Development of a high-titer retrovirus producer cell line capable of gene transfer into rhesus monkey hematopoietic stem cells

DAVID M. BODINE, KEVIN T. MCDONAGH, STEPHEN J. BRANDT, PAUL A. NEY, BRIAN AGRICOLA, ELLEN BYRNE, AND ARTHUR W. NENHUIS

Clinical Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Communicated by Helen M. Ranney, February 12, 1990 (received for review December 19, 1989)

ABSTRACT Retroviral-mediated gene transfer into primitive hematopoietic cells has been difficult to achieve in large-animal models. We have developed an amphotropic producer clone that generates \(10^6\) recombinant retroviral particles (colony-forming units) per ml of culture medium. Autologous rhesus monkey bone-marrow cells were cocultured with either high (\(2 \times 10^5\)) or low (\(5 \times 10^4\)) numbers of producer cells for 4–6 days and reinfused into sublethally irradiated animals. The proviral genome was detected in blood and bone-marrow cells from all three animals 1 week after reinfusion, and low-titer producer cells cocultured with the high-titer producer cells. In contrast, three animals reconstituted with bone marrow cocultured with the low-titer producer clone exhibited no evidence of gene transfer.

Retroviral-mediated gene transfer (1, 2) has been successfully applied to the insertion and expression of various genes in murine stem cells, including the bacterial neomycin resistance \((\text{neo}^r)\) (3–5), human dihydrofolate reductase (6), murine interleukin 3 (IL-3) (7), murine granulocyte–macrophage colony stimulating factor (GM-CSF) (8), and human adenosine deaminase (ADA) (9–11) and human \(\beta\)-globin genes (12–14).

Three large-animal models have previously been used in gene-transfer experiments. In a canine model, six dogs were reconstituted with autologous bone marrow cocultured with retrovirus-producing cells, but only a very low level of drug-resistant progenitor cells were detected in three of the animals (15). Drug-resistant progenitor cells were detected in the peripheral blood of 6/10 newborn lambs that had been infused in utero with autologous infected circulating blood cells. The provirus was detected in DNA from two of the lambs with the polymerase chain reaction (PCR) (16). Gene transfer of the human ADA gene was attempted in a series of 20 cynomolgous (Macaca fascicularis) or rhesus (Macaca mulatta) monkeys. None of the rhesus monkeys exhibited evidence of gene transfer. Two of the cynomolgous monkeys had detectable human ADA in peripheral blood cells at levels of 0.2 and 0.5% of that of the endogenous monkey enzyme, and three other animals had much lower levels of human ADA. The expression of human ADA was transient and could not be detected beyond 90 days after transplantation in any of the animals studied. In situ hybridization and in vitro selection of drug-resistant cells suggested that gene transfer may have been to long-lived T lymphocytes in these animals (17, 18).

The efficiency of retroviral-mediated gene transfer into murine stem cells is influenced by the titer of the recombinant virus (9), preconditioning of the donor marrow with 5-fluorouracil (5-FU) (3, 4, 6) and the addition of the hematopoietic growth factors IL-3 (3, 4) and interleukin 6 (IL-6) (13). Titer is defined as the concentration of infectious retroviral particles that accumulate in the culture medium of producer cells during 24-hr growth from subconfluent to confluent state. Most producer clones useful for gene transfer into murine stem cells have titers of \(10^6\)–\(10^7\) infectious retroviral particles per ml.

We have adapted a superinfection strategy to generate an extremely high-titer producer cell line. When amphotropic and amphotropic producer cell lines are present in the same culture, amphotropic producer cells can be superinfected by virus packaged in the amphotropic partner and vice versa. Bestwick et al. (19) have shown that coculture of amphotropic and amphotropic retrovirus producer cells allows increased expression of genes carried by recombinant retroviruses by amplification of the proviral genome (19). Danos and Mulligan (20) have shown that cocultivation of amphotropic and amphotropic producer cells can amplify replication-competent virus present at undetectable levels in the original cell lines.

We have developed a cell line that secretes gibbon IL-3 and human IL-6 and produces \(10^6\) functional viral particles per ml of culture medium. This cell line has been used to reproducibly transfer genes into bone-marrow stem cells of rhesus monkeys.

METHODS

Cells and Viruses. All pA317 (21) cells and their derivatives were maintained in improved minimal essential medium (IMEM; Biofluids, Rockville, MD) with fetal calf serum (FCS; American Biotechnologies, Columbia, MD). \(\phi\)8S1 (22) and GP+E86 (23) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Biofluids) with 10% newborn calf serum (NCS; Gibco). A pA317 cell line producing \(5 \times 10^6\) infectious particles per ml of the N2 (24) virus was generated by standard techniques (25). This cell line was cotransfected with the plasmids pXM-gII-3 (26), pXM-IL-6 (27), and PMohg, which contain the gibbon IL-3, the human IL-6, and the hygromycin resistance genes, respectively. Transfected cells were isolated by selection in hygromycin at 200 \(\mu\)g/ml (Sigma).

