ABSTRACT

Background. Retroviral-mediated gene transfer stably introduces exogenous genes into normal and neoplastic cells of the hematopoietic system.

Methods. We used two retroviral vectors [the first, FLac, expresses a chimeric protein (Sh-ble::LacZ) between the product of the phleomycin resistance gene (Sh-ble) and the bacterial β-galactosidase encoded by the LacZ gene; the second, NuNL vector, contains a fusion sequence (LacZ::Neo) that expresses the LacZ and the neomycin resistance genes] to transduce T lymphocytes derived from the peripheral blood of healthy human donors. Two lymphocyte activation procedures were employed: a) phytohemagglutinin/interleukin-2 (PHA/IL-2) polyclonal stimulation; b) allogeneic stimulation in a mixed irradiated or non irradiated lymphocyte reaction, both supplemented with IL-2 (MLR/IL-2). Infection was achieved by co-cultivating activated T cells with the producing amphotropic cell line pre-treated with mitomycin C for 96 hours. Infection and transduction efficiency were assayed by LacZ gene expression, which is detected as indigo blue staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

Results. The highest percentage of transduced T cells was reached on the 3rd PHA/IL-2 and on 9th MLR/IL-2 activation days. In these conditions with FLac vector we obtained up to 80% X-Gal+ cells after PHA/IL-2 activation and 66% and 44%, respectively, with non irradiated and irradiated MLR/IL-2, respectively. Up to 40% X-Gal+ cells were obtained with NuNL vector after PHA/IL-2 stimulation, 40% with irradiated and 48% with non irradiated MLR/IL-2 activated cells. In term of transduction efficiency, large variability was observed among patients. There were no immunophenotypical differences between FLac or NuNL vector-transduced cells activated by either of the two techniques and the control cells.

Conclusions. Our results indicate that: a) the use of FLac or NuNL vector retroviral-mediated gene transfer into T-lymphocytes derived from peripheral blood and stimulated by either PHA/IL-2 or a MLR produces a high percentage of transduced T cells; b) MLR is a good system for generating a transduced alloreactive lymphocyte population. The combination of high transduction efficiency and the capacity to obtain alloreactive transduced lymphocytes should open up the possibility of generating new in vitro and in vivo studies with selectable genes for in vivo therapeutic use.

Key words: LacZ, T-lymphocytes, retroviral vectors
could theoretically consider a target for gene therapy with hematopoietic stem cells include immune deficiency disorders (ADA deficiency, X-linked, Wiskott-Aldrich syndrome), lysosomal storage diseases (Gaucher’s disease, Hurler’s disease), hemoglobinopathies (sickle cell disease, thalassemia), Fanconi’s anemia and acquired immune deficiency syndrome (AIDS).

Gene transfer in hematological malignancy encompasses: a) gene marking protocols which use retroviral vectors that encode a marker gene, such as the neomycin resistance gene or the β-galactosidase gene, utilized mainly for studying the source of relapse and evaluating hematopoietic reconstitution; b) gene therapy protocols that employ suicide genes such as the thymidine kinase (TK) gene to render host cells sensitive to the cytotoxic effects of nucleotide analogs like ganciclovir. Another approach could be genetic modification of tumor cells by generating tumor vaccines or altering the malignant phenotype by blocking oncogenes and modifying tumor suppressor genes; c) modification of the drug sensitivity of normal progenitor cells by using the multiple drug resistance gene.¹

