PROTECTION OF BONE MARROW TRANSPLANT RECIPIENTS FROM LETHAL DOSES OF METHOTREXATE BY THE GENERATION OF METHOTREXATE-RESISTANT BONE MARROW

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The ability to generate methotrexate-resistant (MTX^r)[1] hematopoietic cells via gene transfer techniques would have a number of important implications for both basic gene transfer studies in hematopoietic cells and eventual clinical applications of the gene transfer technology. In the case of general studies involving the transfer of genes into stem cells in vitro and the subsequent engraftment of recipients with the transduced cells, a major limitation to the wide application of the technique is the need for very-high-titer virus-producing cell lines in order to obtain efficient gene transfer (1). In an attempt to increase the efficiency of gene therapy, several groups have explored the use of selective schemes in vitro to increase the percentage of transduced stem cells before transplantation (2–4). Thus far, the neo phosphotransferase (neo) gene (5) has been used in conjunction with the drug G418 for these studies. While this selection is a powerful one in vitro, it is unlikely that it could be applied in vivo, due to the dose-limiting toxicity of G418 in other organs. The introduction of methotrexate resistance into bone marrow stem cells, however, may well enable the selection of transduced hematopoietic cells in vivo, and even make possible the transplantation of sublethally irradiated or untreated recipients. In addition to potential use in obtaining efficient engraftment with transduced cells, the generation of recipients engrafted with drug-resistant bone marrow may also facilitate the development of more aggressive chemotherapeutic regimens that otherwise might lead to lethal bone marrow toxicity.

As a first step towards the development of a general method for generating MTX^r bone marrow cells at high efficiency, we have constructed and character-

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[1] *Abbreviations used in this paper:* DHFR, dihydrofolate reductase; MTX, methotrexate; neo, neo phosphotransferase.

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ized the properties of recombinant retroviruses encoding a dihydrofolate reductase (DHFR) enzyme that is highly resistant to methotrexate (DHFR^r) (6). In this report we describe some of the properties of the viruses in vitro, and present data that indicates that mice engrafted with bone marrow cells previously infected with the DHFR^r retroviruses are protected from doses of MTX that are lethal when administered to transplant recipients engrafted with uninfected bone marrow.

Materials and Methods

Infection of Bone Marrow Cells, Transplantation, and MTX Treatment of Recipients. The infection of bone marrow cells in vitro with recombinant viruses and transplantation of the cells in lethally irradiated recipients was performed as described (1), except that freshly explanted CBA/J male bone marrow was used for infection and CBA/J males served as recipients. CBA/J recipients were irradiated with a split dose of 14 Gy before transplantation. Bone marrow cells were infected with neo gene–carrying virus (1), and transplanted to yield control mice. Cell counts and CFU-S (colony-forming units of spleen cells) determinations were made to ascertain that equal numbers of cells and CFU-S were transplanted into each group of mice.

2 d after transplantation, the recipients were subjected to a thrice-weekly schedule of intraperitoneal MTX administration. This consisted of 4 mg/kg in the first week, followed by 10 mg/kg for the following 7 wk. The schedule was shown in preliminary experiments done for previous studies to result in the death of all recipients transplanted with uninfected bone marrow cells. At intervals after the transplant, peripheral hematocrit and total blood counts with differentials were performed on tail vein bleeds. In addition, animals were sacrificed, and both bone marrow cells (provided from the hind limbs of all animals) and spleen cells from individual animals were analyzed for cellular and progenitor content (colony-forming units of granulocytes and macrophages [CFU-GM], and burst-forming units of erythroid cells [BFU-E]) (see below), and for the presence of proviral DNA sequences.

Bone Marrow Progenitor Assays. Bone marrow was isolated as previously described from the hind limbs of animals (1), pooled, and counted. 2×10^5 bone marrow cells/ml were plated in MEM/α-methylcellulose, 24% FCS, 1% BSA (Boehringer Mannheim Biochemicals, Indianapolis, IN), 10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO), 1% penicillin/streptomycin, and L-glutamine with 10% L cell supernatant as colony-stimulating factor for granulocytic colonies (7) or 5% pokeweed mitogen–stimulated spleen-conditioned media as burst-promoting activity with 2 U/ml erythropoietin (Connaught Laboratories, Ltd., Willowdale, Canada) for erythroid colonies (8). Progenitor cocktails were plated in 1-ml culture (in triplicates) in 10×35–mm tissue culture plates (Lux; Miles Scientific, Naperville, IL) and placed in 5% CO_2 at 37°C. Colonies were counted (>50 cells) at day 10 of cultures.