Amplification of Retrovirus Production. N236 and GP+E86 cells were mixed in ratios of 3:1, 1:1, and 1:3. A total of \(5 \times 10^3\) cells from these mixtures were seeded onto 10-cm plates in 10 ml of DMEM/10% newborn calf serum. These cultures were split 1:3 upon reaching confluence, usually every 2–3 days, for a 17-day period. On day-17 of coculture, the supernatant of the culture was assayed for virus production, and the cultures were plated out at limiting dilutions in 1% FCS containing hygromycin at 200 \(\mu\)g/ml, and individual amphotropic clones were isolated. Similar mixtures of \(\phi\)8S1 (amphotropic cell line producing a virus containing the human \(\beta\)-globin gene) and pA317 cells were cocultured exactly as above, and individual ecotropic clones were

Abbreviations: PCR, polymerase chain reaction; IL-3 and IL-6, interleukin 3 and 6, respectively; ADA, adenosine deaminase; 5-FU, 5-fluorouracil; cfu, colony-forming units; GM-CSF, granulocyte–macrophage colony stimulating factor; FCS, fetal calf serum.
isolated by selection in hypoxanthine at 15 µg/ml, xanthine at 250 µg/ml, and mycophenolic acid at 25 µg/ml.

**Retrovirus Titer Assays.** Retrovirus production (titer) by amphotrophic producer cell lines was assayed on HeLa cells, whereas the titer of mixed pools of producer cells or ecotropic producer cells was assayed on NIH 3T3 cells. Six centimeter plates were seeded with 1 x 10⁶ HeLa or NIH 3T3 cells per plate in 5 ml of IMEM/10% FCS. Twenty-four hours later the medium on these plates was replaced with 5 ml of serial 10-fold dilutions of medium conditioned for 24 hr by confluent cultures of producer cells. Polybrene (Sigma) was added to a final concentration of 6 µg/ml. Twenty-four hours later, the virus-containing medium was replaced with fresh medium. After another 24 hr the medium was replaced with 5 ml of medium plus G418 at 0.48 mg/ml (active). Selection was allowed to proceed for 10–14 days. Medium was changed as necessary during the selection process. Macroscopic colonies were stained with crystal violet and counted.

Retroviral RNA for slot blot analysis of retrovirus production was extracted from medium conditioned for 24 hr by producer cells. Nine milliliters of medium was centrifuged for 90 min at 45,000 rpm in a 50Ti rotor. RNA was extracted from the pellet by the method of Berger and Birkenmeier (28), except that the lysate P-40 lysate was omitted. Replication-competent or wild-type virus production was assayed directly on D56 cells by the method of Bassen et al. (29). Alternatively, serial 10-fold dilutions of medium conditioned by producer cells were added to NIH 3T3 cells seeded onto 6-cm plates, as described above, in the presence of Polybrene at 6 µg/ml. The cells were maintained for 14 days to allow wild-type virus to spread in the culture. On day-14, 1 ml of the medium from each culture was assayed directly on D56 cells, as described above.

**Infection Protocol.** On day-1, 10 confluent plates of producer cells were split 1:10 in DMEM/10% FCS (HyClone) containing Polybrene at 6 µg/ml (Sigma). Also on day-1, bone marrow was aspirated from the femurs and ischia of adolescent (≤4 kg) rhesus monkeys in DMEM/2% FCS (HyClone) containing heparin at 10 units/ml. Mononuclear cells were isolated by centrifugation through lymphocyte separation medium (Organon Teknika–Cappel), as directed by the supplier. Typically 3–5 x 10⁶ cells were collected from each animal, and 1–2 x 10⁶ mononuclear cells were isolated for infection. Approximately 1–2 x 10⁶ cells were added to each plate, and cocultivation was allowed to proceed for 3 days. On day-3, an additional 10 confluent plates of producer cells were split 1:10. The nonadherent cells were collected from the original plates and reseeded on the fresh plates for an additional 3 days. On day-3 and -4, the donor animal was given 1 R (1 R = 0.258 mCi/kg) total body irradiation. On day-6, the nonadherent cells were collected, washed in Hanks’ balanced salt solution, and infused into the irradiated donor in 50 ml of phosphate-buffered saline/2% FCS (HyClone) containing heparin at 10 units/ml. In some experiments, human IL-3 (~200 units/ml) was added to the cultures. Forty-eight hours after autologous transplantation, a continuous infusion of GM-CSF (50 units/kg per day) was administered by intravenous injection (experiment 1). The mouse gene-transfer experiments were similar to the protocol described above except for the following: (i) unfraccionated 48-hr post-5-FU bone marrow from C57BL/6J donor mice was used, (ii) the cultures were supplemented with murine IL-3 at 200 units/ml, and (iii) genetically anemic WBB6F/M/W/W mice (The Jackson Laboratory) were used as recipients (3, 22, 31).

