McCoy's supplemented with 20% prescreened heat-inactivated FCS (HyClone Laboratories, Logan, UT), 10% giant cell tumor–conditioned medium (Gibco, Grand Island, NY) as a source of granulocyte/macrophage colony-stimulating factor (GM-CSF), and 0.6% agarose (Gibco). CFU-C were allowed to grow at 37°C and were counted on day 14–17 after plating.

DNA Southern Blotting. High-molecular-weight DNA was prepared by the method of Gross-Bellard (19) and was digested with Kpn I restriction endonuclease (New England Biolabs, Beverly, MA), electrophoresed on a 1.0% agarose gel (FMC Bioproducts, Rockland ME), and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) by the

**Figure 1.** Map of SAX vector. The following regions are indicated: 0–1.5 and 4.7–5.5 kb, Moloney murine leukemia virus sequences; 1.5–2.9 and 4.6–4.7 kb, neomycin-resistance gene (*neo") from Tn 5 transposon (the hatched area is the coding sequence); 2.9–3.3 kb, Kpn I–Hind III fragment of the SV40 early promoter; 3.3–4.6 kb, human ADA cDNA (h-ADA, black box); LTR, viral long terminal repeat; 5',5' donor splice site; P, viral packaging signal. Restriction sites: S, Sac I; K, Kpn I; P, Pst I; E, Eco RI; C, Cla I. Scale (kb) at bottom.
method of Southern (20). The blot was hybridized with a $^{32}$P-labelled neo' (neomycin resistance) gene probe (sp act >$10^6$ dpm/μg).

**Adenosine Deaminase Assay.** The separation of primate from human adenosine deaminase activity using fast protein liquid chromatography (FPLC) has been described in detail elsewhere (21). Briefly, proteins were fractionated on a Pharmacia Mono Q column (HR 5/5) using a linear gradient of 0.05–0.5 M KCl, 20 mM Tris-HCl (pH 7.5). ADA activity in column fractions was determined by measuring the conversion of $[^{38}C]$adenosine (Amersham Corp., Arlington Heights, IL) to $[^{38}C]i$nosine followed by thin-layer chromatographic separation using 0.1 M Na$_2$HP0$_4$ (pH 6.8), saturated ammonium sulfate and n-propylalcohol (100:60:2), according to the method of Soberman and Karnovsky (22).

**Neomycin Phosphotransferase (NPT) Assay.** Cell lysates were made from nucleated cells derived from Ficoll-Hypaque separation. The lysates were electrophoresed for 15 h at 50 V on a nondenaturing polyacrylamide (Pharmacia Fine Chemicals, Piscataway, NJ) gel system and transfer of $^{32}$P from γ$[^{32}P]ATP$ (Amersham) to a Kanamycin (Sigma Chemical Co., St. Louis, MO) substrate was achieved using the method of Riess et al. (23) to detect NPT activity.

**In Situ Hybridization.** Marrow or peripheral mononuclear hematopoietic cells, after Ficoll-Hypaque separation, were adjusted to $2 \times 10^6$ cells/ml in Dulbecco's Minimum Essential Medium (Biofluids, Rockville, MD) containing 10% FCS (Gibco). Cells were transferred onto poly-L-lysine (Miles Laboratories, Naperville, IL)-coated slides by cyto-centrifugation and fixed with freshly made 4% vol/vol paraformaldehyde (Sigma Chemical Co.) in PBS (Biofluids) containing 5 mM MgCl$_2$ (Sigma Chemical Co.) for 15 min at room temperature. The slides were stored at 4°C in 70% vol/vol ethanol/water. The methods of hybridization, washing, and exposure of film emulsion were those of Singer et al. (24). The probe used was an $^{35}$S-labelled neo' probe.

**T Cell Cloning.** T cell cloning was performed using the methods previously described by Kernan et al. (25).

### Results

**Infection of Primate Hematopoietic Cells In Vitro.** Initial studies in our primate model were undertaken to establish whether and to what degree primate hematopoietic cells could be infected by the SAX vector and express the vector-containing neo' (NPT) and h-ADA genes. Conditions were established for in vitro analysis of infection of hematopoietic progenitor cells, based on the resistance of cells expressing the neo' gene to the toxic neomycin analogue G418. As shown in Fig. 2, the growth of normal cynomolgus marrow CFU-C (curve A) is completely suppressed at a G418 concentration of 0.29 mg/ml and above.

