High-Efficiency Gene Transfer into Normal and Adenosine Deaminase-Deficient T Lymphocytes Is Mediated by Transduction on Recombinant Fibronectin Fragments

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Primary human T lymphocytes are powerful targets for genetic modification, although the use of these targets in human gene therapy protocols has been hampered by low levels of transduction. We have shown previously that significant increases in the transduction of hematopoietic stem and progenitor cells with retroviral vectors can be obtained by the colocalization of the retrovirus and target cells on specific fibronectin (FN) adhesion domains (H. Hanenberg, X. L. Xiao, D. Dilloo, K. Hashino, I. Kato, and D. A. Williams, *Nat. Med.* 2:876–882, 1996). We studied the transfer of genes into primary T lymphocytes by using FN-assisted retroviral gene transfer. Activated T lymphocytes were infected for three consecutive days on the recombinant FN fragment CH-296 with a retroviral vector encoding the murine B7-1 protein. Transduced lymphocytes were analyzed for murine B7-1 expression, and it was found that under optimal conditions, 80 to 89% of the CD3⁺ lymphocytes were transduced. Gene transfer was predominantly augmented by the interaction between VLA-4 on the T lymphocytes and the FN adhesion site CS-1. Adenosine deaminase (ADA)-deficient primary T lymphocytes transduced on CH-296 with a retrovirus encoding murine ADA (mADA) exhibited levels of mADA activity severalfold higher than the levels of the endogenous human ADA protein observed in normal human T lymphocytes. Strikingly, the long-term expression of the transgene was dependent on the activation status of the lymphocytes. This approach will have important applications in human gene therapy protocols targeting primary T lymphocytes.

Genetic transduction of hematopoietic stem cells has been the focus of most preclinical gene therapy protocols, since these cells have the capacity for long-term multilineage reconstitution of both the blood and the immune system (5, 7, 8, 12, 16, 17, 30, 34, 35). Although initial clinical gene therapy trials have proven the feasibility and safety of delivering genes to hematopoietic cells via retroviral vectors, the low transduction efficiency of long-term reconstituting stem cells has hampered their use in patients with genetic disorders of the hematopoietic system (7, 12, 26). In certain situations, more-mature long-lived cells, such as T lymphocytes (1, 26), might be alternative targets, e.g., introducing the wild-type gene for genetic diseases (2, 5, 46, 51, 61), delivering immunomodulatory cytokines for cancer therapy (31, 52, 58), or conferring resistance to infection with the human immunodeficiency virus (HIV) (11, 38, 48, 63). For patients who have undergone allogeneic transplantation procedures, T-lymphocyte populations transduced with the herpes simplex virus thymidine kinase cDNA as a suicide gene have been reinfused for a graft-versus-leukemia effect and eradicated by ganciclovir if graft-versus-host disease arises (4).

ADA⁻ SCID patients, who exhibit severe combined immu-

nodeficiency due to adenosine deaminase (ADA) deficiency (29), are ineligible for bone marrow transplantation but receive bovine ADA conjugated to polyethylene glycol for extended periods of time; this results in an increase in their levels of peripheral blood T lymphocytes and in improved cellular immunity (28). In the first clinical gene therapy protocol for a genetic disease, T lymphocytes derived from ADA⁻ SCID patients were transduced in vitro with a cell-free supernatant containing a retroviral vector carrying the human ADA cDNA. Subsequently, transduced populations were repeatedly infused into patients. Gene transfer efficiencies in vitro ranged from 0.1 to 10% for T lymphocytes transduced with a cell-free supernatant (2, 5) and were up to 40% for both peripheral T lymphocytes and hematopoietic progenitors when cocultivated with a retrovirus-packaging line (5). In another approach, Kohn et al. (34) transduced umbilical cord blood CD34⁺ cells with a cell-free supernatant containing a human ADA-expressing retrovirus; this resulted in a 12.5 to 21.5% rate of transfer of clonogenic progenitors in vitro. Although the frequencies of gene-marked peripheral blood T lymphocytes and stem cell-derived progeny in the patients were lower than those measured prior to infusion in vitro, these clinical trials showed that retroviral gene transfer of a human ADA cDNA into human cells from bone marrow (5), umbilical cord blood (34), or human peripheral blood (2, 5) was a safe procedure. However, it remains to be demonstrated whether genetic modification of either hematopoietic stem and progenitor cells or T lympho-

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cytes with retroviral vectors is a therapeutic option for treating ADA⁻ SCID patients.

Cocultivation of retrovirus-packaging cells with target cell populations yields high levels of gene transfer in both murine and human hematopoietic cells. However, safety concerns and concerns about the reproducibility of cocultivation on a large scale make this a less than desirable infection method for clinical gene therapy protocols. To circumvent these problems, we have transduced hematopoietic stem and progenitor cells on chymotryptic or recombinant fragments of human fibronectin (FN) and demonstrated improved efficiency of retrovirus gene transfer (22, 23, 44, 45). This increased gene transfer efficiency was due to the colocalization of retroviral particles and target cells on specific adhesion domains of FN (22), and it obviated the need for cocultivation of the target cells with the packaging cells. FN contains at least three distinct cell adhesion domains, and they can interact with a variety of ligands on hematopoietic cells. These domains are the central cell-binding domain, at which interaction between the tetrapeptide Arg-Gly-Asp-Ser and the integrin VLA-5 on the target cells occurs; the CS-1 sequence, located in the alternatively spliced III_{CS} region that interacts with the integrin VLA-4 on the target cells; and the high-affinity heparin-binding site, located in the type III repeats 12 to 14 that interact with cell surface proteoglycans (27).

Primary T lymphocytes express the two FN receptors VLA-4 and VLA-5 and upon activation, the binding affinity of each of these receptors significantly increases (56, 57). Therefore, the aim of this study was to determine if recombinant FN fragments could be utilized to enhance gene transfer into peripheral-blood-derived T lymphocytes and to determine the feasibility of this approach for future application to clinical gene therapy protocols. Here, we demonstrate that colocalization of retrovirus and T lymphocytes on FN fragments leads to transduction of up to 80 to 90% of normal or ADA-deficient primary T lymphocytes, without any *in vitro* selection. High-level gene transfer into these T lymphocytes was accomplished by activation with CD3/CD28i (coimmobilized CD3 and CD28 monoclonal antibodies [MAbs]) and incubation with a retrovirus-containing supernatant on plates coated with recombinant FN fragments containing the type III repeats 12 to 14 and at least one integrin binding domain. The T-cell repertoire and function were not affected by this transduction protocol. In addition, we demonstrate that the expression of the transgene after transduction correlates with the activation status of the cell, with expression increasing 4- to 11-fold by CD3/CD28i-induced activation compared to interleukin-2 (IL-2)-induced proliferation. Therefore, the results described here strongly suggest that clinical gene therapy protocols for which gene delivery into T lymphocytes is the major aim may profit significantly from the inclusion of specific FN fragments in the transduction protocol.

