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Methotrexate Preconditioning Allows Sufficient Engraftment to Confer Drug Resistance in Mice Transplanted With Marrow Expressing Drug-Resistant Dihydrofolate Reductase Activity

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List of abbreviations: APP, amyloid precursor protein; BMT, bone marrow transplant; DHFR, dihydrofolate reductase; MTX, Methotrexate.

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Abstract

Methotrexate is an effective antitumor agent which has been demonstrated to be particularly useful in the treatment of hematopoietic neoplasms, but which causes substantial hematologic and gastrointestinal toxicity. We previously demonstrated that transplantation with transgenic marrow expressing drug-resistant dihydrofolate reductase (DHFR) into animals preconditioned by irradiation substantially protected recipient mice from the toxic side-effects of methotrexate administration. Here we test the use of methotrexate itself as a preconditioning agent for engraftment of drug-resistant transgenic marrow, subsequently conferring drug-resistance upon recipient animals. Administration of methotrexate beginning one or two weeks prior to or on the same day as transplantation with drug-resistant DHFR transgenic marrow did not allow sufficient engraftment to confer drug-resistance to most unirradiated recipients. A small number of animals were curiously protected from lethal MTX toxicity, but exhibited extremely low hematocrits and were not engrafted with stem cells, as indicated by low engraftment levels assessed in secondary transplant recipients. However, we subsequently found that MTX preconditioning allowed sufficient engraftment of DHFR transgenic marrow to confer drug resistance if MTX administration was withdrawn at the time of BMT and withheld until two weeks post-transplant. Quantitative molecular analysis of primary and secondary recipients indicated a stem cell engraftment level of about 1%, consistent with previous studies demonstrating that a low level of DHFR transgenic cell engraftment was sufficient to confer drug-resistance in recipient animals. We conclude that MTX can be used as a preconditioning agent for subsequent engraftment of hematopoietic stem cells, in this case conferring resistance to MTX.

Introduction

Methotrexate (MTX) is a potent competitive inhibitor of dihydrofolate reductase (DHFR), a key enzyme in the generation of reduced folates crucial for the biosynthesis of purines and thymidylic acid (Blakley, 1984; Blakley, 1995). Due to its substantial antiproliferative activity, MTX has been used effectively as a chemotherapeutic agent in the treatment of both hematopoietic and solid organ neoplasms, particularly acute lymphocytic leukemia, non-Hodgekin's lymphoma, choriocarcinoma, Ewing's sarcoma, and osteosarcoma (Jolivet et al., 1983; Schornagel, 1983; Bertino, 1993). However, the usefulness of MTX as an antitumor agent is limited by toxicity for highly proliferative normal cells and tissues of the hematopoietic system and of the gastrointestinal tract (Margolis, 1971; Rivera et al., 1985).

Chemotherapeutic use of MTX and other antifolates might be rendered more effective if GI toxicity and myelosuppression could be averted by expression of a drug-resistant form of DHFR in normal, drug-sensitive tissues. To this end, several investigators have reported experiments in which transplantation with donor bone marrow, expressing drug-resistant DHFR either by retroviral transduction or by germ line transgenesis, rendered recipient animals resistant to MTX (Williams et al., 1987; Corey et al., 1990; Zhao et al., 1994; Morris et al., 1996; James et al., 1997). As a part of these studies, we recently reported that animals subjected to mild preconditioning and subsequently engrafted with as little as 1% DHFR transgenic cells after bone marrow transplantation were significantly resistant to doses of methotrexate which are lethal for normal animals (James et al., 2000) . MTX administration itself can cause severe myelosuppression, potentially resulting in conditions which are sufficiently cytoreduced to allow engraftment of subsequently transplanted DHFR transgenic marrow and resistance of recipient animals to continued methotrexate administration. In this

study, we tested this possibility and demonstrated that MTX preconditioning does indeed allow engraftment of subsequently transplanted DHFR transgenic marrow. However, such engraftment did not occur when MTX was administered continuously and immediately post-BMT, but rather required withdrawal of MTX administration during the time immediately following BMT. The results from these experiments demonstrate that the myelosuppressive effect of antifolate administration can be used to create hematopoietic space for subsequent engraftment of hematopoietic stem cells, which may in turn be genetically engineered to express drug-resistance genes or other types of genes to confer therapeutic benefit for the patient.

Materials and Methods

Animals and Bone Marrow Transplant Studies. FVB/N mice were obtained from the NIH supply facility at Frederick, MD. The line 04 Arg22 DHFR transgenic mice used in this study have been previously described (Morris et al., 1996; James et al., 1997). APP transgenic mice were used to provide a separate, transgenic non-DHFR donor signal, and were obtained from Dr. Karen Hsiao, Dept. of Neurology, University of Minnesota (Hsiao et al., 1995). Animals were provided with food and water *ad libitum*. Bone marrow transplant experiments were conducted as previously described. Briefly, marrow was flushed from the long bones of the hind limbs of donor DHFR or APP transgenic animals into DMEM, and a single cell suspension was prepared by repeated pipeting and passage through a 27-gauge needle. Ten million donor marrow cells were introduced through the lateral tail vein of unirradiated recipient mice. Recipient animals were administered 4 mg/kg/day methotrexate (amethopterin, Sigma) or phosphate-buffered saline by intraperitoneal injection before and/or after bone marrow transplantation as described in the Results. Control animals were treated similarly to the experimental animals with respect to the timing and volume of materials administered. Peripheral blood was collected weekly from the retro-orbital vein under anesthesia for determination of hematocrit. For secondary transplants, marrow was collected from primary recipients 90 days after drug administration, and 5×10^6 total bone marrow cells were injected intravenously into lethally-irradiated (8.5 Gy) FVB/N mice. Statistical analysis was conducted using the Kaplan-Meier product limit method (Kaplan, 1958), calculating the log-rank statistic (Peto, 1972). All animal studies were carried out under the guidance of the University of Minnesota Institutional Animal Care and Use Committee.

