Serial Transplantation of Methotrexate-Resistant Bone Marrow: Protection of Murine Recipients from Drug Toxicity by Progeny of Transduced Stem Cells

C.A. Corey, A.D. DeSilva, C.A. Holland, and D.A. Williams

Recombinant retroviral vectors have been used to transfer a variety of genetic sequences into hematopoietic stem cells. Although transfer and expression of foreign genetic sequences into reconstituting stem cells is one approach to somatic gene therapy, few studies have shown long lasting phenotypic changes in recipient mice in vivo. In this study, we show successful transfer of a methotrexate-resistant cDNA (DHFR') into reconstituting hematopoietic stem cells using a retroviral vector, FrDHFR', in which the DHFR cDNA is expressed off a hybrid Friend/Moloney long term repeat. Both primary and secondary recipients transplanted with bone marrow infected with this recombinant retrovirus show improved survival and protection from methotrexate-induced marrow toxicity when compared with control animals. These data suggest that retroviral-mediated gene transfer of DHFR' cDNA leads to a stable change in the phenotype of hematopoietic stem cells and progeny derived from those cells in vivo after bone marrow transplantation. Gene transfer using recombinant retroviral vectors seems to be one rational approach to establishing chemotherapy-resistant bone marrow cells.

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Retroviral-mediated gene transfer has lead to the successful introduction of a number of genetic sequences into hematopoietic stem cells. Transplantation of such transduced hematopoietic stem cells into mice leads to reconstitution of all hematopoietic lineages with cells containing the transferred sequences. In some, but not all, instances the transferred DNA sequences have been shown to be expressed by RNA or protein analysis. The mechanisms associated with the lack of expression of some introduced vectors in primary hematopoietic stem cells are unclear, but may relate to vector design, specific promoter/enhancer combinations, or properties of stem cells into which the vectors have been introduced. Therefore, one approach to improve the expression of transferred sequences is the construction of vectors with transcriptional regulatory sequences of genes that are expressed in specific hematopoietic cells. For example, sequences at the 3' end of the nondefective Friend virus genome, including those encompassing the transcriptional enhancers located in the long terminal repeat (LTR), confer some of the erythroid disease specificity in Friend erythroleukemia. Holland et al have shown previously the use of sequences in the Friend LTR in recombinant retroviral vectors improves expression of the neo phosphotransferase (Neo) gene in infected murine (in vitro) hematopoietic progenitor colonies.

One potential application of gene transfer into hematopoietic stem cells is the correction of a disease phenotype by introduction and expression of a functional gene in transplanted hematopoietic cells. However, few previous studies have demonstrated any phenotypic changes in vivo in recipients of transduced hematopoietic stem cells. Our laboratory has previously shown protection of mice from lethal methotrexate (MTX) toxicity by bone marrow cells transduced with a mutant dihydrofolate reductase (DHFR') cDNA expressed off the Moloney murine leukemia virus LTR. However, it was unclear in this study if protection from MTX toxicity was due to gene transfer into more differentiated progenitor and precursor cells or expression of transferred sequences in the progeny of transduced primitive hematopoietic stem cells (HSC). Generation of MTX-resistant primitive HSC is a requisite for the use of in vivo drug selection to enrich transduced cells during reconstitution, since these cells are capable of extensive self-renewal and long-term marrow reconstitution. In addition, such drug-resistant HSC may allow development of more aggressive cancer chemotherapeutic regimens if bone marrow is the dose-limiting organ for such chemotherapy.

In this report, we have used serial transplantation of bone marrow cells to study the capacity of retroviral-mediated gene transfer to alter the phenotype in vivo of HSC and progeny cells derived from HSC. In an effort to improve expression of the transferred DNA sequences in myeloid hematopoietic cells, a hybrid Friend/Moloney LTR containing enhancer sequences from the Friend virus has been used to express the MTX-resistant mutant DHFR (DHFR') cDNA. Infection of murine bone marrow cells with this DHFR' recombinant retrovirus confers MTX protection, which is maintained after serial transplantation. These data suggest that we have generated MTX-resistant primitive HSC capable of long-term reconstitution.

Materials and Methods

Recombinant vector and packaging cell lines. The vector used in this study, FrDHFR', was constructed by replacing the Sau3A-KpnI fragment of the Moloney LTR of Zip DHFR' with the corresponding fragment of the Friend murine leukemia virus LTR. The resulting retrovirus contains a hybrid Friend/Moloney LTR, the psi packaging sequence, abbreviated Gag sequences, and a 660 bp DHFR' cDNA cloned into the BamHI site of Zip SV(X) Neo.