Human T lymphocytes offer a potential target for gene therapy.²⁻⁴ They are readily available from peripheral blood and can be manipulated in tissue culture prior to transduction. Peripheral lymphocytes are more fully differentiated than bone marrow precursor cells, and thus inserted genes should be less susceptible to inactivation than cells passing through many differentiation steps.⁵ The efficiency of transduction and the behavior of transduced cells can be monitored by using retroviral vectors containing a marker gene which encodes for the neomycin resistance (Neo) or the β-galactosidase gene (LacZ). The LacZ gene is a bacterial sequence that codes for a β-galactosidase whose expression can be detected as indigo blue staining with the chromogenic substrate 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-Gal), or as fluorescent staining with the fluorescein-di-galactopyranoside-FACS protocol.⁶⁻⁷ Endogenous mammal β-galactosidases are involved in lactose metabolism and glycolipid catabolism. They are mainly localized in lysosomes.⁸⁻⁹ One advantage of using bacterial β-galactosidases is that they are optimally active at neutral pH, whereas the endogenous form requires a pH of 3-6. Consequently, by controlling the pH of the reaction buffers only the exogenous β-galactosidases will be visualized.⁸⁻⁹ The product of the LacZ gene is localized mainly in the cytoplasm.¹⁰ Studies in CV-1 cells have demonstrated that since the protein has no membrane component it is not expressed on the cell surface.¹¹ Moreover, β-galactosidases are unable to pass through the intercellular junction between a LacZ gene-expressing cell and an adjacent cell.¹² Various forms of the LacZ gene have been engineered to improve the performance of this marker.¹³ Bacterial sequences that induce resistance to antibiotics have been fused in frame to the 3’ terminus or the 5’ terminus of the regular LacZ sequence. The Sh-ble::LacZ gene encodes a bifunctional protein fusion that retains both the marking activity of the LacZ gene and resistance to phleomycin.¹⁴ The LacZ::Neo gene encodes for a bifunctional protein fusion which also conserves expression of the marker gene and resistance to G418 induced by the Neo gene.¹⁵⁻¹⁶

This report demonstrates that retroviral-mediated gene transfer into T lymphocytes derived from peripheral blood and stimulated by either PHA/IL-2 or a mixed lymphocyte reaction (MLR) produces a highly transduced T-cell population. Infection was accomplished by co-cultivating target cells with the producing amphotropic cell line. No step in the infection procedure exerted any cytotoxic effect on the transduced cells; immunophenotypic analysis and growth kinetics were superimposable on the control cells.

**Materials and Methods**

**Cells and media**

Peripheral blood lymphocytes (PBLs) were obtained from 6 healthy donors. Mononuclear cells were separated from 50 mL of buffy coats by Ficoll-Hypaque gradient separation and cultivated in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (ICN Flow Laboratories,
Costa Mesa, CA), 1% penicillin/streptomycin (Gibco BRL) and 2 mM L-glutamine (Gibco BRL). Monocytes were removed from the mononuclear fraction by adherence in Petri dishes for 2 hours at 37°C.

**Retroviral vectors**

pNU vector, a defective retroviral vector derived from the Moloney murine leukemia virus, was produced by ligating the SalI-NotI fragment of pIRV (kindly provided by P. Savatier, ENS, Lyon, France), which contains the 3’ long terminal repeat (LTR), the leader sequence in which both the viral splice donor and the start codon of the gag gene were deleted, and the enhancer located in the gag gene, to the SalI-NotI fragment of pBabePuro (kindly provided by J.L. Darlix, ENS, Lyon, France), which contains the 5’LTR. The pFLac vector (Figure 1a) was constructed by cloning the BamHI fragment of pUT95 (Cayla, France), which contains the Sh-ble::LacZ gene, into the BamHI cloning site of the pNU vector. The pNuNL (Figure 1b) vector was constructed by cloning the XhoI fragment, which contains the LacZ::Neo gene, into the SalI cloning site of the pNU vector.