Southern Hybridization Analysis. Engraftment levels in marrow transplant recipients were determined by Southern hybridization analysis as previously described (Southern, 1975). Briefly, genomic DNA was isolated from marrow and spleen, digested with BglII, electrophoresed through 1% agarose/tris-acetate, and blotted onto Nytran (Scleicher and Schuell, Keene, NH). Blots were probed with either a 485 bp DHFR fragment containing exons 1 and 2 and intron 1 (Morris, 1996), or with a 1.3 Kb APP cDNA fragment (kindly provided by Dr. Hsiao), radiolabelled by random priming. Blots were hybridized and washed as previously described (James et al., 1997). Radioactive signals were quantitated using a 445SI PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For quantitation of DHFR transgene signals (n), the endogenous DHFR gene signal was used as a loading control in the formula; $n = (a \div b)/(c \div d)$, where a and b are the DHFR transgene and endogenous DHFR signals in the sample evaluated, while c and d are the DHFR transgene and endogenous signals from a DHFR transgenic (100%) positive control. APP transgene signals were similarly quantitated, where a and c were the test sample APP transgene signal and APP transgenic (100%) positive control, respectively.

Polymerase chain reaction (PCR). In some cases, engraftment of transgenic cells was determined by PCR analysis of genomic DNA extracted from spleen tissues of test animals. Genomic DNA was isolated from liver tissue (for transgene positive and negative controls) using the Gentra Generation Capture Column Kit (Minneapolis, MN) according to kit instructions. Standard PCR was performed to amplify a DNA sequence specific to the HBV (hepatitis B virus) sequence located within the DHFR transgene. Reactions contained 10 pmoles each of HBV-specific oligonucleotide primers (sense, 5′-ACCTCTCTTTACGCGGTCTC-3′; antisense,

5′-AATGTCCATGCCCCAAAGCC-3′), in a 50 ul reaction mixture with 100 mM Tris-HCl (pH 8.5), 50 mM KCl, 3.5 mM $MgCl_2$, 200 uM of each dNTP, 0.5 U Taq DNA polymerase, and 300 ng genomic DNA template for the control reactions, or 500 ng genomic DNA template for the unknown reactions. The cycling parameters were 30 cycles of 30 seconds at 94°C, 30 seconds at 62°C, and 30 seconds at 72°C, followed by a 10-minute incubation at 72°C. PCR reaction products were electrophoresed in 1% agarose, and the 381 bp HBV-specific product was visualized with 0.5 ug/ml ethidium bromide.

Quantitative real-time PCR. The ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) was used for these assays. A TaqMan probe and primer set was designed using Primer Express software (PE Biosystems, Foster City, CA), and synthesized by Applied Biosystems (Foster City, CA). The sense primer 5′-CCGGTCCGTGTGCACTTC-3′, antisense primer 5′-AGGATCTGATGGGCGTTCAC-3′, and fluorescent FAM dye-labeled probe 5′-ACCTCTGCACGTTGCATGGAGACCA-3′ were designed to amplify the HBV (hepatitis B virus) sequence located within the DHFR transgene (Morris, 1995). Calibration standards consisted of mixtures containing both DHFR transgenic and normal liver genomic DNA, corresponding to varying DHFR-transgene content (0, 0.01, 0.1, 1, 10, 25. 50, 100%). 25 µl reaction mixtures contained TaqMan Universal PCR Master Mix (PE Biosystems – Roche Molecular Systems, Branchburg, NJ), 200nM of each forward and reverse primer, 200 nM probe and 100 ng DNA sample (standard or unknown). PCR reaction conditions consisted of 50°C for 2 minutes and 95°C for 10 minutes, followed by cycling between a melting temperature of 95°C for 15 seconds and an anneal-extension temperature of 60°C for 1 minute, repeated for 40 cycles.

Threshold cycle values obtained for test samples (run in triplicate) were interpolated from the calibration curve to determine DHFR transgene copy number.

Results

We previously reported that FVB/N mice can be protected from methotrexate toxicity by transplantation with marrow obtained from transgenic animals expressing drug-resistant dihydrofolate reductase activity (May, 1995; James et al., 1997). Methotrexate causes severe hematologic toxicity in humans and in animals, so as a part of our ongoing studies on methotrexate resistance mediated by expression of drug-resistant DHFR activity we wondered whether MTX itself might be used as a cytoreductive agent to create hematopoietic space for subsequently infused transgenic marrow expressing drug-resistant DHFR activity. We first tested this possibility by transplanting 10^7 Arg22 DHFR transgenic marrow cells into normal, unirradiated FVB/N animals preconditioned by administration of MTX at 4 mg/kg per day. Methotrexate administration was initiated either two weeks before BMT (Figure 1A), one week before BMT (Figure 1B), or on the same day as BMT (Figure 1C), and continued through day 60 post-BMT. The dose of MTX (4 mg/kg) used in these experiments is a standard dose that we have established in our experimental system and is well tolerated by animals transplanted with Arg22 DHFR marrow while normal animals or animals transplanted with normal marrow succumb to toxicity. It was anticipated that the hematologic toxicity of MTX administered starting at the time of BMT or starting 1-2 weeks prior to BMT would provide sufficient cytoreduction to allow engraftment of DHFR transgenic marrow and, subsequently, protection of recipient animals from MTX toxicity, as previously observed for animals preconditioned with reduced doses of irradiation (James et al., 2000). However, we found instead that only 3 animals survived out of a total of 24 mice transplanted with DHFR drug-resistant marrow in these three study groups (Figure 1), indicating that continuous

methotrexate administration does not allow sufficient engraftment to confer methotrexate-resistance of recipient animals.

