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Retroviral vector-mediated high-efficiency expression of adenosine deaminase (ADA) in hematopoietic long-term cultures of ADA-deficient marrow cells

(gene therapy/severe combined immunodeficiency/gene transfer)

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ABSTRACT **Two recombinant retroviral vectors encoding the cDNA of the human adenosine deaminase (*ADA*; EC 3.5.4.4) gene and the bacterial neomycin resistance (*Neo*) gene have been used to transduce bone marrow cells obtained from four patients affected by the ADA-deficient variant of severe combined immunodeficiency. By utilizing the long-term marrow culture system, freshly isolated bone marrow cells were subjected to multiple infection cycles with cell-free supernatants containing high titers of viral vector and then maintained in long-term marrow culture in the absence of any overt selection pressure. By using this experimental protocol, about 30–40% of the hematopoietic progenitors were productively transduced with the viral vector, as judged by the appearance of G418-resistant colonies derived from granulocyte/macrophage and multipotent hematopoietic progenitor cells. The vector-encoded human *ADA* gene was expressed efficiently in both the myeloid and lymphoid progeny of the cultured bone marrow cells, reaching levels between 15% and 100% as compared to the levels of ADA in normal bone marrow cells. The efficiency of gene transfer and ADA production was proportional to the number of infection cycles. Furthermore, transduction of the ADA vectors into the bone marrow cells derived from an ADA-deficient patient restored the capacity of the cells to respond to phytohemagglutinin and interleukin 2.**

Previous studies in a number of laboratories have demonstrated that retroviral vectors can be used to introduce exogenous DNA sequences into hematopoietic progenitors and pluripotent stem cells. Efficient gene transfer *in vitro* and *in vivo* has been reported in the mouse model system (1–4).

On the other hand, only low levels of gene transfer were achieved in cultured hematopoietic progenitors derived from dog and humans (5, 6) and only a few studies have succeeded in demonstrating significant levels of expression of the transduced gene in cultured progenitors (7, 8) or in long-term reconstituted animals (2, 9).

A long-term objective of our studies is to develop an efficient gene transfer system for the introduction of the human adenosine deaminase (*ADA*; EC 3.5.4.4) gene into hematopoietic stem cells of human origin. For this purpose we have constructed an ADA expression vector based on the N2 retroviral vector (10). This ADA-containing vector, called SAX, was shown to transduce the *ADA* gene with high efficiency into established lymphoid cell lines derived from an ADA-deficient patient (11). On the other hand, low levels of gene transfer and transient human ADA expression were achieved when lethally irradiated cynomolgus monkeys were reconstituted with autologous marrow cells transduced with the SAX vector (12).

In the present study, we have attempted to improve the frequency of progenitor transduction through the use of multiple infection exposures of ADA-deficient bone marrow progenitors under conditions designed to promote their growth *in vitro* in both short- and long-term culture. Our results demonstrate that efficient gene transfer can be achieved by the use of multiple infection cycles, high-titer vector preparations, and high vector particle to target cell ratio, leading to the expression of high levels of the ADA enzyme throughout the life-span of the culture. Efficient expression of the newly introduced ADA enzyme occurred in both myeloid and lymphoid lineages. Furthermore, transfer of the *ADA* gene into the ADA-deficient bone marrow cells resulted in complete restoration of their capacity to respond to proliferative stimuli such as phytohemagglutinin (PHA) and interleukin 2 (IL-2).

MATERIALS AND METHODS

ADA-Deficient Bone Marrow Cells. Mononuclear bone marrow cells were obtained from four patients (AH, JC, PAC, and GB) affected by the ADA-deficient variant of severe combined immunodeficiency (ADA-SCID). The diagnosis was confirmed by immunologic tests revealing severe T lymphocytopenia, the absence of lymphocyte responses to stimulation with mitogens and alloantigens, the absence of antibody response to vaccines, and the absence of ADA activity in the mononuclear peripheral blood cells and in the erythrocytes. Three patients were transplanted with a T-cell-depleted maternal bone marrow graft at Memorial Sloan–Kettering Cancer Center. Prior to their preparative cytoreduction for transplantation and with parental consent, they underwent a bone marrow harvest. Mononuclear bone marrow cells were isolated by separation on Ficoll/Hypaque gradients and utilized for the *in vitro* gene transfer experiments described in this report.