Two protocols for infecting bone marrow progenitor cells in vitro were used. In the first, Ficoll/Hypaque-separated bone marrow mononuclear cells were cocultured for various periods of time with the SAX vector-producing NIH 3T3 cell clone S3A. This producer cell line, derived from the PA12 amphotropic packaging cell line (26), generates infectious SAX virus at concentrations as high as $6.0 \times 10^8$ neo' CFU/ml. Cocultivation of cynomolgus monkey bone marrow mononuclear cells with an S3A monolayer for periods ranging from 4 to 24 h conferred resistance to 1.7 mg/ml G418 in a maximum of 7% (at 24 h) of the CFU-C detected on day 14 (Fig. 2, curve B). In the second protocol, bone marrow cells were exposed to virus-containing supernatants derived from the S3A producer cells, using ratios (SAX virus/bone marrow mononuclear cells) of between 5 and 10 to 1. After 2 h of exposure followed by extensive washing with PBS, >10% of day 7 CFU-C (data not shown) and 28% of the day 14 CFU-C (Fig. 2, curve C) detected were resistant to toxic concentrations of G418.
In Vivo Studies. The in vitro results outlined above were subsequently supported by the results of autologous bone marrow transplants of SAX vector-infected bone marrow cells administered to cynomolgus monkeys. For these experiments, 40–60 ml of heparinized bone marrow was collected from the animal. After gel sedimentation and Ficoll/Hypaque separation to remove red blood cells, the washed bone marrow cells were infected with the SAX vector, either by cocultivation with S3A producer cells for 18–24 h or by incubation with S3A-derived cell-free supernatants for 2 h. During the separation and infection of marrow cells, the animals underwent total body irradiation, receiving a midline dose of 1,000 rad. There is no animal model for ADA deficiency and, therefore, the monkeys required lethal irradiation to ablate their remaining marrow and make space for the treated cells. The SAX-infected bone marrow cells were then infused back into the same animal. After transplantation, the animals were maintained in filtered air reverse isolation, and received transfusions of blood products, antibiotics, and intravenous nutritional support by an indwelling catheter in the superior vena cava until they achieved normal hematopoietic function and reconstitution of all blood cell lines. At intervals after transplantation, blood and bone marrow samples or tissues obtained at autopsy were analyzed for vector DNA and for both h-ADA and NPT activities. The results of the first six bone marrow/gene transplants are summarized in Table 1.

Cocultivation Protocol. The first two monkeys received bone marrow that had been cocultured with a monolayer of S3A producer cells (Table 1). One of these animals (855) died of systemic bacterial infection 30 d after transplant without evidence of recovery of normal hematopoietic function, and was not studied. The other animal (10) experienced a rapid recovery of his white blood cells (achieving an absolute neutrophil count of 1,000 cells/mm³ by day 20), but never achieved a normal platelet count throughout the posttransplant period (Fig. 3). Animal 10 was sacrificed at day 69 and the tissues were analyzed for evidence of
gene transfer. A $^{32}$P-labelled $neo'$ gene probe was used for Southern blot analysis of PBMC, bone marrow, and spleen DNA digested with Kpn I restriction endonuclease. As shown in Fig. 4, a 4.9 kb Kpn I band, representing the known unit length fragment characteristic of the intact SAX vector (see Fig. 1), was detected in blood and marrow but not spleen. This band was also present in the plasmid control lane, but was absent in the lane containing DNA from the PBMC of a noninfected monkey. Analysis of the blot suggests that the equivalent of a single-copy of the vector sequence was present in 5–10% of blood and marrow cells, because these bands are less than one-tenth the intensity of the plasmid control that contained a quantity of DNA equivalent to approximately one copy per cell. Similar results were obtained probing the same blot with a $^{32}$P-labelled h-ADA gene. However, in addition to hybridization of sequences in the bone marrow and peripheral blood, a light band was detected at 4.9 kb in the spleen lane (data not shown).