Interestingly, these three animals survived for several weeks after their hematocrit levels had fallen to around 10 starting at three weeks post-transplant, without otherwise showing signs of deteriorating health (Fig 1B and 1C). In contrast, we have previously observed that animals suffering from such a reduced hematocrit level usually succumb to the effects of MTX administration within 1 to 2 weeks after reaching this low level i.e., less than 15; (May, 1995; Morris et al., 1996; James et al., 2000). We hypothesized that these animals had engrafted with cells which were capable of contributing to protection from MTX toxicity, but which were either incapable of or somehow prevented from contributing to erythropoiesis. MTX was withdrawn, and within two weeks their hematocrit level was normal, indicating that MTX administration was indeed responsible for maintenance of low hematocrit prior to withdrawal from drug administration. To determine whether these animals had engrafted with hematopoietic stem cells, the animals were sacrificed, harvesting marrow and transplanting it into secondary recipients preconditioned by lethal total-body irradiation. Secondary recipients were allowed to engraft for 4 months before harvesting spleen and marrow and carrying out quantitative Southern hybridization studies to determine the level of engraftment (Figure 2). The DHFR transgene signal was very low in spleen and marrow (less than 0.1% transgenic material) from all secondary transplant recipients. We conclude from these studies that the small number of animals surviving MTX administration after MTX preconditioning and transplant with DHFR transgenic marrow must have engrafted with drug-resistant hematopoietic progenitors which were capable of mediating protection from methotrexate, but

which were not primitive enough to mediate long-term regeneration of hematopoietic stem cells in secondary transplant recipients, nor to mediate erythropoiesis.

One possible explanation for the lack of engraftment and drug-resistance in animals preconditioned with methotrexate is that methotrexate administration after BMT may have inhibited engraftment of donor, DHFR transgenic stem cells, preventing their contribution to hematopoiesis post-transplant. We have, in fact, observed decreased engraftment associated with methotrexate administration in animals transplanted with transgenic marrow after preconditioning with sublethal doses of irradiation (James et al., 2000). To test this possibility, we preconditioned normal, FVB/N females with 14 days of methotrexate adminstration at 4 mg/kg/day. The animals were rested for four days, and then transplanted with 10^7 arg22 DHFR transgenic marrow cells. Recipient animals were allowed to recover from BMT for another 14 days, and then tested for drug-resistance by initiating methotrexate administration at a daily dose of 4 mg/kg. In this experiment, animals preconditioned by MTX administration and transplanted with drug-resistant marrow exhibited long-term resistance to MTX that extended out to two months post-transplant (Figure 3A). Reduced survival was observed for untransplanted animals as well as animals transplanted with normal (APP) marrow. The resistance of DHFR transgenic marrow transplant recipients to the toxic effect of MTX administration was further demonstrated in the maintenance of hematocrit levels observed in these animals, i.e., hematocrit levels did not fall below an average of 30 during the entire period of drug administration (Figure 3B).

Quantitative Southern hybridization analysis indicated that these animals were engrafted at a level of approximately 1% donor transgenic marrow (Figure 4). In contrast, in control animals administered PBS rather than MTX both before and after BMT, the

engraftment level was nearly undetectable (less than 0.1%). These results using MTX conditioning are similar to our previous studies, in which we have reported protection from MTX toxicity in animals preconditioned with reduced doses of irradiation (1 to 4 gy) and transplanted with reduced numbers of drug-resistant marrow cells (down to 10^6), resulting in reduced engraftment levels down to 1% (James et al., 2000).

These results demonstrate that MTX administration, using the schedule described above, allows for engraftment of donor transgenic hematopoietic cells with subsequent drug resistance of recipient animals, but that MTX must be withdrawn during the time immediately post-transplant in order for such engraftment to occur. Finally, we tested whether the engraftment observed in MTX-preconditioned animals was attributable to stem cells by transplanting marrow collected from primary recipients into lethally-irradiated secondary transplant recipients. Secondary recipients were sacrificed 4 months post-transplantation, and then DNA was extracted from spleen samples and assayed for engraftment by quantitative PCR (Figure 5). In Figure 5, images from standard PCR/agarose gel electrophoresis are shown along with the Arg22 DHFR transgene copy number (per genome equivalents) as determined by real-time quantitative PCR. DHFR transgene engraftment in animals transplanted with Arg22 transgenic DHFR marrow ranged from 0.02 to 3.03 copies per genome equivalent, while in control animals that received no bone marrow transplant or were transplanted with APP marrow, DHFR transgene levels were essentially undetectable (Figure 5). Thus, as we observed for the primary recipients, engraftment levels in secondary transplant recipients were also at around the 1% level. These results demonstrate that MTX preconditioning allows for engraftment of hematopoietic stem cells capable of serial reconstitution in secondary irradiated transplant recipients.