Derivation of Recombinant Viruses. The structures of the two retroviral vectors containing the *ADA* gene are shown in Fig. 1. The human ADA cDNA (13) was introduced into a Moloney murine leukemia virus-based vector called N2 that also contains the bacterial neomycin resistance (*Neo*) gene used as a selectable marker. In one vector construct the ADA cDNA was fused to the early simian virus 40 (SV40) promoter

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Abbreviations: ADA, adenosine deaminase; SV40, simian virus 40; IL-2, interleukin 2; SCID, severe combined immunodeficiency; LTMC, long-term marrow culture; PHA, phytohemagglutinin; CFU-GM, granuloycte/macrophage colony-forming unit; CFU-GEMM, multipotent hematopoietic progenitor CFU; rhG-CSF, recombinant human granulocyte colony-stimulating factor; E, erythrocyte.

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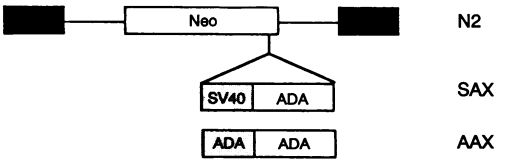

FIG. 1. Structures of retroviral vectors containing the human ADA cDNA. Both vectors are derived from the parental vector N2.

and inserted downstream to the *Neo* gene. The N2 vector and the SAX derivative were previously described (10). The AAX vector construct is similar to the SAX construct except the SV40 promoter is replaced with a 540-base-pair *Xho* I–*Nco* I DNA fragment derived from the human ADA promoter (14). The recombinant constructs were transfected into the amphothropic producer cell line PA 317, as described (15). Supernatants from the producer cell lines containing high titers of viral vector ($\approx 10^6$ neomycin-resistant colony-forming units/ml) were collected and maintained at −80°C until use.

Vector Transduction of ADA-Deficient Bone Marrow Cells. Bone marrow mononuclear cells obtained from ADA-deficient SCID patients were infected in multiple cycles. Each infection cycle consisted of a 2-hr exposure to the vector-containing supernatant in the presence of Polybrene (4 μg/ml); this was followed by an 8-hr expression time in the long-term marrow culture (LTMC) medium. During infection mononuclear marrow cells were maintained in continuous gentle agitation at 37°C. Vector to cell ratios were between 2:1 and 5:1. Viral vector-containing supernatants were utilized undiluted. After the completion of the multiple infection cycles, marrow cells were resuspended in the LTMC medium and maintained in culture for gene transfer and enzyme activity analyses for 4–6 wk.

Hematopoietic Colony Assays. Short-term colony assays utilized for the analysis of gene transfer and the efficiency of long-term culture were the standard assays for granulocyte/macrophage colony-forming units (CFU-GM). The ability of bone marrow to form colonies and clusters of granulocytes and macrophages was assayed in soft agar culture, according to the method of Pike and Robinson (16), 24 hr after completion of the last infection cycle. Marrow cells were plated at 2×10^5 cells in a 1.0-ml layer of 0.3% agar (Difco) in McCoy's medium supplemented with 10% conditioned medium from the 5637 human carcinoma cell line (17). Colonies and clusters were scored after 7–14 days of incubation at 37°C in humidified 5% CO_2/95% air. All assays described were performed with and without increasing concentrations of the neomycin analogue G418.

Hematopoietic Long-Term Cultures. Long-term bone marrow cultures were preformed according to the method of Gartner and Kaplan (18) with minor modifications. Briefly, mononuclear marrow cells were obtained by Ficoll/Hypaque separation, and 20×10^6 marrow cells were resuspended in 10 ml of LTMC medium and were inoculated into T-25 tissue culture flasks. Cultures were gassed with 5% CO_2 in air and incubated at 37°C or 33°C. The LTMC medium consisted of supplemented McCoy's 5A medium containing 12.5% horse serum, 12.5% fetal calf serum, and 1 μM hydrocortisone sodium succinate (Calbiochem–Behring). At weekly intervals, half the cells were removed from the long-term culture and analyzed for gene(s) transfer and expression. At each weekly feeding, half of the cells were removed from the cultures for analyses. After an initial decay (first 3–5 days of culture), the number of cells and progenitors recovered in this fraction remained stable for the following 3–4 wk of culture. These data indicate that regeneration occurred for at least this period of time.

