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Retroviral Transfer and Long-Term Expression of the Adrenoleukodystrophy Gene in Human CD34$^+$ Cells

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ABSTRACT

Adrenoleukodystrophy (ALD) is a demyelinating disease of the central nervous system that results from a genetic deficiency of ALDP, an ABC protein involved in the transport of very long-chain fatty acids (VLCFAs). The cloning of the ALD gene and the positive effects of allogeneic bone marrow transplantation support the feasibility of a gene therapy approach. We report the retroviral transfer of the ALD cDNA to peripheral blood and bone marrow CD34$^+$ cells from control donors and ALD patients. Prestimulation of these cells with cytokines, followed by infection with the M48-ALD retroviral vector, resulted in 20% transduction efficiency (4–40%) and expression of the vector-encoded ALDP in 20% of CD34$^+$ cells (7.3–50%). Long-term culture (LTC) of transduced CD34$^+$ cells from two ALD patients showed efficient transduction (24–28%) and stable expression (25–32%) of ALDP in derived clonogenic progenitors at 3 weeks of culture. The expression of ALDP in CFU cells derived from 5 and 6 weeks of LTC confirmed the effective transduction of LTC-initiating cells. Expression of ALDP was observed in CD68$^+$ CFU-derived cells, suggesting that monocyte-macrophages, the target bone marrow cells in ALD, were produced from transduced progenitor cells. VLCFA content was corrected in LTC and CFU-derived cells in proportion to the percentage of transduced cells, indicating that the vector-encoded ALDP was functional. Although not efficient yet to allow a clinical perspective, these results demonstrate the feasibility of ALD gene transfer into CD34$^+$ cells of ALD patients.

OVERVIEW SUMMARY

Adrenoleukodystrophy (ALD), a severe demyelinating disease of the central nervous system, can be corrected by allogeneic bone marrow transplantation. Because of the limitations of this approach, ALD gene transfer into patient hematopoietic stem cells followed by autologous transplantation could be a valuable alternative. We report the retroviral transfer of the human ALD cDNA into CD34$^+$ cells from peripheral blood or bone marrow from control donors and ALD patients. Expression of vector-encoded ALDP and correction of the biochemical defect of ALD is demonstrated in long-term bone marrow culture (LTC) and monocyte-macrophages derived from progenitor cells of two ALD patients.

INTRODUCTION

X-LINKED ADRENOLEUKODYSTROPHY (ALD), a common neurodegenerative disorder affecting 1 in 20,000 males, is characterized by a progressive demyelination of the central nervous system (CNS) and by adrenal insufficiency (Moser *et al.*, 1995; Aubourg, 1996). Phenotypic variation is frequent within the same kindred. Patients with the cerebral form of ALD remain apparently normal until 5–12 years of age. Thereafter, progressive cerebral demyelination leads to death within 3–5 years. The adult form (adrenomyeloneuropathy, AMN) affects the spinal cord of young adults and leads to severe paraplegia within 5–15 years. Addison's disease may be the only clinical manifestation of ALD. The mechanisms of such phenotypic variation are not known.

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All forms of ALD accumulate excessive amounts of very long-chain fatty acids (VLCFAs) in cerebral white matter, adrenal glands, fibroblasts, and plasma owing to an impairment of the β-oxidation of these fatty acids in peroxysomes (Moser *et al.*, 1995; Aubourg, 1996). The ALD gene encodes a 75-kDa protein, named ALDP, which is a new member of the ATP-binding cassette (ABC) transporter family (Higgins, 1992; Mosser *et al.*, 1993). ALDP is located in the peroxisomal membrane (Contreras *et al.*, 1994; Mosser *et al.*, 1994; Watkins *et al.*, 1995) and may transport the activated VLCFA-CoA esters into peroxisomes (Hettema *et al.*, 1996; Shani and Valle, 1996).

A lipid diet (Lorenzo's oil) that normalizes plasma VLCFA within 2 months (Rizzo *et al.*, 1989; Aubourg *et al.*, 1993; Kaplan *et al.*, 1993) failed to modify neurological deterioration in cerebral ALD or AMN. Attempts to use bone marrow transplantation (BMT) in the advanced stage of the disease were unsuccessful (Moser *et al.*, 1984; Yeager *et al.*, 1986). Bone marrow transplantation was reevaluated in an 8-year-old boy who had early neurologic involvement. Two years after transplantation, the neuropsychological and neurologic deficits disappeared and the magnetic resonance imaging (MRI) scan normalized (Aubourg *et al.*, 1990). Complete reversal of neurologic manifestations is still maintained 9 years later in this patient. Similar positive results were obtained in 28 other ALD patients provided that BMT is performed at an early stage of the disease (Shapiro *et al.*, 1997).

The lack of histocompatible donors, however, limits the efficacy of allogeneic BMT, and the procedure remains associated with significant morbidity and mortality risks. We considered therefore that ALD gene transfer into autologous hematopoietic stem cells followed by transplantation would be a valuable therapeutic alternative.

We previously demonstrated that retroviral-mediated transfer of ALD cDNA is followed by appropriate targeting of the vector-encoded ALDP in peroxisome and restores VLCFA oxidation in ALD fibroblasts *in vitro* (Cartier *et al.*, 1995). The present study reports the transfer of the human ALD cDNA into peripheral blood or bone marrow $CD34^+$ cells from control donors and ALD patients and expression of the vector-encoded ALDP in long-term bone marrow culture.

MATERIALS AND METHODS

Virus production

The vector M48-ALD, containing the full-length ALD cDNA under the control of the phosphoglycerate kinase (PGK) promoter, was constructed and packaged as described (Cartier *et al.*, 1995). The M48-ALD-producing cell line was grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Grand Island, NY) with 10% decomplemented newborn calf serum (GIBCO-BRL). Supernatant from a high-titer Ψ-CRIP clone (determined by ALDP immunostaining of NIH 3T3 murine fibroblasts in a limiting dilution infection assay) was harvested 24 hr after a medium change on confluent monolayers and directly used for infection. This producer was shown to be free of helper virus by a virus mobilization assay (Danos, 1991).

Hematopoietic cells

Peripheral blood (PB) progenitor cells mobilized by chemotherapy and granulocyte colony-stimulating factor (G-CSF) and bone marrow (BM) cells from four control patients without ALD were obtained after informed consent. Bone marrow cells were collected from an autologous rescue sample from one ALD patient who underwent allogeneic bone marrow transplantation and from two ALD patients who provided bone marrow aspirates for diagnostic procedures (investigation of thrombocytopenia due to Lorenzo's oil) (Zinkham *et al.*, 1993). Immunocytochemistry and Western blotting demonstrated a complete absence of ALDP in fibroblasts and/or mononuclear cells from these ALD patients (Feigenbaum *et al.*, 1996).

