Mouse Fetal Liver Cells Lack Functional Amphotropic Retroviral Receptors

By Christine Richardson, Maureen Ward, Silvio Podda, and Arthur Bank

We have been transducing mouse hematopoietic cells with the human MDR1 (MDR) gene in retroviral vectors to determine the optimal conditions for retrovirai gene transfer as a model system for potential human gene therapy. In these studies, we have demonstrated transduction and expression of the human MDR gene using ecotropic and amphotropic MDR-retroviral producer lines. To obtain more mouse hematopoietic cells for detailed study, mouse fetal liver cells (FLC) have been used for MDR transduction and expression, and to reconstitute the ablated marrows of adult mice. FLC contain hematopoietic cells that have a reconstituting capacity comparable to that of adult mouse bone marrow cells. However, to our surprise, FLC can only be transduced with ecotropic retrovirus and not with amphotropic virus. This restriction of transduction of FLC cannot be overcome by higher titer virus. The resistance to amphotropic transduction by FLC may be part of a changing developmental program that results in a different antigen repertoire on FLC as compared with adult bone marrow cells.

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Preparation of MDR lines. GP + E86 ecotropic packaging cells or GP + envAm12 amphotropic packaging cells were transfected with the retroviral vector N2 (containing the neoR gene) by the calcium phosphate precipitate method and selected in medium containing 400 μg/mL of G418, as described. Clones releasing the highest titers (5 \times 10^4 for the ecotropic line and 5 \times 10^5 for the amphotropic line) were maintained as viral producer lines.

Preparation of viral supernatants. Forty percent of confluent viral producer cell cultures were incubated for 24 hours with a minimum essential medium (αMEM) containing 15% fetal calf serum (FCS), 15% WEHI conditioned medium, and 1% penicillin/streptomycin solution. Supernatants were then passed through a 0.45-μm filter.

Fetal Liver Transduction

Harvest of FLC. FLC were harvested from day-15.5 pc C57BL/6J fetuses. Single-cell suspensions were made by forcing FLC through a 0.625-mm wire mesh and then by sequential pipetting with a 5-mL syringe and 20-gauge needle.

Cytospin preparation. A 1 \times 10^6 cell aliquot of the resultant FLC suspension was washed once in 1X phosphate-buffered saline (PBS) and resuspended in 100 μL. The cytopsin preparation was spun at 250 rpm for 3 minutes. The resultant slide was dried and then stained using the Diff-Quik kit (Scientific Products, McGaw Park, IL).

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**FLC transduction.** Cell aliquots of $2 \times 10^7$ were prestimulated for division in T175 flasks (Nunc, Glostrup, Denmark) containing 50 mL αMEM containing 15% FCS, 15% WEHI conditioned medium, 1% penicillin/streptomycin solution (GIBCO, Grand Island, NY), interleukin-6 (200 U/mL), and stem cell factor (2.5 U/mL) in aMEM containing 15% FCS and 0.2% NaNO₃. Cells were incubated for 12 to 24 hours at 37°C. Cultures were then exposed to viral supernatants and polybrene (8 μg/mL) for 24 to 48 hours and incubated at 37°C. Cells were incubated for 12 to 24 hours at 37°C. Cultures were then exposed to viral supernatants and polybrene (8 μg/mL) for 24 to 48 hours and incubated at 37°C. Cells were incubated for 12 to 24 hours at 37°C. Cultures were then exposed to viral supernatants and polybrene (8 μg/mL) for 24 to 48 hours and incubated at 37°C.

**Analysis of Transduced Cells**

**Preparation of transduced FLC for polymerase chain reaction (PCR).** Aliquots of $2 \times 10^7$ cells were washed once with 1X PBS. Cells were incubated in lysis buffer containing proteinase K (0.6 mg/mL) at 55°C overnight. DNA was then phenol extracted, ethanol precipitated, and resuspended in TE, pH 7.0. PCR was performed using 1 μg of DNA with 1 U of AmpliTaq polymerase and appropriate reaction mix (Perkin Elmer/Cetus, Norwalk, CT) in a final volume of 50 μL. For amplification of MDR and N2 sequences, each of 35 cycles included 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, and 60 seconds of extension at 72°C. MDR-specific sequences were amplified using the sense-strand primer CCCATCATTGCAATAGCAGC (residues 2596-2615) and the antisense-strand primer CAGTAAGTGGTGGTACCGAT. Ten microliters of the PCR reaction product was examined on a 4% agarose/NuSieve gel for the presence of the expected band. For amplification of MDR and N2 sequences, each of 35 cycles included 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, and 60 seconds of extension at 72°C. MDR-specific sequences were amplified using the sense-strand primer CCCATCATTGCAATAGCAGC (residues 2596-2615) and the antisense-strand primer CAGTAAGTGGTGGTACCGAT. Ten microliters of the PCR reaction product was examined on a 4% agarose/NuSieve gel for the presence of the expected band.

