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Genetics

Efficient retrovirus-mediated transfer and expression of a human adenosine deaminase gene in diploid skin fibroblasts from an adenosine deaminase-deficient human

(gene therapy/drug resistance/severe combined immunodeficiency/gene transfer)

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ABSTRACT **Skin fibroblasts might be considered suitable recipients for therapeutic genes to cure several human genetic diseases; however, these cells are resistant to gene transfer by most methods. We have studied the ability of retroviral vectors to transfer genes into normal human diploid skin fibroblasts. Retroviruses carrying genes for neomycin or hygromycin B resistance conferred drug resistance to greater than 50% of the human fibroblasts after a single exposure to virus-containing medium. This represents at least a 500-fold increase in efficiency over other methods. Transfer was achieved in the absence of helper virus by using amphotropic retrovirus-packaging cells. A retrovirus vector containing a human adenosine deaminase (ADA) cDNA was constructed and used to infect ADA⁻ fibroblasts from a patient with ADA deficiency. The infected cells produced 12-fold more ADA enzyme than fibroblasts from normal individuals and were able to rapidly metabolize exogenous deoxyadenosine and adenosine, metabolites that accumulate in plasma in ADA-deficient patients and are responsible for the severe combined immunodeficiency in these patients. These experiments indicate the potential of retrovirus-mediated gene transfer into human fibroblasts for gene therapy.**

Adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) deficiency is associated with a severe combined immunodeficiency disease that is invariably fatal unless effectively treated (1, 2). Major pathogenic mechanisms involve the ADA substrates deoxyadenosine and adenosine, which are cytotoxic to T and B lymphocytes (2). The most satisfactory therapy for this disease is bone marrow transplantation from a normal histocompatible donor (2); however, for the majority (about two-thirds) of patients there are no suitable donors. Enzyme replacement by repeated erythrocyte transfusion has resulted in improved immune function in some patients (3, 4) but has disadvantages for long-term therapy (4). The severity of the disease and the lack of suitable bone marrow donors for most patients have made gene transfer an attractive potential therapy. Since the cytotoxic metabolites deoxyadenosine and adenosine circulate in plasma and can traverse cell membranes, introduction of a functioning ADA gene into some of the patient's somatic cells should reduce their levels and allow lymphocyte function to develop.

Current approaches to gene therapy have focused on the introduction of genes into pluripotent stem cells present in bone marrow. Transfer of functionally active genes into hemopoietic stem cells should result in continued expression of the desired gene in many types of blood cells derived from stem cells, including T and B lymphocytes. Retrovirus-mediated transfer and expression of genes introduced into hemopoietic progenitor cells of humans (5) and other animals (6–11) has been demonstrated in culture. Gene transfer into hemopoietic stem cells of mice has also been demonstrated (6–8, 10), but gene expression in stem-cell progeny appears to be low and variable (10). An explanation for this might involve suppression of gene expression during the extensive differentiation of transplanted hemopoietic cells. Study of the factors responsible for poor expression is complicated by the complexity of the hemopoietic system and the scarcity of hemopoietic stem cells in bone marrow.

Skin fibroblasts provide an alternative target tissue for gene therapy. These cells are easily obtained and reintroduced and can be propagated for a short time in culture. Since fibroblasts show little if any differentiation after transplantation, genes introduced into fibroblasts should not be subject to suppression as a result of alteration in gene-expression patterns during differentiation. Studies on transfer and expression of genes in normal human diploid fibroblasts have been severely limited by the short *in vitro* replicative life span of these cells and inefficient gene-transfer techniques (12, 13). Amphotropic murine retroviruses can infect immortalized human fibroblasts (14); however, it is not known whether retroviral infection of normal human fibroblasts would be inhibited due to their different growth characteristics. We show here that retroviruses provide an efficient vehicle for gene transfer into normal human diploid skin fibroblasts.