$CD34^+$ cell enrichment

Light-density mononuclear cells (MNCs) were obtained by centrifugation on a Diatrizoate-Ficoll gradient (Feigenbaum *et al.*, 1996). A total of $0.6–1 \times 10^9$ nucleated BM or PB cells from the sources described above were incubated at room temperature with the anti-CD34 monoclonal antibody 12-8 IgM (CellPro, Bothell, WA) and passed through an avidin–biotin immunoadsorption column (CEPRATE; CellPro) according to the manufacturer recommendations. Ninety-one to 94% of cells purified from PB and 71–85% of cells purified from BM were shown to be $CD34^+$ by flow cytometric analysis. Cells were divided into aliquots of $4–6 \times 10^6$ cells that were either cryopreserved using standard procedures in 10% dimethyl sulfoxide (DMSO) until subsequent transduction experiments or placed directly into stimulation medium.

Flow cytometric analysis

Cell surface antigens were detected using mouse anti-human CD34 monoclonal antibody (HPCA-2; Becton Dickinson, San Jose, CA) and mouse anti-human CD45 monoclonal antibody (Becton Dickinson) conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Isotype controls were FITC- or PE-labeled mouse immunoglobulins (IgG_1; Becton Dickinson). Five to 10×10^4 cells were incubated at 4°C for 20 min in 0.1 ml of phosphate-buffered saline (PBS) with 1% bovine serum albumin and 2 μl of the undiluted antibodies. After washing twice in ice-cold PBS containing 0.01% sodium azide, cells were analyzed on a Coulter (Hialeah, FL) Epics Profile II flow cytometer.

Prestimulation and transduction of $CD34^+$ cells

$CD34^+$-enriched cells were placed in DMEM with 10% newborn calf serum (GIBCO-BRL), recombinant human interleukin 3 (rhIL-3; 10 ng/ml), rhIL-6 (10 ng/ml), recombinant human stem cell factor (rhSCF, 50 ng/ml) (Pepro Tech, Canton, MA) at a density of 10^5 cells/ml. The cells were placed in T-25 flasks and incubated at 37°C with 5% CO_2 for 16 hr.

Stimulated $CD34^+$-enriched cells were transduced by addition of cell-free virus-containing supernatant (5×10^4 $CD34^+$ cells/ml) supplemented with rhIL-3, rhIL-6, rhSCF in the presence of Polybrene (8 μg/ml) (Sigma, St. Louis, MO). Fresh supernatant and cytokines were replaced four to five times in 30 hr (see Results). Twelve hours after the addition of the last virus-containing supernatant, cells were removed, centrifuged, and then directly analyzed or plated for long-term culture (LTC).

Long-term culture

Infected and noninfected CD34$^+$ cells (2.5×10^4 cells/ml) were seeded in 24-well plates on irradiated (1000 rads) preestablished allogeneic stromal layers in long-term bone marrow cell (LTBMC) premixed medium (MyeloCult; Stem Cell Technologies, Vancouver, British Columbia, Canada). Cells were incubated at 33°C, in 5% CO_2. Half of the medium and nonadherent cells were removed each week and fresh medium was added (Sutherland and Eaves, 1994). Cultures were terminated at 1, 3, 5 and 6 weeks; nonadherent cells were removed, washed in PBS, and directly analyzed by immunocytochemistry or plated in a standard CFU-GM assay.

Colony-forming unit granulocyte-macrophage assay

CFU-GM assays were performed essentially as described (Sutherland and Eaves, 1994). Nonadherent cells from the LTC were plated in triplicate at 10^4 to 5×10^4 cells/ml of ready-mix methylcellulose (MethoCult; Stem Cell Technologies) in 35-mm plates and incubated at 37°C in 5% CO_2. Colonies (with more than 50 cells) were scored 15 and 21 days later according to morphology as CFU-G, -M, and -GM. Individual colonies were isolated for subsequent polymerase chain reaction (PCR) assay and pooled colonies were analyzed by immunocytofluorescence.

Polymerase chain reaction and Southern blot

DNA from uninfected and infected CD34$^+$ and LTC cells (2×10^5) was directly extracted on Qiagen columns (Qiagen, Chatsworth, CA) and resuspended in 400 μl of Tris-HCl, 10 m*M* (pH 9).

Colonies grown in methylcellulose were individually picked, washed in PBS, and incubated for cell lysis in 30 μl of Tween 20–0.45% proteinase K (6 μg/ml) at 56 and 90°C for two 10-min periods.

PCR was performed on a Perkin-Elmer (Norwalk, CT) 2400 thermocycler using a primer pair designed specifically to detect vector provirus DNA, the 5′ (sense) primer (5′-AAGTTCGA-GAAGCTGGACTCAGCT-3′) located in the 3′ end of the ALD cDNA (nucleotides [nt] 2424–2447) (Mosser *et al.*, 1993) and the 3′ (antisense) primer (5′-GACCACTGATATCCTGTCTT-TAAC-3′) in the 3′ long terminal repeat (LTR) region of the Moloney murine leukemia virus (Mo-MuLV) vector backbone. PCR reactions were performed in buffer containing 10 m*M* Tris (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$ with 10-μl DNA samples, 1.25 U of *Taq* DNA polymerase (Perkin-Elmer), 20 pmol of the two primers, 10 mmol of each dNTP (Pharmacia, Uppsala, Sweden) in a final volume of 50 μl. Amplification conditions included initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 64°C for 30 sec, and 72°C for 45 sec and a final elongation at 72°C for 10 min. To standardize DNA concentrations in the above-described PCR assay, control amplification was performed with two primers located in exon 1 (nt 466–483 and 702–721) of the ALD gene (5′-ATGGAGCC-CA-CAAAGTCT-3′ and 5′-GCCACATACACCGA-CAGGAA-3′), using the same conditions except that the reaction was supplemented with 5% DMSO and the annealing temperature was 58°C.

For semiquantitative PCR assay, a vector copy number per cell standard curve was established: different DNA dilutions of a Ψ-CRIP clone carrying one copy per genome of M48-ALD provirus in 3T3 cell DNA were amplified with specific provirus primers. To standardize the amount of DNA used in PCR assay of provirus integration, we adjusted the quantity of DNA to obtain equivalent signal intensity of ALD exon 1 amplification product in all samples and in the Ψ-CRIP reference cells.

After extraction and amplification, DNA products were separated on 1% agarose gels, blotted onto Hybond N+ (Amersham, Arlington Heights, IL) membranes, and hybridized to a [α-^{32}P]dCTP (Amersham) M48-ALD or ALD exon 1 probe (Ready Prime; Amersham). Membranes were washed with 0.1 SSC/0.1 SDS buffer (1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate; SDS, sodium dodecyl sulfate) at 42°C for 20 min, at 50°C for 20 min, and at 70°C for 10 min and exposed to Hyperfilm MP (Amersham) for 2 hr at room temperature.