**Producing cell line**

High-titer FLac and NuNL vector-producing cell lines were obtained using a ping-pong amplification procedure according to a modified protocol. Vectors were first transferred into GP+E86 packaging cells by transfection. Then 5×10⁶ GP+E86 producing cells were plated in a 60 mm² dish and adherent cells were treated with 10 µg/mL mitomycin C (Sigma, St. Louis, MO) for 3 hrs. GP+envAm12 packaging cells were co-cultivated with mitomycin-treated GP+E86-producing cells for four days and then expanded and assayed for viral titer. Co-cultivation was repeated for several rounds until the viral titer reached a plateau. Producing cells were subcloned and assayed for transfer of β-galactosidase activity. Viral titers were carried out by testing the capacity of serial dilutions of retrovirus supernatants to transfer β-galactosidase activity to Rat-2 cells. Supernatants were collected by expanding producer cells to confluence on plates and adding 5 mL fresh medium for 24 hours. Supernatants were filtered through a 0.45 µm filter, and dilutions from 10⁻² to 10⁻⁵ were added to 60 mm² plates of Rat-2 cells grown to approximately 50 to 60% confluence and cultured with 4 µg/mL polybrene (Sigma) for 1 hour. Cultures were stained with X-Gal substrate two days after infection as previously described. Briefly, cell aliquots were placed in 96-well plates, centrifuged and fixed for 15 minutes at room temperature in 0.2% glutaraldehyde (Sigma), 1% formaldehyde (Sigma), 0.02 NP40 (Sigma) in phosphate-buffered saline (PBS) (Bichrom KG); then they were washed with PBS and overlaid with a reaction mixture containing 0.5 mg/mL X-Gal (Sigma), 5 mM potassium ferrocyanide (Sigma), 5 mM potassium ferricyanide (Sigma), 0.01% sodium deoxycholate (Sigma), 0.02% NP40 (Sigma), 0.1 phosphate buffer, pH 7.3. X-Gal was separated from β-galactosidases in galactosium and 5-bromo-4-chloro-3-indolyl. The addition of potassium ferricyanide and potassium ferrocyanide catalyzes the oxidation of 5-bromo-4-chloro-3-indolyl to an indigo soluble derivative that is visible at light and electron microscopy. Viral titers in the FLac and NuNL-producing cell lines obtained using the ping-pong amplification procedure were about 1×10⁸ LacZ-positive colony forming units per mL (c.f.u. mL⁻¹) and 1×10⁶ LacZ-positive c.f.u. mL⁻¹, respectively.
T cell activation techniques

The T cell rich population was stimulated with: 1) phytohemagglutinin (PHA, 0.25 mg/mL) (Biochron Kg, Berlin, Germany) and recombinant human interleukin-2 (IL-2, 100 U/mL) (Eurocetus B.V., Amsterdam, Netherlands); 2) irradiated (3000 rad for 10 min) allogeneic mononuclear cells and IL-2 (100 U/mL); 3) non irradiated allogeneic mononuclear cells and IL-2 (100 U/mL). GP+E86-derived cells, GP+envAm12-derived cells, and RAT-2 cells (ATCC CRL1764) were grown in complete Dulbecco’s modified Eagle’s medium (DMEM) (BioWitthaker Inc, Walkersville, MD) with 10% new-born calf serum (Gibco), 2 mM glutamine, and 1% antibiotics. Lymphocyte activation was detected and quantified by measuring the incorporation of tritiated thymidine (3H-TdR), which was taken as an indicator of activated lymphocytes. Briefly, cells were incubated with 10 µL 3H-TdR (1 mCi/mL; specific activity 25 Ci/mmL; Amersham, UK) for 6 hours before harvesting the DNA and counting in a scintillation counter (Beckman Ls 3801, Galway, Ireland). The results were expressed as counts per minute (cpm) and presented as the means of triplicate cultures ± standard deviation.

The PBLs cell cycle was determined by flow cytometry analysis of cellular DNA content using DNA-Prep reagents (Coulter Co., Hialeah, FL). Briefly, 1×10^6 cells, after permealizing and DNA staining (50 µg/mL propidium iodide), were analyzed on an Epics Profile II (Coulter Co.) and the data elaborated with the Multicycle Software program (Phoenix Flow Systems, San Diego, CA). The proliferative activity is the sum of all cells in the S+G2/M phases of the cell cycle.

Transduction procedures

Transduction was carried out by co-cultivating cells in presence of 4 µg/mL polybrene (Sigma) and IL-2 (100 U/mL); 2×10^5 producing cells were treated with 10 µg/mL mitomycin C for 3 hr and washed three times with PBS1X. From the 1st to the 10th activation day 2×10^5 lymphocytes were added and co-cultivation continued for 96 hr. Non-adherent cells were gently harvested from the plate and seeded onto a new dish in the absence of producing cells. Two days later, aliquots of the cells were examined for β-galactosidase activity by staining with X-Gal. Polybrene was added during the transduction procedure. Gene transfer efficiency was expressed as a percentage of X-Gal+ cells. Retroviral transduction of T lymphocytes was also performed by co-cultivating target cells on a layer of producing cells separated by a transmembrane with a pore diameter of 0.45 µm.

There was no evidence of replication competent viruses in mobilization assays.