PBMC obtained from animal 10 on day 69 were also analyzed for h-ADA and NPT activities. Fractionation of cell proteins by FPLC MonoQ ion-exchange chromatography effectively separates human from primate ADA as assessed by starch gel analysis of species specific ADA isozymes (21). In animal 10, 1% conversion of $^{14}$C-labelled adenosine to $^{14}$Cinosine can be detected in the fractions in which human but not monkey ADA elutes. The human ADA activity detected was calculated to be $<0.01\%$ of the endogenous primate ADA activity (see legend, Table I). NPT activity (which elutes from the column later, see Fig. 5) was also detected in the PBMC at low levels at the same time.

Supernatant Infection Protocol. In the first two transplants there was poor recovery of bone marrow cells from the tissue culture dishes after the 24-h
Figure 3. Hematopoietic reconstitution of primates after transplantation. A: the hematopoietic reconstitution of white blood cells (open symbols) and platelets (closed symbols) of primates 10 (squares) and 855 (circles), whose marrow cells were cocultivated for 24 h on a monolayer of SAX-producing NIH 3T3 cells. After coculture, the nonadherent cells were washed with PBS and infused back into the animal. B: the hematopoietic reconstitution of primates 56 (squares) and 57 (circles) that received autologous bone marrow cells infected during a 2-h incubation with a cell-free supernatant containing SAX vector at a titer of $2 \times 10^6$ neo⁺ CFU/ml. Two additional primates described in this paper were also transplanted with a similar protocol (Table 1, primates 77 and 78); their pattern of reconstitution was similar to those of primates 56 and 57.

In an attempt to increase the number of cells recovered after transfer manipulations and to eliminate the possibility of S3A cell contamination, the subsequent two vector transfers (animals 56 and 57) were performed by incubating bone marrow cells with a filtered (0.22 μm) supernatant collected from the SAX vector-producing S3A cells. Infection was for 2 h, a time that had been determined to be adequate to infect CFU-C in vitro (Fig. 2) and to yield a good recovery of viable bone marrow progenitor cells (Table 1). The ratios of total infectious SAX virus (as measured by G418-resistant CFU/ml) to total bone marrow cells were 8.5:1 and 4.9:1 in monkeys 56 and 57, respectively. The 14-d CFU-C analysis of an aliquot of the treated marrow cells showed that 25% of the colonies were resistant to 1.7 mg/ml G418 for animal 56 (data not shown) and 28% for animal 57 (Fig. 2, curve C). Both primates achieved a rapid and sustained return of normal hematopoietic function with full reconstitution of all blood cell lines entirely analogous to that observed after transplants of untreated autologous bone marrow cells. These animals survived and are healthy more than 1 year after transplantation. Hematopoiesis remains normal.
in these animals and no evidence of retroviremia has been detected by S"L- assay.

Southern blot analyses (of DNA extracted from PBMC obtained on days 34 and 52, as well as of DNA from bone marrow on day 104) for evidence of SAX vector sequences in these two animals were repeatedly negative. This suggests that the vector, if present, was inserted in <5% of cells, that is, below the limits of detection of the method used. PBMC were also analyzed for h-ADA activity and for NPT activity. Both monkeys demonstrated low but readily detectable levels of h-ADA at several times during the posttransplant course (Table 1). PBMC from animal 56 contained a level of h-ADA activity equivalent to 0.2% of the endogenous primate activity at day 104; in animal 57, the peak levels of h-ADA on day 69 brought about a 66% conversion of [14C]adenosine to [14C]inosine (Table 1), which was calculated to be 0.5% of endogenous primate ADA activity. The separation of human (fractions 2-4) from primate (fractions 16-25) ADA using FPLC and the levels of human and primate ADA activity detected in animal 57 at day 69, expressed as percent adenosine to inosine conversion, are presented in the top panel of Fig. 5. These results are contrasted with those obtained from a control animal that received uninfected autologous bone marrow: this animal (as well as several other control animals) had no detectable activity in the column fractions that would contain h-ADA (Fig. 5, bottom) despite greater total protein being loaded onto the column. Sequential assays of PBMC from animals 56 and 57 for h-ADA (Fig. 6) demonstrated h-ADA activity between days 60 and 120. h-ADA was no longer detectable after day 160 in either animal. NPT activity was also detected early in the posttransplant period (day 52) but was not detected at day 104 at a time when h-ADA was still discernible (Fig. 7).