Images of Southern blot filters were recorded on an Imager (Appligene-Oncor) and the intensity of each signal was scanned and quantified with the public domain NIH image program. The percentage of cells showing provirus integration was calculated on the basis that positively transduced cells contained one copy per genome of the provirus. For every assay, samples of DNA extracted from uninfected cells were tested in parallel to detect contamination of reagents with PCR product.

Immunocytochemistry

Cells were centrifuged at $100 \times g$ for 10 min as described (Feigenbaum *et al.*, 1996). The mouse anti-human ALDP monoclonal antibody (MAb 1D6) has been previously characterized (Mosser *et al.*, 1994). Labeling of ALDP was revealed with anti-mouse antibodies directly conjugated to Cy3 (Jackson ImmunoResearch, Westgrove, PA) (Cartier *et al.*, 1995). The percentage of ALDP-positive cells was calculated by counting an average of about 200 cells on each slide. Results were expressed as the mean value of two counts made blindly by two investigators.

For ALDP and catalase colocalization experiments, cells were first treated with MAb 1D6 and rabbit anti-human catalase polyclonal antibody (Chemicon, Temecula, CA). Cells were further incubated with donkey anti-mouse IgG (H + L) antibody directly conjugated to Cy3 and goat anti-rabbit IgG (H + L) antibody directly conjugated to fluorescein (Vector Laboratories, Burlingame, CA).

Expression of ALDP in monocytes derived from pooled CFU colonies was analyzed using double immunostaining with anti-ALDP and anti-CD68 antibodies (Pulford *et al.*, 1989). Cells were first treated with a rabbit anti-human ALDP polyclonal antibody (1693) and a mouse anti-human CD68 KP1 monoclonal antibody (Dako, Carpinteria, CA), then further incubated with horse anti-mouse IgG (H + L) antibody directly conjugated to fluorescein (Vector Laboratories) and anti-rabbit (H + L) IgG antibody directly conjugated to Cy3 (Chemicon). A mouse $MsIgG_1$ isotype antibody (Coulter) was used as a control.

The anti-human ALDP polyclonal antibody (1693) was obtained by immunization of two rabbits with a synthetic peptide as described (Fouquet *et al.*, 1997). This synthetic peptide comprises the last 19 carboxy-terminal amino acids of human ALDP (Mosser *et al.*, 1993). The 1693 polyclonal antibody does not cross-react with the 70-kDa peroxisomal membrane protein (PMP) (Kamijo *et al.*, 1990) and the ALD-related protein (Lombard-Platet *et al.*, 1996).

Appropriate filters for each fluorochrome, and a combined filter for fluorescein and Cy3, were used on a light microscope equipped for fluorescence (Nikon Optiphot-2).

Measurement of very long-chain fatty acids in long-term culture- and CFU-derived cells

N-tert-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) with 1% *tert*-butyldimethyl-chlorosilane (TBDMCS) was purchased from Fluka (Buchs, Switzerland). [3,3,5,5-$^2H_4$]docosanoic ($C_{22:0}$-d_4) and [3,3,5,5-$^2H_4$]hexacosanoic ($C_{26:0}$-d_4) acids were a gift from H.J. ten Brink (Amsterdam, The Netherlands). Natural docosanoic ($C_{22:0}$) and hexacosanoic ($C_{26:0}$) acids were obtained from Sigma.

Cells (5×10^4) were suspended in 200 μl of twice-distilled water, sonicated, and added to 2 ml of chloroform–methanol (1:1; v/v) containing 100 p*M* $C_{22:0}$-d_4 and 25 p*M* $C_{26:0}$-d_4 (internal standards) that had been evaporated to dryness. After evaporation to dryness, the pellet was resuspended in 2 ml of acetonitrile with 400 μl of hydrochloric acid (12 *M*), heated for 1 hr at 110°C, and extracted twice with 5 ml of hexane after cooling. Hexane phases were pooled, transferred into vials, and evaporated to dryness. The butyldimethylsilyl (TBDMS) derivatives of fatty acids were obtained by dissolving the residues in 20 μl of pyridine–(MTBSTFA/MTDMCS; 99:1) (1:1, v/v) and heating at 60°C for 10 min. Samples were stored at −20°C until analysis.

To establish a calibration curve, variable amounts of natural $C_{22:0}$ (0 to 100 pmol) and $C_{26:0}$ (0 to 7.5 pmol) were mixed and added to $C_{22:0}$-d_4 (100 pmol/liter) and $C_{26:0}$-d_4 (25 pmol/liter) as internal standards. After extraction, the fatty acid mixture was converted into TBDMS derivatives.

TBDMS derivatives of fatty acids were separated on a gas chromatograph (Varian 3400, Sunnyvale, CA) coupled with a quadrupolar mass spectrometer (Incos XL-50; Finnigan MAT, San Jose, CA). Chromatographic conditions were as follows: after an initial isotherm of 1 min at 80°C and purge split at 1 min, the temperature was programmed from 80 to 290°C (15°C/min) and from 290 to 340°C (3°C/min) on a BPX-5 capillary column (5% phenyl polysiloxane; 30 m × 0.33 mm; film thickness, 0.25 μm; Scientific Glass Engineering, Melbourne, Australia). Ions at *m/z* 397, *m/z* 401, *m/z* 453, and *m/z* 457, corresponding to the [M-57] ions of 22:0, 22:0-d_4, 26:0, and 26:0-d_4 TBDMS derivatives, were selectively monitored using electron impact. The corresponding peaks were integrated and the content of $C_{22:0}$ and $C_{26:0}$ fatty acids was calculated according to the calibration curve using C_{22}-d_4 and C_{26}-d_4 as internal standards. Results were expressed as $C_{26:0}/C_{22:0}$ ratios.

RESULTS

Retroviral transduction of ALD cDNA in control CD34$^+$ cells

We first evaluated transduction efficiency of M48-ALD retroviral vector in CD34$^+$ cells from four control individuals.

The efficiency of transduction was determined by measuring, with a semiquantitative PCR assay, the percentage of cells containing the M48-ALD provirus and by studying, with immunocytochemistry, the percentage of cells expressing recombinant ALDP. Immunocytofluorescence analysis with MAb 1D6 of control untransduced CD34$^+$ cells, stimulated or not by cytokines, revealed consistently a weak punctate fluorescence signal suggesting a low expression of ALDP (Fig. 1A and B, arrowheads). We previously demonstrated by Western blot analysis that transduction with M48-ALD vector increased the expression of ALDP in normal or ALD fibroblasts by fivefold (Cartier *et al.*, 1995). This increase was correlated with higher expression of ALDP in fibroblasts, using immunocytochemistry (see Fig. 3 in Cartier *et al.*, 1995). We observed similar overexpression of ALDP in transduced normal CD34$^+$ cells (compare the expression of ALDP in nontransduced normal CD34$^+$ cells [Fig. 1A and B, arrowheads] and in transduced CD34$^+$ cells [Fig. 1B, arrow]).