MATERIALS AND METHODS

Retroviral vectors and producer cell lines. The mB7-1 retroviral producer line PA317-LNCmB7-1 (mB7-1) was a generous gift from Randy Hock (Indiana University, Indianapolis) and has been described elsewhere (23). The producer clone used in experiments reported here had titers of 1.7×10^6 G418-resistant CFU/ml as measured on NIH 3T3 cells when the supernatant was collected at 37°C and of 5×10^7 G418-resistant CFU/ml when the supernatant was collected at 32°C. The vector MSCV2.1, a generous gift from R. G. Hawley (University of Toronto, Toronto, Ontario, Canada), contains the neomycin phosphotransferase gene under the control of the human phosphoglycerate kinase (PGK) promoter (25) and was packaged in GP + envAM12 cells (39). The clone used had a titer of 2×10^6 G418-resistant CFU/ml as measured on NIH 3T3 cells. The GP + envAM12 producer line containing Zip-PGK-mADA (PGK-mADA; clone 55/6) has been described elsewhere (43) and expresses the murine ADA (mADA) cDNA under the control of the human PGK promoter. All retroviral producer

FIG. 1. Composition of recombinant FN fragments derived from sequences located within the A chain of FN. The binding sites for the integrins VLA-5 and VLA-4 are marked as CELL and CS-1, respectively. The CS-1 site is composed of the first 25 amino acids of the alternatively spliced III_{CS} region. The binding site for proteoglycans is marked as HEPARIN for the heparin-binding domain spanning the type III repeats 12 to 14 (III₁₂₋₁₄).

lines were maintained in Dulbecco's modified Eagle's medium (GIBCO-BRL, Gaithersburg, Md.) containing 10% Cosmic Calf serum (HyClone Laboratories, Inc., Logan, Utah). Retrovirus-containing supernatant for transduction experiments was collected by adding 10 ml of RPMI 1640 medium (Gibco-BRL) containing 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc.) to confluent 10-cm-diameter plates overnight. Supernatants were harvested from confluent plates for three consecutive days. In some experiments, supernatants were collected at 32°C as recently reported (9). Harvested medium was filtered through 0.45-μm-pore-size filters (Nalge Company, Rochester, N.Y.) and either used fresh or aliquoted and stored at -80°C.

Retroviral transduction protocol. Non-tissue culture 24-well plates (Falcon, Franklin Lakes, N.J.) were coated with recombinant FN fragments containing different combinations of binding domains for VLA-4, VLA-5, and surface proteoglycans (Fig. 1) (33) supplied by Takara Shuzo Ltd. (Otsu, Japan) at a predetermined saturating concentration of 100 pmol/cm² (60a). Plates were coated either at 4°C overnight or at 37°C for 2 h, subsequently blocked with 1% bovine serum albumin (BSA) for 20 min at 25°C, and then washed twice with phosphate-buffered saline (PBS) prior to use. All peripheral blood samples from normal healthy donors and one ADA⁻ SCID patient were collected in heparinized tubes in accordance with protocols approved by the Institutional Review Board of Indiana University School of Medicine. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Ficoll-Hypaque (density, 1.077 g/ml; Pharmacia, Piscataway, N.J.) for 30 min at 25°C and washed twice with PBS. PBMCs were activated with either phytohemagglutinin M (5 μg/ml; Calbiochem, San Diego, Calif.), IL-2 (100 U/ml; Chiron Corporation, Emeryville, Calif.), soluble CD3 MAb (CD3s) (1 μg/ml; clone OKT3; Ortho Biotech, Inc., Raritan, N.J.), immobilized anti-CD3 MAb (CD3i), or CD3i/CD28i (clone-CD28.2; PharMingen, San Diego, Calif.). For the immobilization of antibodies, 24-well non-tissue culture-treated plates were coated with antibody (1 μg/ml in PBS) at 0.5 ml/well for 2 to 4 h at 37°C. The coated plates were then blocked with 1% BSA in PBS for 20 min at 37°C, washed once with PBS, and then used for activation. PBMCs (10^6 per well) were incubated in complete medium (CM) (RPMI 1640 supplemented with 10% [vol/vol] FBS, 50 μM 2-mercaptoethanol, 1% L-glutamine, and 1% penicillin-streptomycin [GIBCO-BRL]). Tissue culture plates (Costar, Corning, N.Y.) were used for all T-cell activation methods not requiring immobilized antibodies. At days 2 to 3 postactivation, the cells were harvested and counted. Flow cytometric analysis indicated that these populations were routinely >90% CD3⁺ and hence were highly enriched for T lymphocytes. T lymphocytes were then incubated on recombinant FN fragments at 0.5×10^6 /well with 2.8 ml of retrovirus-containing supernatant supplemented with 50 U of IL-2 per ml. T lymphocytes were also incubated with retrovirus-containing supernatant on BSA-coated wells containing 8 μg of Polybrene per ml supplemented with 50 U of IL-2 per ml (Sigma, St. Louis, Mo.). No Polybrene was added to cultures transduced on FN fragments (23). After 4 h, cells were harvested from the wells by vigorous pipetting and the wells were washed once with PBS. Cells were resuspended in medium supplemented with 50 U of IL-2 per ml and placed on CD3i/CD28i-coated plates overnight. In some experiments, the procedure for transduction on FN was repeated on two consecutive days. After transduction, cells were expanded at 10^5 /well on freshly coated CD3i/CD28i plates for the next 3 to 4 days. At this point in time, ≥98% of the cells were CD3⁺ T lymphocytes. At days 5 to 6 posttransduction, manipulated T cells were harvested and analyzed for phenotype and mB7-1 expression. During the second week of culture, T lymphocytes were either maintained on CD3i/CD28i MAbs or, for long-term expansion, placed in 100-U/ml IL-2. Cultures were split every 2 to

FIG. 2. Optimization of efficiency of gene transfer into primary T lymphocytes. CD3i/CD28i-activated T lymphocytes were transduced with the mB7-1 retrovirus, previously collected at 37°C, for one to three consecutive days on FN CH-296-coated plates or in the presence of Polybrene on BSA-coated plates. Flow cytometric analysis of mB7-1 was monitored on days 5 (A) and 13 (B) posttransduction. This experiment is representative of four independent experiments. (C) In subsequent experiments CD3i/CD28i-activated T lymphocytes were transduced with an mB7-1 retrovirus supernatant, previously collected at 32°C, for one to three consecutive days on FN CH-296-coated plates or in the presence of Polybrene on BSA-coated plates. Flow cytometric analysis of mB7-1 was monitored on day 13 posttransduction. This experiment is representative of two independent experiments. Percent mB7-1⁺ expression is the percentage of transduced T lymphocytes staining positive for mB7-1 minus the percentage of nontransduced cells staining positive for mB7-1. The background of the nontransduced cells stained with the mB7-1 MAb was routinely $\leq 2.5\%$.

formed on FN CH-296 (Table 1). This was in contrast to the low gene transfer efficiency observed on BSA-coated plates and with other methods of T-cell activation (Table 1 and data not shown). Therefore, all subsequent experiments were performed with CD3i/CD28i-activated T lymphocytes.

Optimization of gene transfer into T lymphocytes. To optimize retroviral gene transfer into T lymphocytes, cells were infected with an mB7-1 supernatant, previously collected at 37°C for one to three consecutive days on either FN CH-296- or BSA-coated plates, and analyzed at 5 days (Fig. 2A) or 13 days (Fig. 2B; Table 2) posttransduction for expression of the mB7-1 transgene. Strikingly, 5 days after the start of transduction, more than 80% of the T lymphocytes expressed mB7-1 if lymphocytes had been transduced with retroviruses two to three times on consecutive days (Fig. 2A). Two parameters, the percentage of transgene-expressing cells and the mean fluorescence intensity (MFI), were monitored to assess the gene transfer efficiency in long-term cultures of transduced T lymphocytes. Gene transfer on FN CH-296 was consistently three- to ninefold more efficient than by supernatant infection on BSA with Polybrene (Table 2). The MFI values for the transduced populations indicated that a three- to eightfold enhancement of mB7-1 transgene expression occurred when T lymphocytes were transduced on FN CH-296, compared to expression with the BSA system (Table 2). For long-term expression (Fig. 2B), mB7-1 expression clearly correlated with the length of exposure to retroviral particles, with transductions on three consecutive days rendering the highest levels of gene transfer. Approximately 37 to 46% of the cells expressed mB7-1 in long-term culture, suggesting that the higher levels of mB7-1-positive cells observed at day 5 posttransduction might not be due to integrated provirus (Fig. 2 and Table 2). Analysis of infected cells for coexpression of mB7-1 with either CD4 or CD8 indicated that both CD4⁺ and CD8⁺ T-cell subsets were transduced to similar levels with the mB7-1 retrovirus (data not shown).