In an attempt to determine the frequency of cells expressing the vector-encoded RNA, in situ hybridization was performed using a 35S-labelled neo' probe on PBMC obtained from animal 57 on day 127 (Fig. 8). Hybridization was detected in 28 of the 3415 (0.8%) cells counted.

To define the lineage(s) of cells containing the vector, we examined marrow
FIGURE 5.  FPLC fractionation of hematopoietic mononuclear cell lysates from animal 57 and a noninfected control animal. ADA activity (both endogenous monkey and vector-derived human) in primate 57 bone marrow cells 69 days after transplantation is shown in the upper panel. An uninfected control is shown in the lower panel. The graph represents the absorbance at 280 nm of bone marrow cell lysate fractionated by ion-exchange chromatography on an FPLC Mono Q column using a KCl salt gradient. Stippled bars represent ADA activity expressed as the percent conversion of $[^{14}C]$adenosine to $[^{14}C]$inosine (%$A \rightarrow I$). After thin-layer chromatographic separation of adenosine and inosine, the raw data for fraction 3 (66% conversion) was 9,045 cpm in the $[^{14}C]$adenosine spot, and 17,889 cpm in the $[^{14}C]$inosine spot. Background counts were: 407 cpm for $[^{14}C]$adenosine after 100% conversion (that is, fraction 17-18), and 426 cpm for $[^{14}C]$inosine after 0% conversion (fraction 1). Fractions containing human ADA, monkey ADA or neo$^+$ activity are indicated.

CFU-C and clonal peripheral blood T cells for evidence of G418 resistance. Assays of marrow from both animals 57 and 58 for G418-resistant CFU-C performed on days 28, 104, and 169 failed to demonstrate myeloid colonies resistant to the same high concentration (1.7 mg/ml) of G418 that was used to detect CFU-C cultured from vector-infected marrow at the time of transplantation (Fig. 2, Table II). However, with concentrations of G418 lower than maximal (0.29 mg/ml), but still toxic for noninfected controls, resistant colonies were obtained from monkey 56 at day 28 and 169 posttransplant, and from monkey 57 at day 169 posttransplant (Table II).
In limiting-dilution analyses of peripheral blood lymphocytes, which are used as a means of quantitating the frequency of clonable T lymphocytes expandable in the presence of mitogen (PHA) and IL-2, clonable T cells resistant to concentrations of 0.1 mg/ml G418 were detected in animal 56 at a frequency of 1:98 on day 181 posttransplant (Fig. 9). By comparison, the overall frequency of clonable T cells detected without selection in the PBMC from this animal was one in seven. Thus, ~8% of the clonable T cells detected in the circulation were
FIGURE 8. In situ hybridization of peripheral blood from animal 57 at day 127. Two positive cells (one, in a cluster of negative cells in the lower left of the figure, and the other at the upper edge on the right) showing overlying silver grains produced by hybridization of an 35S-labeled neo' probe to vector-derived mRNA are shown. There were no positive cells found on slides made from uninfected control primate PBMC.

|     | G418 (mg/ml) |     |     |     |     |
|-----|--------------|-----|-----|-----|-----|
|     | CFU-C before transplant | CFU-C 28 d after transplant | CFU-C 169 d after transplant |
|     | 0 | 0.29 | 1.71 | 0 | 0.29 | 1.71 |
| Uninfected control | 37 | 0 | 0 | 0 | 26 | 0 |
| Monkey 56 | 56 | 17 | 14 | 153 | 5 | 0 |
| Monkey 57 | 41 | 12 | 12 | ND | ND | ND |

Bone marrow cells were harvested, processed, and incubated with the supernatant from S3A producer cells that generate viral particles (containing the SAX retroviral vector) at a titer of 2 x 10⁶ CFU/ml, as described in the legends of Figs. 2 and 3. After infection, cells were immediately plated in increasing concentrations of G418-containing CFU-C medium. For the posttransplant times, bone marrow mononuclear cells were collected and prepared as described and directly plated into the G418-containing CFU-C medium. The values of colonies are reported for 10⁵ cells. The number of cells plated were 3 x 10⁵ for the controls (G418) and 1.2 x 10⁶ for G418-containing points.