**FACS analysis.** Posttransduction aliquots of $1 \times 10^8$ cells were stained with 1 μg of a human MDR monoclonal antibody, 17F9, for 20 minutes on ice. The 17F9 antibody was a gift of Dr David Ring (Chiron, Emeryville, CA). Cells were washed once with 1X PBS with 3% FCS and 0.2% NaNO₃. Cells were then stained with 1 μg of an IgG2b secondary antibody conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) for 20 minutes on ice. Cells were washed once and resuspended in 300 μL of 1X PBS with 3% FCS and 0.2% NaNO₃. Dead cells were excluded from analysis by propidium iodide staining. Analysis was performed on a FACStar Plus (Becton Dickinson, Mountain View, CA).

**Progenitor assays.** Aliquots of $2.5 \times 10^7$ postransduction cells were plated in Iscove’s modified Dulbecco’s medium with 30% FCS, 50 mmol/L 2-mercaptoethanol, 10% WEHI conditioned medium, erythropoietin (2 U/mL), and stem cell factor (2.5 U/mL). Cultures were incubated for 10 days and hemoglobinized BFU-E and CFU-GM colonies were scored and picked under an inverted microscope. Harvested colonies were washed once with 1X PBS. Cells were incubated in lysis buffer containing proteinase K (0.6 mg/mL) at 55°C for 1 hour, and then at 94°C for 10 minutes to inactivate the proteinase K. PCR on the lysates was performed as described above with 25 μL of the resultant lysate used for each reaction.

**Spleen colony analysis.** C57BL/6 female mice were gamma irradiated with 1,100 rad administered in two doses 3 hours apart. Mice were injected via tail vein with aliquots of $1 \times 10^7$ FLC transduced with amphotropic MDR supernatant or $1 \times 10^7$ FLC transduced with ecotropic MDR supernatant or $1 \times 10^7$ FLC transduced with amphotropic MDR supernatant or $1 \times 10^7$ FLC transduced with ecotropic MDR supernatant. On day 10 posttransplantation, the mice were killed and spleens were harvested. Individual spleen colonies indicative of the seeding of CFU-S were dissected out and incubated in lysis buffer containing proteinase K (0.6 mg/mL) at 55°C overnight. DNA was then phenol extracted, ethanol precipitated, and resuspended in TE, pH 7.0. PCR analysis was performed as described above.

**Analysis of Amphotropic Receptor mRNA**

Poly A+ RNA was prepared from aliquots of $5 \times 10^7$ FLC using Micro-FastTrack Kit (Invitrogen, San Diego, CA). Reverse transcription and PCR (RT-PCR) were performed on varying dilutions...
of the poly A+ RNA from FLC, murine adult tissues, and yeast using a StrataScript RT-PCR Kit (Stratagene, LaJolla, CA) and the supplied random primers. Using recently published sequences for the amphotropic Moloney receptor gene, appropriate primers for RT-PCR analysis were designed. Amphotropic receptor cDNA specific sequences were amplified using the sense-strand primer TGAGACTGCACTTCTGCTG (residues 401-420) and the antisense-strand primer ACTCTCATGATTCTG (residues 1001-1020), which yield a 619-bp product. Control primers specific for murine β-actin cDNA (Clontech, Palo Alto, CA) yield an approximately 500-bp product, and primers for yeast endogenous sequences were amplified using the sense-strand primer CAGAGCATCT- CAAAATGAAACC and the antisense-strand primer TTTGGAATA- TACGGAGACG, yielding a 600-bp product. For amplification of cDNA sequences, each of 35 cycles included 60 seconds of denaturation at 94°C, 60 seconds of annealing at 60°C, and 120 seconds of extension at 72°C. Ten microliters of the PCR reaction product was examined on a 4% agarose/NuSieve gel for the presence of the MDR cDNA, as expected (data not shown). These cells again show an enhanced signal for the endogenous murine homolog. We have previously shown with this antibody that MDR producer lines express high levels of p-glycoprotein, whereas untransfected NIH3T3 and untransduced murine cells show no expression. In cells exposed to ecotropic MDR virus, 10%, 21%, and 41% of cells in three different experiments contained significantly increased levels of p-glycoprotein (Fig 3). These transduced cells that express high levels of p-glycoprotein were collected by FACS and PCR was performed on the DNA of the sorted population (data not shown). These cells again show an enhanced signal for the presence of the MDR cDNA, as expected (data not shown). By contrast, as expected, FLC exposed to amphotropic MDR virus do not demonstrate any fluorescence levels above background, indicating a lack of MDR expression (Fig 3). The small number of amphotropic MDR-transduced cells demonstrating fluorescence levels above the set FACS analysis gate were sorted and PCR was performed; no PCR signal was seen (data not shown).