To further optimize gene transfer, the mB7-1 retrovirus supernatant was harvested at 32°C to increase the titer (9) and used in the transduction protocol. Analysis of mB7-1 expression at day 13 posttransduction indicated that a single infection on FN CH-296 yielded 63 to 68% mB7-1⁺ primary lymphocytes while infection on FN CH-296 for two to three consecutive days resulted in at least 80% of primary cells expressing mB7-1 (Fig. 2C and Table 3). The MFI values for the transduced populations indicated that a 2.7- to 10-fold enhancement of mB7-1 transgene expression occurred when T lymphocytes were transduced on FN CH-296, compared with expression with the BSA system (Table 3).

Contribution of VLA-4 and VLA-5 to gene transfer efficiency. To further analyze the relative contribution of VLA-4 and VLA-5 on T lymphocytes in FN-assisted retroviral gene transfer, we used five different recombinant FN fragments which contained, in various combinations, the heparin, VLA-5 (cell), and VLA-4 (CS-1) binding sites (Fig. 1). Prestimulated

TABLE 2. Effect of multiple infections on efficiency of gene transfer with supernatant collected at 37°C^a

Expt	Days	% mB7-1 expression on ^b		Fold increase in % mB7-1 expression ^d	MFI on ^c		Fold increase in MFI ^e
		BSA	CH-296		BSA	CH-296	
I	1-3	10.0	37.1	3.7	13.1	78.1	6.0
II	2-4	15.4	45.9	3.0	37.4	127.4	3.4
III	2-4	4.4	41.5	9.4	27.0	211.9	7.8
IV	2-4	13.6	45.4	3.3	16.8	55.6	3.3

^a Human T lymphocytes were activated with CD3i/CD28i MAb for 1 or 2 days and then transduced with the mB7-1 retrovirus for three consecutive days on BSA or CH-296. T lymphocytes were analyzed for mB7-1 expression on day 13 posttransduction.

^b Data are represented as the percentage of transduced cells expressing mB7-1 minus the percentage of nontransduced cells expressing mB7-1. Nonspecific binding of mB7-1 MAb to nontransduced cells was always less than 2.5%.

^c Data are represented as the MFI of the transduced population stained with the mB7-1 MAb minus the MFI of the transduced population stained with the isotype control MAb.

^d The fold increase in mB7-1 transgene expression on T cells transduced on CH-296 versus those transduced on BSA was calculated by dividing the percent expression on CH-296-transduced T cells by the percent expression on T cells transduced on BSA.

^e The fold increase in MFI was calculated by dividing the MFI for T cells for T cells transduced on CH-296 by the MFI for T cells transduced on BSA.

TABLE 3. Effect of multiple infections on efficiency of gene transfer with supernatant collected at 32°C^a

Expt	Day(s)	% mB7-1 expression on ^b :		Fold increase in % mB7-1 expression ^d	MFI on ^c :		Fold increase in MFI ^e
		BSA	CH-296		BSA	CH-296	
I	2	27.0	68.0	2.5	25.0	125.0	5.0
	2-4	62.4	87.2	1.4	105.04	441.0	4.2
II	2	9.9	62.9	6.4	22.1	218.5	9.9
	2-3	35.6	76.0	2.1	97.5	407.1	4.2
	2-4	48.9	80.1	1.6	154.7	415.3	2.7

^a Human T lymphocytes were activated with CD3i/CD28i MABs for 2 days and then transduced with the mB7-1 retrovirus on days 2 to 4 postactivation on BSA or CH-296. T lymphocytes were analyzed for mB7-1 expression at day 13 posttransduction.

^b Data are represented as the percentage of transduced cells expressing mB7-1 minus the percentage of nontransduced cells expressing mB7-1. Nonspecific binding of mB7-1 MAB to nontransduced cells was always less than 2.5%.

^c Data are represented as the MFI of the transduced population stained with the mB7-1 MAB minus the MFI of the transduced population stained with the isotype control MAB.

^d The fold increase in mB7-1 transgene expression on T cells transduced on CH-296 versus those transduced on BSA was calculated by dividing the percent expression on CH-296-transduced T cells by the percent expression on T cells transduced on BSA.

^e The fold increase in MFI was calculated by dividing the MFI for T cells transduced on CH-296 by the MFI for T cells transduced on BSA.

T lymphocytes were transduced on FN-coated plates with the mB7-1 retrovirus previously collected at 37°C. Analysis of mB7-1 expression (Fig. 3) revealed that neither the FN cell binding site (C-274) nor the FN heparin binding site (H-271) was sufficient to promote high-level gene delivery to T lymphocytes. Efficient gene transfer required the presence of a binding site for retroviruses and at least one binding site for the target cells (CH-271, H-296, and CH-296). Transduction efficiency on FN CH-296 or FN H-296 was superior to that on FN CH-271, suggesting that VLA-4, and not VLA-5, was the major mediator in adhesion of activated T lymphocytes to FN and therefore predominantly assisted in genetic transduction of T lymphocytes. The standard approach of supernatant infection with Polybrene (BSA) was far less efficient than using FN in the transduction protocol.

Efficiency of gene transfer from normal donors and an ADA⁻ SCID patient into T lymphocytes, utilizing the PGK-mADA retrovirus. No obvious changes in the proliferation of mB7-1-expressing cells and nontransduced cells were observed (data not shown). However, there existed the possibility that expression of the mB7-1 receptor could influence the proliferation or function of transduced T cells, leading to a selective advantage for these cells compared to nontransduced cells. Therefore, we performed similar experiments with the PGK-mADA retrovirus, which has been used previously to efficiently transduce human clonogenic hematopoietic progenitor cells (43), primitive human cord blood cells capable of repopulating SCID/NOD mice (36), and primate long-term repopulating stem cells (3). The resulting data suggest that the intracellular expression of the mADA protein does not affect the proliferation or function of transduced human or primate cells. As described above, CD3i/CD28i-activated T lymphocytes were transduced with the PGK-mADA retrovirus on FN CH-296- or BSA-coated plates for three consecutive days; 12 days later, cells were analyzed for proviral integration by Southern blot analysis and for protein expression by ADA enzyme assay. Southern blot analysis indicated that transduction of primary T cells was extremely efficient on FN CH-296, since multiple

provirus copies were detected in cells after retroviral infection for three consecutive days (Fig. 4A, lane 3). The actual proviral copy number per cell was larger than that of the standard, containing the equivalent of five provirus copies per cell (Fig. 4A; compare lanes 3 and 4). In contrast, a proviral band was barely detectable for infections performed in parallel on BSA-coated plates in the presence of Polybrene (Fig. 4A, lane 2). Increased proviral integration of T lymphocytes transduced on FN CH-296 versus that of cells transduced on BSA also correlated with an increase in functional mADA activity, as shown by in situ gel analysis of protein extracts (Fig. 4B, normal control; compare BSA and CH-296 lanes).