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The Neo cassette has been deleted with the XhoI and ClaI cloning sites intact in the 3' end of the virus. This virus, FrDHFR', differs from Zip DHFR' only in the substituted Sau3A-KpnI sequences of the LTR. Total LTR-LTR proviral size is 2.8 kb. Psi-2 producer clones of FrDHFR' were generated by infection of Psi-2 cells with transient harvest of transfected PA317 cells, as previously described. Infected Psi-2 cells were selected in 0.25 μmol/L MTX (Lederle, Carolina, Puerto Rico) in α-modified Eagles Medium (αMEM) supplemented with 5% fetal calf serum (FCS) and 5% calf serum (CS) (dialyzed versus phosphate buffered saline 8,000/mol wt cut-off tubing), 100 units/mL penicillin, and 100 μg/mL streptomycin (both GIBCO, Grand Island, NY). After selection for 14 days, clones were picked and expanded, and the titer of MTX-resistant colony-forming units (CFU)/mL was determined. Psi-2 FrDHFR'1 was maintained in αMEM 5% FCS-5% CS and used for subsequent bone marrow infections.

**Infection of bone marrow cells, transplantation, and MTX treatment of recipients.** Bone marrow cells were harvested as previously described from the hind limbs of 10- to 15-week-old male C3H/HeJ (Jackson Laboratories, Bar Harbor, ME) mice 48 hours after a single intraperitoneal injection of 5-fluorouracil (5-FU). Nucleated bone marrow cells (3 x 10^6) were prestimulated for 48 hours with 10% conditioned media from Wehi-3b cells (Wehi-CM) in αMEM with 20% FCS, then cocultivated with mitomycin-c-treated (10 μg/mL for 2 hours) Psi-2 FrDHFR'-1 producer cells for 48 hours in the presence of 10% Wehi-CM and 8 μg/mL polybrene (Aldrich Chemical, Milwaukee, WI). Subsequently, 2 x 10^6 nonadherent cells were injected into lethally irradiated (total dose: 1,150 Gys, at 145 rads/min split doses with ≥3 hours between doses) C3H/HeJ recipients. Control mice received bone marrow cells infected in identical fashion with a recombinant retrovirus containing the neo phosphotransferase (Neo) gene.2

Two days after transplantation, mice were injected thrice weekly with MTX (1.4 mg/kg) for 1 week, followed by 4 mg/kg for 7 weeks. In preliminary studies, this dose regimen had been shown to be lethal to the majority of control mice. Tail vein hematocrits and weights were followed weekly. At 8 weeks posttransplantation, surviving animals were sacrificed, bone marrow and spleen cellularity were determined, and DNA was prepared from spleen cells. Bone marrow cells from individual primary animals (5 x 10^6) were transplanted into secondary irradiated recipients to determine the MTX resistance of the stem cell compartment. Two days after secondary transplantation, animals were started on thrice weekly intraperitoneal MTX injections (2.4 mg/kg) for 1 week followed by 6 mg/kg thereafter. Tail vein hematocrits, weights, and survival were monitored in these secondary transplant recipients.

**Progenitor assay.** Cells (2 x 10^4/mL) were plated in MEM/10% methycellose, 24% horse serum, 1% bovine serum albumin (BSA) (Boehringer Mannheim Biochemicals, Indianapolis, IN), 10^-2 mol/L 2-mercaptoethanol, 1% penicillin/streptomycin, 1% glutamine, 7% pokeweed mitogen/spleen conditioned medium. In order to show the presence of MTX-resistant progenitor colonies, MTX was used in the progenitor cocktail at concentrations of 5 x 10^-8 mol/L and 5 x 10^-7 mol/L. Cultures were plated in duplicate in 1 mL aliquots in 10 x 35 mm tissue culture dishes (Lux; Miles Scientific, Naperville, IL) and placed in 5% CO₂ at 37°C. Colonies (greater than 50 cells) were counted at day 10 of culture. Southern blot analysis. High molecular weight DNA was prepared as previously described, digested with SacI (New England Biolabs, Beverly, MA), electrophoresed through a 1% agarose gel, and transferred to nylon filter (Zetabind, AMF/Boehringer Mannheim). The filter was probed with a 3P-labeled HindIII-BglII fragment isolated from pSV.DHFR. Prehybridization, hybridization, and post-hybridization washes were carried out as described by the manufacturer. Filters were exposed to X-ray films at -70°C in the presence of calcium tungstate intensifying screens.