Discussion

We conducted experiments to test the effectiveness of methotrexate administration as a preconditioning regimen for engraftment of DHFR transgenic marrow and subsequent MTX-resistance of recipient animals. Continuous administration of MTX starting either before or at the same time as transplantation with drug-resistant transgenic marrow did not allow sufficient engraftment to confer drug-resistance in recipient animals. However, when MTX was administered for two weeks and then withdrawn for two weeks immediately following BMT, animals were found to be engrafted at the 1% level with DHFR transgenic marrow and to exhibit substantial resistance to MTX administration. Engraftment of transgenic hematopoietic stem cells in MTX preconditioned animals was further demonstrated by serial marrow transplantation and maintenance of engraftment levels in secondary transplant recipients. We conclude that MTX can be used as a preconditioning agent for engraftment of hematopoietic stem cells (although with more limited efficiency than other cytoreductive treatments) and that this engraftment can be used to mediate drug-resistance in recipient animals.

Numerous investigations have reported the introduction and expression of drug-resistant forms of DHFR in hematopoietic cells, resulting in substantial resistance of test animals to subsequent antifolate administration (MTX or trimetrexate, TMTX) (Williams et al., 1987; Corey et al., 1990; Zhao et al., 1994; Morris et al., 1996; James et al., 1997; Allay et al., 1997; Sorrentino, 1999; Warlick et al., 2002). In these studies, recipient animals were transplanted either with retrovirally-transduced normal hematopoietic cells (Williams et al., 1987; Corey et al., 1990; Zhao et al., 1994) or with DHFR transgenic hematopoietic cells

(Morris et al., 1996; James et al., 1997) after preconditioning with total-body irradiation. In most cases, lethal TBI has been used in order to maximize the engraftment level of donor hematopoietic cells. This is particularly important for experiments using retrovirally transduced donor material, since the frequency of transduced cells achieved in the test animal would be compromised by a reduced level of overall donor cell engraftment. A key question in these experiments is the level of DHFR transgenic or transduced cell engraftment that is necessary in order to confer antifolate resistance in recipient animals. We recently reported experiments in which animals were transplanted with DHFR transgenic marrow after preconditioning with sublethal doses of TBI, and in which rescue from lethal MTX administration post-BMT was observed in animals engrafted with as little as 1% donor transgenic marrow (James et al., 2000). In the experiments described in this paper, we similarly found that MTX itself can provide preconditioning to allow engraftment of DHFR transgenic marrow at approximately the 1% level, sufficient to protect animals from subsequent MTX administration. These results imply that, in a gene therapy procedure intended to protect the recipient from MTX toxicity by drug-resistant DHFR gene transfer and expression, preconditioning other than that provided by MTX administration itself may not be necessary. The apparent low-level requirement for preconditioning and engraftment in this system is an important consideration, as *ex vivo* gene therapy protocols have in general avoided using myeloablative conditions.

Although MTX is known to be acutely myelosuppressive and to cause hematopoietic toxicity (Schornagel, 1983; Bertino, 1993), it has not been previously used as a preparative agent for the purpose of hematopoietic stem cell transplantation. This is perhaps because MTX is not known to be particularly toxic for stem cells on its own. Blau reported that MTX

administered as a single injection caused no toxicity for stem cells in mice (Blau, 1996). Allay et al reported that the antifolate trimetrexate was not toxic for stem cells when administered on its own, but rather required co-administration of a nucleoside transport inhibitor (nitrobenzyl-mercaptopurine riboside-phosphate) to prevent salvage of nucleosides and rescue from antifolate toxicity (Allay et al., 1997; Allay et al., 1998). However, reduced engraftment has been reported in patients undergoing methotrexate therapy as GVHD prophylaxis (Atkinson et al., 1983). Additionally, we have recently reported that MTX inhibited engraftment of DHFR transgenic marrow when administered starting immediately post-transplant (James et al., 2000). In this study, we found that MTX administered at a moderate dose (4 mg/kg/day) over a period of two weeks, while not extremely toxic for stem cells, created sufficient hematopoietic space to allow subsequent low-level engraftment of drug-resistant HSC. The ability of drug-resistant marrow to engraft in MTX pre-conditioned animals must have resulted either from toxicity of this dose of MTX for stem cells to some extent, or from the effect of MTX on the character of the marrow microenvironment, rendering it susceptible to the establishment of newly introduced stem cells in the marrow (Srour et al., 2001).

The results reported here support the concept that drug-resistance conferred by DHFR gene transfer and expression requires only a low level of DHFR gene transfer and cellular engraftment. Results from the experiments described in this paper also demonstrate that this level of cellular engraftment is achievable without any further cytoablative procedure than that provided by MTX administration itself. Under these conditions, MTX could be administered first for the purpose of providing cytoreductive preparation for engraftment of DHFR-transduced hematopoietic stem cells, and subsequently MTX could be administered as

an antitumor chemotherapeutic agent with reduced toxicity for normal tissues resulting from expression of drug resistant DHFR activity. Furthermore, under the appropriate pharmacologic conditions (i.e. in combination with nucleoside transport inhibitor), DHFR-expressing HSC can be expanded *in vivo* (Allay et al., 1997; Allay et al., 1998; Warlick et al., 2002). Vectors containing a therapeutic gene in addition to a DHFR gene as a selectable marker may thus be initially established at a low level of engraftment following MTX administration, and subsequently expanded *in vivo* to increase the representation of transduced cell numbers in the blood and hematopoietic organs. Such expanded numbers of transduced stem cells may be necessary in the treatment of diseases which would require an increased frequency of transduced cells in the blood in order to be effective. Drug-resistant DHFR gene transfer and expression thus has potential applications in the treatment of anti-folate sensitive tumors through improved chemotherapy, and in the treatment of hematologic disorders through the use of DHFR as a selectable marker (Karlsson, 1991; Halene, 2000).