Assays for ADA Activity. ADA enzyme activity was analyzed by the [^{14}C]adenosine conversion assay, performed essentially as described by Van der Weyden and Bailey (19). Briefly, individual samples of cell lysate were mixed with [^{14}C]adenosine, incubated for different times (15, 30, and 60 min), and separated on thin-layer chromatography (TLC) plates. The individual migration spots were measured for [^{14}C]adenosine conversion to [^{14}C]inosine in a scintillation counter. The results were expressed as percent conversion and as nmol/min per 10^8 cells. Exact standardization of the cell lysates was obtained by normalization for total protein by the Bio-Rad protein assay (Bio-Rad).

RESULTS

Infection of Hematopoietic Cells and Short-Term Culture Analysis. Bone marrow cells were obtained from normal individuals and from patients affected with the ADA-deficient variant of SCID. The levels of residual enzymatic activity detectable in the peripheral blood and marrow mononuclear cells of the ADA-deficient patients (0.5–2.8% of normal individuals) represent the baseline control to which the expression of the transduced cells was compared.

Since our previous data *in vitro* and *in vivo* indicated low frequency of infection as one of the most important limitations in achieving good levels of long-term expression, we have pursued several different approaches in an attempt to increase efficiency of gene transfer. By utilizing high viral particle to target cell ratios, we introduced two modifications of the original gene transfer protocols: (*i*) multiple infection cycles and (*ii*) cycling of the target cells by *in vitro* exposure to hematopoietic growth factors.

Initially, efficiency of gene transfer was tested by determining the frequency of G418-resistant hematopoietic CFU-GM and multipotent hematopoietic colony-forming units (CFU-GEMM) (6) 24 hr after infection. As indicated by the data reported in Fig. 2*A*, repeated short courses of infection increased proportionally the efficiency of gene transfer up to five cycles of infection, as measured by the number of G418-resistant colonies. The efficiency of gene transfer was similar in the different patients studied. However, it could not be enhanced past 30–40%, regardless of the number of infection cycles utilized. Similarly, a proportional enhancement of ADA activity was observed after each infection cycle (Fig. 2*B*). In this experiment, 24 hr after the last infection cycle, ADA activity increased from 4.6 to 32.4 nmol/min per 10^8 cells. In all of these experiments the recovery of total nucleated cells and of hematopoietic progenitors (CFU-GM) was >90% after each infection cycle, indicating the low toxicity of the procedure on marrow cells.

To further increase the efficiency of gene transfer, we attempted to increase the proportion of stem cells cycling by short exposure *in vitro* to recombinant hematopoietic growth factors. As indicated by the experiment reported in Fig. 2*C*, bone marrow cells pretreated *in vitro* with optimal concentrations of rhG-CSF (10 units/ml per 1×10^6 cells) showed G418 resistance that was proportional to the number of infection cycles utilized. However, the maximum efficiency observed was markedly increased over that achieved in cultures without G-CSF. Bone marrow cells were exposed to the hematopoietic growth factor for 8 hr prior to the first infection cycle and during expression periods between infection cycles. In this experiment rhG-CSF was not added to the viral vector-containing supernatants during infection cycles.

Infection of Hematopoietic Cells in LTMC. An analysis of the capacity of these approaches to enhance the efficiency of gene transfer and long-term ADA expression in less differentiated progenitor cells was performed by long-term culture of vector-infected bone marrow cells obtained from the four different ADA-SCID patients. After each infection cycle,