MATERIALS AND METHODS

Cell Culture. Cells were grown in Dulbecco–Vogt modified Eagle's medium (DMEM) with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum. Mouse cell lines included PA317 (15), PA12 (16), and NIH 3T3 TK⁻ (15). Human diploid fibroblast (HDF) lines were isolated from skin biopsies of normal human donors using standard methods (17) and were maintained in culture for 22–31 population doublings. They were HDF 78-18, from a 92-day female fetus; HDF 1-85, from newborn foreskin; HDF 79-81, from a 26-year-old man; HDF 72-46, from a 29-year-old man; and HDF SF03, derived from a patient with ADA deficiency. HDF lines 78-18, 79-81, and 72-46 were generously provided by P. Rabinovitch (University of Washington).

Retroviral Vector Construction and Production. Retroviral vectors (Fig. 1) contain selectable genes inserted between viral long terminal repeats (LTRs) and replication signals. The 3′ LTRs and adjacent sequences are from Moloney murine leukemia virus (Mo-MuLV). The 5′ LTRs and adjacent sequences of LHL, N2, and LNSAL are from Mo-MuLV, whereas those of LSHL and LHL2 are from Moloney murine sarcoma virus (Mo-MuSV). The normal splice donor

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Abbreviations: HDF, human diploid fibroblast; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; LTR, long terminal repeat; cfu, colony-forming units.

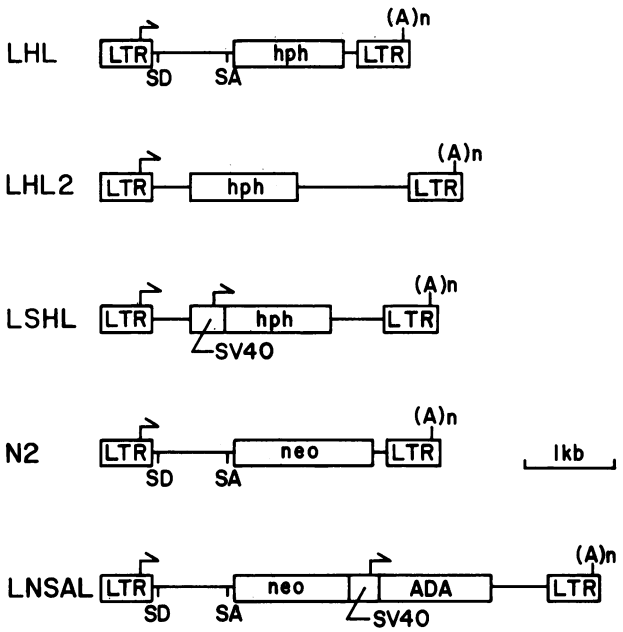

FIG. 1. Retroviral vectors. Arrows indicate promoters, SV40 indicates the early-region enhancers and promoter of simian virus 40, (A)n indicates polyadenylylation signals, and SD and SA are splice donors and splice acceptors, respectively. kb, Kilobase.

in Mo-MuSV at position 206 (18) was removed by a single base substitution in LHL2 and LSHL (AGGT to AGGC). N2 (7) and LNSAL contain the gene coding for neomycin phosphotransferase (*neo*) (19). LHL, LHL2, and LSHL contain the hygromycin phosphotransferase gene (*hph*) from pLG90 (20). N2, LHL, and LNSAL contain ≈400 base pairs of the *gag* coding region of Mo-MuLV, while LHL2 and LSHL do not contain this region. Cell lines producing amphotropic helper-free viral vectors were generated as described (21). The vectors N2, LHL, and LNSAL were produced by using the packaging line PA317 (15), whereas vectors LHL2 and LSHL were produced by using PA12 cells (16). No helper virus (<1 per ml) was produced from these clonal cell lines as measured by using the S^+L^- assay (16).