To assess whether this transduction protocol is suitable for clinical application, peripheral blood from an ADA⁻ SCID patient was also utilized. As previously reported (59), in situ gel analysis of ADA activity in primary T lymphocytes from this patient revealed no demonstrable activity (Fig. 4B). Proliferation assays indicated that the patient's lymphocytes could be activated in a fashion similar to normal-donor lymphocytes on CD3i/CD28i in the presence of FBS-containing medium (data not shown). Activated T lymphocytes from the patient and those from a normal donor were transduced with either the mB7-1 or PGK-mADA retrovirus on BSA-coated plates in the presence of Polybrene or on FN CH-296-coated plates in the absence of Polybrene. Flow cytometric analysis of mB7-1-transduced cultures indicated that the transduction efficiency of ADA-deficient T lymphocytes was not significantly different from that of normal-donor T cells (data not shown). T lymphocytes from a normal donor and those from the ADA-deficient patient were transduced with the PGK-mADA retrovirus in parallel. Analysis of mADA activity in the ADA-deficient patient's T lymphocytes 12 days after transduction demonstrated that the activity of the mADA transgene transduced on FN CH-296 was similar to the activity of the endog-

FIG. 3. Relative role of VLA-4 and VLA-5 binding sites during gene transfer. CD3i/CD28i-activated T lymphocytes were transduced with an mB7-1 retrovirus supernatant, previously collected at 37°C, in the presence of Polybrene on BSA-coated plates or on alternative recombinant FN fragments. Transductions were performed three times on three consecutive days. As a second control, one group of activated T lymphocytes was transduced with the MSCV 2.1 retrovirus. Transduced and nontransduced cells were analyzed for mB7-1 expression 12 days posttransduction. Percent mB7-1⁺ expression is the percentage of transduced T lymphocytes staining positive for mB7-1 minus the percentage of nontransduced cells staining positive for mB7-1. The background of the nontransduced cells stained with the mB7-1 MAB was routinely $\leq 5\%$. This is representative of two independent experiments.

FIG. 4. Efficiency of gene transfer utilizing the PGK-mADA retrovirus. (A) CD3i/CD28i-activated T lymphocytes were transduced with the mB7-1 retrovirus as a control (Mock) or with the PGK-mADA retrovirus, either in the presence of Polybrene on BSA-coated plates (BSA) or on FN CH-296-coated plates (CH-296), and analyzed by genomic Southern blotting for proviral integration at day 12 posttransduction. The arrow indicates the 3.4-kb PGK-mADA provirus. (B) CD3i/CD28i-activated T lymphocytes from a normal donor and from an ADA⁻ SCID patient were transduced with the PGK-mADA retrovirus for the presence of mADA enzyme activity and analyzed at day 12 posttransduction. These experiments are representative of two (for the ADA⁻ SCID patient) or three (for the normal donor) independent experiments.

enous hADA protein observed in normal human T lymphocytes (Fig. 4B; compare ADA⁻ SCID to hADA in the normal control [all lanes]). In addition, as seen previously with the mB7-1 retrovirus, infection on FN CH-296 consistently led to much higher gene transfer efficiencies than those achieved by the standard approach of supernatant plus Polybrene (Fig. 4B, ADA⁻ SCID; compare BSA and CH-296 lanes).

Functional analysis of genetically transduced T-lymphocyte populations. To assess the functional capacity of cells following ex vivo manipulation and gene transfer, T lymphocytes were transduced with the PGK-mADA retrovirus on FN CH-296-coated plates by the 3-day transduction protocol after prestimulation on CD3i/CD28i for 2 days. At 7 days posttransduction, the cultures were rested in CM and used as responders in an MLC (Fig. 5A). T cells transduced with the PGK-mADA retrovirus on FN CH-296 mounted a proliferative response against HLA-disparate PBMCs comparable to the response observed in nontransduced cells (no virus) and in cells incubated on FN CH-296 without retrovirus (CH-296, no virus). Next, the ability of genetically modified T lymphocytes to specifically lyse an OKT3 hybridoma was determined (Fig. 5B). T lymphocytes transduced on FN CH-296 with either retrovirus, PGK-mADA or mB7-1, were able to effectively lyse OKT3 hybridoma cells. This lysis was CD3 restricted, since the genetically modified cells did not kill K562 cells, a target population used for assessing natural killer cell activity (Fig. 5B).

A V_β RT-PCR analysis performed on T-lymphocyte populations collected on day 0, after CD3i/CD28i activation, and after CD3i/CD28i activation and transduction with mB7-1 on FN CH-296 (collected on day 6 posttransduction) revealed that the V_β repertoire of the targeted T-lymphocyte population was

unchanged (Fig. 5C). Similar results were seen for V_β repertoire analyses performed on transduced T lymphocytes 13 days posttransduction (data not shown). Collectively, these functional studies indicated that, at least on a polyclonal level, populations of T lymphocytes activated on CD3i/CD28i and transduced on FN CH-296 with retroviruses have no apparent loss of function. The functional capacity of transduced T lymphocytes derived from the ADA-deficient patient also appeared to be normal, since these cells proliferated similarly to control lymphocytes, maintained their V_β repertoire, and were capable of CD3-mediated cell lysis of the OKT3 hybridoma (data not shown).

Activation status of transduced primary T lymphocytes significantly affects expression of the transgene. In preliminary studies, we noted that cultures of transduced lymphocytes maintained in IL-2 after transduction appeared to express less transgene than cells maintained on CD3i/CD28i. To examine this apparent difference in detail, T lymphocytes were transduced on FN CH-296 for 3 days and maintained in IL-2. On day 11 posttransduction, only low levels of mB7-1 were expressed. The culture was subsequently divided and maintained in parallel cultures containing either IL-2 alone or CD3i/CD28i for an additional 2 days. After either a single infection on day 2 or after multiple infections on days 2 to 4 (Fig. 6A, Table 4, and data not shown), the level of expression of mB7-1, on a percentage level, was 4- to 11-fold higher in transduced lymphocytes reactivated with CD3i/CD28i than in those maintained in IL-2. The MFI of the CD3i/CD28i-reactivated lymphocytes transduced with the mB7-1 retrovirus increased 30- to 122-fold compared to that of transduced lymphocytes maintained in IL-2 (Table 4). Subsequent reactivation of IL-2-maintained cultures with CD3i/CD28i for 2 days led to high levels of mB7-1 expression, demonstrating that these differences were not due to preferential loss of transduced cells in IL-2 cultures (data not shown). Confirmation of the existence of similar numbers of transduced cells in each culture was obtained by Southern blot analysis of transduced T lymphocytes maintained in IL-2 or reactivated with CD3i/CD28i; proviral copy numbers were virtually identical (data not shown). This observation did not appear to be specific for the mB7-1 retrovirus, for PGK-mADA retrovirus-transduced lymphocytes from a normal donor and those from an ADA⁻ SCID patient showed much higher mADA protein activities when reactivated with CD3i/CD28i than those maintained in IL-2, for both groups (BSA and CH-296) (Fig. 6B). These observations suggested that transgene expression in T lymphocytes may be regulated by the activation status of the T lymphocyte.

DISCUSSION

The efficacy of gene modification of autologous cells as a therapeutic alternative for the treatment of genetic diseases has been limited by the low efficiency of gene transfer into relevant target cell populations (8). In hematopoietic stem cell transductions, the cell cycle status of the target cells and the level of the amphotropic receptor may be limiting (41, 47). Concerns that ex vivo manipulation of reconstituting stem and progenitor cells with cytokines, a procedure that has been shown to increase gene transfer via retrovirus vectors, may decrease the ability of these cells to engraft have also been raised. In this regard, when highly transduced populations of CD34⁺ cells were transplanted into SCID/NOD mice, only 1.4% of the transduced human cells were found in the bone marrow of these mice after repopulation (36). In addition, the constitutive expression of a transgene in hematopoietic stem and progenitor cells could be detrimental to the function of