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Footnotes

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Figure Legends

Figure 1. Effect of DHFR marrow transplant on health (hematocrit) and survival of normal animals administered 4 mg/kg/day MTX. (A). Schematic representation of treatment protocol. Animals were administered MTX intraperitoneally starting at day 0 and continuing until animal death or until drug withdrawal after day 60. 10^7 Arg-22 DHFR transgenic marrow cells (line 04) or normal FVB/N marrow cells were transplanted into recipient animals on day 0 (B), day 7 (C), or on day 14 (D) after initiation of MTX administration. Top panels (B-D). Kaplan-Meier plots showing the fraction of animals surviving over the two month period of MTX administration. Bottom panels: Mean hematocrit values $\pm$ S.D. were assessed for each group on a weekly basis.

Figure 2. Southern hybridization analysis of secondary transplant recipients. Bone marrow was harvested from the three surviving animals depicted in Fig. 1B and 1C, and each marrow sample was transplanted into three lethally-irradiated secondary recipients. After four months, the secondary recipients were sacrificed, harvesting marrow and spleen for Southern analysis as described in Materials and Methods. The locations of DHFR-hybridizing BglII fragments corresponding to the DHFR transgene (Tg) and the endogenous DHFR gene (En), used here as a loading control, are shown. Animal numbers correspond to the primary recipient marrow source: #71 was a control animal transplanted with DHFR transgenic marrow and administered PBS; #47 and #48 were the two survivors shown in Fig. 1B; #67 was the single survivor shown in Fig. 1C. Samples from animals transplanted with normal or DHFR transgenic marrow are shown as negative and positive controls, respectively.

Figure 3. MTX preconditioning allows engraftment of subsequently transplanted normal or transgenic marrow, and drug-resistance of DHFR transgenic marrow recipients. (A). Schematic representation of treatment protocol. Normal FVB/N animals were preconditioned with 14 days of MTX administration, 4 mg/kg/day. Drug administration was withdrawn for 4 days, and then some of the animals were transplanted either with 10^7 DHFR transgenic marrow (line 04) cells or with 10^7 normal marrow cells bearing a different transgene (APP) as a molecular marker (as described in Materials and Methods). MTX administration (4 mg/kg/day i.p.) was resumed starting 14 days after BMT. (B). Kaplan-Meier plot showing the fraction of animals surviving over the two month period of MTX administration for each of the three groups. (C). Mean hematocrit values $\pm$ S.D. were assessed for each group on a weekly basis.

Figure 4. Southern hybridization analysis to assess engraftment level in MTX preconditioned animals. Bone marrow was harvested from the surviving animals depicted in Fig. 3B, as well as from PBS-administered controls. DNA was extracted and subjected to Southern hybridization analysis as described in Materials and Methods, probing for both DHFR- and APP-hybridizing BglII fragments. In each set of samples, the type of marrow transplanted (DHFR transgenic, "Tg", APP transgenic, or none) is indicated underneath the solution administered (PBS or MTX). Control marrow samples included DHFR transgenic marrow (T), normal marrow (N), and APP transgenic marrow (A). The locations of BglII fragments corresponding to the DHFR transgene (Tg) and the endogenous DHFR gene (En), and the APP transgene (APP), are shown.

Figure 5. Standard PCR and real-time quantitative PCR analysis to assess engraftment levels in secondary transplant recipients. Marrow was harvested from each primary recipient (indicated by numbers) and transplanted into 3 secondary recipient mice. DNA was extracted from the spleen and subjected to PCR analysis as described in Materials and Methods. Control samples consisted of normal mouse liver DNA mixed with transgenic DHFR marrow ranging from 0.01% to 3% in the standard PCR analysis. The type of marrow transplanted into the primary recipients and the conditioning received is indicated next to the animal numbers. QPCR values are expressed as DHFR transgene copies per diploid genome equivalent and represent the mean of at least three reactions.