Measurement of Virus Titer and Infection Efficiency. Cells to be infected were seeded at 5×10^5 per 60-mm dish. The next day the culture medium was replaced with 4 ml of medium containing Polybrene (Sigma) at 4 μg/ml, and various amounts of medium that had been exposed to virus-producing cells were added. After 16 hr, the infected cells were suspended by use of trypsin; the NIH 3T3 cells were divided 1:10 into 60-mm dishes containing selective medium, while the HDF lines were divided 1:10 and 1:100 into 100-mm dishes containing selective medium to prevent overlap of the loosely organized colonies of migrating HDF. Resistant colonies were scored after 10–12 days. Since the plating efficiency of HDF cell lines is often far less than 100%, viral titers were corrected by dividing the observed viral titer by the plating efficiency. Plating efficiency equals the number of colonies growing without selection divided by the number of viable cells plated. Exposure to virus had no effect on plating efficiency. Infection efficiency for an infected cell population was measured by seeding equal numbers of cells into selective or nonselective medium and dividing the number of drug-resistant colonies by the number of colonies growing in the absence of selection.

Enzyme Assays. ADA and purine nucleoside phosphorylase (PNP; purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) were measured in cell extracts by spectrophotometric assays (22, 23). Electrophoresis of ADA was performed in starch gels (22).

High-Performance Liquid Chromatography. Purine nucleosides and bases were measured by reversed-phase column chromatography using a Waters Associates chromatograph equipped with detectors set at 254 nm and 280 nm and a dual-channel integrator (24).

RESULTS

Drug Sensitivity of HDFs. We tested three drugs for their ability to kill HDFs, the neomycin analogue G418, hygromycin B, and methotrexate. The drug sensitivities of the human fibroblasts were significantly different from established NIH 3T3 mouse fibroblasts (Table 1). For example, all of the HDF cell lines tested were resistant to killing by 10 μM methotrexate, and most were resistant to >1 mM methotrexate, whereas the mouse cells were efficiently killed by 0.1 μM methotrexate. Human cell lines were about 10-fold more sensitive to killing by hygromycin B than the mouse cells, and cell death occurred quickly. Only G418 showed similar toxicity for both human and mouse cells. These results showed that G418 or hygromycin, but not methotrexate, were suitable selective agents for gene-transfer experiments in HDF cells.

Transfer of Drug-Resistance Genes into HDFs. We used retroviral vectors carrying one of two drug-resistance genes, the neomycin phosphotransferase gene (*neo*) (19) or the hygromycin phosphotransferase gene (*hph*) (20), which confer resistance to G418 or hygromycin B, respectively (Fig. 1). Two HDF lines were infected with these vectors to determine which configuration of sequences within the vector conferred drug resistance most efficiently. We measured the ability of limiting dilutions of virus to induce drug-resistant colony formation (virus titer) and the proportion of cells converted to drug resistance in one exposure to a large amount of virus (infection efficiency). The highest efficiency of gene transfer occurred using N2 and LHL vectors; up to 87% of the infected cells became drug resistant (Table 2). In the HDF lines, we noted that the infection efficiency for each vector reached a plateau at the same dilution of virus containing medium (1:10 dilution of medium exposed to virus-producing cells, data not shown). This suggests that the important factor for efficient infection of cells is the quality and not the quantity of virus.

The growth characteristics of human fibroblasts depend on the age of the cell donor (25). To determine whether these

Table 1. Drug concentrations needed for complete cell killing

Drug	Fetal HDF 78-18	Newborn HDF 1-85	Adult HDF 79-81	Adult HDF 72-46	Mouse NIH 3T3
G418 (μg/ml)	500	500	1000	500	1000
Hygromycin B (μg/ml)	12	50	50	25	400
Methotrexate (μM)	>1000	>1000	>10	>1000	0.1

Cells were seeded at 10^4 per 3.5-cm dish in culture medium plus 10% fetal bovine serum and drug, except for methotrexate selection where 10% dialyzed serum was used. Drug-resistant cells were scored after 10 days. G418 and hygromycin B concentrations are by weight of dry powder, with activities of 50% and 950 units/mg, respectively. With the exception of HDF 78-18, all of the HDF lines formed colonies at the highest concentration of methotrexate used. HDF 78-18 did not form colonies, but viable cells (by the trypan blue exclusion test) remained after 15 days of selection.