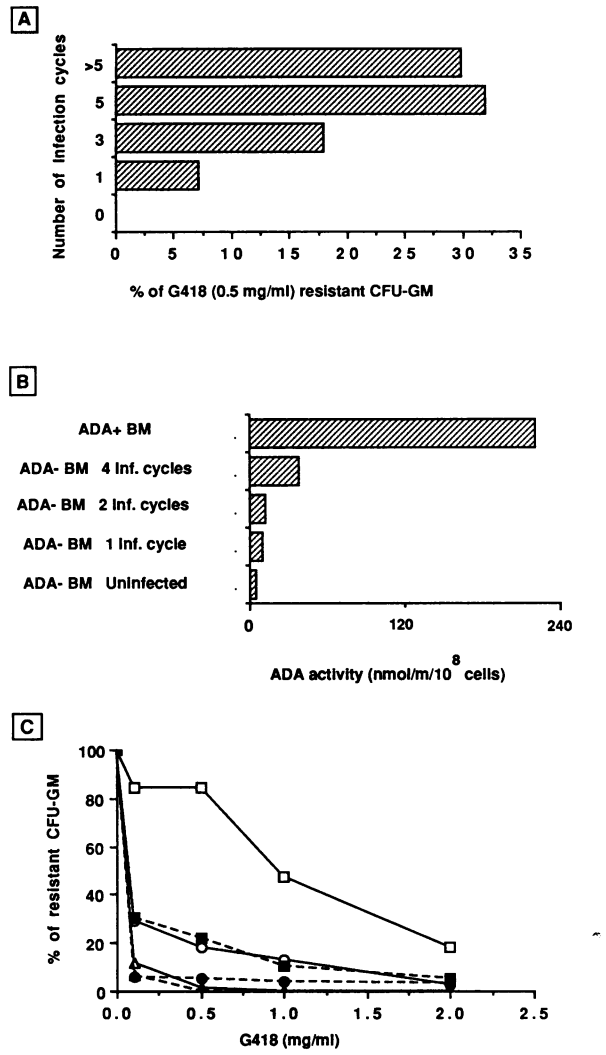

FIG. 2. (*A*) Frequency of resistant CFU-GM at a concentration of G418 of 0.5 mg/ml after multiple infection cycles with SAX vector of freshly isolated marrow mononuclear cells. (*B*) [^{14}C]adenosine to [^{14}C]inosine conversion of cell lysates obtained from ADA$^-$ bone marrow (BM) cells transduced with SAX vector multiple infection cycles. ADA activity is expressed as nmol/min per 10^8 cells. (*C*) Increased efficiency of SAX vector-mediated gene transfer in recombinant human granulocyte colony-stimulating factor (rhG-CSF)-treated bone marrow cells. Shown are the percentages of resistant CFU-GM at the different concentrations of G418 of uninfected cells (triangles) and cells that had undergone one (circles) or four (squares) cycles of vector infection. Open and closed symbols represent results with cells infected after preincubation in medium with or without rhG-CSF, respectively.

bone marrow cells were placed in the long-term culture system. This culture technique supports replication of primitive progenitor cells capable of differentiation in multiple lineages. Furthermore, in this culture system, the spontaneous production of several hematopoietic lymphokines has been reported (20), thus potentially further increasing the efficiency of gene transfer.

Presence of proviral DNA in transduced LTMC cells. High molecular weight DNA was prepared from LTMC cells transduced with SAX vector and analyzed for the presence of the vector DNA by Southern analysis (21). As shown in Fig. 3, digestion of cellular DNA with the restriction enzyme *Xba* I and utilization of a *Neo*-specific probe revealed the presence of a 4.4-kilobase DNA fragment that comigrated with a similar band generated by the digestion of purified SAX vector DNA. Moreover, the intensity of the vector-specific band and, consequently the amount of proviral DNA in the LTMC cells, is proportional to the number of infection cycles used. To rule out a prevalent infection of fibroblast contained in the adherent layers of the LTMC, DNA was prepared from nonadherent cells.

Efficiency of vector-derived ADA expression. Fig. 4 *A* and *B* show the ADA activity in cell lysates obtained after 4 wk of culture in the LTMC system. Prior to undergoing the gene transfer procedure, the marrow mononuclear cells from both patients had undetectable levels of ADA activity. As expected, after 4 wk of culture, the uninfected controls still did not show any increase in ADA activity. However, when marrow cells were infected with the SAX vector, they showed detectable levels of ADA activity. Multiple cycles of infection resulted in a proportional increase in the level of enzymatic activity, as shown by quantitative analysis of ADA activity in the cell lysates. Results presented from the analyses of the two patients (Fig. 4*D*) demonstrate that bone marrow cells exposed to three infection cycles developed levels of ADA activity that were comparable to the normal ADA$^+$ marrow cells (up to 466.5 and 483.1 nmol/min per 10^8 cells, with the normal control at 566.4 nmol/min per 10^8 cells).

Increased expression of ADA activity in marrow cells infected over four cycles was also observed in a third patient in this study, but vector-mediated ADA expression did not reach levels comparable to those of normal individuals. In two separate experiments multiple infection cycles (3 and 4) increased levels of ADA activity from 0.5% to 11% and 17% of normal controls. These levels were comparable or superior to those expressed by the mononuclear cells of the patient's heterozygous parents (data not shown).