PCR analysis of the Northern-blot step was specific for the N2 provirus was amplified using the following primers: left-hand primer, 5’-GGACCTTGACAGATAGCGT-3’ (hybridizes to the neo’ gene) and right-hand primer, 5’-CTGGTTCCTGACCTTGGTGTG-3’ (hybridizes to the residual envelope-coding sequences in the provirus). A 193-bp sequence specific for the ecotropic Moloney murine leukemia virus envelope genome was amplified by using the following primers: left-hand primer, 5’-CACCTGGAGATATCGTGCAATGG-3’ and right-hand primer, 5’-TGTCGCCCTGAGCAACGGT-3’. A 227-bp sequence specific for the amphotropic Moloney murine leukemia virus envelope genome was amplified by using the following primers: left-hand primer, 5’-GGATGGAGATGCGCAGAGC-3’ and right-hand primer, 5’-CTGTCCTCCTGGGTCATTCT-3’. Five hundred nanograms of DNA was amplified according to the specifications of the manufacturer (United States Biochemical), except that 1 µl of [α-32P]dCTP (800 mCi/mmol; Amersham) was added to each reaction. Analysis was performed for 20 cycles with hybridizing temperature of 52°C. The amplified products were visualized by autoradiography.

**RESULTS**

**Generation of High-Titer Producer Cell Line.** Amphotropic retroviruses can infect cells from a wide variety of species, including mice and primates (21). A pA317 cell line producing 5 x 10⁸ infectious particles per ml of the N2 (24) virus was cotransfected with plasmids containing the gibbon IL-3, human IL-6, and hybrid retrovirus genes. A hygromycin-resistant population, designated N263, was isolated, which produced gibbon IL-3 at 10 units/ml, human IL-6 at 50 units/ml, and 5 x 10⁶ colony-forming units (cfu)/ml. To increase recombinant retrovirus production and minimize the generation of replication-competent virus, amphotropic N263 cells were cocultivated with ecotropic GP+EB6 cells for 14 days. The plasmid was used to infect the GP+ cell line of N263 and GP+866 cell lines required resistance to the amphotropic virus only. Similar results were obtained when an ecotropic producer cell line, pSFR1, was cocultivated with pA317 cells in a separate experiment (experiment 2).

**Characterization of the High-Titer Cell Line.** One high-titer amphotropic producer cell line (designated N263A2) was identified that secreted gibbon IL-3 at <10 units/ml, human IL-6 at 50 units/ml, and 2 x 10⁶ cfu/ml. Southern blot analysis of DNA from N263A2, N263, and NIH 3T3 cells with Sst I revealed that the parental N263 cell line had a 3.2-kilobase (kb) band representing a single unrearranged copy of the provirus (Fig. 1A and data not shown), and the N263A2 cell line contained >20 unrearranged copies of the N2 genome. Increased production of recombinant retrovirus was documented by analysis of retroviral RNA extracted from medium conditioned for 24 hr by the parental N263 and N263A2 cell lines. Southern blot analysis of RNA from these cells using a neo’ probe showed that the expanded retrovirus production events were retained. N263A2 cell line produced >10-fold more retroviral RNA than the parental N263 cell line (Fig. 1B).

**Recombinant-competent virus production by N263 and N263A2 cells was assayed by mobilization of a sarcoma virus genome in D-56 cells, resulting in plaque formation (29). Direct infection of hygromycin resistance genes.**
passaged for 14 days to allow helper virus to spread and amplify in the cultures. Medium conditioned by these cells was then assayed directly for replication-competent virus as above. The greatest dilution of N263 and N263A2-conditioned medium that contained replication-competent virus was 10^{-1} and 10^{-4}, respectively (Table 1).