FIG. 5. Determination of the functional capacity of transduced T-lymphocyte populations. (A) MLC utilizing transduced T lymphocytes. T lymphocytes transduced with the PGK-mADA retrovirus on FN CH-296 for three consecutive days (CH-296 PGK-mADA), nontransduced T lymphocytes incubated on FN CH-296 without virus (CH-296 no virus), or nontransduced T lymphocytes (no virus) were incubated in CM for 3 days, harvested, and analyzed for BrdU incorporation by MLC. T lymphocytes were incubated in CM or with irradiated allogeneic PBMCs (allo-PBMC). This is representative of two independent experiments. (B) Determination of the ability of transduced and nontransduced T lymphocytes to mediate lysis of the OKT3 hybridoma. T lymphocytes transduced on FN CH-296 or nontransduced T lymphocytes were incubated at an effector/target ratio of 12.5:1 with ⁵¹Cr-labeled OKT3 hybridoma or K562 cells. This is representative of three independent experiments. (C) Comparison of the V_β repertoires of transduced and nontransduced T lymphocytes. V_β RT-PCR analyses were performed on T-lymphocyte populations collected on day 0 (D0), CD3i/CD28i-activated noninfected T lymphocytes (NI), and T lymphocytes transduced with the PGK-mADA retrovirus on FN CH-296 at day 6 posttransduction (CH-296/mADA). This is representative of three independent experiments.

these target cell populations. For example, Kaina et al. recently demonstrated that the overexpression of the DNA repair enzyme methylpurine-DNA glycosylase in human cells led to an imbalance in the base excision repair pathway, chromosomal aberrations, and sister chromatid exchange in hematopoietic cells (32).

In some situations, T lymphocytes may be useful as an alternative to hematopoietic stem and progenitor cells for genetic modification (26). Preclinical studies performed with mice (13, 18, 19) and nonhuman primates (10, 14, 60) demonstrated that transduced T lymphocytes were long-lived and functioned normally. In initial human gene therapy trials for ADA⁻ SCID patients, transduced peripheral blood T lymphocytes (2, 5) and bone marrow-derived T lymphocytes (5) were detectable by PCR analysis for at least a year after discontinuation of gene therapy treatment.

We recently demonstrated that the colocalization of retrovirus and target cells on recombinant FN fragments improved

the efficiency of gene transfer into both human and murine hematopoietic stem cells and progenitors (22, 23, 45). Since T lymphocytes express both VLA-4 and VLA-5 and the transduction efficiency of primary T lymphocytes derived from normal donors or ADA⁻ SCID patients had been low when conventional methods were used (2, 5, 6, 10, 13, 14, 18, 19, 40, 46, 60), T lymphocytes were a logical target cell population for investigation of the contribution of FN in the context of gene delivery. Several parameters influenced the gene transfer levels obtained by transduction on recombinant FN fragments. First, a comparison of prestimulation conditions indicated that activation of lymphocytes with CD3i/CD28i MABs resulted in more efficient gene transfer than did activation in parallel with CM, IL-2, PHA, CD3s MAB, or CD3i MAB (Table 1 and data not shown). Effective cross-linking of CD3 and CD28 molecules on T cells has been reported to dramatically enhance the adhesion of T cells to FN via VLA-4 and VLA-5 (56). The increased adhesion of T cells to FN presumably results in an increase in the proximity of T lymphocytes and retroviruses, effectively increasing the titer of the virus, even in the absence of polycations such as Polybrene. Since the vast majority of T lymphocytes enter the cell cycle when maintained on CD3i/CD28i (53), this could also facilitate integration of proviral DNA.

The timing of exposure to retrovirus particles was also critical. The highest levels of gene transfer were observed when T lymphocytes were exposed to virus for three infection cycles on

FIG. 6. Comparative analysis of transgene expression on IL-2- and CD3i/CD28i-activated T lymphocytes. T lymphocytes were transduced with the mB7-1 retrovirus (A) or the PGK-ADA retrovirus (B) by the optimized protocol and maintained in IL-2 until day 11 posttransduction. The culture was subsequently divided and maintained in parallel cultures containing IL-2 alone or on plates coated with CD3i/CD28i for an additional 2 days. (A) The percent mB7-1⁺ expression is the percentage of transduced T lymphocytes staining positive for mB7-1 minus the percentage of nontransduced cells staining positive for mB7-1. The background of the nontransduced cells stained with the mB7-1 MAb was routinely $\leq 2.5\%$. This is representative of five independent experiments. (B) Analysis of mADA activity in T lymphocytes from a normal donor and an ADA⁻ SCID patient. This is representative of two independent experiments. Mock, samples transduced with the m37.1 retrovirus as a control for the transduction protocol.

consecutive days in culture (Fig. 2; Tables 2 and 3). From the data of experiments that are not described here, it was clear that after each infection cycle, incubation of the lymphocytes overnight without exposure to virus was instrumental in obtaining optimal gene transfer. For instance, if lymphocytes were exposed to retrovirus three times in a 24-h period, the level of gene transfer was significantly lower than for cells exposed to retrovirus three times on consecutive days (48a). We are currently investigating the kinetics of amphotropic receptor expression and downmodulation of the receptor on CD3i/CD28i-activated T lymphocytes in an effort to clarify the mechanism behind these results.

As expected, the titer of the retroviral supernatant correlated to some degree with the gene transfer efficiency obtained with CD3i/CD28i-activated T lymphocytes. When using mB7-1 supernatant collected at 37°C, 37 to 42% of the T lymphocytes continued to express mB7-1 (Fig. 2B and Table 2). When the mB7-1 titer was increased by collecting supernatant at 32°C,

higher levels of gene transfer (approaching 90%) were observed at day 13 posttransduction when T lymphocytes were transduced on FN CH-296 (Fig. 2C, Table 3, and Fig. 6A). Therefore, it is possible to achieve high transduction efficiencies in nonselected T-lymphocyte populations in which the vast majority of primary human T lymphocytes express the introduced transgene in long-term culture, indicative of integrated provirus. Most significantly, transduction of T lymphocytes from normal donors or an ADA⁻ SCID donor on FN CH-296 with the PGK-mADA retrovirus conferred high levels of gene transfer 12 days posttransduction in nonselected T-cell populations (Fig. 4). In contrast, transductions performed with these T-cell populations by the standard approach (cell-free supernatant in the presence of Polybrene) yielded relatively low levels of mADA activity. Collectively, these data show that high levels of gene transfer into ADA-deficient T lymphocytes are possible with the transduction protocol described here.

In addition, our studies indicated that at least in primary human T lymphocytes, the expression level of the introduced transgene may be modified by the activation status of the lymphocyte population. This observation did not appear to be dependent on the transgene, the retroviral backbone, or the promoter element utilized, since expression of two different

TABLE 4. Effect of CD28i/CD3i reactivation on transgene expression^a

Expt	% mB7-1 expression with activation on ^b :		Fold increase in % mB7-1 expression ^d	MFI with activation on ^c :		Fold increase in MFI ^e
	IL-2	CD3i/CD28i		IL-2	CD3i/CD28i	
I	20.4	87.2	4.3	11.6	441.0	38
II	15.6	80.1	5.1	13.7	415.3	30
III	7.1	76.2	10.7	6.9	416.0	60
IV	7.4	81.0	10.9	3.1	377.6	122

^a Human T lymphocytes were activated with CD3i/CD28i MABs for 1 or 2 days and transduced with the mB7-1 retrovirus on days 2 to 4 postactivation on CH-296. At 10 days posttransduction, T lymphocytes were reactivated for 2 days on CD3i/CD28i or maintained in 100 U/ml IL-2 and then analyzed for mB7-1 expression.

^b Data are represented as the percentage of transduced cells expressing mB7-1 minus the percentage of nontransduced cells expressing mB7-1. Nonspecific binding of mB7-1 MAB to nontransduced cells was always less than 2.5%.

^c Data are represented as the MFI of the transduced population stained with mB7-1 MAB minus the MFI of the transduced population stained with the isotype control MAB.

^d The fold increase in mB7-1 transgene expression on CD3i/CD28i-reactivated T cells versus those maintained in IL-2 was calculated by dividing expression on CD3i/CD28i-activated T cells by expression on IL-2-activated T cells.