**RESULTS**

Construction of vector and generation of producer lines. The substituted Kpn I-Sau3A fragment of the Friend LTR contains sequences that have been identified in other retroviruses as the viral enhancer. These sequences have been shown to determine, at least in part, the erythroid pathogenic specificity of Friend murine leukemia virus. This hybrid Friend/Moloney enhancer/promoter was used to express the DHFR' cDNA encoding a mutant enzyme that is highly resistant to MTX. The FrDHFR' vector is identical to Zip DHFR' used in our previously reported in vivo studies except for the substituted sequences located in the LTR. A similar vector with this hybrid LTR expressing the Neo gene has been previously shown to yield a higher percentage of G418' in vitro progenitor colonies when compared with an identical Neo virus containing the Moloney enhancer sequences. The FrDHFR' provirus contains SacI sites in each LTR and additional SacI site in the DHFR' cDNA. Psi-2 producer cells infected with transient viral supernatants from transfected PA317 were selected in 0.25 μmol/L MTX. Multiple MTX-resistant clones were analyzed for recombinant viral titers on NIH/3T3, and one clone, Psi-2 FrDHFR'-1, was selected for infection of bone marrow cells. The titer of this clone, 2 x 10^4 MTR' CFU/mL, was at least 100-fold lower than the highest titer Zip DHFR' producer clone (500.8.4). This apparent reduction in titer in producer cells could result from the enhancer substitution, if the Friend promoter/enhancer is less active in fibroblasts. However, we saw no large decrement in the transient titer of PA317 cells transfected with this construct compared with Zip DHFR' (not shown). In addition, the producer clone 500.8.4 was generated by multiple infection rounds followed by selection in extremely high levels of MTX (500 μmol/L) and contains 30 to 50 ZipDHFR' proviral copies. Thus, this difference in titer could be due to the method used to generate 500.8.4. Southern blot analysis of DNA obtained from infected NIH/3T3 cells showed no rearrangement of the recombinant provirus (see below).

**Infection and transplantation of bone marrow cells.** Infection of murine bone marrow cells was carried out using a modification of previously described methods that includes prestimulation of 5FU-resistant bone marrow for 48 hours in Wehi-3b CM before infection by cocultivation. Such prestimulation improves the efficiency of gene transfer into hematopoietic stem cells using low-titer viral producers without reliance on any in vitro selection. Southern blot analysis of DNA from animals reconstituted for 2 months with bone marrow cells infected with FrDHFR'-1 using this protocol showed the presence of unrearranged provirus in 100% of transplanted animals (see below). The absence of helper virus was determined by overlaying 0.2 mL of serum (obtained from each primary transplant recipient at the time of sacrifice) on NIH/3T3 cells. No transplant recipient sera generated MTX-resistant NIH/3T3 colonies when cells were selected in 0.25 μmol/L MTX.

Treatment of primary transplant recipients during the
immediate posttransplant period with MTX resulted in significant anemia (Fig 1), wasting, and mortality (Fig 2) in the control group. In contrast, animals reconstituted with bone marrow infected with FrDHFR'-1 maintained normal weight, higher hematocrits, and significantly less mortality than control animals (Figs 1 and 2). In MTX-treated control mice, hematocrits fell to 13 ± 1% by day 17, while mice transplanted with FrDHFR'-1–infected bone marrow fell to only 24 ± 1.9%. Hematocrits were also significantly lower in the control groups at days 7 and 13 (Fig 1). In addition, mortality was as high as 60% in the control group but consistently below 20% in the animals transplanted with FrDHFR'-1–infected bone marrow cells (Fig 2). Analysis by log rank test shows that the difference in survival of the two groups was significant at $P < .003$ (Fig 2).25,26 These results represent three independent experiments. As previously reported, the nadir of MTX-induced anemia was seen between 2 and 3 weeks posttransplant, after which hematocrits gradually returned to normal values even with continued MTX administration.17 Animals transplanted with bone marrow cells infected with FrDHFR' virus and treated with MTX showed higher hematocrits (24 ± 1.9% versus 19 ± 1.1% at day 17; $P < .05$) and slightly higher survival (81% versus 56%, n = 16) than animals transplanted with bone marrow cells infected with Zip DHFR' virus (in spite of the higher titer of the latter virus), although the difference in survival did not reach statistical significance. Comparison of bone marrow cellularity at 8 weeks posttransplant between FrDHFR' animals and surviving control animals also revealed a significant difference in cellularity ($4.14 \pm 0.4 \times 10^7$ versus $2.91 \pm 0.2 \times 10^7$), with the treated FrDHFR' bone marrow containing normal numbers of cells (Table 1).

