Marking and Gene Expression by a Lentivirus Vector in Transplanted Human and Nonhuman Primate CD34+ Cells

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Recently, gene delivery vectors based on human immunodeficiency virus (HIV) have been developed as an alternative mode of gene delivery. These vectors have a number of advantages, particularly in regard to the ability to infect cells which are not actively dividing. However, the use of vectors based on human immunodeficiency virus raises a number of issues, not the least of which is safety; therefore, further characterization of marking and gene expression in different hematopoietic lineages in primate animal model systems is desirable. We use two animal model systems for gene therapy to test the efficiency of transduction and marking, as well as the safety of these vectors. The first utilizes the rhesus animal model for cytokine-mobilized autologous peripheral blood CD34+ cell transplantation. The second uses the SCID-human (SCID-hu) thymus/liver chimeric graft animal model useful particularly for human T-lymphoid progenitor cell reconstitution.

In the rhesus macaques, detectable levels of vector were observed in granulocytes, lymphocytes, monocytes, and, in one animal with the highest levels of marking, erythrocytes and platelets. In transplanted SCID-hu mice, we directly compared marking and gene expression of the lentivirus vector and a murine leukemia virus-derived vector in thymocytes. Marking was observed at comparable levels, but the lentivirus vector bearing an internal cytomegalovirus promoter expressed less efficiently than did the murine retroviral vector expressed from its own long terminal repeats. In assays for infectious HIV type 1 (HIV-1), no replication-competent HIV-1 was detected in either animal model system. Thus, these results indicate that while lentivirus vectors have no apparent deleterious effects and may have advantages over murine retroviral vectors, further study of the requirements for optimal use are warranted.

A number of groups have recently exploited the substantial available data regarding human immunodeficiency virus type 1 (HIV-1) molecular biology and pathogenesis to develop vehicles for gene delivery based on HIV-1 and other lentiviruses (3, 9, 27, 30, 39, 40, 42, 43, 45, 47, 48, 51, 54). The simplest of these vectors consists of the minimal cis-acting sequences required for HIV-1 replication. Other HIV-1 genes are expressed in trans with a packaging plasmid. The gene to be expressed, either a therapeutic gene or a reporter gene, is expressed as part of an internal transcriptional unit inserted between the long terminal repeats (LTRs) of the vector. Other modifications of these vectors have resulted in the generation of tat-dependent or inducible expression of the gene to be expressed. A tat-dependent vector may be particularly suitable for HIV-1 disease since it will be expressed only in the presence of HIV-1 infection (5, 9). In some cases, HIV-1 vectors are themselves capable of inhibiting HIV-1 replication (5, 10, 15). Other therapeutic genes have been inserted into HIV-1 vectors, and it is likely that there will be increasing emphasis on the potential use of these vectors for treatment of various human conditions (22, 24, 29, 36, 52). The primary advantage of lentivirus vectors is that by virtue of nucleic localization signals present in HIV-1 proteins that are part of the preintegration complex (11, 21, 26, 56), these vectors can efficiently infect some nondividing cells (39, 41, 43, 48, 54), provided they reside or progress through at least the G1b state of the cell cycle (32). Other retroviral vectors based on murine retroviruses require passage of the cell through mitosis in order to integrate (34, 38, 49). Another advantage of lentivirus vectors is that they have evolved to efficiently replicate in human cells. However, the latter factor also underscores the need to carefully assess the properties of lentivirus vectors, particularly those derived from HIV-1, prior to use in humans.

Several animal model systems have been used to evaluate retroviral vector delivery systems (2, 6, 8, 12, 13, 18, 41, 43). Of particular relevance to lentivirus vector systems is the ability to test transduction, reconstitution, gene expression, and marking and ultimately therapeutic efficacy in model systems for human and/or nonhuman primates. The rhesus macaque model system has been shown to be amenable to transduction of CD34+ cells and transplantation and is arguably the closest model system for human gene therapy (7, 18, 20, 28, 53, 57). In addition, for some diseases such as AIDS, the rhesus macaque will also allow for testing of therapeutic efficacy against simian immunodeficiency virus (17, 23). Use of this transplantation model, however, is expensive and does not lend itself to the type of experimental manipulation required to test multiple variables as do other small-animal model systems, such as the SCID-NOD (50) and SCID-human (SCID-hu) chimeric mouse models (37). In the SCID-NOD system, human CD34+ cells are used to reconstitute an irradiated SCID mouse, resulting in the production of human granulocytes, monocytes, and B cells (16, 25). In the SCID-hu system, human CD34+ cells can be transplanted following irradiation of a chimeric human thymus/liver (thy/liv) organ previously implanted into the mice to mimic human thymopoiesis (2, 6, 8). Typically, the SCID-NOD mouse is used to analyze non-T-lymphoid progenitor CD34+
cell transplant, whereas the SCID-hu mouse is utilized to investigate T-lymphoid progenitor CD34⁺ cell transplant.