Preliminary studies using 10^5 cells/ml and one to three infection cycles led to low provirus integration (<2%) (Fig. 2, lane *) and expression of recombinant ALDP in less than 10% of transduced cells (data not shown). When cells were plated at 5×10^4/ml retroviral supernatant and submitted to four or five cycles of 6-hr infection, 25% of the PB or BM cells showed M48-ALD provirus integration (Table 1 and Fig. 2). Immunocytofluorescence showed that 22 to 25% of these cells overexpressed ALDP (Table 1 and Fig. 1B).

Retroviral transduction of ALD cDNA in BM CD34$^+$ cells of ALD patients

These optimized transduction conditions were used to infect BM CD34$^+$ cells of three ALD patients. Two CD34$^+$ aliquots from two ALD patients and three CD34$^+$ aliquots from the third patient were transduced in a total of seven experiments. Transduction efficiency was similar to that observed with PB or BM control CD34$^+$ cells (Table 1). Semiquantitative PCR assay demonstrated that 20% of transduced cells (calculated as a median of the seven experiments, range, 4–40%) integrated the M48-ALD provirus (Table 1, Fig. 2). After transduction, a marked punctate fluorescent staining was detected in CD34$^+$ cells of the three ALD patients as compared with uninfected cells (Fig. 1C and D). Colocalization studies with anti-catalase antibody confirmed that the localization of recombinant ALDP was peroxisomal (data not shown). The absence of cytosolic aggregates in the transduced ALD CD34$^+$ cells indicated that most, if not all, recombinant ALDP had been correctly incorporated into peroxisomal membrane. Expression of vector-encoded ALDP correlated with the percentage of M48-ALDP provirus integration (Table 1). While no BM CD34$^+$ cells of the three ALD patients expressed ALDP before transduction, a median 20% (range, 7.3–50%) of cells expressed ALDP after five cycles of infection at 5×10^4 cells/ml supernatant (Table 1).

Transduction of long-term culture initiating cells from BM CD34$^+$ cells of ALD patients

To determine whether CD34$^+$ cells of ALD patients could be used as potential targets for gene transfer into hematopoietic progenitors, transduced and nontransduced BM CD34$^+$ cells of two ALD patients were maintained in LTC for 5 weeks (patient 1, experiment 1) and 6 weeks (patient 2, experiment 2). At different time points, infected and uninfected cells were collected for assessment of clonogenic potential and gene transfer efficiency. The clonogenic potential of ALD BM cells (determined on CFU-G, -M, and -GM count) was identical in infected and uninfected cells (data not shown). Gene transfer

efficiency was evaluated by the presence of M48-ALD provirus on cells derived from CFU-GM assays and by the expression of ALDP on cells directly collected from the LTC.

The analysis of clonogenic progenitors (CFU-M and -GM) obtained immediately after infection showed M48-ALD proviral integration in 32% (experiment 1) and 18% (experiment 2) of ALD cells (Table 2). These percentages remained stable at 3 weeks of culture (28 and 24%) (Table 2 and Fig. 3). Expression of the transduced ALD cDNA was analyzed by immunocytofluorescence on cells directly harvested from the LTC. Less than 5% of ALDP-positive cells was found in the control uninfected culture (Table 2). These cells were likely contaminating cells originating from the allogeneic irradiated stroma used to support the LTC. Whereas 15.5% (experiment 1) and 20%