Cell surface phenotyping

PBLs cell surface phenotype was determined by using a panel of mouse monoclonal antibodies (MoAbs) and an indirect fluorescence labelling method. Briefly, 1×10^6 lymphocytes were washed and then incubated for 15 minutes at room temperature in PBS containing 2% human AB serum (ICN Flow Laboratories). Cells were incubated with MoAbs directed against the following antigens: CD2, CD3, CD19 (Coulter Co.), CD4, CD8, CD71, HLA-DR (Ylem, Rome, Italy), CD20, CD25 (Immunotech, Marseille, France), CD45 (Dako, Glostrup, Denmark). After incubation for 30 minutes at 4°C, cells were washed twice with PBS and resuspended with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG. Negative controls were assessed using isotype-matched, irrelevant MoAbs. Stained cells were analyzed on an Epics Profile II.

Results

Retroviral vector infection requires the presence of dividing cells. Two different activation techniques, PHA/IL-2 and MLR/IL-2 with a pool of irradiated or nonirradiated lymphocytes as allogeneic stimulation, were employed to induce T lymphocyte proliferation. ^2^H-TdR incorporation studies revealed a maximum peak between the 2nd and 5th days after stimulation with PHA/IL-2 (Figure 2a). Following activation with MLR/IL-2, the maximum peak occurred between the 6th and 10th days in both the irradiated (Figure 2b) and non irradiated pools (Figure 2c).
Parallel cell cycle analyses gave superimposable results. The proliferative index reached a peak on the 2nd day after PHA/IL-2 stimulation (Figure 2a), while after activation with MLR/IL-2 the maximum peak occurred in both the irradiated and non irradiated pools between the 6th and 8th days (Figure 2b, 2c).

Only low efficiency transduction was obtained when infection experiments were performed with a transmembrane (1-2% of X-Gal+ cells with the FLac or NuNL vector).

To increase gene transfer efficiency, the infection protocol was performed by co-cultivating T lymphocytes with FLac or NuNL-producing cell lines pre-treated with mitomycin C. When T lymphocytes were stimulated with PHA/IL-2, transduced with the FLac vector and stimulation was started on the 3rd activation day and prolonged for 96 hrs (Figure 2a), X-Gal+ cells increased up to 80% (range 43-99%). The percentage of X-Gal+ lymphocytes after MLR stimulation was 44% (range 30-69%) in the irradiated and 66% in the non irradiated (range 18-88%) pool, in both cases when co-cultivation was begun on the 9th day and prolonged for 96 hrs (Figure 2b, 2c).

Transduction efficiency was 40% X-Gal+ cells (range 19-71%) in T lymphocytes stimulated with PHA/IL-2 and transduced with the NuNL vector when co-cultivation was initiated on the 3rd activation day and prolonged for 96 hrs. In cells activated with MLR, efficiency was 40% (range 27-56%) in the irradiated and 48% (range 16-87%) in the non irradiated pool when co-cultivation was begun on the 9th day and prolonged for 96 hrs.

As expected, the highest percentage of X-Gal+ transduced cells was seen at the moment of maximum lymphocyte activation, irrespectively of whether stimulation occurred with PHA/IL-2 or MLR, as documented by 3H-TdR incorporation studies and proliferative index analysis.

As expected, T cells exhibited cytoplasmic localization of staining with both the FLac and NuNL vectors (Figure 3) and, as demonstrated by increasing cell volume, which is considered a morphological criterion of lymphocyte activation, most X-Gal+ transduced cells were activated T cells.

X-Gal staining analysis of transduction effi-
ciency revealed that the degree of lymphocyte infection varied from one healthy donor to another. There was no evidence of morphological alterations in the T lymphocyte population after co-cultivation or of modifications associated with the β-galactosidase expression induced by the LacZ gene.

Immunophenotypic analysis of transduced T lymphocytes was performed to demonstrate that the entire transduction procedure and gene integration had not in any way modified the behavior of transduced T cells. There were no significant differences in the surface membrane markers of the FLac and NuNL vector-transduced cells.

Discussion

Retroviral vectors are the most efficient means of transducing and expressing exogenous genes within hemopoietic cells. Ongoing clinical bone marrow and peripheral blood gene transfer protocols are based on the use of retroviral vectors. However, although gene transfer into hemopoietic stem cells remains the goal of gene therapy for many congenital and acquired disorders, there is at present no clear evidence that retroviral vectors can adequately transduce these cells and maintain stable gene expression in their progeny.