Table 2. Titer and infection efficiencies of four vectors on two HDF lines

Vector	Fetal HDF 78-18		Adult HDF 79-81		Mouse NIH 3T3
	Virus titer, cfu/ml	Infection efficiency, %	Virus titer, cfu/ml	Infection efficiency, %	virus titer, cfu/ml
N2	1.1×10^6	87	8.8×10^5	73	3.2×10^7
LHL	1.6×10^6	55	2.1×10^6	52	2.4×10^6
LSHL	4.6×10^5	17	4.2×10^5	19	1.0×10^6
LHL2	2.0×10^3	<1	1.4×10^3	<1	1.0×10^4

Plating efficiencies in this experiment were 0.09, 0.08, and ≈1.0 for HDF 78-18, HDF 79-81, and NIH 3T3 cells, respectively. cfu, Colony-forming units.

differences affect the efficiency of infection, we infected four HDF lines from donors of different ages with the two high-titer vectors N2 and LHL (Table 3). The infection efficiency was similar for all of the HDF lines and was not related to donor age.

Transfer of ADA into ADA⁻ HDFs. To determine whether a gene with therapeutic importance could be introduced into and expressed well in normal fibroblasts, we constructed a retroviral vector containing both the ADA gene and the selectable gene *neo*. The ADA vector LNSAL was made by inserting an ADA cDNA (26) linked to a simian virus 40 (SV40) early region enhancer/promoter downstream of the *neo* gene in N2 (Fig. 1). Helper-free virus was made from LNSAL by using PA317 amphotropic retrovirus-packaging cells (15). The titer of LNSAL virus produced by PA317/LNSAL cells was 10^6 G418-resistant cfu/ml when assayed on NIH 3T3 cells, and no helper virus was detected (<1 per ml). LNSAL virus was used to infect HDF skin cell line SF03, which was isolated from a patient with ADA deficiency. SF03 cells lack detectable ADA activity (<0.01 μmol per hr per mg of protein) and are killed by 2 mM adenosine, a concentration that has no effect on normal HDFs. Using either G418 or adenosine, we could assay for expression of either *neo* or the ADA gene in LNSAL-infected cells. The number of drug-resistant HDF colonies formed in G418 or adenosine was essentially the same, about 10^5 per ml. The efficiency of SF03 cell infection with a large amount of LNSAL virus was about 50%. Thus, even though the titer of LNSAL virus was lower than those of the N2 or LHL vectors, efficient HDF infection was still obtained with the ADA-containing vector.

We examined the structure of the viruses present in genomic DNA from cells containing the LNSAL virus, to check for potential virus rearrangement. DNAs were cleaved with the restriction endonuclease *Sac* I, which should cleave in each LTR of the integrated provirus, and the products were analyzed by the method of Southern (27) (Fig. 2), using a probe that hybridized to the *neo* gene present in the virus. Two LNSAL-virus-producing cell lines (PA317/LNSAL c3 and c11) displayed a single band at about 5.3 kb, which matched the size of the band produced by the original plasmid containing the LNSAL virus. Three infected ADA⁻ HDF clonal cell lines displayed the same 5.3-kb band. These cells are not immortal and were near senescence when DNA was prepared, due to the time involved in expanding these clonal cell lines. Only a small amount of DNA was obtained for HDF/LNSAL c1 and c2, explaining the relative weakness of the bands. Thus, no gross rearrangement of the virus was detected in the virus-producing cells or in infected HDF cell lines.