Figure 1

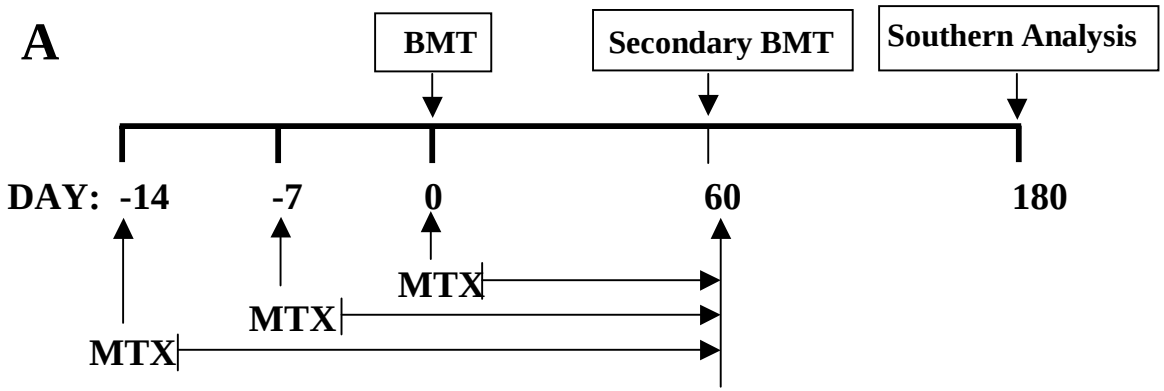

Figure 1

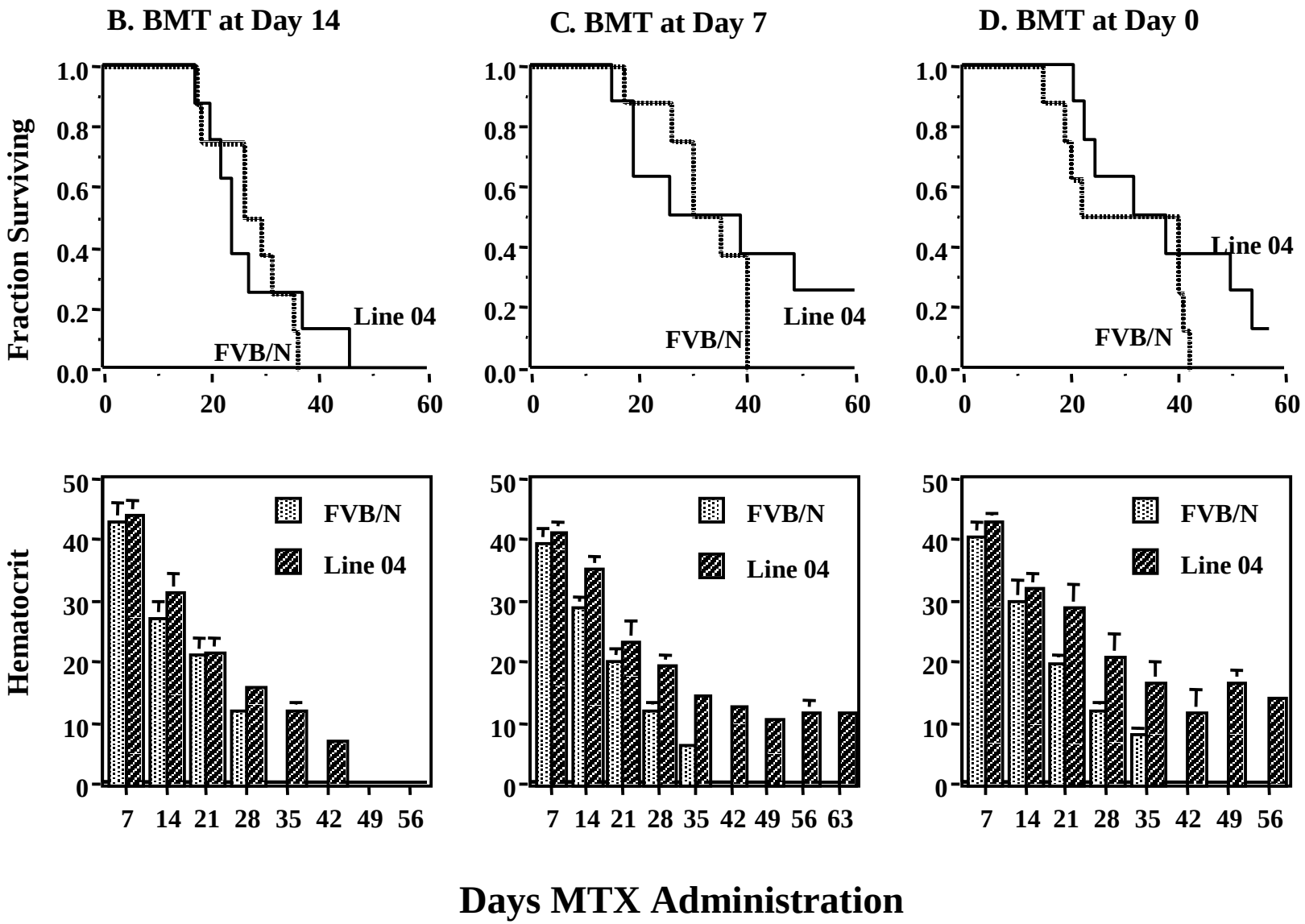