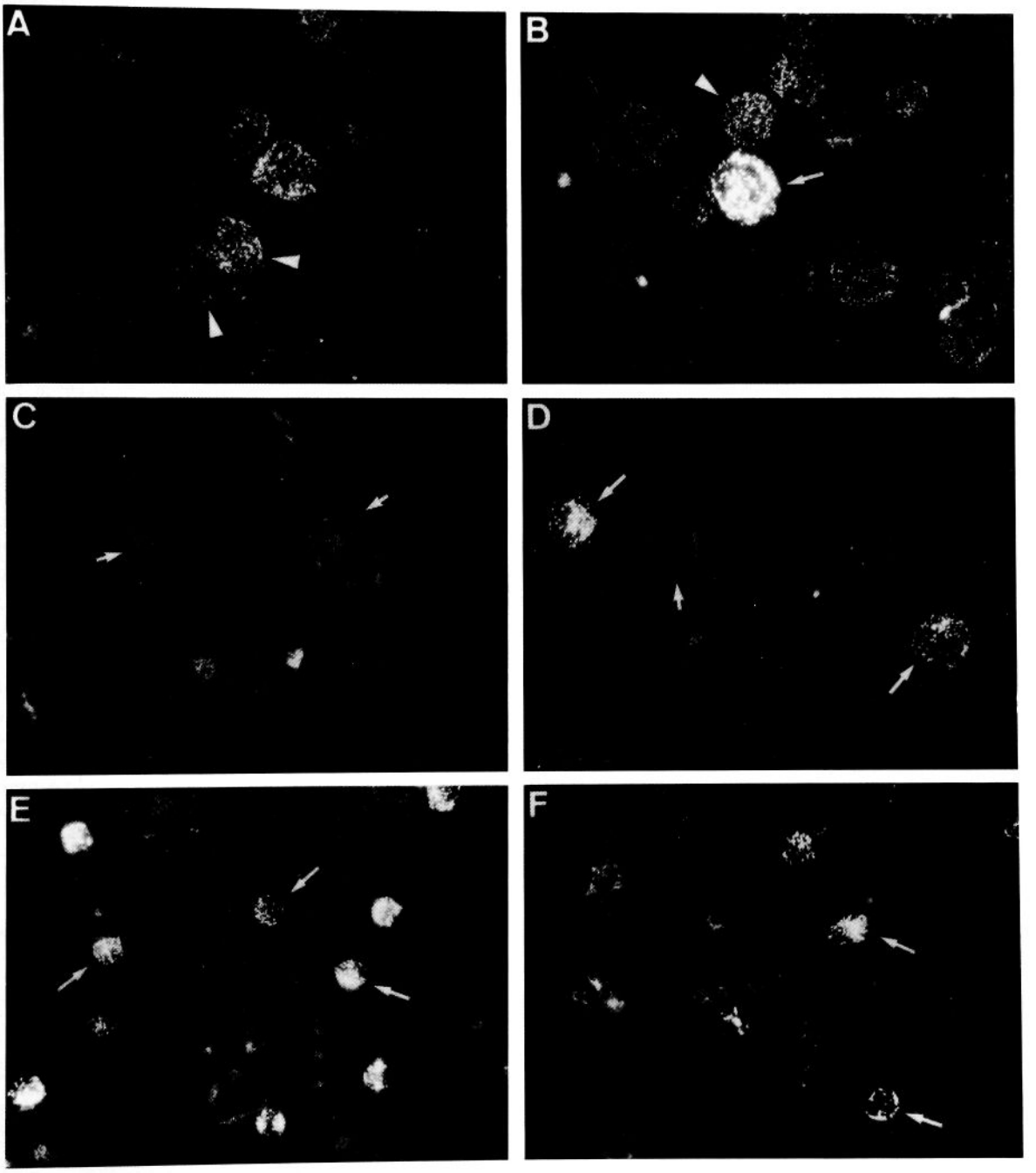

FIG. 1. Expression of ALD protein in hematopoietic cells after transduction with the M48-ALD vector. Nontransduced and transduced CD34+ cells from control (A and B) and ALD patients (C and D) were analyzed at the end of the infection cycle. Arrowheads in (A) and (B) show the weak endogenous expression of ALDP in nontransduced control cells, contrasting with stronger expression of ALDP in transduced control cells (B, arrow). The absence of ALDP expression in nontransduced ALD cell is shown in (C) and in (D) (short arrows). Note in comparison the expression of ALDP in transduced ALD cells (D, arrows). Expression of vector-encoded ALDP in LTC cells of two ALD patients: (E) nonadherent cells at 23 days of LTC (experiment 1); (F) nonadherent cells at 42 days of LTC (experiment 2).

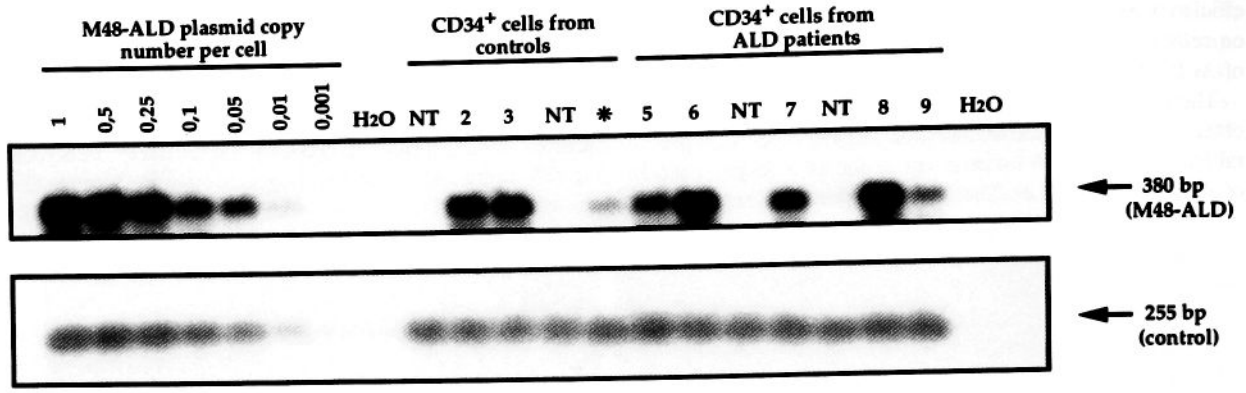

FIG. 2. M48-ALD proviral integration in control and ALD CD34$^+$ cells. DNA extracted from 2×10^5 cells was used to amplify a specific provirus DNA fragment (380 bp) and a control fragment (255 bp) from exon 1 of the ALD gene (see Materials and Methods). Signal intensities of the provirus and control DNA fragments were scanned simultaneously and compared with the intensity of the M48-ALD copy number standard curve established by serial dilutions of a Ψ-CRIP clone containing one copy per genome of the M48-ALD provirus in 3T3 murine fibroblasts. Density of plated cells per milliliter of retroviral supernatant was 5×10^4; asterisk corresponds to a preliminary experiment with control CD34$^+$ cells, in which cell density per milliliter of supernatant was 10^5. NT, Nontransduced cells.

(experiment 2) of transduced ALD cells expressed ALDP on day 0 of the LTC, 25% (experiment 1) and 32% (experiment 2) still expressed ALDP on day 23 (Fig. 1E). This result was correlated with the percentage of proviral integration in the CFU-M and -GM colonies (Table 2).

A decrease in ALDP expression to 3% was observed in cells directly collected from the LTC in experiment 1 at 5 weeks; this was correlated with the decrease in the percentage of M48-ALD provirus integration in the CFU assay (6%) (Table 2). This result contrasted with the stability of ALDP expression observed in ALD cells derived from the LTC in the second experiment. Twenty percent of these cells expressed ALDP on day 0, whereas 22.5 and 17.6% expressed ALDP at 5 and 6 weeks of culture (Table 2 and Fig. 1F).

ALDP expression was also analyzed at different time points in CFU cells. Preliminary experiments revealed that immunocytochemistry analysis of normal (i.e., 100% ALDP positive) individual CFU-M and -GM colonies was not reliable because of the very small number of cells obtained from each colony; in order to characterize ALDP expression in CFU assay of infected cells, we thus decided to perform this analysis on CFU cells pooled from methylcellulose plates. After 5 weeks (ex-

TABLE 1. TRANSDUCTON EFFICIENCY OF PB AND BM CD34+ CELLS FROM CONTROLS AND ALD PATIENTS[a]

	CD34+ cell density per milliliter	*Number of viral infections*	*Percentage of cells showing provirus integration*	*Percentage of cells expressing vector-encoded ALDP*
PB (control)				
1	5×10^4	4	ND	22
2	5×10^4	4	25	25
3	5×10^4	4	25	25
BM (control)				
4	5×10^4	5	25	25
BM (ALD)				
5	5×10^4	5	15	20
6	5×10^4	5	25–30	30
7	5×10^4	5	20	15.5
8	5×10^4	5	30–40	30
9	5×10^4	5	4	7.3
10	5×10^4	5	ND	50
11	5×10^4	5	ND	10

[a]Four control CD34$^+$-enriched cell samples (three PB and one BM sample, 1 to 4) and seven BM CD34$^+$-enriched cell samples (5 to 11) of ALD patients were infected with retroviral supernatant. Transduction efficiency was assessed by measuring, with a semiquantitative PCR assay (see Materials and Methods), the percentage of CD34$^+$ cells showing M48-ALD provirus integration and by studying, after immunostaining with anti-ALDP antibody, the percentage of cells expressing (ALD CD34$^+$ cells) or overexpressing (control CD34$^+$ cells) vector-encoded ALDP.

Abbreviation: ND, Not determined.