Analysis of ADA in Infected Cells. To confirm that the LNSAL-infected SF03 cells produced active ADA, random populations and clonal lines of G418- or adenosine-resistant cells were assayed for ADA activity. After infection, SF03 cells produced 12-fold more ADA than normal HDF (Table 4). No differences were seen in the ADA levels from cells selected in G418 or adenosine, indicating that both genes in LNSAL are expressed concurrently (Table 4). This result is in contrast to results obtained with other vectors carrying two genes, where selection for one of the genes results in suppression of expression of the other gene (28). When isolated clones of G418-resistant cells were analyzed for ADA expression, as much as 23 times the ADA activity of normal fibroblasts was found (data not shown). Furthermore, these genetically modified fibroblasts produced relatively high levels of ADA in comparison with other human cell types (Table 4).

We determined the Michaelis constant and electrophoretic pattern of the ADA from the infected SF03 cells. The K_m measured with adenosine as substrate was 26.2 ± 1.9 μM, which is the same as the normal value of 29.2 ± 2.6 μM (22). The starch gel electrophoretic pattern was typical of normal skin fibroblasts and showed the ADA 1 erythrocyte phenotype and fibroblast tissue-specific ADA isozyme (29) (data not shown). Mixed homogenates of normal erythrocyte ADA and ADA⁻ skin fibroblasts also generate the tissue-specific ADA isozyme (29). Hence, the ADA produced from the transferred gene possesses normal kinetic and structural properties and can bind to the ADA-complexing protein present in ADA⁻ fibroblasts. These results demonstrate faithful transfer and high-level expression of the ADA cDNA by the LNSAL virus.

Table 3. HDF donor age does not affect virus titer and infection efficiency

Vector	Exp.	Fetal HDF 78-18		Newborn HDF 1-85		Adult HDF 79-81		Adult HDF 72-46	
		Virus titer, cfu/ml	Infection efficiency, %	Virus titer, cfu/ml	Infection efficiency, %	Virus titer, cfu/ml	Infection efficiency, %	Virus titer, cfu/ml	Infection efficiency, %
N2	1	1.1×10^6	87	8.9×10^5	74	8.8×10^5	73	ND	ND
	2	1.7×10^6	31	3.1×10^6	48	4.1×10^6	35	7.3×10^6	50
LHL	1	1.1×10^6	55	6.0×10^5	46	2.1×10^6	52	ND	ND
	2	1.7×10^5	33	4.5×10^5	41	1.5×10^6	19	1.2×10^6	24

N2 or LHL virus was used to infect four HDF lines. Values from two experiments are ordered by row. Plating efficiencies were as follows (cell line, exp. 1, exp. 2): 78-18, 0.09, 0.09; 1-85, 0.23, 0.42; 79-81, 0.08, 0.03; 72-46, ND, 0.03. ND, not determined.

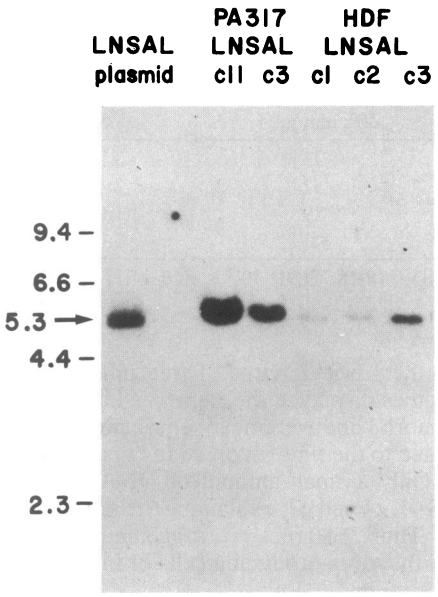

FIG. 2. Analysis of DNA from LNSAL-infected cells. Ten-microgram samples of genomic DNA were analyzed, with the exception of HDF/LNSAL c1 and c2, for which about 2 μg of DNA was analyzed. LNSAL plasmid (2 pg) was also analyzed. Numbers at left represent sizes in kb; 9.4, 6.6, 4.4, and 2.3 represent fragments of *Hin*dIII-digested λ DNA used as size markers.