Southern Blot Analysis. High-molecular-weight cellular DNA was extracted, digested with Eco RI or Xba I (New England Biolabs, Beverly, MA) and electrophoresed through a 1% agarose gel. The DNA was transferred to a Zetabind filter (AMF/Voit, Meriden, CT) and hybridized to ^{32}P-labeled Hind III–Bgl II DHFR fragment isolated from pSV_2 DHFR as previously described. Filters were washed as described by the manufacturer and exposed to x-ray films at −70°C in the presence of intensifying screens.

Results

Generation of Cell Lines Producing High Titers of Virus Encoding MTX Resistance. The source of DHFR-coding sequences used in the construction of a recombinant retroviral genome encoding MTX resistance was the plasmid pR400-12 (kindly provided by Genentech, Inc., South San Francisco, CA). This plasmid carries DNA sequences that encode a mutant murine DHFR enzyme

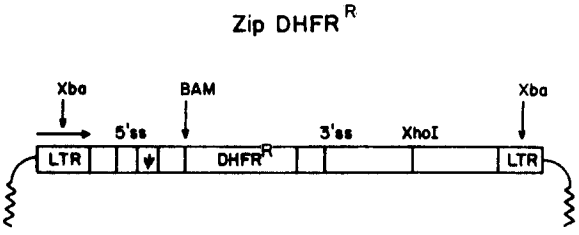

FIGURE 1. Map of Zip-DHFR^r. Sequences include the Moloney long terminal repeats and sequences necessary for the efficient encapsidation of the viral genome (designated ψ). The 5′ splice site used in the generation of the Moloney murine leukemia virus *env* mRNA is designated *5′ss*, and the 3′ splice acceptor is designated *3′ss*. Restriction sites are as shown, including a unique Xho I cloning site 3′ of the selectable marker. The total size of the Zip-DHFR^r construct is 2.7 kb. Note that Xba I cleaves once in each long terminal repeat, and that Eco RI does not cleave the provirus.

(DHFR^r) that has 1/270 the binding affinity for MTX that the wild type enzyme possesses. A 660 bp Fnu 4 HI–Bgl II DNA segment encompassing the DHFR coding sequence was isolated from pR400-12 and inserted into the Bam HI cloning site of the retrovirus vector pZIPNeoSV(X) (9). Subsequently, a portion of SV(X) vector sequences (including pBR322ori, SV40ori, and neo sequences) was deleted from the construct by cleavage of the plasmid with Xho I, removal of the smaller Xho I fragment, and recirculation of the plasmid sequences. The structure of the resulting plasmid, termed pZIP-DHFR^r is shown in Fig. 1.

In an attempt to generate cell lines producing titers of DHFR virus suitable for the infection of bone marrow cells, conditions were established so that multiple Zip-DHFR^r proviral copies could be introduced into the ψ2 cells. First, the Zip-DHFR^r construct was introduced by DNA transfection into ψAM cells (10) to generate virus with amphotropic host range. Selections of stable transformants in the presence of 0.25 μm MTX yielded cell lines that produced 10^2–10^4 MTX^r colonies/ml when assayed on 3T3 cells cultured in 0.25 μm MTX and dialyzed calf serum (8–10 kD cutoff vs. PBS). While ψ2 cells are resistant to superinfection with ecotropic virus, they can be readily infected with amphotropic virus. Accordingly, irradiated (14 Gy) ψAM cells producing Zip-DHFR^r virus were cocultured with ψ2 cells to achieve a highly efficient infection. After selection of infected ψ2 cells that were highly resistant to MTX (500 μM), one clone 500.8.4, was selected for use, based on its high-titer virus production (10^6–10^7 MTX^r CFU/ml). Southern blot analysis of this cell line indicates the presence of multiple integrated provirus copies, as evidenced both by the smear of fragments obtained after cleavage of the DNA with enzymes that yield bands indicative of unique integrations (Fig. 2, lanes *6* and *7*), and also by the intensity of the band generated by cleavage of the DNA with Xba, which cleaves once in each LTR (Fig. 2, lane *3*) compared to a single-copy control (Fig. 2, lane *2*). In addition, comparison of the Xba-digested DNA with the original Zip-DHFR^r plasmid DNA also indicated that the proviral sequences were transmitted intact to the ψ2 cells (not shown).