Figure 2

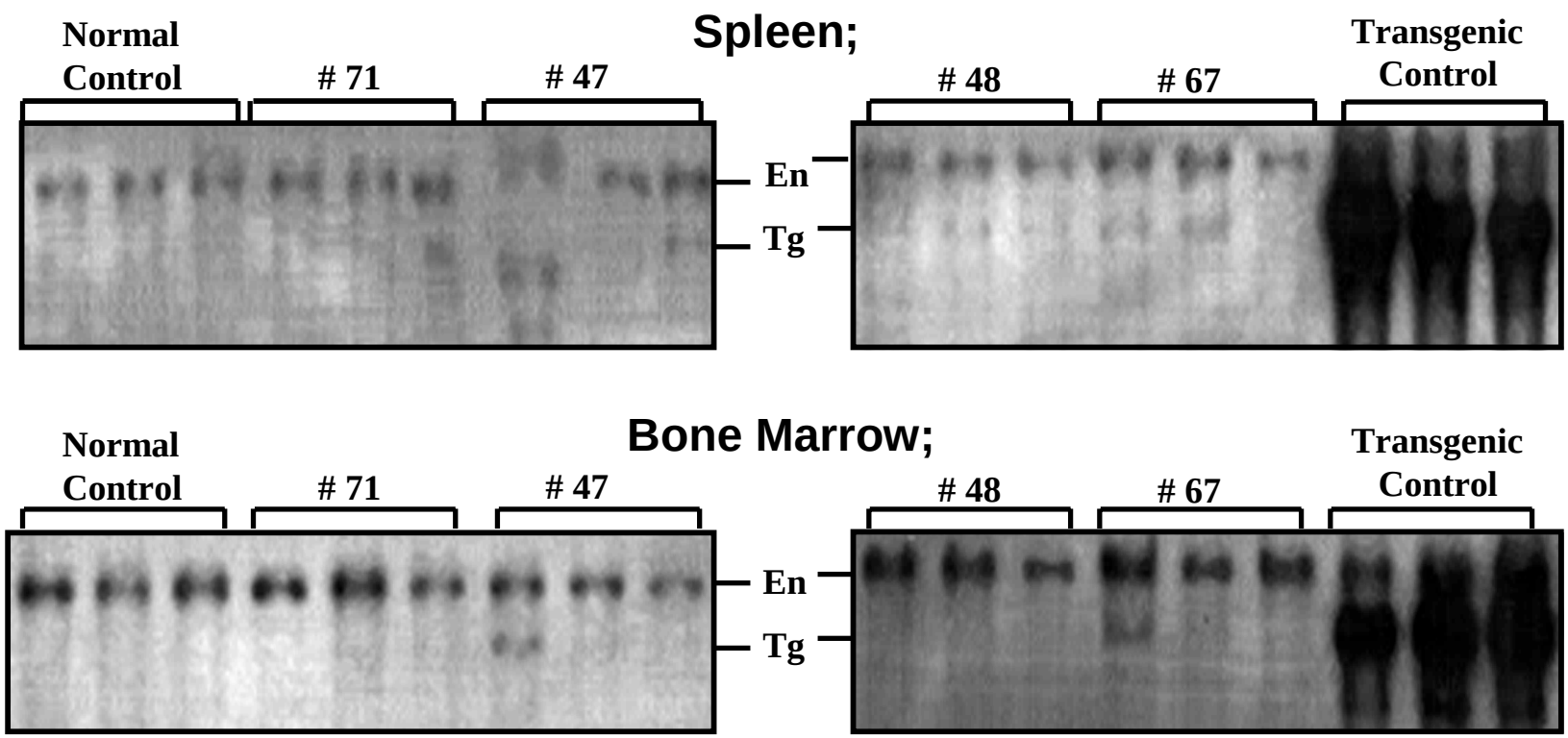

Figure 3

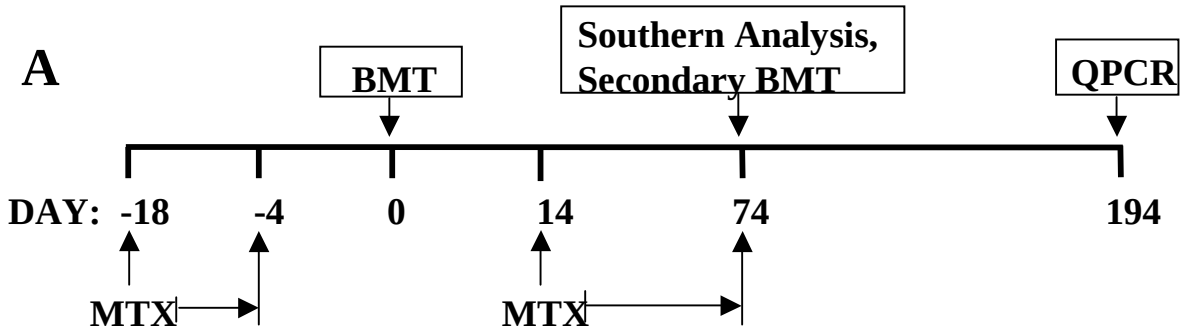

Figure 3