Since protection of transplant recipients in the early posttransplantation period (1 to 3 weeks) could be due to drug resistance in the precursor and progenitor compartments, we elected to perform serial transplantation of bone marrow from primary recipients 2 months posttransplantation. Bone marrow from individual recipients was harvested and counted, and $5 \times 10^6$ cells were injected into secondary irradiated mice. Control animals who had survived the initial drug toxicity and had recovered normal hematocrits were

Table 1. Bone Marrow Cellularity in Animals Surviving Chronic MTX Administration

<table>
<thead>
<tr>
<th>Transplant Group</th>
<th>Bone Marrow Cellularity</th>
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<tr>
<td>FrDHFR' infected</td>
<td>$4.14 \pm 0.4 \times 10^7$</td>
</tr>
<tr>
<td>Control</td>
<td>$2.91 \pm 0.2 \times 10^7$</td>
</tr>
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Transplanted animals received bone marrow cells infected with recombinant retrovirus FrDHFR' or Zip Neo (2) as control, as described in Materials and Methods. Bone marrow was harvested from both hind limbs of animals surviving 8 weeks of MTX administration and nucleated cellularity determined. Results represent nucleated cells ± SEM. FrDHFR', n = 20; Zip Neo, n = 10.
used as control donors for secondary transplants. Thus, this group controls for any selection of MTX-resistant progenitor or stem cells in treated animals that have received bone marrow cells infected with the Neo virus.

Secondary transplants were treated with thrice weekly MTX beginning 2 days posttransplant. Again, the presence of bone marrow cells containing the FrDHFR' provirus was associated with significantly better survival (Fig 3) and hematocrits (39 ± 0.8% [n = 7] versus 25% [n = 2] at 60 days posttransplant) than MTX-treated control animals. The mortality of secondary control animals was greater than 70% by 10 weeks posttransplant, while animals transplanted with bone marrow containing the FrDHFR'-1 provirus showed less than 20% mortality. Analysis by log rank test showed the difference in survival of those two groups was highly significant (P < .000003).

Analysis of bone marrow cells from both primary and secondary transplant recipients revealed the presence of MTX-resistant progenitors in animals transplanted with bone marrow cells infected with the FrDHFR' virus. Figure 4A shows that at both MTX concentrations tested (5 x 10^{-8} and 5 x 10^{-7} mol/L), significantly more MTX-resistant progenitor colonies were present in the FrDHFR' group (74 ± 10 versus 22 ± 7, and 29 ± 7 versus 7 ± 6%) than in animals transplanted with neo virus-infected bone marrow cells. Likewise, secondary transplant recipients also show the presence of MTX-resistant progenitors at both drug concentrations (55 ± 9.8 versus 27 ± 9.2, and 49 ± 13 versus 6 ± 2; Fig 4B). These data provide independent evidence that the transferred DHFR' cDNA was expressed in vivo in transplant recipients.

**Molecular analysis of transplant recipients.** Southern blot analysis of DNA obtained from spleen cells of primary and secondary transplants showed the presence of unrearranged provirus in each recipient (Fig 5A and 5B). As seen in Fig 5A, DNA obtained from spleen cells harvested 4 weeks (lanes 1 and 2) or 8 weeks (lanes 3 and 4) after transplantation of mice with FrDHFR'-1-infected bone marrow cells and probed with {sup 32}P-labeled DHFR cDNA showed the presence of three hybridizing endogenous murine DHFR bands and two hybridizing bands corresponding to the transferred FrDHFR'-1 provirus at 0.9 and 1.9 kb (arrows). Southern blot analysis of individual colony forming unit-spleen (CFU-S)-derived spleen colonies from primary transplant recipients that were either treated or untreated with MTX showed a slightly higher proportion of FrDHFR positive colonies in the MTX-treated group (36% versus 12%; Table 2). However, Southern blots of DNA obtained from whole spleens of MTX-treated versus untreated primary animals revealed no consistent differences in the intensity of DHFR-hybridizing bands (date not shown). In addition, as seen in Fig 5B, the intensity of the DHFR-hybridizing exogenous bands is similar in DNA from secondary animals compared with DNA from primary animals (compare lanes P [primary mouse] with lanes A, B, C [secondary mice]) in representative groups of primary and secondary transplants. This data implies that there was no increase in the copy number of the transferred FrDHFR'
after 8 additional weeks of MTX administration in the secondary mice. Therefore, it appears that with a thrice weekly exposure to MTX, no detectable in vivo selection of transduced HSC has occurred.