^e The fold increase in MFI was calculated by dividing the MFI for CD28i/CD3i-activated T cells by the MFI for IL-2-activated T cells.

transgenes under the control of different promoters showed a similar effect depending on whether the T-lymphocyte population was maintained on IL-2 or reactivated for a short time on CD3i/CD28i. In these experiments, there was a significant increase in transgene expression in transduced T lymphocytes reactivated on CD3i/CD28i compared to those maintained on IL-2. Even at 31 days posttransduction, mB7-1-transduced T lymphocytes reactivated with CD3i/CD28i showed three- to fivefold-higher levels of mB7-1 expression than those maintained on IL-2 (48a). These observations raise the possibility that relying on high levels of protein expression (as detected by flow cytometry) as an indicator of gene transfer may not necessarily be an accurate assessment of transduction efficiency if the majority of the target cell population is not appropriately activated.

Transgene expression can be regulated in both a positive and a negative fashion. For example, transgene expression driven by commonly used promoters and enhancers (e.g., CMV, Rous sarcoma virus, simian virus 40, or Moloney murine leukemia virus long terminal repeat) was downregulated by proinflammatory cytokines such as gamma interferon and tumor necrosis factor alpha (21, 24, 49), while reporter gene expression increased when driven by the major histocompatibility complex class I promoter (24). In a murine heterotopic, nonvascularized cardiac transplant model, reporter gene expression from an adenovirus vector containing the human CMV immediate-early 1 promoter increased in the presence of a neutralizing anti-gamma-interferon MAB (49). Recently, Bunnell et al. reported that transgene expression was not detectable in nonstimulated CD4⁺ cell-transduced rhesus lymphocytes reisolated from rhesus macaques. Transgene expression in retrovirally marked cells could be detected by RT-PCR only when lymphocytes were cultured in IL-2 (10). Recently, in another study, rhesus macaques were infused with autologous lymphocytes transduced with a vector expressing an antisense *tat* or *rev* gene and subsequently infected with the simian immunodeficiency virus. Lymphocytes taken from these infected monkeys expressed the transgene without any in vitro culturing, consistent with the idea that activation of the immune system may increase trans-

gene expression in vivo (15). Regulation of transgene expression may be instrumental in determining the efficacy of gene therapy in a variety of settings. In studies investigating the feasibility of a dominant-negative mutant of the HIV *rev* transactivator protein (RevM10), it was clear that only highly activated, transduced T-cell populations showed resistance to HIV replication (48). Our results extend these observations by demonstrating that the activation status of the transduced lymphocyte dramatically influences transgene expression. In preliminary experiments, Northern analysis of transduced cells has indicated that there are increased levels of mB7-1 transgene RNA in T lymphocytes reactivated with CD28i/CD3i compared to those maintained in IL-2 (48a). Experiments to determine whether transgene regulation is occurring at the transcriptional level or at the level of RNA stability are in progress. Further studies will be necessary to clarify the relationship between the activation status defined in vitro and expression of transgene in lymphocytes in vivo. The ability to regulate transgene expression may have important implications for gene therapy protocols.

Improvements in producer lines (9, 55) have been reported to increase the gene transfer efficiency. For example, supernatants containing retroviruses pseudotyped with the vesicular stomatitis virus glycoprotein could be concentrated to titers approaching 10⁹ retroviral particles. However, the levels of gene transfer documented in primary T lymphocytes when using high-titer vesicular stomatitis virus glycoprotein-pseudotyped retroviruses did not reach the transfer levels obtained in our present study using amphotropic retroviruses and FN CH-296. In a report of Bunnell et al. (9), the use of the alternative packaging line PG13, derived from the gibbon ape leukemia virus envelope, led to increased gene transfer rates in human and primate T lymphocytes. In that study, a PG13-derived retroviral supernatant, metabolic induction of the gibbon ape leukemia virus receptor, low-temperature incubation, and centrifugation were all used to increase the gene transfer efficiency. They reported >50% lymphocyte transduction with PG13-packaged vectors and >25% transduction efficiency with amphotropic-packaged retroviral vectors at 72 h posttransduction. After two cycles of transduction utilizing ADA-deficient T lymphocytes, 42% of the target cells were transduced with a PG13-derived retrovirus while only 3% of the target cells were transduced with the amphotropic pseudotyped retrovirus. These studies suggested that PG13 packaging cells may be a useful alternative to amphotropic packaging lines. Since in our current gene transfer protocol T lymphocytes must be transduced on two to three consecutive days for high-efficiency gene transfer with amphotropic retroviruses, the development of protocols utilizing a single infection cycle would be beneficial in the clinical setting. Currently, we are comparing the ability of retroviruses derived from PG13 packaging cells to transduce T lymphocytes on FN CH-296-coated plates with that of retroviruses derived from amphotropic packaging cells.

Utilizing this gene transfer protocol, we have obtained the highest levels of retroviral gene transfer reported to date in primary human T lymphocytes. With the development of protocols to achieve maximal gene transfer into human T lymphocytes, acquiring transduced T-lymphocyte populations comprising a full immune repertoire may be possible. Our transduction protocol provides a simple and reliable way of delivering genes at high efficiency to human T cells by using retroviral supernatants derived from amphotropic producer lines. The gene transfer strategy reported here should be useful in gene therapy trials for ADA⁻ SCID patients, since we also demonstrated that ADA-deficient T lymphocytes were transduced efficiently on FN CH-296-coated plates. Furthermore, if

100% transduction of the target population is necessary (4), the selection of transduced cells by a retrovirally encoded cell surface marker, such as the truncated nerve growth factor receptor, could be facilitated by initially transducing on recombinant FN fragments. Larger numbers of transduced cells could thereby be obtained by a combination of transduction on FN CH-296 followed by selection of surface-marked cells. However, in some situations, a simplified vector expressing only the therapeutic gene may be optimal. Studies have shown that long-term gene expression can be turned off in constructs expressing more than one gene (62). The efficient delivery of genes to human primary T cells designed to modulate the immune response may lead to novel treatments for a variety of acquired and inherited immunodeficiency diseases (26, 35, 51), lysosomal disorders (61), and cancers (52).