The level of ADA activity in marrow cells infected over three cycles detected after 1 and 3 wk in culture is presented in Table 1. As can be seen, continuous expansion of the progenitor population was associated with a significant increment in ADA activity of vector-infected ADA-negative populations. Enzyme levels in normal control and noninfected ADA-deficient marrow cells were not substantially modified over this time course. This increase in ADA activity with time cannot be explained by the presence of replication-competent virus in the LTMC. ADA vector-infected cultures showed no evidence of helper virus using a sensitive reverse transcriptase method that would detect one infectious viral particle in 1 ml of supernatant.

Finally, the electrophoretic properties of the enzyme produced by the transduced cells confirmed the vector origin of the product as demonstrated by Cellogel analysis (22) (data not shown).

ADA Expression in Terminally Differentiated Cells. Analysis of lineage-specific ADA expression was performed by selective stimulation and expansion of lymphoid and myeloid cells in subcultures derived from progenitor populations grown for 1 wk in long-term culture. T lymphocytes were

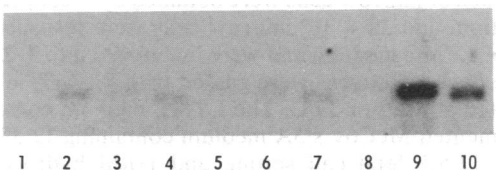

FIG. 3. Southern blot analysis of high molecular weight DNA from cells derived from transduced progenitors maintained in long-term culture, hybridized to a *Neo*-specific probe. Lanes 2 and 4, samples from patient JC LTMCs subjected to three infection cycles of SAX vector; lane 3, two infection cycles; lanes 5, 6, and 7, one, two, and three infection cycles of patient PAC; lanes 9 and 10, positive controls (the producer cell line) at one copy per cell and a 1:4 dilution, respectively; lanes 1 and 8, uninfected samples from patient JC as negative control.

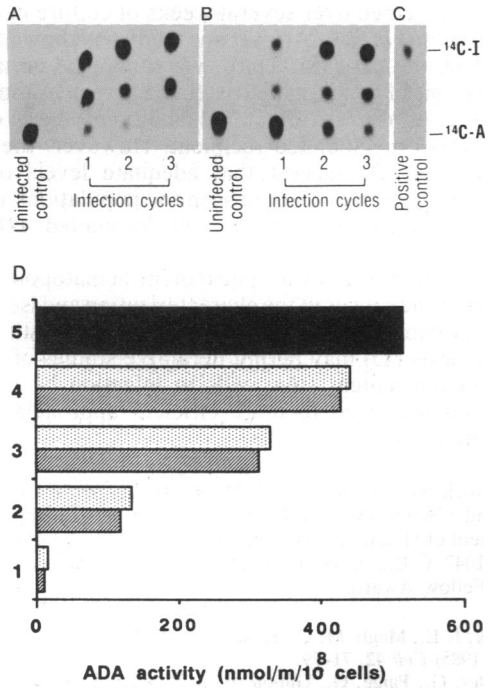

FIG. 4. (*A* and *B*) TLC analysis of [^{14}C]adenosine (^{14}C-A) to [^{14}C]inosine (^{14}C-I) conversion in cell lysates obtained after 4 wk of culture in the long-term system of bone marrow cells from two ADA-deficient patients. LTMCs were established with marrow cells uninfected or infected with one, two, and three cycles of exposure to SAX vector. (*C*) Normal control. (*D*) Quantitative results of [^{14}C]adenosine to [^{14}C]inosine conversion obtained from cell lysates of the two patients reported in *A* and *B* as compared to a normal control marrow cells (column 5). Columns labeled 1 represent uninfected controls; columns 2, 3, and 4 represent one, two, and three infection cycles, respectively. ADA activity is expressed as nmol/min per 10^8 cells.