In this study, we used the rhesus macaque and SCID-hu system to investigate the properties of transplanted CD34⁺ cells transduced with a lentivirus vector bearing an internal cytomegalovirus (CMV) promoter. We demonstrate that the lentivirus vector can result in multilineage hematopoietic cell marking. However, comparative studies using SCID-hu mice indicate that the levels of gene expression are substantially lower than that of a murine retroviral vector using the LTR as a promoter. We find no evidence for replication competent HIV-1 in either transplanted rhesus macaques or SCID-hu mice.

**MATERIALS AND METHODS**

**HIV-1 vector construction and production.** An HIV-1-based vector, pHR-LTR-go fp S V C2 (43), was modified to contain the enhanced green fluorescent protein (EGFP) cDNA with expression driven by a CMV promoter (HR/CMV EGFP). A packaging plasmid for the HIV-1-based vector, pCMVR8.2DVPR, was derived from pCMVR8.2 (43, 44) by deleting the vpr gene from nucleotide positions 5625 to 5731 by oligonucleotide-directed mutagenesis. Numbering of nucleotides starts at the 5’ end of HIV-1 NL4-3 provirus (1). All vector stocks were generated by calcium phosphate-mediated transfection of 293T cells (American Type Culture Collection, Manassas, Va.). 293T cells were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. 293T cells (2 × 10⁶) were plated on 175-cm² flasks in 25 ml of the medium and transfected the following day with 5 μg of pHCMVG, 12.5 μg of pCMVR8.2DVPR, and 12.5 μg of HR/CMV-EGFP for the HIV-1-based vector. For the murine leukemia virus (MLV)-based vector (SRαLegFP with expression of EGFP from the 5’ LTR), 5 μg of pHCMVG (14), 12.5 μg of pSFr-env-MLV (33), and 12.5 μg of SRαLegFP were used (5). At 8 h posttransfection, the medium was replaced with 35 ml of fresh medium. At 36 and 60 h posttransfection, the medium was harvested, centrifuged at 1,500 rpm for 5 min (Sorvall RT 6000B; Ivan Sorvall, Norwalk, Conn.), and filtered through a 0.45-μm-pore-size filter. Further vector concentration was achieved by ultracentrifugation at 50,000 × g for 90 min at 4°C. The vectors were concentrated 100-fold and kept in liquid nitrogen until use. Stocks of vectors were titrated by infecting HeLa cells (10⁴), with various amounts of the virus and analyzing for EGFP expression by flow cytometry on day 3 postinfection. The titers of vectors were 10⁸ infectious units/ml for the HIV-1 vector and 2 × 10⁹ infectious units/ml for the MLV-based vector.

**Rhesus leukapheresis procedure.** Young adult rhesus macaques (Macaca mulatta) that were serologically negative for simian T-cell lymphotropic virus, simian immunodeficiency virus, simian AIDS-related type D virus, and herpes B virus were used. Experimental animals were quarantined and housed in accor-

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**TABLE 1. Outcome of G-CSF- and SCF-mobilized peripheral blood transplantation using immunoselected CD34⁺ cells**

<table>
<thead>
<tr>
<th>Animal (cytokine[s] used in vitro, frequency of transduction)</th>
<th>No. of CD34⁺ cells reinfused (10⁷)</th>
<th>No. of CD34⁺ cells/kg (10³)</th>
<th>% EGFP-expressing cells reinfused</th>
<th>Day leukocyte count &gt; 1,000</th>
<th>Day platelet count &gt; 50,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>95E008 (SCF, BID)</td>
<td>4.0</td>
<td>1.0</td>
<td>3.2</td>
<td>12</td>
<td>Did not fall below 50,000</td>
</tr>
<tr>
<td>95E009 (SCF, BID)</td>
<td>1.6</td>
<td>0.4</td>
<td>13.2</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>RC504 (SCF + IL-6, SID)</td>
<td>1.4</td>
<td>0.4</td>
<td>9</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>RC505 (SCF + IL-6, SID)</td>
<td>1.6</td>
<td>0.4</td>
<td>6</td>
<td>11</td>
<td>Did not fall below 50,000</td>
</tr>
</tbody>
</table>

| a SID: once a day; BID: twice a day. |
| b Percentage of CD34⁺ cells expressing EGFP on the day of infusion. |