Resistant Bone Marrow Colonies (CFU-C) Assayed at Time of Infection and after Transplantation

Bone marrow cells from cynomolgus monkeys have consistently failed to detect clonable T cells resistant to this concentration (0.1 mg/ml) of G418. Further analysis of these G418-resistant clones for h-ADA activity and/or vector DNA sequences could not be performed because of the limited lifetime and number of available cells.
The bone marrow cells of two additional animals, 77 and 78, were infected with a modified protocol: the viral supernatant was at pH 7.4 rather than 6.8, and the polybrene was mixed with the supernatant before addition of the marrow cells rather than afterwards. Although both animals reconstituted rapidly (see Fig. 2), animal 77 showed no evidence of h-ADA activity and animal 78 had an h-ADA level equivalent to only 0.05% of endogenous monkey activity (Table 1). In neither case were vector sequences or NPT activity detected. Preliminary analysis using the modified protocol to measure gene transfer into murine CFU-S suggests that both modifications decrease the number of spleen foci that acquire vector DNA sequences (data not shown). Whether these modifications can account for the primate results is still not clear.

Discussion

These studies were undertaken to establish a large animal model to evaluate protocols that might ultimately be used for gene therapy in humans. We have used an amphotropic retroviral vector containing the h-ADA cDNA, SAX, to infect the marrow of cynomolgus primates for subsequent infusion back into donor animals after total body irradiation. Our studies with primates have demonstrated that adequate numbers of autologous bone marrow cells will survive after in vitro culture and exposure to a retroviral vector to provide the animal with full hematopoietic reconstitution and long-term survival. The reconstituted animals have also provided evidence for a low level of successful gene transfer together with expression of the inserted genes.

As detailed in Fig. 2 and Table I, the method of infection of bone marrow...
cells is an important variable. In previous studies with the mouse, we found that
the infection efficiency was 86% for hematopoietic progenitor cells (CFU-S)
when these cells were cocultivated with a vector-producing (namely, N2, the
parent vector of SAX) cell line (6). Accordingly, we initially used a cocultivation
protocol for our early primate transplants. Cocultivation of primate bone marrow
cells with the vector-producing cell line for periods of 4–24 h, however, did not
achieve as efficient a transfer into marrow progenitors as did incubation with
virus-containing supernatants. This was reflected by the consistently lower fre-
quency of G418-resistant CFU-C detected after cocultivation with the producer
cell lines. Furthermore, cocultivation with the producer line was associated with
bone marrow cell loss, and accordingly with a marked reduction in the total
number of bone marrow progenitor cells subsequently available for transplanta-
tion. This loss of cells after cocultivation is evident in the transplantation of
monkeys 10 and 855, wherein only 15–50% of cells were recovered after
cocultivation. This lower recovery of cells undoubtedly contributed to the poor
reconstitution of hematopoietic function (particularly in platelet number)
achieved in these two monkeys. The four subsequent animals were transplanted
with bone marrow cells cultured for 2 h with virus-containing supernatant. Each
of these animals achieved full reconstitution of hematopoietic function in the
same manner observed in animals receiving autologous, unmanipulated marrow.
In vitro studies also indicated that the frequency of G418-resistant colonies
transplanted was much higher in the marrow infected with the virus-containing
supernatants. Taken together, these data suggest that short-term infection with
cell-free virus-containing supernatants provides several advantages for gene
transfer experiments in this species. However, to obtain a higher rate of infection,
additional modifications of the infection protocol will be necessary.

Our results demonstrate that infection and integration of the retroviral vector
SAX in primate marrow cells can be obtained under conditions that favor the
recovery of viable cells, and that expression of low levels of the gene products,
both h-ADA and NPT enzymatic activities, can be detected for short periods in
circulating blood and marrow cells from some reconstituted animals. Although
the levels of expression in the PBMC and bone marrow are low, they may
represent moderate levels of expression on a per-cell basis. It is unfortunate that,
for logistic reasons, we do not have more complete data for animals 56 and 57
during the critical period from 60 to 120 d. The data for animal 57 are
summarized in Table III. Because the limit of resolution on our Southern blots
is ~1 copy in 20 cells, the negative Southern blots on days 34, 52, and 104 for
animal 57 would indicate that vector DNA was present on those days in <5% of
the PBMC. The presence of neo' RNA in 28 of 3,415 cells as detected by in situ
hybridization on day 127 indicates that at that time at least 0.8% of the cells
contained vector (more may have had nonexpressing vector DNA sequences).
The number of cells containing vector on day 69, when the maximum level of
h-ADA was found (0.5% of endogenous monkey ADA activity), is unknown. If
5% of PBMC contained vector on day 69, then infected cells may produce h-
ADA at ~10% of endogenous monkey ADA levels (in addition to endogenous
monkey ADA already being produced in the cell). If <5% of the cells contained
vector on day 69, then the amount of h-ADA produced on a per-cell basis could exceed 10%.