In addition to yielding a titer of 10^6–10^7 MTX^r CFU/ml on 3T3 cells selected in the presence of 0.25 μM MTX, 500.8.4 also yielded titers of 5×10^2 MTX CFU/ml on 3T3 cells selected in 100 μM MTX (i.e., 400 times the normal selection concentration). Southern blot analysis of infected 3T3 cells in all cases confirmed that the Zip-DHFR^r sequences were transmitted intact to cells, and

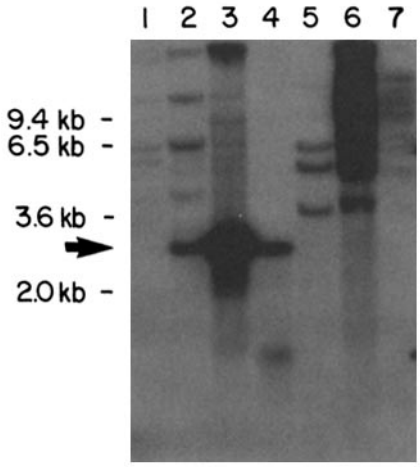

FIGURE 2. Southern blot analysis of $\psi 2$ producer 500.8.4. Lanes *1–4*; Xba digests. Lane *1*, 10 μg uninfected 3T3 DNA; lane *2*, 10 μg DNA from 3T3 cells infected with a low multiplicity of Zip-DHFR^r virus and selected in 0.25 μM MTX; lane *3*, 10μg 500.8.4 DNA; lane *4*, 1 μg 500.8.4 DNA; lanes *5–7*, Eco RI digests; lane *5*, 10 μg uninfected 3T3 DNA; lane *6*, 10 μg 500.8.4 DNA; lane *7*, 1 μg 500.8.4 DNA. See Fig. 1 for map of construct. Xba cleaves once in each long terminal repeat; Eco RI does not cleave in the proviral genome. Arrow denotes proviral band.

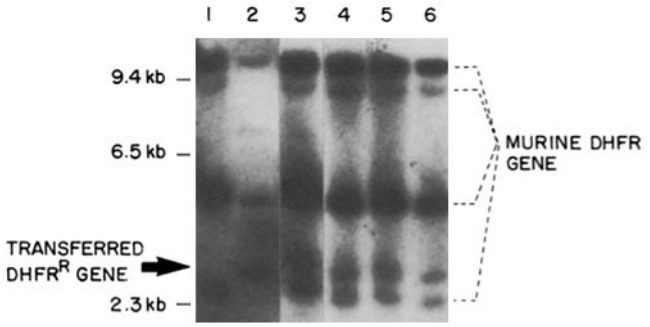

FIGURE 3. Southern blot analysis of Xba I–digested genomic DNA isolated from pooled bone marrow or spleen of transplant recipients engrafted with ZIP-DHFR^r-infected bone marrow cells and treated with MTX. Lane *1*, uninfected bone marrow cell DNA; *2*, 3T3 cell DNA containing a single copy of ZIP-DHFR^r; *3*, infected bone marrow cell DNA (pooled from four animals); *4–6*, infected spleen DNAs from three separate mice. Endogenous murine DHFR bands are as noted; the transferred DHFR^r gene is seen as an additional 2.7 kb band in lanes *2–6 (arrow)*.

northern analysis demonstrated expression of the expected 2.7 kb DHFR-containing viral RNA transcript (data not known).