Deoxyadenosine and Adenosine Metabolism. We measured the metabolism of exogenous deoxyadenosine and adenosine by skin fibroblasts from an ADA-deficient patient, before and after gene transfer, and from a normal donor. A nucleoside concentration of 10 μM was chosen to approximate the deoxyadenosine concentration in ADA-deficient patients (2). Cells were incubated in serum-free medium with added nucleoside for 0–120 min and the concentration of nucleoside in the culture medium was determined by HPLC (Fig. 3). The genetically modified ADA$^-$ cells rapidly metabolized the exogenous deoxyadenosine; about 50% disappeared after 4 min, whereas the normal skin fibroblasts required about 40 min to achieve 50% metabolism of the deoxynucleoside. This result parallels the approximately 10-fold difference in ADA activity between these cell lines (Table 4). In the incubations with unmodified ADA$^-$ HDF, about 90% of the deoxyadenosine remained after 2 hr. When the three cell lines were incubated with adenosine, essentially identical results were obtained (data not shown). In these incubations with deoxyadenosine and adenosine, hypoxanthine was the major product, and incubations with radiolabeled nucleosides showed that <5% of the nucleosides were converted to intracellular nucleotides (data not shown). These results show that ADA$^+$ skin fibroblasts can rapidly metabolize exogenous deoxyadenosine and adenosine and convert these toxic compounds primarily into hypoxanthine, which would not be toxic to ADA-deficient patients.

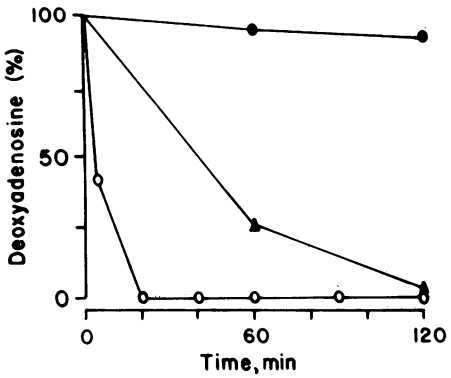

FIG. 3. Deoxyadenosine metabolism by normal HDF (▲) and ADA$^-$ HDF before (●) and after (○) gene transfer. Fibroblasts were harvested at 80% confluence and aliquots (10^5 cells) were incubated in 0.5 ml of serum-free RPMI 1640 medium containing 10 μM deoxyadenosine at 37°C with agitation. At 0–120 min the cells were separated by centrifugation, and the supernatant fluid was treated with perchloric acid and analyzed for unmetabolized deoxyadenosine by HPLC. Cell viability determined by trypan blue dye exclusion was >95% after the 2-hr incubations. Data points are the mean of duplicate determinations.

DISCUSSION

We have shown that genes can be efficiently transferred into fibroblasts by using retroviral vectors. Comparison of different vectors revealed that the design used in the N2 and LHL vectors promoted the highest efficiency of gene transfer. Similar results were obtained for infection of canine (9) and human (ref. 5 and R.A.H. and A.D.M., unpublished results) hemopoietic progenitor cells. Additional evidence suggests that the high titer and high efficiency of gene transfer achieved with these vectors is due to the presence of *gag* sequences, which allow more efficient packaging of vector RNA into virions (unpublished data).

Fibroblasts infected with the ADA gene-containing vector produced 12-fold more ADA enzyme than normal fibroblasts. The infected cells rapidly metabolized deoxyadenosine and adenosine at concentrations observed in the plasma of ADA-deficient patients (2). We can estimate the number of fibroblasts needed to effectively treat a human subject, based on the production of enzyme in infected fibroblasts and the amount of enzyme provided by existing therapies. ADA-deficient patients can be treated by transfusion of one unit of erythrocytes to provide a temporary source of the missing enzyme (3, 4). ADA activity in erythrocytes is 0.036 μmol per hr per mg of cell protein, and in LNSAL-infected ADA$^-$ fibroblasts was about 12 μmol per hr per mg of cell protein. Assuming that 1 mg of protein is present in 2.5×10^6 fibroblasts or 4×10^7 erythrocytes, then ADA activity produced by 4×10^8 fibroblasts is equivalent to one unit of erythrocytes (2×10^{12} cells). Transfer of this number of fibroblasts is feasible. Factors that might influence this calculation include the consideration that transplanted fibroblasts will have less access to plasma than erythrocytes,