FIG. 1. PCR analysis of rhesus macaque cell fractions following transplant. DNA from rhesus macaque granulocytes, lymphocytes, and whole PBMN were analyzed for HIV-1 vector transduction by PCR 10 weeks after reconstitution of rhesus macaque 95E008 and 16 weeks after reconstitution of rhesus macaque RC505. Granulocyte and lymphocyte populations were sorted from PBMN to, respectively, 98 and 99% purity, based on forward and side scatter. HIV-1 vector DNA-specific signal was compared with that of amplified β-globin DNA sequences to determine the number of vector copies per cell (%HIV-1 vector DNA copies/cell, calculated as number of HIV-1 vector DNA copies/number of cell equivalents × 1/100 × 100). For PCR amplification, 10-fold less DNA was used for β-globin DNA PCR in order to obtain quantitative β-globin DNA signals. Quantitative HIV-1 vector DNA and β-globin DNA standards (std) were assayed along with DNA from a nontransduced rhesus (negative control) in parallel. No signals were detected for the negative control (data not shown). Percent EGFP expression was determined by flow cytometric analysis at the same time point as PCR. tRNA served as the negative control for the PCR assay.
log fluorescent intensity of EGFP; the y cytocyte populations but did not demonstrate any detectable fluorescence in erythroplanted animals had EGFP expression in granulocytes, monocytes, and lymphocytes by flow cytometry in all the subpopulations in RC505. The other three transduced rhesus macaques RC505 and 95E008 are shown. EGFP expression was detected scatter) and analyzed for EGFP expression. Results for HIV-1 vector-transduced (PLAT) were gated according to size (forward scatter) and granularity (log 90° monocytes (MONO), lymphocytes (LYM), erythrocytes (RBC), and platelets (PLAT)) were gated according to size (forward scatter) and granularity (log 90° scatter) and analyzed for EGFP expression. Results for HIV-1 vector-transduced rhesus macaques RC505 and 95E008 are shown. EGFP expression was detected by flow cytometry in all the subpopulations in RC505. The other three transduced animals had EGFP expression in granulocytes, monocytes, and lymphocyte populations but did not demonstrate any detectable fluorescence in erythrocytes and platelets. Data for rhesus macaque 95E008 are shown. The x axis is log fluorescent intensity of EGFP; the y axis represents the gated population based on forward and side scatter (logarithmic fluorescence intensity).

FIG. 2. Flow cytometric analysis of rhesus macaque PBMC at 30 weeks. Circulating leukocytes at 30 weeks were evaluated by flow cytometry in animals transduced with the HIV-1 vector (HR CMVEGFP). Granulocytes (GRAN), monocytes (MONO), lymphocytes (LYM), erythrocytes (RBC), and platelets (PLAT) were gated according to size (forward scatter) and granularity (log 90° scatter) and analyzed for EGFP expression. Results for HIV-1 vector-transduced rhesus macaques RC505 and 95E008 are shown. EGFP expression was detected by flow cytometry in all the subpopulations in RC505. The other three transduced animals had EGFP expression in granulocytes, monocytes, and lymphocyte populations but did not demonstrate any detectable fluorescence in erythrocytes and platelets. Data for rhesus macaque 95E008 are shown. The x axis is log fluorescent intensity of EGFP; the y axis represents the gated population based on forward and side scatter (logarithmic fluorescence intensity).

TABLE 2. EGFP expression after activation of PBMN

<table>
<thead>
<tr>
<th>Animal</th>
<th>% EGFP</th>
<th>[3H]Thymidine incorporation (cpm)</th>
<th>% EGFP</th>
<th>[3H]Thymidine incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95E008</td>
<td>0.03</td>
<td>113,650</td>
<td>0.02</td>
<td>1,964</td>
</tr>
<tr>
<td>95E009</td>
<td>0.09</td>
<td>53,135</td>
<td>0.21</td>
<td>291</td>
</tr>
<tr>
<td>RC504</td>
<td>0.05</td>
<td>88,973</td>
<td>0.03</td>
<td>362</td>
</tr>
<tr>
<td>RC505</td>
<td>0.15</td>
<td>35,913</td>
<td>0.13</td>
<td>170</td>
</tr>
<tr>
<td>Mock</td>
<td>0.00</td>
<td>64,415</td>
<td>0.00</td>
<td>325</td>
</tr>
</tbody>
</table>

* Rhesus macaque PBMN (10^6) were obtained from reconstituted rhesus macaques at 23 weeks (98E008 and 98E009) or 18 weeks (RC504 and RC505) postreconstitution. These cells were cultured for 3 days in the presence of human IL-2 (500 U/ml), immobilized anti-monkey CD3 (200 μg/ml). As a control, PBMN were cultured in parallel in the absence of growth-stimulating agents. [3H]Thymidine incorporation was measured by pulse-labeling cells for 6 h on day 3.
DNA per ml. The copy number of HIV-1 vector included in the standard curve ranged from 3 to 1,000. Standard curves for rhesus macaque β-globin DNA were obtained by amplification of 0.001 to 0.3 μg of rhesus macaque cellular DNA (100 to 30,000 cell equivalents) from rhesus macaque PBMN.