Detection of Proviral Sequences in Recipients Transplanted with DHFR-Infected Bone Marrow Cells. Freshly explanted bone marrow cells from CBA/J male mice were infected with Zip-DHFR^r and transplanted into lethally irradiated recipients as described in the Materials and Methods. Similarly, a number of recipients were transplanted with bone marrow cells infected by DHFR-neo (1), a construct that confers G418 resistance upon cells in culture yet does not transmit an intact or functional copy of the DHFR gene. To establish the efficiency of gene transfer in these experiments, DNA was isolated from the bone marrow and spleen cells of a number of recipients 8 wk after transplantation and selection with MTX, and was analyzed by the method of Southern (Fig. 3). In each spleen and bone marrow DNA sample from the recipients transplanted with infected cells and selected with MTX, proviral sequences of the expected size could be detected (Fig. 3, lanes *3–6*). It was difficult to ascertain the efficiency

TABLE I

Survival of Recipients Transplanted with Bone Marrow Cells Infected with DHFR^r or Neo Virus and Subsequently Treated with MTX for 4 wk

Group infected with:	MTX treatment	Survival
DHFR^r gene	−	12/12
DHFR^r gene	+	11/12
Neo gene (control)	+	0/12

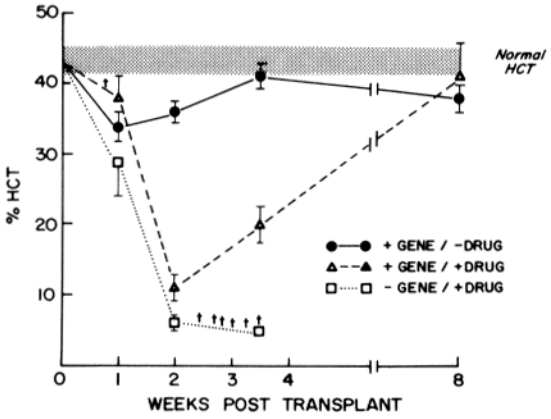

FIGURE 4. Results of peripheral hematocrit determinations. Blood was obtained by tail vein bleeds and spun in microhematocrit tubes with heparin. Mean ± SD. Numbers of determinations: week 1, $n = 8$; week 2, $n = 8$; week 3.5, $n = 6$, except neo-treated, $n = 1$; week 8, DHFR^r-untreated $n = 6$, DHFR^r-treated $n = 4$. (●) Transplanted with DHFR^r containing bone marrow; no MTX treatment. (△) Transplanted with DHFR^r containing bone marrow; treated with MTX. (□) Transplanted with neo-containing bone marrow, treated with MTX.

of gene transfer with much accuracy, due to the fact that much less DNA representing single copy proviral sequences was loaded in comparison with the spleen and bone marrow DNA samples (compare Fig. 3 lane 2 to lanes *3–6*). Nevertheless, based on the relative intensity of bands representing the endogenous DHFR sequences and the proviral sequences in each lane, ~10–20% of bone marrow and spleen cells appeared to harbor the proviral sequences. This level of infection is consistent with our previous experience with the particular infection protocol used here (D. A. Williams, unpublished data).

Effects of MTX Treatment of Transplant Recipients. As expected from pilot experiments, the treatment of recipients transplanted with bone marrow cells infected with the neo gene–containing virus with MTX resulted in 100% mortality by 3.5 wk. In contrast, the presence of the DHFR^r gene was associated with a significant increase in survival (Table I). Treatment of transplant recipients with MTX was associated with dramatic changes in hematological parameters, both in control animals as well as in those transplanted with cells infected with Zip-DHFR^r (Fig. 4). While transplantation of DHFR^r or neo-containing cells into recipients followed by no drug treatment resulted in a mild anemia that resolved by 3.5 wk after transplantation, MTX treatment of recipients transplanted with marrow infected either with the DHFR^r or neo constructs led to a dramatic fall in peripheral hematocrit to a plateau at 2 wk after transplant. During the period between 2 and 3.5 wk all mice transplanted with neo gene–containing virus–infected marrow died, with premorbid hematocrits of 5–6%. In recipients containing DHFR^r gene–containing virus–infected marrow, significantly higher