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REFERENCES

- Blaese, R. M. 1993. Development of gene therapy for immunodeficiency: adenosine deaminase deficiency. *Pediatr. Res.* 33(Suppl. 1):S49-S55.
- Blaese, R. M., K. W. Culver, A. D. Miller, C. S. Carter, T. Fleisher, M. Clerici, G. Shearer, L. Chang, Y. Chiang, P. Tolstoshev, J. J. Greenblatt, S. A. Rosenberg, H. Klein, M. Berger, C. A. Mullen, W. J. Ramsey, L. Muul, R. A. Morgan, and W. F. Anderson. 1995. T lymphocyte-directed gene therapy for ADA⁻ SCID: initial trial results after 4 years. *Science* 270:475-480.
- Bodine, D. M., T. Moritz, R. E. Donahue, B. D. Luskey, S. W. Kessler, D. I. K. Martin, S. H. Orkin, A. W. Nienhuis, and D. A. Williams. 1993. Long-term *in vivo* expression of a murine adenosine deaminase gene in rhesus monkey hematopoietic cells of multiple lineages after retroviral mediated gene transfer into CD34⁺ bone marrow cells. *Blood* 82:1975-1980.
- Bonin, C., G. Ferrari, S. Verzeletti, P. Servida, E. Zappone, L. Ruggieri, M. Ponzoni, S. Rossini, F. Mavilio, C. Traversari, and C. Bordignon. 1997. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 276:1719-1724.
- Bordignon, C., L. D. Notarangelo, N. Nobili, G. Ferrari, G. Casorati, P. Panina, E. Mazzolari, D. Maggioni, C. Rossi, P. Servida, A. G. Ugazio, and F. Mavilio. 1995. Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. *Science* 270:470-475.
- Braakman, E., V. W. van Beusechem, B. A. van Krimpen, A. Fischer, R. L. H. Bolhuis, and D. Valerio. 1992. Genetic correction of cultured T cells from an adenosine deaminase-deficient patient: characteristics of non-transduced and transduced T cells. *Eur. J. Immunol.* 22:63-69.
- Brenner, M. K., D. R. Rill, M. S. Holladay, H. E. Heslop, R. C. Moen, M. Buschle, R. A. Krance, V. M. Santana, W. F. Anderson, and J. N. Ihle. 1993. Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet* 342:1134-1137.
- Brenner, M. K. 1996. Gene transfer into hematopoietic cells. *N. Engl. J. Med.* 335:337-339.
- Bunnell, B. A., L. M. Muul, R. E. Donahue, R. M. Blaese, and R. A. Morgan. 1995. High-efficiency retroviral-mediated gene transfer into human and non-human primate peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA* 92:7739-7743.
- Bunnell, B. A., M. Metzger, E. Byrne, R. A. Morgan, and R. E. Donahue. 1997. Efficient *in vivo* marking of primary CD4⁺ T lymphocytes in non-human primates using a gibbon ape leukemia virus-derived retroviral vector. *Blood* 89:1987-1995.
- Chinen, J., E. Aguilar-Cordova, D. Ng-Tang, D. E. Lewis, and J. W. Belmont. 1997. Protection of primary human T cells from HIV infection by Trev: a transdominant fusion gene. *Hum. Gene Ther.* 8:861-868.
- Crystal, R. G. 1993. Transfer of genes to humans: early lessons and obstacles to success. *Science* 270:404-410.
- Culver, K., K. Cornetta, R. Morgan, S. Morecki, P. Aebersold, A. Kasid, M. Lotze, S. A. Rosenberg, W. F. Anderson, and R. M. Blaese. 1991. Lymphocytes as cellular vehicles for gene therapy in mouse and man. *Proc. Natl. Acad. Sci. USA* 88:3155-3159.
- Culver, K. W., R. A. Morgan, W. R. A. Osborne, R. T. Lee, D. Lenschow, C. Able, K. Cornetta, W. F. Anderson, and R. M. Blaese. 1990. *In vivo* expression and survival of gene-modified T lymphocytes in rhesus monkeys. *Hum. Gene Ther.* 1:399-410.
- Donahue, R. E., B. A. Bunnell, M. C. Zink, M. E. Metzger, R. P. Westro, M. R. Kirby, T. Unangst, J. E. Clements, and R. A. Morgan. 1998. Reduction of SIV replication in rhesus macaques infused with autologous lymphocytes engineered with antiviral genes. *Nat. Med.* 4:181-186.
- Dunbar, C. E., M. Cottler-Fox, J. A. O'Shaughnessy, S. Doren, C. Carter, R. Berenson, S. Brown, R. C. Moen, J. Greenblatt, F. M. Stewart, S. F. Leitman, W. H. Wilson, K. Cowan, N. S. Young, and A. W. Nienhuis. 1995. Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood* 85:3048-3057.
- Emmons, R. V. B., S. Doren, J. Zujewski, M. Cottler-Fox, C. S. Carter, K. Hines, J. A. O'Shaughnessy, S. F. Leitman, J. J. Greenblatt, K. Cowan, and C. E. Dunbar. 1997. Retroviral gene transduction of adult peripheral blood or marrow-derived CD34⁺ cells for six hours without growth factors or on autologous stroma does not improve marking efficiency assessed *in vivo*. *Blood* 89:4040-4046.
- Ferrari, G., S. Rossini, R. Giavazzi, D. Maggioni, N. Nobili, M. Soldati, G. Ungers, F. Mavilio, E. Gilboa, and C. Bordignon. 1991. An *in vivo* model of somatic cell gene therapy for human severe combined immunodeficiency. *Science* 251:1363-1366.
- Ferrari, G., S. Rossini, N. Nobili, D. Maggioni, A. Garofalo, R. Giavazzi, F. Mavilio, and C. Bordignon. 1992. Transfer of the ADA gene into human ADA-deficient T lymphocytes reconstitutes specific immune functions. *Blood* 80:1120-1124.
- Genevee, C., A. Diu, J. Nierat, A. Caignard, P.-Y. Dietrich, L. Ferrandini, S. Roman-Roman, F. Triebel, and T. Hercend. 1992. An experimentally validated panel of subfamily-specific oligonucleotide primers (V α 1-w29/V β 1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. *Eur. J. Immunol.* 22:1261-1269.
- Ghazizadeh, S., J. M. Carroll, and L. B. Taichman. 1997. Repression of retrovirus-mediated transgene expression by interferons: implications for gene therapy. *J. Virol.* 71:9163-9169.
- Hanenberg, H., X. L. Xiao, D. Dilloo, K. Hashino, I. Kato, and D. A. Williams. 1996. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med.* 2:876-882.
- Hanenberg, H., K. Hasino, H. Konishi, R. Hock, I. Kato, and D. A. Williams. 1997. Optimization of fibronectin-assisted gene transfer into human CD34⁺ hematopoietic cells. *Hum. Gene Ther.* 8:2193-2206.
- Harms, J. S., and G. A. Splitter. 1995. Interferon- γ inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHCI promoter. *Hum. Gene Ther.* 6:1291-1297.
- Hawley, R. G., A. Z. C. Fong, B. F. Burns, and T. S. Hawley. 1992. Transplantable myeloproliferative disease induced in mice by an interleukin 6 retrovirus. *J. Exp. Med.* 176:1149-1163.
- Hege, K. M., and M. R. Roberts. 1996. T-cell gene therapy. *Curr. Opin. Biotechnol.* 7:629-634.
- Hemler, M. E. 1990. VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu. Rev. Immunol.* 8:365-400.
- Hershfield, M. S. 1995. PEG-ADA replacement therapy for adenosine deaminase deficiency: an update after 8.5 years. *Clin. Immunol. Immunopathol.* 76:S228-S232.
- Hirschhorn, R. 1995. Adenosine deaminase deficiency: molecular basis and recent developments. *Clin. Immunol. Immunopathol.* 76:S219-S227.
- Hoogerbrugge, P. M., V. W. van Beusechem, A. Fischer, M. Debre, F. le Deist, J. L. Perignon, G. Morgan, B. Gaspar, L. D. Fairbanks, C. H. Skeoch, A. Moseley, M. Harvey, R. J. Levinsky, and D. Valerio. 1996. Bone marrow gene transfer in three patients with adenosine deaminase deficiency. *Gene Ther.* 3:179-183.
- Hwu, P., J. Yannelli, M. Krieger, W. F. Anderson, C. Perez, Y. Chiang, S. Schwarz, R. Cowherd, C. Delgado, J. Mule, and S. A. Rosenberg. 1993. Functional and molecular characterization of tumor-infiltrating lymphocytes transduced with tumor necrosis factor- α cDNA for the gene therapy of cancer in humans. *J. Immunol.* 150:4104-4115.
- Kaina, B., G. Fritz, and T. Coquerelle. 1993. Contribution of O⁶-alkylguanine and N-alkylpurines to the formation of sister chromatid exchanges, chromosomal aberrations, and gene mutations: new insights gained from studies of genetically engineered mammalian cell lines. *Environ. Mol. Mutagen.* 22:283-292.
- Kimizuka, F., Y. Taguchi, Y. Ohdate, Y. Kawase, T. Shimojo, K. Hashino, I. Kato, K. Sekiguchi, and K. Titani. 1991. Production and characterization of functional domains of human fibronectin expressed in *Escherichia coli*. *J. Biochem.* 110:284-291.
- Kohn, D. B., K. I. Weinberg, J. A. Nolte, L. N. Heiss, C. Lenarsky, G. M. Crooks, M. E. Hanley, G. Annett, J. S. Brooks, A. El-Khourey, K. Lawrence,