Transduction and immunoselection of gene-transduced human fetal liver-derived CD34<sup>+</sup> cells by flow cytometry. Human fetal liver-derived CD34<sup>+</sup> cells were purified from a fetal liver as previously described [6]. Cells were cultured in Iscove's modified Dulbecco's medium with 100 ng each of IL-3, IL-6, and SCF (kindly supplied by Amgen) per ml, 20% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Fetal liver-derived CD34<sup>+</sup> cells (2 × 10<sup>6</sup>) were transduced with VSV-G-pseudotyped HR<sub>9</sub>CMVEGFP or SR<sub>a</sub>LEGFP vector by incubating 2 ml of a 40-fold dilution of the HR<sub>9</sub>CMVEGFP vector stock or 10-fold dilution of the SR<sub>a</sub>LEGFP vector stock in the presence of Polybrene (8 μg/ml) at 37°C for 2 h on day 1 or 2 after CD34<sup>+</sup> cell purification from fetal liver, respectively. On day 3 after CD34<sup>+</sup> cell purification from fetal liver, vector-transduced CD34<sup>+</sup> cells were stained with a MAb to human CD34 (Becton Dickinson, Mountain View, Calif.) conjugated with phycoerythrin. EGFP and CD34<sup>+</sup> cells were sorted on a FACStarplus (Becton Dickinson). Thy/liv implants of irradiated (300 rads) animals were directly injected with 10<sup>5</sup> EGFP and CD34<sup>+</sup> cells on day 4 after CD34<sup>+</sup> cell purification from fetal liver.

Flow cytometric analysis for human thymocytes. Thymocytes were obtained by biopsy from reconstituted thy/liv implants of SCID-hu mice 4 weeks postreconstitution. Thymocytes were stained with a MAb to human CD1, CD3, CD4, CD5.

FIG. 3. Kinetics of EGFP expression following transplant. The percentage of EGFP-expressing granulocytes (○), lymphocytes (●), and monocytes (▲) was determined at various time points as described in the legend to Fig. 2 for all four animals receiving the lentivirus-transduced immunoselected CD34<sup>+</sup> cells. The percentage of EGFP-expressing cells is shown over a 30- to 40-week evaluation period.

**TABLE 3. Cocultivation with activated human PBL**

<table>
<thead>
<tr>
<th>Sample</th>
<th>p24 ELISA (ng/ml)</th>
<th>MAGI cell assay (no. of blue cells in a field)</th>
<th>% EGFP expression Before cocultivation</th>
<th>% EGFP expression After cocultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMN from HIV-1 vector-transduced macaque 95E008</td>
<td>&lt;0.008</td>
<td>3</td>
<td>0.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PBMN from HIV-1 vector-transduced macaque RC505</td>
<td>&lt;0.008</td>
<td>2</td>
<td>1.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PBMN from mock-transduced macaque 96E025</td>
<td>&lt;0.008</td>
<td>2</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HIV-1 uninfected MAGI cell culture supernatant</td>
<td>&lt;0.008</td>
<td>6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HIV-1 (NL4-3)-infected human PBL</td>
<td>1,303</td>
<td>&gt;800</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*a* PBMN (10<sup>7</sup>) from lentivirus vector-transduced rhesus macaques were cocultured with PHA-activated human PBL (10<sup>7</sup>) for 4 weeks. Every 7 days, activated human PBL (10<sup>7</sup>) were added to the culture. At 4 weeks postcocultivation, culture supernatants were harvested, measured for p24, and subjected to MAGI assay as described in Materials and Methods. Blue cells in a field were counted. Magnification was 100-fold (objective lens [10×] and eyepiece lens [10×]). Approximately 2,500 MAGI cells could be observed in a field when the cells were confluent. NA, not applicable.
FIG. 4. (A) EGFP expression 4 weeks after reconstitution in human CD45^+ thymocytes of reconstituted thy/liv implants in SCID-hu mice. EGFP expression at 28, 14, and 0.9% was detected in human CD45^+ thymocytes in three SCID-hu mice receiving a thy/liv transplant transduced with the HIV-based vector and at 39, 11, and 1.2% in three SCID-hu mice receiving a thy/liv transplant transduced with an MLV-based vector 4 weeks postimplant. Less than 0.2% of EGFP expression was detected in thymocytes from a SCID-hu mouse receiving nontransduced cells as a thy/liv implant (data not shown). Ten thousand events were analyzed. (B) Distribution of...
CD6, or CD45 directly conjugated with phycoerythrin or peridinin chlorophyll protein (Becton Dickinson). Samples were run on a FACScan flow cytometer, and data analyzed with the CellQuest program (Becton Dickinson). Ten thousand events were acquired for analysis.