It should be noted that vectors that do not express genes in one species may be capable of doing so in others. We have used the SAX vector to efficiently transfer the ADA gene into mouse hematopoietic cells but have not detected any ADA expression in vivo in the mouse. We are currently investigating whether this is a vector- and/or promoter-specific phenomenon.

Our studies indicate that the expression obtained appears to be transient, although in both animals, G418-resistant T cells could be obtained as late as 5–7 mo after transplant. The transient expression of vector-derived genes in blood cells obtained from the reconstituted animals could be explained by any of several possibilities. For example, infection might have been restricted to the more differentiated bone marrow cells, which have a limited life span. Alternatively, infection might have been achieved only in a very small number of stem cells, with subsequent progressive dilution of these cells in the absence of a positive selective growth advantage. It is also possible that infected cells undergo cell membrane modification or express extraneous neoantigens that could elicit a response from activated macrophages, NK cells, or immune lymphocytes that are regenerating in their radiated, marrow-reconstituted host. Finally, the decline in h-ADA activity could be explained by the selective loss of vector sequences due to vector instability, by a decrease in the level of expression of the vector genes in individual cells over time, or by overproduction of ADA, thereby inhibiting or killing positive cells.

Our failure to detect CFU-C equally G418-resistant after transplant as were found before transplant is also consistent with any of the above hypotheses. The detection of G418-resistant clonable T cells in the circulation of animal 56 at
day 181 (Fig. 9) and of T cells capable of growth in G418 from animal 57 at day
230 (Table III) also raises the possibility that long-lived T lymphocytes infected
with the SAX vector survive for extended periods in these animals.

Vector gene expression among the animals in our series has not been consistent.
For example, in monkey 10, h-ADA activity was detected at levels 20–50-fold
lower than in monkeys 56 and 57, despite the fact that Southern blot analysis of
circulating cells detected vector DNA in this animal. Thus, animal 10 had a
higher proportion of cells containing the integrated vector DNA, but expressed
the vector at a much lower level. It appears that vector expression in vivo is
dependent on variables that are not yet understood. Permanent engraftment of
cells containing an expressing gene will probably require the successful infection
of pluripotent self-replicating stem cells and a means to maintain positive selective
pressure in vivo. Selective pressure may occur for hematopoietic cells containing
an expressing ADA gene in patients with ADA deficiency (8).

In conclusion, the data presented indicate that we have been able to obtain h-
ADA expression in four out of five reconstituted animals. Four of these animals
are still alive >9 mo after transplant. These animals have shown no sign of
retroviremia (S'L assay of serum), marrow dysfunction, hematopoietic malign-
nancies, solid tumors, or other signs of pathology. We will continue to study the
animals. The S3A cell line produces, in addition to the SAX viral particles,
~0.1% helper virus. Whether or not this low level of helper virus affects the
infection frequency is not established (27). It is unlikely, however, that an ongoing
productive viral infection was established in vivo, because no evidence of retro-
viremia has been detected in any of the animals. A complementary gene transfer
program using the SAX vector into rhesus monkeys has shown low levels of h-
ADA activity (in two out of three animals) and neo' activity (in one out of three
animals). In addition, these animals have shown no evidence of retroviremia or
marrow pathology. Clinical application of this gene transfer protocol will require,
we believe, a higher and more stable level of expression of the inserted h-ADA
gene as well as greater reproducibility among animals than has been achieved to
date.

Summary

Primate bone marrow cells were infected with a retroviral vector carrying the
genes for human adenosine deaminase (h-ADA) and bacterial neomycin resis-
tance (neo'). The infected cells were infused back into the lethally irradiated
donor animals. Several monkeys fully reconstituted and were shown to express
the h-ADA and neo' genes at low levels in their recirculating hematopoietic cells
for short periods of time.

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