Gene transfer is more easily achieved in peripheral blood lymphocytes, a valid alternative to bone marrow, at least as far as congenital and acquired immune system disorders are concerned. In order to obtain a population that is really representative of the entire immune system, one must establish the quantity of PBLs needed to transduce and ascertain that expression remains stable over time, since both are fundamental requirements if gene transfer is to have clinical relevance. For this reason, gene transfer efficiency and new vectors that assure high levels and stability of expression of exogenous genes are crucial for the future development of gene therapy. Gene transfer into T lymphocytes has been described in tumor-infiltrating lymphocytes and human peripheral blood-derived lymphocytes but the percentage of infected cells has been low (5 to 20% transduced cells, as assessed by detection of the presence of the provirus).

Using co-cultivation techniques, Finer et al. obtained a retroviral transduction efficiency of up to 40% with the CD4 coding sequence in primary CD\(^8\)\(^+\) lymphocytes. In a recent study, Imbert et al. reported that they achieved up to 78% transduction with the same FLac vector in human peripheral blood lymphocytes stimulated with PHA/IL-2 or activated through the CD2/CD28 molecule.

The aim of our study was to compare different retroviral vectors, all containing the same gene marker, and to determine their capacity to infect and express the exogenous gene in human lymphopoietic cells. We also tested whether substituting the PHA/IL-2 activated polyclonal lymphocyte system with allogeneic stimulation in a mixed lymphocyte culture could mimick in vivo conditions and, at the same time, produce highly alloreactive transduced cells with therapeutic potential. Since no DNA is synthesized during the first 30-36 hrs after lymphocyte stimulation, no activation signals can be obtained from \(^3\)H-TdR or cell cycle cytofluorimetry. The lymphocyte activation obtained by PHA stimulation is already detected by \(^3\)H-TdR incorporation at 48 hrs. Because only a small fraction of cells are activated when lymphocytes are stimulated by MLR, a longer time period (usually 5-10 days) is necessary before the number of proliferating cells is sufficient to be quantified by \(^3\)H-TdR. Our results agree with those already published...
in the literature and show that whereas 48 hrs are sufficient to reveal lymphocyte activation after PHA stimulation, a longer period, at least 5 days, is required for the MLR.

Per cent infection was always very low when it was mediated by supernatant derived from producing cells or transmembranely, probably because only close contact between target and producing cells during co-cultivation guarantees high transduction efficiency. An efficient producing line can mark more than 80% of peripheral blood T lymphocytes and thus allow expression of the transduced gene to be followed without having to subclone the transduced population. By employing the GP+envAm12/FLAC packaging cell line we obtained up to 80% X-Gal+ cells after activation with the PHA/IL-2 system, and about 66% after mixed lymphocyte culture activation. The other vector used, NuNL, was less efficient; transduction was 40% with the PHA/IL-2 system and 48% with the mixed lymphocyte culture. These results demonstrate that, when activation conditions are equal and the lymphocytes are derived from the same donor, the percentage of cells transduced differs with the vector employed and with the respective retroviral titers.

Surface marker expression studies and the lymphocyte growth curve provided evidence that neither the co-cultivation procedure nor the transduced gene expression had any deleterious effect on the transduced population.

The use of the LacZ gene marker opens up interesting perspectives for setting up gene therapy protocols, since transgene expression and its maintenance can be followed for the medium and long term through a simple cytochemical reaction, an indispensable condition for monitoring the fate of transduced cells. The simple cytochemical reaction of β-galactosidase would permit the localization of transduced cells to be checked. In the case of lymphocytes, a bone marrow biopsy or a peripheral blood sample is all that would be required.28

Our results indicate that: a) by using FLac or NuNL vectors retroviral-mediated gene transfer into T-lymphocytes derived from peripheral blood and stimulated by either PHA/IL-2 or through a MLR produces highly transduced T cells; b) the MLR is a good system for generating a transduced alloreactive lymphocyte population. The high transduction efficiency documented in this study should open up new horizons for the marking and later insertion of functional genes. In AIDS, enzyme deficient and cancer patients, with high transduction efficiency one could contemplate inserting acquired drug resistant genes by transducing genes that encode the cytokines. However, in vivo monitoring of the destiny of the transduced population by gene marking is the most important step in gene therapy protocols.

References

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