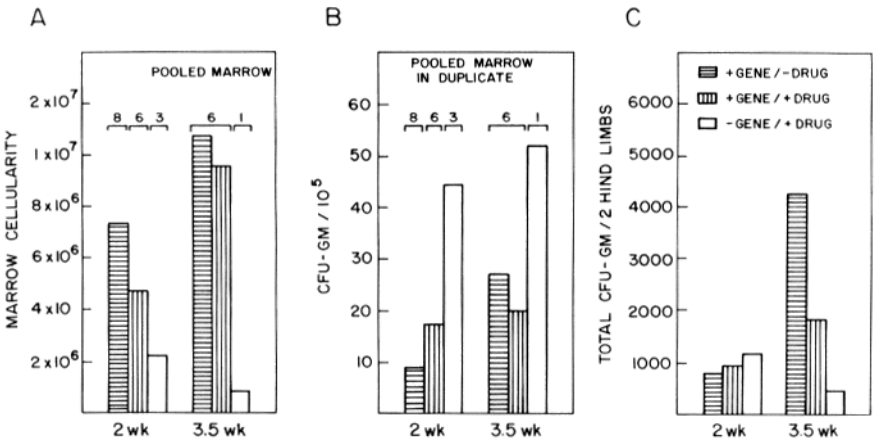

FIGURE 5. (*a*) Bone marrow cellularity and (*b* and *c*) progenitor content of mice treated with MTX. Numbers above bars represent number of animals examined.

($p < 0.01$) hematocrits were observed at 1, 2, and 3.5 wk after transplant and normal hematocrits were obtained by 8 wk in spite of continued MTX treatment during this entire period. This apparent protective effect of the DHFR^r gene was also reflected in peripheral blood absolute granulocyte and absolute lymphocyte counts, and spleen cellularity (data not shown), and in the bone marrow cellularity of these mice (Fig. 5*a*). Mild bone marrow hypoplasia in transplanted mice not treated with MTX largely recovered by 3.5 wk after transplant. In mice transplanted with neo gene–containing virus–infected bone marrow that were treated with MTX, severe hypoplasia at 2 wk worsened during the period of animal death between 2 and 3.5 wk. Animals receiving bone marrow containing the DHFR^r gene and treated with MTX were less hypoplastic at 2 wk and bone marrow cellularity recovered to near that of untreated mice by 3.5 wk.

Additional experiments carried out revealed significant changes in the progenitor compartment of bone marrow (Fig. 5, *b* and *c*) in these animals as well. Bone marrow progenitors are stem cells with limited self-renewal and restricted differentiation capacity that can be cultured in vitro. Both granulocyte-macrophage (CFU-GM) (7) and erythroid (BFU-E) (8) progenitors in mice containing the DHFR^r gene were intermediate between control treated and untreated mice. In mice containing the neo gene and treated with MTX, a relative increase in the concentration of CFU-GM progenitors (44 per 10^5 compared to 9 per 10^5 in untreated mice) (Fig. 5*b*) led to maintenance of the total number of these progenitors per two hind limbs (one animal) at 2 wk after transplant. However, with continued MTX treatment, the number of these progenitors was nearly exhausted by 3.5 wk after transplant (Fig. 5*c*). The increase in CFU-GM concentration during MTX treatment has been previously reported (11). In contrast, animals in which the DHFR^r gene was present showed little increase in the concentration of CFU-GM progenitors (17 per 10^5 vs. 9 per 10^5 in untreated mice) (Fig. 5*b*), and by 3.5 wk the total number of CFU-GM in these mice was four times that of control, treated mice (1,900 CFU-GM per two hind limbs vs.

400 CFU-GM per hind limb) (Fig. 5*c*). We observed similar results in the number of erythroid progenitors (BFU-E) (data not shown).

Discussion

We have constructed a recombinant retroviral genome encoding a MTX-resistant DHFR enzyme (DHFR^r), and shown its ability to efficiently confer a high level of MTX-resistance upon cells in vitro. To generate cell lines producing Zip-DHFR^r virus at sufficient titers for bone marrow infection studies, a protocol was developed for generating ψ2 virus producing cell lines containing multiple integrated Zip-DHFR^r proviral copies. ψ2 cells possessing multiple proviral copies could be selected in very high levels of MTX and yielded cell lines producing uniformly high viral titers. A similar strategy for generating high titer producer cell lines has recently been described (12).