- S. Wells, R. C. Moen, J. Bastian, D. E. Williams-Herman, M. Elder, D. Wara, T. Bowen, M. S. Hershfield, C. A. Mullen, R. M. Blaese, and R. Parkman. 1995. Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nat. Med.* **1**:1017–1023.
35. Kohn, D. B. 1997. Gene therapy for haematopoietic and lymphoid disorders. *Clin. Exp. Immunol.* **107**:54–57.
36. Larochelle, A., J. Vormoor, H. Hanenberg, J. C. Y. Wang, M. Bhatia, T. Lapidot, T. Moritz, B. Murdoch, X. L. Xiao, I. Kato, D. A. Williams, and J. E. Dick. 1996. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat. Med.* **2**:1329–1337.
37. Lim, B., D. A. Williams, and S. H. Orkin. 1987. Retrovirus-mediated gene transfer of human adenosine deaminase: expression of functional enzyme in murine hematopoietic stem cells in vivo. *Mol. Cell. Biol.* **7**:3459–3465.
38. Lisiewicz, J. 1996. TAR decoys and trans-dominant gag mutant for HIV-1 gene therapy. *Antibiot. Chemother.* **48**:192–197.
39. Markowitz, D., S. Goff, and A. Bank. 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* **62**:1120–1124.
40. Mavilio, F., G. Ferrari, S. Rossini, N. Nobili, C. Bonini, G. Casorati, C. Traversari, and C. Bordignon. 1994. Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. *Blood* **83**:1988–1997.
41. Miller, A. D. 1996. Cell surface receptors for retroviruses and implications for gene transfer. *Proc. Natl. Acad. Sci. USA* **93**:11407–11413.
42. Miller, D. G., M. A. Adam, and A. D. Miller. 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* **10**:4239–4242.
43. Moritz, T., D. C. Keller, and D. A. Williams. 1993. Human cord blood cells as targets for gene transfer: potential use in genetic therapies of severe combined immunodeficiency disease. *J. Exp. Med.* **178**:529–536.
44. Moritz, T., V. P. Patel, and D. A. Williams. 1994. Bone marrow extracellular matrix molecules improve gene transfer into human hematopoietic cells via retroviral vectors. *Clin. Invest.* **93**:1451–1457.
45. Moritz, T., P. Dutt, X. L. Xiao, D. Carstanjen, T. Vik, H. Hanenberg, and D. A. Williams. 1996. Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. *Blood* **88**:855–862.
46. Mullen, C. A., K. Snitzer, K. W. Culver, R. A. Morgan, W. F. Anderson, and R. M. Blaese. 1996. Molecular analysis of T lymphocyte-directed gene therapy for adenosine deaminase deficiency: long-term expression in vivo of genes introduced with a retroviral vector. *Hum. Gene Ther.* **7**:1123–1129.
47. Orlic, D., L. J. Girard, C. T. Jordan, S. M. Anderson, A. P. Cline, and D. M. Bodine. 1996. The level of mRNA encoding the amphotropic retrovirus receptor in mouse and human hematopoietic stem cells is low and correlates with the efficiency of retrovirus transduction. *Proc. Natl. Acad. Sci. USA* **93**:11097–11102.
48. Plavce, I., M. Agarwal, K. E. Ho, M. Pineda, J. Auten, J. Baker, H. Matsuzaki, S. Escaich, M. Bonyhadi, and E. Bohnlein. 1997. High transdominant RevM10 protein levels are required to inhibit HIV-1 replication in cell lines and primary T cells: implication for gene therapy of AIDS. *Gene Ther.* **4**:128–139.
- 48a. Pollak, K., and D. A. Williams. Unpublished data.
49. Qin, L., Y. Ding, D. R. Pahud, E. Chang, M. J. Imperiale, and J. S. Bromberg. 1997. Promoter attenuation in gene therapy: interferon- γ and tumor necrosis factor- α inhibit transgene expression. *Hum. Gene Ther.* **8**:2019–2029.
50. Roe, T. Y., T. C. Reynolds, G. Yu, and P. O. Brown. 1993. Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* **12**:2099–2108.
51. Rosen, F. S., M. D. Cooper, and R. J. P. Wedgwood. 1995. The primary immunodeficiencies. *N. Engl. J. Med.* **333**:431–440.
52. Roth, J. A., and R. J. Cristiano. 1997. Gene therapy for cancer: what have we done and where are we going? *J. Natl. Cancer Inst.* **89**:21–39.
53. Rudd, C. E. 1996. Upstream-downstream: CD28 cosignaling pathways and T cell function. *Immunity* **4**:527–534.
54. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
55. Sharma, S., M. Cantwell, T. J. Kipps, and T. Friedmann. 1996. Efficient infection of a human T-cell line and of human primary peripheral blood leukocytes with a pseudotyped retrovirus vector. *Proc. Natl. Acad. Sci. USA* **93**:11842–11847.
56. Shimizu, Y., G. A. van Severter, K. J. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA (β_1) integrin receptors on T cells. *Nature* **345**:250–253.
57. Shimizu, Y., G. A. van Severter, E. Ennis, W. Newman, K. J. Horgan, and S. Shaw. 1992. Crosslinking of T cell-specific accessory molecules CD7 and CD28 modulates T cell adhesion. *J. Exp. Med.* **175**:577–582.
58. Treisman, J., P. Hwu, S. Minamoto, G. E. Shafer, R. Cowherd, R. A. Morgan, and S. A. Rosenberg. 1995. Interleukin-2-transduced lymphocytes grow in an autocrine fashion and remain responsive to antigen. *Blood* **85**:139–145.
59. Umetsu, D. T., C. M. Schlossman, H. D. Ochs, and M. S. Hershfield. 1994. Heterogeneity of phenotype in two siblings with adenosine deaminase deficiency. *J. Allergy Clin. Immunol.* **93**:543–550.
60. van Beusechem, V. W., A. Kukler, P. J. Heidt, and D. Valerio. 1992. Long-term expression of human adenosine deaminase in rhesus monkeys transplanted with retrovirus-infected bone marrow cells. *Proc. Natl. Acad. Sci. USA* **89**:7640–7644.
- 60a. van der Loo, J. C. M., X. L. Xiao, D. McMillin, K. Hashino, I. Kato, and D. A. Williams. VLA-5 is expressed by mouse and human hematopoietic repopulating cells and mediates adhesion to recombinant extracellular matrix peptides derived from fibronectin. Submitted for publication.
61. Whitley, C. B., R. S. McIvor, E. L. Aronovich, S. A. Berry, B. R. Blazer, S. R. Burger, J. H. Kersey, R. A. King, A. J. Faras, R. E. Latchaw, J. McCullough, D. Pan, N. K. C. Ramsay, and D. F. Stroncek. 1996. Retroviral-mediated transfer of the iduronate-2-sulfatase gene into lymphocytes for treatment of mild Hunter syndrome (mucopolysaccharidosis type II). *Hum. Gene Ther.* **7**:537–549.
62. Williams, D. A. 1990. Expression of introduced genetic sequences in hematopoietic cells following retroviral-mediated gene transfer. *Hum. Gene Ther.* **1**:229–239.
63. Woffendin, C., V. Ranga, Z. Yang, L. Xu, and G. J. Nabel. 1996. Expression of a protective gene prolongs survival of T cells in human immunodeficiency virus-infected patients. *Proc. Natl. Acad. Sci. USA* **93**:2889–2894.