We thought that these results might be caused by relative differences in retroviral titer between the amphotropic (5 x 10^5) and ecotropic (5 x 10^5) MDR producer lines. The negative results of transduction with amphotropic MDR virus could be caused by low titer. To eliminate this possibility, transduction of the FLC was repeated using high-titer amphotropic and ecotropic lines containing the neoR gene instead of the MDR gene; these neoR producer lines have titers of 10^6 and 5 x 10^6, respectively. The results of PCR on cells exposed to the neoR viral particle-containing supernatants are similar to the results seen with MDR virus (Fig 4 and Table 1). Cells exposed to ecotropic viruses show a strong PCR signal, indicating integration of the neoR DNA. By contrast, no PCR signal is seen when cells are exposed to amphotropic virus (Fig 4 and Table 1).

The negative results of transduction of total FLC with samples exposed to amphotropic virus do not show a PCR signal (Fig 1 and Table 1). Cell samples tested immediately and 72 hours after transduction show the same results (data not shown). The MOI was increased from 1 to 10, but no PCR signal is seen (Table 1), suggesting that the lower titer of amphotropic MDR virus was not rate-limiting for transduction. By contrast, adult bone marrow cells exposed to the same amphotropic MDR retroviral supernatants give a strong positive PCR signal immediately after transduction, and the peripheral blood of 7 of 10 mice transplanted with these transduced cells give a positive MDR PCR signal 50 days posttransplant (data not shown).

To ensure that the cells exposed to the retroviral supernatants were hematopoietic, a cytospin preparation of the harvested FLC suspension was made. Figure 2 shows that the majority of the cells exposed to retrovirus are hematopoietic and possess a blast morphology. Hepatocytes are very fibrous, but hematopoietic cells readily form a single-cell suspension. Thus, the nonhematopoietic cells are largely left behind when the midgestational fetal livers are forced through the wire mesh.

Expression of the human MDR gene product, p-glycoprotein, on the surface of the transduced cells was analyzed by FACS. The monoclonal antibody 17F9 recognizes an external epitope of the human p-glycoprotein without any cross-reactivity to the endogenous murine homolog. We have previously shown with this antibody that MDR producer lines express high levels of p-glycoprotein, whereas untransfected NIH3T3 and untransduced murine cells show no expression. In cells exposed to ecotropic MDR virus, 10%, 21%, and 41% of cells in three different experiments contained significantly increased levels of p-glycoprotein (Fig 3). These transduced cells that express high levels of p-glycoprotein were collected by FACS and PCR was performed on the DNA of the sorted population (data not shown). These cells again show an enhanced signal for the presence of the MDR cDNA, as expected (data not shown).

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The negative results of transduction of total FLC with
amphotropic MDR virus do not exclude the possibility that some rare FLC in the population express the amphotropic receptor. To examine this possibility, we first evaluated the sensitivity of the PCR reaction using the MDR-specific primers. In several experiments, the sensitivity of the PCR reaction in which MDR-positive and -negative cells were mixed was shown to be 1:200 (data not shown). Thus, any rare FLC population expressing the amphotropic receptor must comprise less than 0.5% of the isolated FLC population. Furthermore, after exposure to MDR viral particle-con-
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Fig 4. PCR analysis of N2-transduced FLC. PCR was performed on 1 μg of DNA from transduced populations as described in Materials and Methods. The arrow at the left indicates the expected band size of 349 bp. Lane 1, untransduced cells; lane 2, ecotropic N2-transduced cells; lane 3, amphotropic N2-transduced cells; lane 4, N2 plasmid; lane 5, 1× PCR buffer; lane 6, H2O; lane 7, Phi X marker.