**Cell culture for in vitro activation studies.** Human thymocytes and rhesus macaque PBMN were cultured at a concentration of 10^5/m in flat-bottom culture plates. Culture plates were coated with goat anti-mouse immunoglobulin G (GAM; Tago, Burlingame, Calif.). GAM (10 g/ml) in phosphate-buffered saline (PBS; pH 7.4) was added to wells and incubated for 2 h at 37°C. Plates were then washed three times with PBS. Anti-human CD3 MAb (T3; 4 mg/ml in PBS; Coulter, Hialeah, Fla.) or anti-monkey CD3 MAb (1 mg/ml; BioSource International, Camarillo, Calif.) was added on to the GAM-coated culture plates and incubated at 37°C for 1 h. The immobilized GAM provides a solid phase for binding of anti-human or monkey CD3 MAb as previously described (35, 55).

After washing, thymocytes obtained from th/lym implants of SCID-hu mice or rhesus macaque PBMN were cultured in the presence of IL-2 (10 U/ml; Amgen), anti-human CD28 MAb, and immobilized anti-human CD3 MAb or anti-monkey CD3 MAb in Iscove modified Dulbecco’s medium—20% FCS supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml), respectively. For control, cells were cultured in parallel in the absence of IL-2, anti-CD28 MAb, and immobilized anti-CD3 MAb. After 3 days of stimulation in vitro, thymocytes were analyzed by flow cytometry for EGFP expression. [3H]thymidine incorporation was measured by pulse-labeling cells for 6 h on day 2 as previously described (58).

**Coculture of rhesus macaque PBMN and activated human PBL.** PBMN (10^5) from lentivirus vector-transduced or mock-transduced rhesus macaques were cocultured with mitogen-stimulated human PBMN (PHA-activated cell lines or PHA-activated human PBL). Every 7 days, 10^5 activated human PBL were added to the culture. At 4 weeks postcocultivation, culture supernatants were harvested, marked, and analyzed by flow cytometry for EGFP expression. EGFP expression was detected in both CD4 and CD8 single-positive cells as well as CD4 CD8 double-positive and CD4 CD8 double-negative thymocytes. EGFP expressing thymocytes showed a CD4 and CD8 profile similar to that of non-gene-transduced reconstituted implant controls (mock).

**Results.**

**Transduction and transplantation of immunoselected mPB CD34+ cells in rhesus macaques.** We used an HIV-1 vector bearing an internal CMV immediate-early promoter for expression of the gene encoding EGFP. We evaluated this HIV-1 vector in the rhesus macaque transplantation model. Nonhuman primate immunoselected hematopoietic growth factor mPB CD34+ cells were transduced on non-tissue-coated sixwell plates treated with the recombinant fibrogenic fragment CH-296 (RetroNectin) either once or twice a day for 2 consecutive days following collection. Because lentiviruses can transduce nonmitotic cells, the CD34+ cells were stimulated with combinations of cytokines (SCF alone or SCF plus IL-6) that induced less cell division than combinations typically used and may therefore induce less lineage-specific differentiation. On average, 7.6% (range, 3.2 to 13.2%) of the CD34+ cells expressed EGFP following transduction with the lentivirus vector (Table 1). This transduction efficiency is superior to that observed for murine retroviral vectors, as most cells cultured under these conditions at various time points are not yet susceptible to murine retroviral infection (data not shown).

**Presence and expression of vector in rhesus PBMN subpopulations.** Four animals were transplanted with autologous CD34+ cells transduced with the HIV-1 vector. All animals had uneventful hematopoietic reconstitution following totalbody γ irradiation and autologous transplant, with leukocyte counts returning to 1,000 cells/μl within 15 days of transplant and platelet counts returning to greater than 50,000/μl within 11 days of transplant (Table 1). We monitored the presence of vector in different hematopoietic lineages by fluorescence-activated cell sorting followed by PCR analysis as well as by direct flow cytometric analysis for EGFP in gated cell populations. As determined by PCR analysis, approximately 0.1 to 1% of circulating leukocytes contained vector DNA in sorted lymphocytic and granulocytic cell fractions 10 weeks (animal 95E008) or 16 weeks (animal RC505) following transplant (Fig. 1). In one animal, RC505, we observed a higher level of vector DNA in the granulocyte subpopulation.