**DISCUSSION**

Our laboratory has previously demonstrated protection of primary transplant recipients after transfer to the DHFR' cDNA using the Zip DHFR' retroviral vector. Due to the interval posttransplantation during which MTX was given in these studies, we postulated that such protective effects might be due to expression of the transferred sequences in relatively short-lived progenitor cells. However, our laboratory and other investigators have more recently shown stable long-term expression of transferred sequences for up to 6 months posttransplantation (References 23, 23a, R.C. Mulligan, personal communication, September, 1989; A.D. Miller, personal communication, September, 1989). Such stable expression shows that retrovirus-mediated transfer of new genetic sequences will allow continued expression of the transferred sequences in progeny of transduced HSC capable of reconstitution of recipient animals.

In the present study, we show maintenance of an altered cell phenotype (MTX-resistance) after serial transplantation of transduced bone marrow cells. The protective effect of transduced bone marrow cells is clearly not due to short-lived progenitor cells or infected CFU-S stem cells, since neither would effectively contribute hematopoietic cells in secondary transplants following serial transplantation after two months reconstitution in primary mice. From these data, we conclude it is possible to transfer and express foreign genetic sequences

<table>
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<tr>
<th>Transplant Group</th>
<th>DNA + CFU-S*</th>
<th>CFU-S Examined</th>
<th>+ CFU-S (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>9</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>No methotrexate</td>
<td>3</td>
<td>25</td>
<td>12</td>
</tr>
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*DNA from individual spleen colonies positive by Southern blot analysis for transferred FrDHFR provirus.
in a population of HSC such that transplant recipients demonstrate a stable and long-lived altered phenotype in vivo. Thus, the use of recombinant retroviral vectors for gene transfer into HSC may be a feasible approach to the stable correction of a disease phenotype in these same cells.

We have constructed a hybrid Friend/Moloney promoter/enhancer to direct DHFR cDNA expression in an attempt to improve expression in myeloid cells. Holland et al have previously demonstrated an increase in G418-resistant in vitro progenitor-derived colonies compared with Moloney LTR when bone marrow cells were infected with a recombinant retrovirus containing the neo phosphotransferase gene expressed off this hybrid LTR. In the present study, we compared the Moloney promoter/enhancer expressing DHFR cDNA (Zip DHFR) with FrDHFR-1 in vivo. Animals transplanted with bone marrow cells infected with FrDHFR-1 and treated with MTX showed higher hematocrits and slightly higher survival than animals transplanted with bone marrow cells infected with Zip DHFR virus. Taken together, these data suggest that there is a small advantage to using the Fr/Moloney hybrid LTR for expression in myeloid hematopoietic cells.

Although a potential application of the use of DHFR vectors is the in vivo selection of transduced stem cells, our data suggest that intermittent injections of MTX are not sufficient to accomplish such selection. Southern blot analysis of spleen DNA from primary and secondary MTX-treated FrDHFR recipients demonstrates no consistent increase in gene copy number after 8 and 16 weeks of MTX exposure (Fig 5B). In addition, we have analyzed multiple CFU-S-derived spleen colonies derived from treated versus untreated primary recipients by Southern blot analysis and have seen only a slight increase in the number of transduced CFU-S stem cells in the treated animals. Since the half-life of MTX is short (≈2 hours), it is likely that continuous MTX infusion may be necessary for effective selection of transduced cells in vivo. Studies using constant infusion pumps are currently underway in our laboratory.

Previous work by Kwok et al has shown transfer of DHFR into canine hematopoietic progenitor cells in vitro. Together, these studies are encouraging with respect to the potential use of gene transfer to establish chemotherapeutic-resistant bone marrow cells. The use of DHFR as a dominant selectable market allows in vivo treatment with MTX, an approach not possible with neo phosphotransferase (G418)-gene. DHFR-transfer might be useful in developing more aggressive cancer chemotherapy protocols if bone marrow is the dose-limiting organ for that specific chemotherapeutic agent, and could be useful as a model for such studies with future application to several chemotherapy-resistance genetic sequences.

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REFERENCES