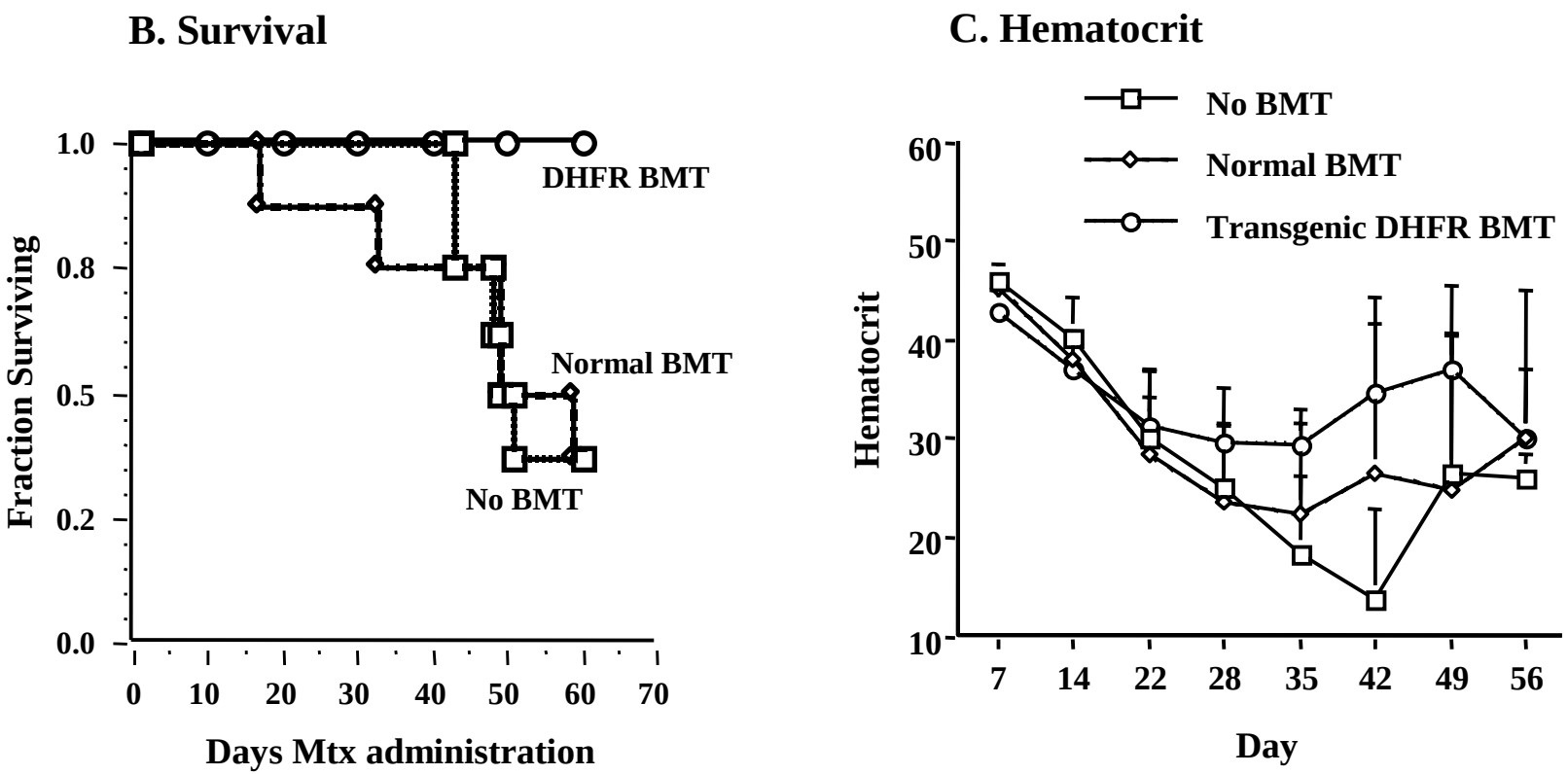

Figure 4

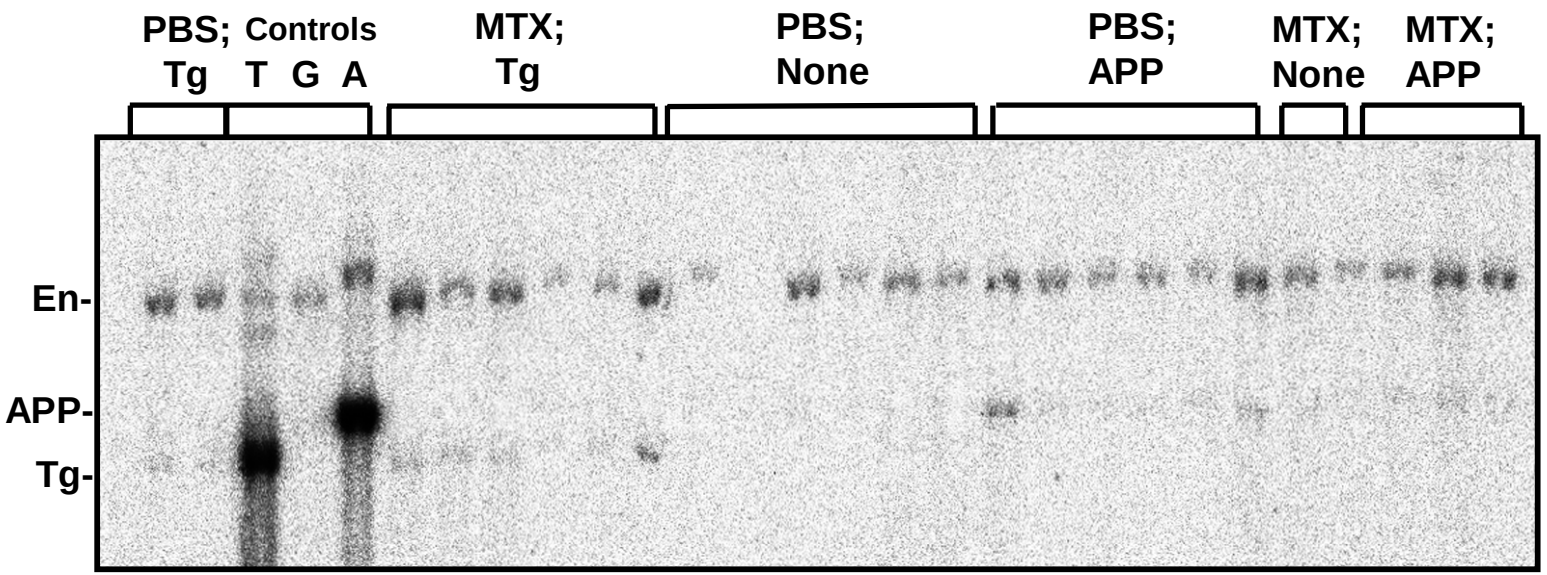

Figure 5