EGFP expression was detected in PBMN beginning at 2 weeks after transplant. EGFP expression at 30 weeks posttransplant in various PBMN subpopulations is shown in Fig. 2. EGFP is mainly detected in granulocyte, monocyte, and lymphocyte subpopulations. As determined by the mean fluorescence intensity, the expression in lymphocytes was the least efficient. Further analysis indicated that both B and T lymphocytes (CD20 and CD2 positive, respectively) as well as NK cells (CD16 CD56 double positive) expressed the EGFP gene (data not shown). The percent of cells expressing EGFP was monitored over a period of 34 to 39 weeks (Fig. 3) in each of four transplanted rhesus macaques. EGFP expression was observed in granulocyte, lymphocyte, and monocyte subsets. Although there was variation in the level of marking over time, we observed a general increase in marking between 5 to 10 weeks following transplant. In the animal (RC505) with the highest levels of marking, the proportions increased over time to between 1 to 2% of PBMN. To date, 14 months following transplant, the proportion of PBMN expressing EGFP has remained relatively stable, with the highest levels ranging from 1 to 3%.

**EGFP expression in erythrocytes and platelets.** Of particular interest is the observation that low but significant levels of EGFP could be detected 30 weeks posttransplant in the erythrocytes and platelets of one animal, RC505, with the highest overall proportion of marked PBMN (Fig. 2). Marking of these cell types has not previously been observed in rhesus macaques transplanted with CD34+ cells transduced with a murine retrovirus (murine stem cell virus [MSCV]-based) vector (46). Since the majority of these cells would not be expected to harbor vector DNA, we presume that we were detecting persisting EGFP protein. We cannot formally exclude the possibility that we were detecting EGFP only in nucleated precursor cells. However, this appears unlikely since if it were the case,
FIG. 5. EGFP expression in human thymocytes 4 weeks after direct injection of HIV-1 vector in thy/liv implants of SCID-hu mice. Prior to vector injection, SCID-hu thy/liv mice were irradiated (200 rads) to induce progenitor cell proliferation and to kill resident thymocytes. At 24 h (implants a and b) or 4 days (implants c and d) postirradiation, 100-fold-concentrated HIV-1 vector was directly injected into irradiated thy/liv implants of SCID-hu mouse. Four irradiated implants (a through d) were each injected with approximately 50 µl of the vector stock. The titer of HIV-1 vector was 10^8 infectious units/ml. (A) At 4 weeks after direct injection of HIV-1 vector into thy/liv implants, EGFP expression was analyzed in human CD45^+ thymocyte populations by flow cytometry. (B) EGFP expression was analyzed in human CD1, CD3, CD4, CD5, and CD8 thymocyte subpopulations. Representative data from implant a are shown.

A

Uninjected implant

HIV-1 vector (HR'CMVEGFP) injected implants

CD45

EGFP

0.01%

3.5%

3.0%

2.2%

4.7%

1.1%

1.4%

1.8%

2.6%

1.6%

0.01%

CD5

EGFP

CD8

EGFP

1.2%

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the majority of circulating erythroid precursor cells would have to express vector, inconsistent with the frequency of marked cells in the other hematopoietic lineages.

**Activation of rhesus PBMN does not enhance the levels of vector expression.** The mean fluorescence intensity of EGFP in lymphocytes was about fivefold weaker than that in granulocytes (Fig. 2). We tested whether activation of PBMN cells with IL-2 and anti-CD3 MAb could enhance expression. Treatment of rhesus PBMN with IL-2 and anti-CD3 resulted in an approximately 100-fold increase in [3H]thymidine incorporation. Activation of PBMN did not appear to significantly increase the proportion of cells expressing EGFP (Table 2) or EGFP fluorescence intensity (data not shown).

Replication-competent HIV-1 is not evident. To date, more than 50 weeks posttransplant, all four animals remain healthy and have not demonstrated circulating antibody to HIV p24 by Western blotting, circulating p24 antigen by ELISA, or evidence of circulating HIV-1 virus by quantitative HIV-1 ultra-sensitive reverse transcription-PCR (data not shown). We further confirmed that there was no evidence of replication-competent or latent HIV-1 in the cells by coculture of 10^7 human fetal liver CD34^+ human cells, indicating that progenitor cells common to CD4 and CD8 cells for HIV-1 or MLV vector-transduced cells were analyzed by flow cytometry for EGFP expression. [3H]thymidine incorporation was measured by pulse-labeling cells for 6 h on day 3.