Primers specific for ecotropic or amphotropic envelope sequences were used in a PCR to identify these genomes in cellular DNA. Amphotropic envelope sequences were detected at similar intensities in pA317, N263, and N263A2 cell DNA, suggesting no amplification of the amphotropic genome during cocultivation with GP+E86 cells (Fig. 2A). Ecotropic envelope sequences were detected in ψ-2 and N263A2 cell DNAs, indicating transfer of ecotropic envelope sequences during amplification. The amount of amphotropic envelope protein expressed on the surface of pA317, N263, and N263A2 cells was analyzed with monoclonal antibodies provided by L. Evans (Laboratory of Persistent Viral Infections; Hamilton, MT). Fluorescence-activated cell sorter analysis showed that N236A2 cells had 4- to 5-fold more amphotropic envelope protein on the cell surface than either pA317 or N263 cells (Fig. 2B). A small (<1%) that seen on ψ-2 cells) amount of ecotropic envelope protein was detected on the surface of N263A2 cells (data not shown).

High Retrovirus Titer Increases Gene Transfer to Mouse Stem Cells. To test the effects of extremely high concentrations of recombinant virus on gene-transfer efficiency, bone-marrow cells isolated from mice treated 48 hr previously with 5-FU were cocultivated with N263 or N263A2 cells for 6 days in the presence of murine IL-3 at 200 units/ml. Because both the N263 and N263A2 cell lines secrete ~50 units of human IL-6 per ml of conditioned medium, these conditions are nearly identical to those we had previously shown adequate for gene transfer into murine stem cells (13). Three months after transplant, DNA extracted from the peripheral blood and bone marrow of these animals was analyzed by PCR for the N2 provirus. Only 2 of 13 mice reconstituted with cells cocultivated with N263 cells were positive for the provirus (Fig. 3A).

### Table 1. Generation of high-titer producer cell lines

<table>
<thead>
<tr>
<th>Ecotropic producer</th>
<th>Pool, cfu/ml</th>
<th>Recombinant virus</th>
<th>Replication-competent virus, cfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP+E86</td>
<td>1 × 10^6</td>
<td>Exp. 1</td>
<td>A1: 6 × 10^3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A2: 2 × 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A3: 1 × 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A4: 3 × 10^3</td>
</tr>
<tr>
<td>ψPS1</td>
<td>5 × 10^8</td>
<td>Exp. 2</td>
<td>E3: 6 × 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E7: 6 × 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E8: 8 × 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E12: 4 × 10^4</td>
</tr>
</tbody>
</table>

Exp., experiment. A1–E12, individual cell lines isolated from large pool of cells; A, amphotropic; E, ecotropic.

*5 × 10^6 cfu/ml.

†2 × 10^6 cfu/ml.

### Fig. 1. (A) Southern blot analysis of the N263 and N263A2 cell lines. Ten μg of DNA was digested with Sst I (which cuts once in each proviral long terminal repeat), run on 1% agarose gel, blotted, and probed with a neo' probe. (B) Slot blot analysis of RNA extracted from medium conditioned by the N263 and N263A2 cell lines. Serial 10-fold dilutions of RNA were blotted and probed with a neo' probe. UD, undiluted.

### Fig. 2. (A) PCR detection of amphotropic (Left) and ecotropic (Right) envelope genomes in various cell lines. Primers specific for each envelope were used to amplify sequences specific for the amphotropic or ecotropic envelope genome in DNA isolated from the indicated cell lines. The ecotropic sequence amplification is slightly off center; bands appear in the ψ-2 and N263A2 lanes. (B) Fluorescence-activated cell sorter analysis of amphotropic envelope gene expression on N263 (Left) and N263A2 (Right) cells. ---, Profile of cells treated with an irrelevant antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG. ---, Profile of cells treated with a monoclonal antibody against amphotropic Moloney murine leukemia virus envelope protein and fluorescein isothiocyanate-conjugated goat anti-mouse IgG.
Our experiments support the utility of recombinant retroviral vectors for insertion of genes into primate hematopoietic stem cells. Three successive animals have exhibited evidence of gene transfer into cells capable of contributing to long-term hematopoietic reconstitution. There are several differences between our protocol for gene transfer in large animals and those used previously. We cocultivated our bone-marrow cells with producer cells for 2-6 days, exposing them to retrovirus particles for much longer times than did previous protocols. In the canine model, bone-marrow cells were cocultivated with producer cells for 24 hr before reinfection into the recipient (15). In the fetal sheep model, circulating progenitor cells were cultured for 24 hr in medium already conditioned by producer cells for 24 hr (16). In previous primates both 24-hr cocultivation or 2- to 24-hr culture in producer cell-conditioned medium were used (17, 18). Lim et al. (10) and we (13) have shown that gene transfer to murine stem cells is enhanced by extending the infection period, presumably by allowing time for stem cells to enter the cell cycle. The producer cell lines used in the canine, fetal sheep, and previous primate studies had generated 5 × 10^6 and 2 × 10^6 retroviral particles per ml, ~500-fold lower than the titer of the virus produced by N263A2 cells. In summary, prior protocols using shorter culture periods and lower titer viruses gave inconsistent infection, and in some cases the transduced cell may have been other than hematopoietic cells capable of long-term reconstitution.