taining supernatants, FLC were placed into methylcellulose cultures and day-10 progenitor colonies were characterized by PCR for the presence of the human MDR gene. Eight of 10 colonies assayed after exposure to ecotropic virus give a strong positive PCR signal (data not shown). However, none of 15 BFU-E and none of 30 CFU-GM colonies assayed after exposure to amphotropic virus are positive for a PCR signal (data not shown). The amphotropic supernatants were shown to efficiently transduce NIH3T3 cells, as expected. Thus, these progenitor populations in the total FLC population cannot be transduced by amphotropic virus. To ensure that the lack of PCR signal from cells exposed to amphotropic virus was not caused by some agent in the DNA preparations inhibiting the PCR reaction, primers for the endogenous murine HPV5 locus were used for a PCR control. All 55 colonies show a strong positive PCR signal (data not shown).

A second population of rare cells in FLC that may express the amphotropic receptor are those capable of forming CFU-S colonies. To examine the ability of this progenitor population to be transduced, FLC were exposed to ecotropic or amphotropic MDR viral particle-containing supernatants and injected into lethally irradiated mice via the tail vein. Day-10 spleen colonies were assayed by PCR for the presence of the human MDR gene. Three of 18 spleen colonies dissected out from mice injected with FLC exposed to ecotropic MDR supernatants are positive by PCR for MDR (data not shown). By contrast, none of 7 of the examined spleen colonies from mice injected with FLC exposed to amphotropic MDR supernatants are positive by PCR (data not shown).

Murine amphotropic Moloney virus receptor cDNA sequences were used to design primers for use in RT-PCR to assess the presence of the receptor poly A+ RNA in FLC (Fig 5). A positive amphotropic receptor RT-PCR signal is seen using FLC, whereas the appropriate controls, including yeast mRNA, are negative (Fig 5). Using poly A+ RNA derived from 5 × 10⁶ FLC, a dilution analysis was performed to attempt to estimate the least number of cells required to obtain a positive RT-PCR signal (Fig 5). This number was calculated to be one cell in 2.5 × 10³ or 2.5 × 10⁴ cells, assuming a 10% to 100% recovery of poly A+ RNA from FLC, respectively (see legend to Fig 5).

DISCUSSION

The results presented here indicate that FLC can be efficiently transduced by ecotropic virus but not by amphotropic virus, even when high-titer retroviruses are used. These results are observed both in the total FLC population and in the rare progenitor subsets such as BFU-E and CFU-S. These results are in marked contrast to those seen with adult bone marrow hematopoietic precursors, which are clearly transduced by the same amphotropic virus. These data are consistent with previous studies that demonstrate that FLC at day 20 pc transduced with amphotropic 4070 wild-type retrovirus as helper show no development of autonomous cell lines, whereas FLC transduced with ecotropic virus as helper do show development.¹⁸

The mouse ecotropic viral receptor (ecoR) is expressed on most murine cells. Sequence analysis of the receptor indicates that it contains 622 amino acids and 14 hydrophobic potentially membrane-spanning regions.¹⁷ The gene for ecoR has been localized to chromosome 5.²⁰ It has been shown that the ecoR also functions as the previously described y+ basic amino acid transporter.²¹,²² The mouse and human amphotropic viral receptors have been localized to chromosome 8 in both the mouse²⁰ and human.²³

The murine and human amphotropic Moloney receptors (Ram-1 and GLVR-2, respectively) have recently been cloned and are related in structure to the gibbon ape leukemia virus (GALV) receptor.¹⁷,²⁴ The two receptors show 92%
a later time in mouse development than the ecoR. There are other functions that do not appear until after day 15.5 pc as well. For example, the rearrangement of the Ig gene segments is not initiated until hematopoietic cells have seeded the fetal liver. In the rearranged Igs present in fetal liver, a single D gene segment is used in greater than 50% of the DJH junctions, indicating that the fetal repertoire is restricted in its antigen-binding potential.25 Other studies show a lack of class II major histocompatibility antigens on midgestational macrophages.26,27 Furthermore, both midgestational liver- and placental-derived macrophages display a deficit in their ability to present antigens to T cells, and the investigators suggest that this may be caused by an alteration in the antigen processing pathway at this time.28 Thus, the expression of the amphotropic receptor appears to be developmentally regulated and to be one of a larger repertoire of proteins only expressed late in mouse development.

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