**HIV-1 vector-transduced CD34^+ cells can reconstitute irradiated SCID-hu thyliv grafts.** We previously used human thy/liv chimeric grafts transplanted into SCID-hu mice as a model for progenitor cell gene therapy using murine retroviral vectors (2, 6). This small-animal model system allows the investigation of marking of T-cell lineages in the human thymus throughout thymopoiesis, not easily addressed experimentally in the rhesus macaque model. SCID-hu thy/liv chimeric grafts transplanted 3 to 4 months previously were irradiated and injected with 5 × 10^5 human fetal liver CD34^+ cells transduced with an HIV-1 or MLV-based vector (SRLEGFP). Both the HIV-1 and MLV vector-transduced CD34^+ cells can reconstitute SCID-hu thy/liv mice (Fig. 4). At 4 weeks after introduction of CD34^+ cells into thy/liv implants, the percentage of EGFP-expressing cells ranged from 0.9 to 28% for HIV-1 vector-transduced implants and from 1.2 to 39% for MLV vector-transduced implants (Fig. 4A). We confirmed that the EGFP-expressing cells were human cells by using anti-CD45, a marker for human lymphocytes. Three-color flow cytometric analysis determined that thymocytes were first gated on EGFP-positive cells and subsequently analyzed for expression of CD4 and CD8, the distribution of CD4^+ and CD8^+ cells for HIV-1 or MLV vector-transduced cells was similar to that for mock-infected cells, indicating that progenitor cells common to CD4^+ and CD8^+ cell lineages were likely to have been transduced with both vectors (Fig. 4B, compare d and e with c).

The MLV vector resulted in gene expression higher than that of the HIV-1 based vector. In comparing the levels of EGFP expression for the MLV and HIV-1 vectors in transduced thymocytes, we noted that the HIV-1 vector expressed at levels of fluorescence intensity approximately 10-fold lower than that of the MLV vector. This difference was consistent among reconstituted implants (Fig. 4). Although we did not directly compare MLV and HIV-1 vector expression in rhesus macaques, the fluorescence intensity of the HIV-1 vector in SCID-hu thymocytes is similar to the fluorescence intensity in PBL in the rhesus macaques when analyzed with similar flow cytometric settings (data not shown).

**Activation of thymocytes does not induce increased level of EGFP expression.** In transplanted rhesus macaques, we found that EGFP expression could not be further induced following activation of PBMN (see above). We similarly tested whether we could induce EGFP expression following activation of the thymocytes. Thymocytes from transplanted SCID-hu mice were stimulated in vitro with IL-2 and anti-CD3 and anti-CD28 MABs for 3 days. The level of thymidine incorporation of the stimulated cells increased approximately 100-fold over that of nonstimulated cells. The level of EGFP expression, however, was only slightly induced following activation with anti-CD3 and IL-2 (Table 4). These results are consistent with our previously published studies with an MLV vector (6).

**The HIV-1 vector is expressed throughout thymopoiesis.** We previously demonstrated that a murine retroviral vector used to transduce human CD34^+ cells and transplanted to SCID-hu mice was expressed in various thymocyte subpopulations throughout thymopoiesis (6). We assessed whether gene expression directed by the HIV-1 vector was similarly expressed. For these studies, we modified the transduction and transplant protocol by using a new and more rapid approach to assess the HIV-1 vector. The vector was directly injected into irradiated thyliv implants of SCID-hu mice that had been irradiated to kill resident thymocytes and induce progenitor cell function. At 4 weeks after transplant of the transduced CD34^+ cells into thyliv implants, EGFP expression was detected in CD45^+ thymocyte populations (Fig. 5A). Similar percentages of EGFP-expressing cells were detected in all thymocyte subpopulations tested (CD1, CD3, CD4, CD5, and CD8), indicating vector expression throughout thymopoiesis (Fig. 5B). These results are consistent with that previously observed for the MLV vector (6).

**Reconstituted SCID-hu implants did not show any deleterious effects.** Human thymocytes of the SCID-hu thyliv mouse are highly susceptible to death when challenged with infectious replication-competent HIV-1. No toxic effects were observed in those implants receiving the HIV-1 or MLV vector-transduced CD34^+ cells. The percentages of CD4^+ , CD8^+ , and CD4^+ CD8^+ cells expressing EGFP remained similar to that of both mock-transduced and MLV vector-transduced animals (Fig. 4B). Thus, no HIV-1-associated pathogenicity was observed in the transduced animals, indicating that no replication-competent HIV-1 was present in the viral stocks or generated after transduction, consistent with the results observed in transplanted rhesus macaques.