obtained by erythrocyte (E) rosetting and 3 days of stimulation with optimal concentrations of rIL-2 and PHA. Myeloid cells of the granulocyte/macrophage lineage were obtained by 24 hr of stimulation of T-depleted cells from the long-term cultures with rhG-CSF. The resulting cells were >99% in the granulocyte lineage (metamyelocytes, bands, and mature neutrophils). As shown in Fig. 5, TLC analysis of the two populations revealed that ADA expression was comparable in both lineages in the progeny of cells transduced with the AAX vector that utilizes the human ADA promoter. In the progeny of cells transduced with SAX (SV40 early promoter) the level of ADA activity in lymphoid cells was <50% of that expressed by myeloid cells. Infection with SAX increased the levels of ADA expression from 1.7% (14.1 nmol/min per 10^8 cells) of uninfected cells to 11.1% (92.4 nmol/min per 10^8 cells) in the lymphoid lineage and to 26.3% (219.1 nmol/min per 10^8 cells) in the myeloid cells, whereas infection with AAX increased ADA activity to 23.3% (194.1 nmol/min per 10^8 cells) in the lymphoid lineage and 28.2% (234.9 nmol/min per 10^8 cells) in myeloid cells. Normal controls in this experiment were 81.0% (674.7 nmol/min per 10^8 cells) in lymphoid cells and 68.9% (573.9 nmol/min per 10^8 cells) in myeloid cells.

Table 1. Increased ADA activity in SAX-infected marrow cells in culture

BM cells	Time in culture	
	1 wk	3 wk
AH uninfected	4.2	7.5
AH SAX infected	58.3	141.6
JC uninfected	4.2	12.5
JC SAX infected	76.6	491.5
PAC uninfected	5.0	11.7
PAC SAX infected	99.9	449.8
Normal control	633.1	566.4

At the reported culture times 1 ml of bone marrow (BM) cells suspension was collected from the marrow cultures, and cell lysates from 1 × 10^5 cells were assayed for [^{14}C]adenosine to [^{14}C]inosine conversion and reported as nmol/min per 10^8 cells.

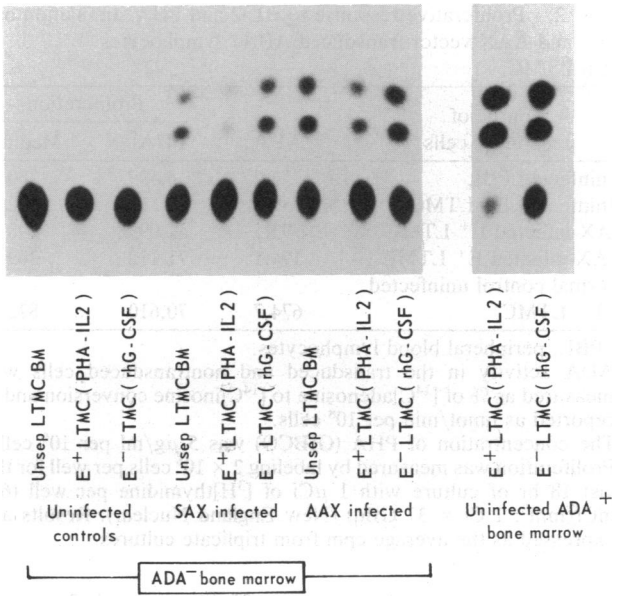

FIG. 5. Autoradiography of TLC analysis of [^{14}C]adenosine to [^{14}C]inosine conversion produced by cell lysates obtained after 1 wk of culture in the long-term system, followed by sheep E rosette separation and stimulation with PHA/IL-2 or rhG-CSF. T lymphocytes were obtained by E rosetting and 3 days of stimulation with optimal concentrations of rIL-2 (Cetus, 100 units/ml per 10^6 cells) and PHA (GIBCO, 5 μg/ml per 10^6 cells). Myeloid cells of the granulocyte/macrophage lineage were obtained by 24 hr of stimulation of T-depleted cells from the long-term cultures with rhG-CSF (Amgen Biologicals, 10 units/ml per 1 × 10^6 cells). BM, bone marrow.

The effect of vector infection on the function of T lymphocytes expanded from the long-term cultures is presented in Table 2. The responses of IL-2 and PHA-stimulated E-positive lymphocytes grown from AAX- or SAX-infected cultures were equal to, or only slightly higher than, that of normal ADA-positive lymphocytes and significantly greater than the response of either E-positive lymphocytes derived from LTMC of uninfected ADA-negative marrow cells or ADA-negative lymphocytes derived directly from the patient.

DISCUSSION

Limited efficiency of retroviral vector-mediated gene transfer and/or limited expression of the transduced gene have been cited as the major obstacles to the success of somatic cell gene therapy in all experimental model systems reported to date (23, 24). Long-term reconstitution with vector-infected hematopoietic grafts has been successful in only a very limited number of experiments in murine systems (1–3, 9). Our reported experience with SAX vector-infected autologous marrow grafts in a non-human primate model for *ADA* gene therapy confirmed and underlined some of these problems, suggesting that conventional protocols of retroviral gene transfer lead primarily to infection of committed hematopoietic progenitors but fail to affect stem cells capable of long-term hematopoietic reconstitution (12).