TABLE 2. TRANSDUCTION EFFICIENCY AND ALDP EXPRESSION IN LTC ESTABLISHED FROM BONE MARROW OF TWO ALD PATIENTS[a]

	Day 0		Day 10		Day 23		Day 35		Day 42	
	PCR^+ CFU (%)	IF^+ cells (%)	PCR^+ CFU (%)	IF^+ cells (%)	PCR^+ CFU (%)	IF^+ cells (%)	PCR^+ CFU (%)	IF^+ cells (%)	PCR^+ CFU (%)	IF^+ cells (%)
Experiment 1										
Transduced	32 (16/50)	15.5	40 (20/50)	25	28 (14/50)	25	6 (3/50)	3	ND	ND
Untransduced	0 (0/15)	0	0 (0/15)	0.5	0 (0/15)	0	0 (0/15)	0	ND	ND
Experiment 2										
Transduced	18 (9/50)	20	ND	ND	24 (12/50)	32	ND	22.5	ND	17.6
Untransduced	0 (0/15)	0	ND	ND	0 (0/15)	5	ND	4	ND	2

[a]Long-term cultures were initiated from transduced and nontransduced $CD34^+$ BM cells of two ALD patients. Cells were harvested on day 0 (experiments 1 and 2), day 10 (experiment 1), days 23 and 35 (experiments 1 and 2), and day 42 (experiment 2) after infection. Nonadherent cells were assayed in a standard CFU-GM assay. Individual CFU-GM colonies were picked and tested for M48-ALD provirus integration. *PCR^+ CFU* columns indicate the percentage of PCR-positive colonies showing the presence of M48-ALD provirus (the number of colonies analyzed at each point is also shown). Cells were also directly harvested from the LTC and analyzed for ALDP expression by immunocytochemistry. *IF^+ cells* columns indicate the percentage of cells, harvested from the LTC at each time point, expressing the vector-encoded ALDP.

Abbreviation: ND, Not determined.

periment 1) and 6 weeks (experiment 2) of LTC, respectively, 2 and 6.1% of the pooled CFU-GM cells from the clonogenic assay still expressed vector-encoded ALDP (Table 3 and Fig. 4). Together with the demonstration of M48-ALD proviral integration in 5-week LTC-derived colonies, these results demonstrate that LTC-initiating cells were transduced.

Expression of recombinant ALDP was analyzed in $CD68^+$ cells derived from pooled CFU colonies. Anti-CD68 KP1 antibody strongly stains monocytes and macrophages, while granulocytes are weakly or negatively labeled (Pulford *et al.*, 1989). Most CFU-derived cells pooled from methylcellulose at 42 days of LTC were $CD68^+$, whereas few cells were negative or weakly marked (Fig. 4B). Expression of recombinant ALDP in $CD68^+$ ALD cells derived from the CFU assay at 42 days of the LTC demonstrates that progenitor-derived monocyte-macrophages were transduced by the M48-ALD vector (Fig. 4).

Correction of VLCFA metabolism in vector-treated ALD LTC- and CFU-derived cells

Accumulation of saturated VLCFAs ($>C_{22:0}$) is the principal biochemical abnormality in the tissues, cells, and body flu-

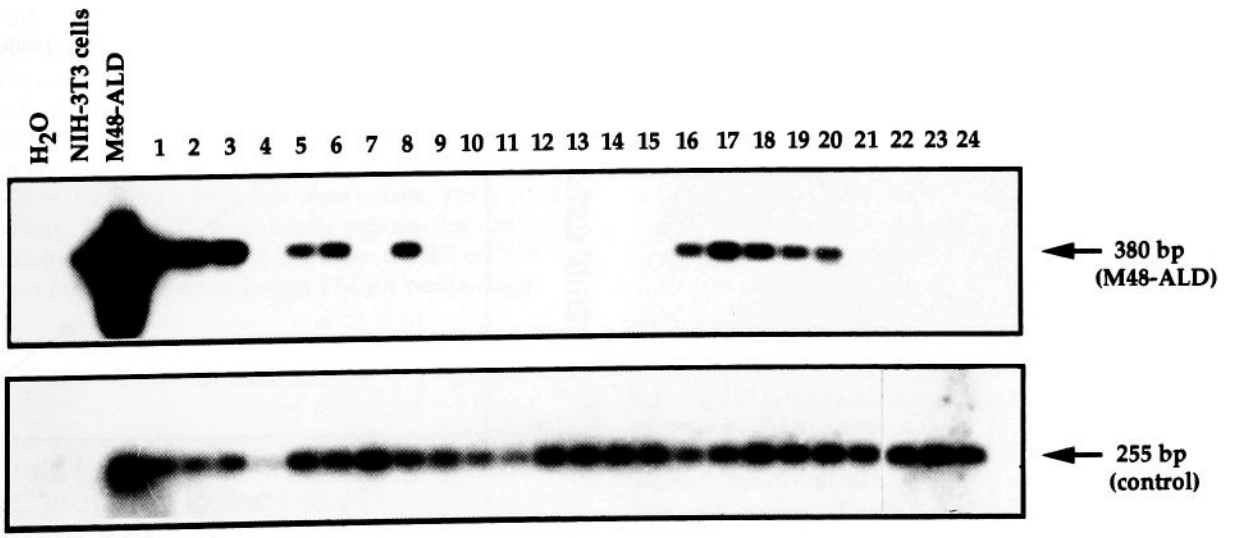

FIG. 3. M48-ALD proviral integration in CFU-GM assay derived from the LTC of ALD $CD34^+$ cells. Colonies from the CFU assay performed at various times during the LTC were individually picked. Shown here is the analysis by CFU assay of cells taken on day 23 of the LTC (experiment 1). PCR amplification of proviral (380 bp) and control (255 bp) DNA fragments is shown for 20 colonies derived from transduced LTC cells (lanes 1–20) and 4 colonies derived from untransduced LTC cells (lanes 21–24).

TABLE 3. PERCENTAGE OF CELLS EXPRESSING ALDP IN POOLED CFU-DERIVED CELLS FROM THE CLONOGENIC ASSAY AT DIFFERENT TIMES OF THE LTC[a]

	Day 0 of LTC (% IF$^+$ cells)	*Day 10 of LTC (% IF$^+$ cells)*	*Day 23 of LTC (% IF$^+$ cells)*	*Day 35 of LTC (% IF$^+$ cells)*	*Day 42 of LTC (% IF$^+$ cells)*
Experiment 1					
Transduced	2	5.8	5.2	2	ND
Untransduced	0	0	0	0	ND
Experiment 2					
Transduced	7	ND	6.1	ND	6
Untransduced	0	ND	0	ND	0

[a]CFU-GM cells were pooled from methylcellulose plates on days 0, 10, 23, 35, and 42 of LTC and the percentage of cells expressing ALDP was studied by immunocytofluorescence (IF) with MAb 1D6.