### Table 4. EGFP expression after activation of thymocytes

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Stimulated thymocytes</th>
<th>Unstimulated thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% EGFP expression</td>
<td>[3H]thymidine incorporation (cpm)</td>
</tr>
<tr>
<td>HR1</td>
<td>20.0</td>
<td>64,410</td>
</tr>
<tr>
<td>HR2</td>
<td>15.3</td>
<td>250,660</td>
</tr>
<tr>
<td>HR3</td>
<td>7.6</td>
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</tr>
<tr>
<td>SR1</td>
<td>28.9</td>
<td>73,593</td>
</tr>
<tr>
<td>SR2</td>
<td>3.4</td>
<td>42,156</td>
</tr>
<tr>
<td>SR3</td>
<td>1.3</td>
<td>22,706</td>
</tr>
<tr>
<td>SR4</td>
<td>1.0</td>
<td>23,533</td>
</tr>
</tbody>
</table>

* Human thymocytes (10^7) obtained from reconstituted thyliv implants with HIV-1 vector (HR) or MLV vector (SR) at 4 weeks postreconstitution were cultured for 3 days in the presence of IL-2, anti-human CD28 MAB, and immobilized anti-human CD3 MAB. As a control, thymocytes were cultured in parallel in the absence of growth-stimulating agents. After 3 days of stimulation in vitro, cells were analyzed by flow cytometry for EGFP expression. [3H]thymidine incorporation was measured by pulse-labeling cells for 6 h on day 3.
DISCUSSION

Our results demonstrate that successful transplantation and marking are obtained in rhesus macaque and SCID-hu mice that have been transplanted with nonhuman and human CD34+ cells, respectively, transduced with an HIV-1-based lentivirus vector. Infusion of myeloablative rhesus macaques resulted in reconstitution and marking of lymphoid, myeloid, and granulocyte lineages in all animals. In the case of one animal with the greatest overall levels of marking, EGFP expression was observed in erythrocytes and platelets, not previously observed with MLV vectors (46). Parallel experiments conducted with human CD34+ cells in SCID-hu mice demonstrated reconstitution of thymopoiesis and evidence of vector throughout various stages of T-cell differentiation. Other investigators have demonstrated multilineage marking in a SCID-NOD model system (41); however, that model system did not examine T-lineage gene transfer and marking, critical for evaluation of efficacy and potential deleterious effects of a vector derived from HIV-1.

Gene expression in different cell types is dependent on the relative strength of promoters used. The HIV-1 vector uses an internal CMV promoter, whereas the murine retroviral vector uses the vector LTR. HIV-1 vector expression of EGFP as monitored by the mean fluorescence intensity in lymphocyte subpopulations was relatively weak. This level of expression was at least 10-fold lower than that for the MLV-based vector in SCID-hu thymocytes. Although rhesus macaque lymphocytes were marked, only a low fluorescence intensity of expression from the HIV-1 vector was detected following transplant, about fivefold lower than expression in rhesus macaque granulocytes, consistent with the lower level of expression observed in the SCID-hu thy/liv thymocytes. For both human and rhesus macaque lymphoid populations, no increase in expression was observed following ex vivo T-cell activation.

We cannot say with certainty whether we have successfully transduced in rhesus macaques a pluripotent hematopoietic stem cell, but several lines of evidence suggest that the HIV-1 vector may have transduced an early progenitor cell prior to commitment to the myeloid or lymphoid pathway. First, the relative degrees of marking for both lymphoid and granulocyte compartments are similar for each rhesus macaque as determined by PCR. Second, these relative proportions have either remained stable or even increased in certain instances 30 weeks following reconstitution (data not shown). Third, in the animal with highest overall levels of marking, EGFP expression could be observed in multiple hematopoietic lineages, including erythrocytes and platelets. Additional studies, however, will be required to improve transduction efficiency and the level of EGFP expression in desired subpopulations of hematopoietic cells.

Concerns have been raised regarding the use of vectors derived from HIV-1 in humans (4). Since these vectors are defective for HIV-1 envelope and generated by cotransfection with a VSV-G-expressing envelope vector, it is highly unlikely that a replication-competent virus could be formed from recombination between members of two distinct families of viruses. Nevertheless, we formally showed in both transplanted rhesus macaques and SCID-hu mice that replication-competent HIV-1 did not result. Therefore, we believe that lentivirus vectors in principle are suitable for use in humans; however, further refinements in vector design including enhanced expression will be necessary before lentivirus vectors can be used effectively for gene delivery.

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REFERENCES