In addition to demonstrating the use of the DHFR^r virus for generating MTX resistance in vitro, which has also been reported by others (12), we showed the ability of Zip-DHFR^r-infected bone marrow cells to protect transplant recipients from otherwise lethal doses of MTX and alter a number of hematological parameters affected by MTX administration. While these findings certainly imply that the Zip-DHFR^r construct can be reasonably well expressed in hematopoietic cells, the interpretation of these results must be tempered by the fact that the evidence for expression is indirect and the nature of the cells responsible for the protective effect unknown. Previous studies in our laboratory and by other investigators have indicated that a number of standard retroviral constructs show dramatic differences in their ability to be expressed in cell lines in vitro versus primary hematopoietic cells (13). In particular, we have recently reported that while a Zip-DHFR^r construct containing human adenosine deaminase (ADA) cDNA sequences is efficiently expressed in cultured fibroblasts and B and T cell lines, only low levels of viral transcript can be detected in cells derived from infected CFU-S (14). A number of other investigators have also reported very low levels of expression of retroviral construction after their transfer to hematopoietic cells in vivo (15, 16). In addition, Dick et al. (2) have reported the decline of expression of viral LTR (long terminal repeat)-promoted constructs in hematopoietic cells in vivo over time. These data may suggest that the specific retroviral construct used may be a critical determinant of the capacity of the integrated proviral sequences to be subsequently expressed in more mature cells derived from the infected stem cells. Taken together, the available data would suggest that it is unlikely that the Zip-DHFR^r construct is efficiently expressed in the progeny of infected totipotent hematopoietic stem cells. We suggest that the protective effect of the Zip-DHFR^r virus observed in transplant recipients may be due to infection and subsequent expression of the provirus in a class of reasonably short-lived progenitor cells, and may depend upon the unique sensitivity of posttransplant hematopoiesis to chemotherapeutic agents such as MTX. Such sensitivity to MTX has also recently been observed in dogs in the immediate posttransplant period (17). The results demonstrated in the experiments here could therefore result from protection mediated during a critical time after transplantation, and do not necessarily imply permanent reconstitution of treated animals with MTX^r bone marrow cells. Clearly, experiments such as those

presented here will need to be extended to include the analysis of transplant recipients after longer periods of reconstitution. Most important, however, will be the development of vectors that result in the efficient and stable expression of inserted sequences in hematopoietic cells derived from transduced stem cells capable of long-term engraftment of recipients.

In general, the availability of retrovirus vectors encoding MTX resistance may allow the development of protocols for the selection in vivo of transduced hematopoietic stem cells and/or stem cell progeny. This method would be of value when the efficiency of gene transfer is limited by low-titer recombinant producers. In addition, the ability to systemically treat animals receiving the $DHFR^r$ gene in vivo will allow study of the effects of selective pressure on the number and function of hematopoietic stem and progenitor cells and the possible development of more aggressive chemotherapeutic protocols involving MTX. Finally, several fatal inherited diseases are manifest primarily in bone marrow–derived cells, and as such are likely candidates for somatic gene correction using retrovirus-mediated gene transfer (18). Because success in somatic gene therapy may depend on introducing a foreign gene in a self-renewing stem cell that will then be exposed to selective pressure in the patient, such basic data could be of critical importance in the successful use of gene transfer for the treatment of bone marrow diseases.

Summary

To develop a highly efficient means for generating methotrexate resistant (MTX^r) hematopoietic cells in vivo, a recombinant retroviral genome was constructed that encodes a MTX^r dihydrofolate reductase ($DHFR^r$). Cell lines producing high titers of virus capable of transmitting the DHFR gene were generated and used to infect mammalian cells in vitro. Analysis of infected fibroblasts indicated that the $DHFR^r$ gene was transmitted intact and conferred a high level of MTX^r upon cells. Based on these findings, $DHFR^r$-containing virus was used to infect murine bone marrow cells in vitro. Following infection, the transduced cells were introduced into lethally irradiated recipients via bone marrow transplantation techniques. The presence of the proviral sequences in cells of the spleen and bone marrow of engrafted recipients was associated with significantly increased survival of mice treated with otherwise lethal doses of MTX.

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