Table 4. ADA and PNP activity in normal human cells and infected ADA$^-$ HDF

Enzyme	Activity, μmol per hr per mg of total protein					
	Normal HDF	ADA$^-$ HDF	Peripheral blood T cells	Erythrocytes	LNSAL ADA$^-$ HDF G418^r	LNSAL ADA$^-$ HDF Adenosine^r
ADA	0.9 ± 0.4	<0.01	7.6 ± 1.4	0.04 ± 0.01	12 ± 6	11 ± 3
PNP	0.7 ± 0.3	0.7 ± 0.1	2.3 ± 0.3	1.6 ± 0.2	1.0 ± 0.5	0.9 ± 0.3

The ADA$^-$ HDF line SF03 was infected with LNSAL virus and selected with G418 (1 mg/ml) or adenosine (2 mM) in Dulbecco's modified Eagle's medium plus 10% horse serum (horse serum contains little ADA, in contrast to fetal bovine serum) and 1 mM uridine. Drug-resistant clones were pooled and assayed for ADA activity. ADA activities in other normal human cell types are listed for comparison. PNP activity was measured as an internal control. Values are means ± SD ($n \geq 4$).

which might increase the number of fibroblasts needed. On the other hand, experiments in rats have shown that transplanted fibroblasts will persist for over 13 months (30). When compared to the 20- to 30-day half-life of ADA in transfused erythrocytes (4), continued production of ADA in fibroblasts might allow smaller doses of genetically modified cells.

In a recent report (31), a retrovirus vector nearly identical in structure to the ADA expression vector LNSAL described here was used to transfer and express ADA in cultured ADA^- human T and B lymphocytes. Mixture of enzymatically corrected T cells with ADA^- T cells resulted in protection of the ADA^- cells from deoxyadenosine toxicity. Again, these results suggest that ADA need not be expressed in all cells, or even cells that are sensitive to nucleoside toxicity, to have a beneficial effect for ADA-deficient patients.

Fibroblasts have been used previously in an attempt to cure genetic disease. HLA-matched fibroblasts from normal donors were subcutaneously injected into patients suffering from various forms of mucopolysaccharidosis (32, 33). Although the normal fibroblasts were able to reverse the accumulation of metabolic intermediates in tissue culture, there were only minor, transient alterations in metabolic products in the patients' serum and there were no changes in the patients' overall condition. However, the allogeneic fibroblasts used in these experiments produced only normal levels of enzyme and may have been subject to graft rejection. In addition, it is not known whether the pathophysiological effects of the enzyme deficiency in mucopolysaccharidosis patients are reversible.

In addition to subcutaneous injection, genetically modified fibroblasts could be reintroduced into patients as part of a full-thickness skin-equivalent structure, an artificial skin of cultured fibroblast and epidermal cells that is quickly vascularized when transplanted onto freshly prepared graft beds (30, 34). Of importance in deciding which route of introduction to use will be accessibility of the fibroblasts to circulating plasma.

The demonstration that retroviral vectors can be used to efficiently transfer the ADA gene into human skin fibroblasts allows consideration of other genetic diseases that might be treated using this procedure. Hemophilia caused by reduced clotting factor activity is currently treated by repeated injection of purified clotting factors. A permanent cure could be achieved by modification of some of the patient's cells to make the missing clotting factor. We have recently found that enzymatically active factor IX can be made in fibroblasts following transfer of a factor IX gene using a retrovirus vector (35). Thus, the procedures described here may have more general application to the treatment of human genetic disease.

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