Our initial studies have identified several problems that must be resolved. All our animals infused with bone-marrow cells cocultured with virus-producing cells required an average of 20 days to reach a neutrophil count of 1000/mm^3. Previous studies have shown that by 3 weeks after irradiation, significant regeneration of endogenous marrow cells can be expected (32), even without the administration of GM-
CSF. Therefore, our transplanted autologous cells are likely to be diluted by cells derived from stem cells that survived the irradiation protocol. Complications of the transplant procedure and prolonged panmyelopenia necessitated sacrifice of three positive animals 32, 54, and 99 days after transplantation, limiting the period of observation with respect to the long-term persistence of the transfused gene. The optimum conditions to maintain and infect rhesus stem cells in culture must be defined.

Secondly, the large number of cells required to reconstitute a primate species, including man, makes the infection of unfractionated bone-marrow cells an impractical approach for gene transfer into stem cells. With current protocols, the 2–4 × 10^8 cells required to reconstitute a human would require injection in 30–60 liters of medium. Murine and primate hematopoietic stem cells have been purified and retain their ability to reconstitute irradiated animals (33, 34). Recent studies have shown that a high percentage of partially purified mouse bone-marrow stem cells can be marked with retroviruses with a titer of 10^6 cfu/ml (35). Purification or partial purification of primate or human stem cells would reduce the number of cells to be infected while increasing the multiplicity of infection.

Finally, superinfection by coculturing ecotropic and amphotropic producer cells has been shown to increase the production of virus by these cells. We have shown that individual clones producing exceptionally high numbers of retroviral particles can be isolated from a pool of producer cells. The role of replication-competent virus in increasing retroviral production by producer cells is not clear. All of the high-titer cell lines we have examined also produce replication-competent virus, but the level of helper virus production does not correlate with the titer of the recombinant virus. Amplification of replication-competent virus production by cocultivation will inhibit superinfection by the recombinant virus if ecotropic replication-competent virus infects amphotropic producer cells or vice versa. Packaging cell lines designed to prevent replication-competent virus formation have been developed and should be valuable in this context (20, 36).

The authors thank Steve Clark for the plasmids pXM-gIL-3 and pXM-IL-6, recombinant/human GM-CSF, recombinant/human IL-3, and for help with the IL-3 assays. We also thank Nancy Seidel for expert technical assistance and L. Evans for providing the monoclonal antibodies against amphotropic and ecotropic envelope proteins.

---

**Table 2. Time of reconstitution in animals infused with cocultured bone-marrow cells**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Producer cell line</th>
<th>Days in culture</th>
<th>Cells infused, no.</th>
<th>Neutrophils &gt;1000/mm³</th>
<th>Platelet &gt;35,000</th>
<th>Reticulocytes &gt;2%</th>
<th>Provirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>001E</td>
<td>N263</td>
<td>2</td>
<td>1.5 × 10⁹</td>
<td>13</td>
<td>22</td>
<td>23</td>
<td>–</td>
</tr>
<tr>
<td>0511</td>
<td>N263</td>
<td>6</td>
<td>1.0 × 10⁹</td>
<td>18</td>
<td>26</td>
<td>19</td>
<td>–</td>
</tr>
<tr>
<td>0457</td>
<td>N263</td>
<td>4</td>
<td>1.8 × 10⁹</td>
<td>8</td>
<td>23</td>
<td>22</td>
<td>–</td>
</tr>
<tr>
<td>273E</td>
<td>N263A2</td>
<td>6</td>
<td>1.2 × 10⁹</td>
<td>19</td>
<td>29</td>
<td>29</td>
<td>+</td>
</tr>
<tr>
<td>0466</td>
<td>N263A2</td>
<td>6</td>
<td>1.3 × 10⁹</td>
<td>22</td>
<td>*</td>
<td>*</td>
<td>+</td>
</tr>
<tr>
<td>0504</td>
<td>N263A2</td>
<td>6</td>
<td>2.5 × 10⁹</td>
<td>25</td>
<td>31</td>
<td>29</td>
<td>+</td>
</tr>
</tbody>
</table>

Average of five animals in a previous study 0 1.2 × 10⁹ 9 18

*Persistent infections hampered the analysis of this animal.

*This animal was pretreated with 5-FU (150 mg/kg).