Abbreviation: ND, Not determined.

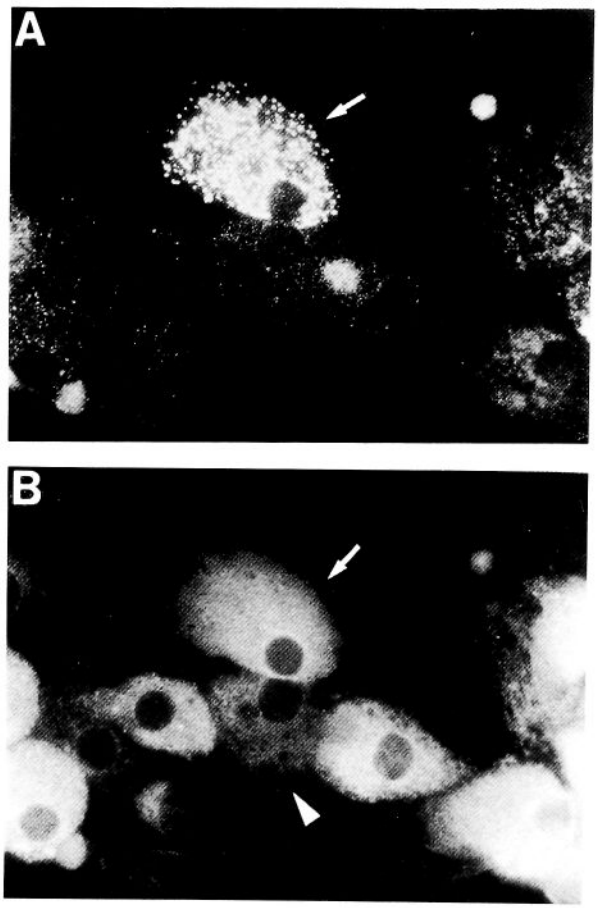

FIG. 4. Expression of vector-encoded ALDP in CD68$^+$ ALD cells derived from the CFU assay at 42 days of LTC. Cells from the CFU assay performed at 42 days of LTC (experiment 2) were harvested from methylcellulose on day 25 and analyzed by immunocytochemistry, using double staining with anti-human ALDP polyclonal antibody (A) and anti-CD68 monoclonal antibody (B). Most cells were CD68$^+$ (B, arrow), whereas few cells were shown to be CD68$^-$ (B, arrowhead). One CD68$^+$ cell (B, arrow) shows marked expression of ALDP (A, arrow).

ids of patients with ALD. The accumulation of VLCFAs involves mainly hexacosanoic ($C_{26:0}$) acid whereas the concentration of docosanoic ($C_{22:0}$) acid remains normal. Therefore, the $C_{26:0}/C_{22:0}$ ratio reflects the ability of cells to metabolize VLCFA (Moser *et al.*, 1995) and is dependent on the presence of functional ALDP involved in VLCFA transport.

Because of the limited number of LTC- or CFU-derived cells and the large fraction of noncorrected ALD cells in our experiments, we first designed a mixing experiment to check that a modification of the $C_{26:0}/C_{22:0}$ ratio can be detected in a small population of cells expressing ALDP. The $C_{26:0}/C_{22:0}$ ratio was determined in a pool of ALD monocytes/lymphocytes (5×10^4 cells) supplemented with 5, 10, 20, 30, 40, 50, and 70% of control cells. The $C_{26:0}$ concentration and $C_{26:0}/C_{22:0}$ ratio of normal or ALD monocytes/lymphocytes are within the range observed in control or ALD LTC cells. As the proportion of control cells increased, the $C_{26:0}/C_{22:0}$ ratio decreased proportionally ($r^2 = 0.998$) (Fig. 5). Thus a large number of noncorrected cells did not hinder our ability to detect a change in the $C_{26:0}/C_{22:0}$ ratio in the transduced ALD LTC or CFU cells. The linearity of the

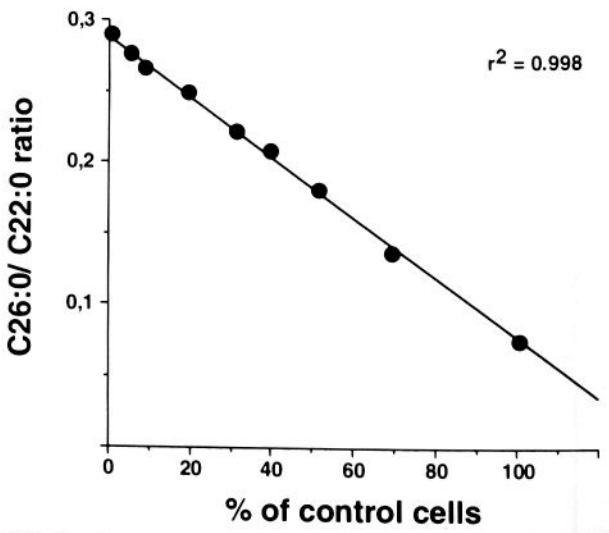

FIG. 5. $C_{26:0}/C_{22:0}$ ratio in mixed cell population. Control and ALD lymphocytes/monocytes were isolated by Ficoll gradient, counted, and then mixed in the ratios indicated. The $C_{26:0}/C_{22:0}$ ratio per 5×10^4 cells is plotted versus the percentage of control cells in the mixture.

TABLE 4. CORRECTION OF VLCFA METABOLISM IN LTC AND CFU-DERIVED CELLS FROM ALD PATIENTS

	N	*Observed C26:/C22:0*[a]	*Expected C26:0/C22:0 for the number of expressing cells (%)*[b]
Control LTC cells (day 12)	3	0.120 ± 0.018	0.120
ALD LTC cells (experiment 1)			
Untransduced (day 0)	2	0.383 ± 0.015	
Transduced (day 0)	2	0.317 ± 0.010	0.349 (15.5%)
Untransduced (day 10)	2	0.391 ± 0.0021	
Transduced (day 10)	2	0.273 ± 0.012	0.323 (25%)
Untransduced (day 23)	2	0.375 ± 0.018	
Transduced (day 23)	2	0.268 ± 0.023	0.311 (25%)
Control CFU-derived cells (day 0)	2	0.304 ± 0.021	0.304
ALD CFU-derived cells (day 0)			
Untransduced (experiment 1)	2	0.825 ± 0.019	
Transduced (experiment 1)	2	0.805 ± 0.011	0.815 (2%)
ALD CFU-derived cells (day 23)			
Untransduced (experiment 2)	1	0.511 ± 0.016	
Transduced (experiment 2)	1	0.486 ± 0.012	0.499 (6.1%)

[a]The $C_{26:0}/C_{22:0}$ ratio is given as the mean ± SD of three determinations of the number (N) of studied samples.