Table 2. Proliferative response to rIL-2 and PHA stimulation of SAX and AAX vector-transduced ADA$^-$ lymphocytes from LTMC

Source of responder cells	ADA*	Proliferation	
		PHA†	Medium
Uninfected PBL	14.2	4,599	360
Uninfected E$^+$ LTMC	39.2	17,512	602
SAX-infected E$^+$ LTMC	92.5	52,093	498
AAX-infected E$^+$ LTMC	194.1	71,112	309
Normal control uninfected E$^+$ LTMC	674.7	70,610	822

PBL, peripheral blood lymphocytes.

*ADA activity in the transduced and nontransduced cells was measured as % of [^{14}C]adenosine to [^{14}C]inosine conversion and is reported as nmol/min per 10^8 cells.

†The concentration of PHA (GIBCO) was 5 μg/ml per 10^6 cells. Proliferation was measured by labeling 2×10^5 cells per well for the last 18 hr of culture with 1 μCi of [^{3}H]thymidine per well (6.7 mCi/mm^3; 1 Ci = 37 GBq) (New England Nuclear). Results are expressed as the average cpm from triplicate cultures.

In this report we describe an efficient retroviral vector-based gene transfer system for the introduction of the human *ADA* gene into bone marrow cells derived from ADA-deficient SCID patients. By using the LTMC technique, which promotes the continuous growth of cells capable of multilineage differentiation, we have shown that the retroviral vector was introduced into a significant proportion of myeloid progenitors and that the *ADA* gene was expressed at high levels in the cultured cells, reaching levels of ADA enzyme activity comparable to those detected in normal bone marrow cells. Our study further suggests that the efficiency of gene transfer and expression of vector-derived genes can be improved by modification of the infection protocols to include multiple infection cycles and the utilization of recombinant hematopoietic growth factors.

In the present study, use of multiple infection cycles resulted in a proportional increase in the frequency of infected progenitors. Moreover, this increase in the proportion of infected cells correlated with a similar increase in the level of ADA activity in these cells. Subcultures of cells derived from LTMC in medium conditions designed to selectively promote growth of lymphoid and myeloid progenitors indicated that the *ADA* gene was efficiently transferred to and expressed in both lineages (Fig. 5). Bone marrow cells derived from ADA-deficient SCID patients are deficient in their ability to respond to proliferative stimuli such as IL-2 and PHA. The restoration of PHA and IL-2 response upon transduction with ADA vectors presents additional evidence as to the physiological relevance of this gene transfer system (Table 2).

Although these studies have demonstrated efficient transfer and expression of the *ADA* gene in ADA-deficient SCID marrow, it remains to be determined whether the results achieved with this *in vitro* system can be translated into a more effective approach for gene transfer *in vivo*. First, it is not possible to determine from this study whether the ADA vector was introduced into pluripotent stem cells capable of long-term reconstitution *in vivo*. However, the initial generation of a high-frequency G418-resistant CFU-GM and CFU-GEMM after infection and the subsequent increases in the expression of the *ADA* gene in LTMC over 4–6 wk of culture, coupled with the efficient expression of the *ADA* gene in lymphoid and myeloid cells subcultured from LTMC, provide encouraging data suggesting that, at least, very early progenitors were transduced in this system. Second, it does not necessarily follow that the efficient expression of the *ADA* gene detected over several weeks of culture in marrow cells will persist *in vivo*. At least one study has shown that this may not be the case (8). Third, we cannot be certain that manipulations of hematopoietic stem cells such as those used in our studies do not affect the ability of these cells to reconstitute a myeloblated recipient. However, the results taken together do suggest that adequate levels of ADA activity can be achieved in progenitor populations infected with vectors containing an internally promoted *ADA* gene and suggest potentially useful approaches for increasing the rate of infection of relevant pluripotent hematopoietic stem cells. Recent advances in the characterization and isolation of stem cells capable of inducing long-term reconstitution of hematopoiesis (25) may permit definitive studies of the sensitivity of pluripotent stem cells to infection by retroviral vector and may lead to more efficient approaches in the immediate future.

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