[b]The expected $C_{26:0}/C_{22:0}$ ratio is calculated by linear regression for the fraction (given in percentage in parentheses) of LTC- or CFU-derived cells expressing vector-encoded ALDP, as detected by immunocytofluorescence.

assay allowed us to calculate the expected $C_{26:0}/C_{22:0}$ values of transduced ALD cells by simple linear regression (see Table 4).

To evaluate the correction of VLCFA metabolism after M48-ALD infection, the $C_{26:0}/C_{22:0}$ ratio was measured in ALD LTC- and CFU-derived cells. Table 4 shows that the $C_{26:0}/C_{22:0}$ ratio of untransduced LTC cells from ALD patient (experiment 1) is threefold that of normal LTC cells. Following infection with M48-ALD vector, we observed a small but consistent decrease in the $C_{26:0}/C_{22:0}$ ratio in the transduced ALD cells at 0, 10, and 23 days of long-term culture. The correction of the $C_{26:0}/C_{22:0}$ ratio was slightly greater than expected after normalization of the $C_{26:0}/C_{22:0}$ ratio with respect to the number of cells expressing vector-encoded ALDP, as determined by immunofluorescence (Table 4). Overexpression of ALDP most likely increases the import of VLCFAs in peroxisomes of transduced cells and leads to an increased degradation of these fatty acids in LTC- or CFU-derived cells. A similar observation has been made in transfected (K. Smith, personal communication, 1997) or transduced (our own observations, 1997) ALD fibroblasts. Altogether, these results indicate that the vector-encoded ALDP localized in peroxisomes of LTC or CFU ALD cells is functional and can restore VLCFA metabolism.

DISCUSSION

The characteristic lesions of ALD consist of diffuse demyelination and marked loss of oligodendrocytes (Schaumburg *et al.*, 1975). There is, however, indirect evidence that dysfunction of microglia, which comprise 10–20% of all glial cells and resemble macrophages (Perry and Gordon, 1991; Altman, 1994), may affect oligodendrocytes of ALD patients. Within the central nervous system, microglia are the predominant cell type expressing the ALD protein (Fouquet *et al.*, 1997). In addition, predominant microglial activation is constantly observed in the cerebral demyelinating lesions (Powers *et al.*, 1992). The importance of microglial dysfunction in ALD is also supported by the observation that bone marrow transplantation can reverse demyelination in ALD patients (Aubourg *et al.*, 1990; Krivit *et al.*, 1995a,b; Shapiro *et al.*, 1995, 1997). Although the origin of microglia is still debated, it is generally considered that microglial cells derive from hematopoietic precursors (Hickey *et al.*, 1992; Lassman and Hickey 1993; Lassman *et al.*, 1993; Ling and Wong, 1993; Perry *et al.*, 1993). The improvement of demyelinating lesions after heterologous BMT is possibly due to the replacement of abnormal microglia by normal hematopoietic donor-derived cells (Krivit *et al.*, 1995b). In normal mice, up to 20% of microglia can be replaced by transplanted syngeneic bone marrow cells expressing the human glucocerebrosidase gene from a retroviral vector (Krall *et al.*, 1994). These findings support a gene therapy approach based on the *ex vivo* transfer of the ALD gene into hematopoietic stem cells followed by autologous transplantation.

A recombinant retroviral vector carrying the human ALD cDNA was used to transduce $CD34^+$ cells purified from bone marrow of ALD patients in the presence of IL-3, IL-6, and SCF (Dick *et al.*, 1991; Crooks and Kohn, 1993; Nolta *et al.*, 1995; Xu *et al.*, 1995). Our results with the M48-ALD retroviral vector are comparable to those reported using cell-free virus in the absence of stromal support (Moore *et al.*, 1992; Nimgaonkar *et al.*, 1994; Ward *et al.*, 1994; Nolta *et al.*, 1995; Xu *et al.*, 1995; Fairbairn *et al.*, 1996). Proviral integration in CFU at 5 weeks of LTC and expression of vector-encoded ALDP in CFU-derived cells at 6 weeks of LTC suggest that LTC-initiating cells were transduced (Sutherland *et al.*, 1990; Sutherland and Eaves, 1994). The expression of vector-encoded ALDP in $CD68^+$ ALD cells derived from the CFU assay demonstrated that cells from the monocytic lineage, the target BM cell pop-

ulation in ALD, were transduced. Finally, VLCFA metabolism was corrected in LTC- and CFU-derived cells, indicating that the vector-encoded ALDP was functional in these cells.

These preliminary results are encouraging but still far from allowing a phase I trial in ALD patients. Our results indicate that approximately 50 to 60% of ALD CD34$^+$ cells would need to be corrected to normalize the VLCFA concentration. As highlightened by clinical trials, the success of retroviral gene therapy using hematopoietic precursors remains strongly limited by the efficiency of *ex vivo* transduction of hematopoietic stem cells (Bordignon *et al.*, 1995; Dunbar *et al.*, 1995; Kohn *et al.*, 1995). Two conditions may improve the efficiency of the procedure: (1) bone marrow ablation followed by reconstitution with transduced cells, and/or (2) a selective *in vivo* growth advantage conferred to transduced cells by the therapeutic gene, as in the case of adenosine deaminase deficiency (Bordignon *et al.*, 1995; Kohn *et al.*, 1995). In our study, we did not observe that normal or ALD-corrected cells had a survival advantage relative to ALD-deficient cells *in vivo* and *in vitro*. Accordingly, the study of ALDP expression in lymphocytes or cultured fibroblasts of heterozygous ALD women does not show skewed X inactivation favoring the normal allele (Watkiss *et al.*, 1993; Feigenbaum *et al.*, 1996). We are now attempting to select ALD-transduced cells by using a bicistronic construct containing the multidrug resistance (MDR) gene (Medin *et al.*, 1996; Sokolic *et al.*, 1996; Ward *et al.*, 1994). Preselection of MDR-expressing cells prior to marrow reconstitution by fluorescence-activated cell sorting using an antibody to external epitope of MDR could increase the number of ALD-expressing microglial cells in the brain. Alternatively, optimization of the viral titer through the use of the vesicular stomatitis virus G envelope protein might allow better transduction rates of hematopoietic stem cells (Agrawal *et al.*, 1996; Ory *et al.*, 1996). In fact, the need to target nondividing cells remains today the main limitation of retroviral gene transfer into hematopoietic stem cells. Studies have shown that both adeno-associated virus (Zhou *et al.*, 1994) and retroviral vectors modified with lentivirus components (Naldini *et al.*, 1996) are able to infect nondividing cells. These vectors might be useful for human use, once large-scale production and safety problems are solved.

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