Protection of Mice From Lethal Doses of Methotrexate by Transplantation With Transgenic Marrow Expressing Drug-Resistant Dihydrofolate Reductase Activity

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Marrow cells from previously established lines of transgenic mice expressing either of two different methotrexate (MTX)-resistant dihydrofolate reductases (DHFRs) were transplanted into recipient animals to determine the resultant in vivo protective effect against toxicity associated with MTX administration. Sublethally irradiated, untransplanted animals were first used to establish conditions of low-dose MTX administration resulting in substantial hematopoietic toxicity with undetectable gastrointestinal toxicity. Under these conditions, low survival rates were observed for normal or transgenic animals not expressing drug-resistant DHFR activity, whereas transgenic animals expressing either the arg22 (line 04) or trp31 (line 03) DHFR variants survived. Transplantation of 10^7 marrow cells from either transgenic lines 03 or 04 rescued normal FVB/N recipient animals from low-dose MTX administration, which was lethal for animals transplanted with 10^6 normal FVB/N marrow cells. Reduced survival of transgenic line 04 marrow recipients was observed when twofold or fourfold doses of MTX were administered. However, when 10^7 transgenic line 04 marrow cells were infused, the recipients were found to be resistant to a MTX dose that was not only lethal for animals transplanted with 10^7 normal FVB/N marrow cells, but also lethal for normal, untransplanted FVB/N mice. Histologic analysis showed protection of both marrow and gastrointestinal tissues from MTX toxicity in transgenic line 04 marrow transplant recipients. Thus, exclusive expression of MTX-resistant DHFR activity in the marrow had a substantial, systemic chemoprotective effect in animals, which could be applied for improved utilization of MTX for antitumor chemotherapy.

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MATERIALS AND METHODS

Animals and bone marrow transplantation (BMT). FVB/N mice were obtained from the National Institutes of Health animal supply facility at Frederick, MD. DHFR transgenic animals used in this study were established in the FVB/N strain as previously described (Morris et al, submitted). Animals were provided food and water ad libitum. The work described in this paper was reviewed and approved by the University of Minnesota Animal Care Committee. BMT was conducted essentially as described previously for C3H mice. Briefly, marrow was flushed from the long bones of the hind limbs of donor mice into Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% newborn calf serum. Marrow cells were washed twice by centrifugation at 2,000 rpm in a Beckman TJ-6 (Beckman Instruments, Fullerton, CA) for 15 minutes at 4°C and then reuspended in DMEM at a final concentration of 1 to 5 × 10^7

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viable cells/mL. Appropriate dilutions were then injected through the tail vein into female FVB/N mice irradiated 18 to 24 hours previously with 950 rads cesium, a dose previously determined to eliminate endogenous spleen colony formation.

**Methotrexate (MTX) administration.** MTX (amethopterin; Sigma Chemical Co, St Louis, MO) stock solutions were prepared in 10 mmol/L TRIS, pH 7.5, and diluted in phosphate-buffered saline (PBS; 137 mmol/L NaCl, 3 mmol/L KCl, 10 mmol/L potassium phosphate pH 7.4). Animals were weighed and administered MTX daily by intraperitoneal injection, beginning 1 day posttransplant (see figure legends for dose schedules of individual experiments). Selected animals were killed for histopathologic analysis as described below. Animal health and survival were monitored on a daily basis, and hematocrit was determined weekly. Survival was analyzed by the Kaplan-Meier product limit method, using the log-rank statistic to test differences among groups.31

**Histopathologic analysis.** Liver, ileum, sternum, and femur were procured from selected animals and fixed in 10% phosphate-buffered formalin (bone samples were subsequently decalcified in 10% formic acid), embedded in paraffin, sectioned, mounted, and stained (hematoxylin and eosin). Analysis of tissue samples was undertaken without prior knowledge of animal identity.

**Southern hybridization analysis.** Southern hybridization analysis52 of marrow samples was performed for presence of the transgene as previously described (Morriss et al, submitted). Briefly, DNA was extracted from mouse bone marrow samples53 and then digested with restriction endonuclease overnight. Samples were electrophoresed on 1% agarose and blotted onto Nytran (Schleicher and Schuell, Keene, NH) according to the manufacturer’s instructions. Prehybridizations and hybridizations were conducted as previously described.15 The probe was a 485-bp DHFR fragment spanning the region between the 5' untranslated region and the exon/intron 2 junction of the DHFR transgene, generated as previously described (Morriss et al, submitted) and labeled using the oligolabeling kit from Pharmacia (Piscataway, NJ). Blots were washed twice in 6x SSPE (0.18 mmol/L NaCl, 5 mmol/L Na2HPO4, 1 mmol/L disodium EDTA, pH 7.0) for 15 minutes at room temperature and once in 0.1 mmol/L SSPE for 30 minutes at 60°C, followed by autoradiography.

**RESULTS**

Low-dose MTX is selectively toxic for hematopoietic tissues in sublethally irradiated animals. In previous studies, we established lines of transgenic mice which exhibited either partial or substantial resistance to MTX at doses that normally cause severe marrow aplasia, disruption of gastrointestinal tissue and eventual death (Morriss et al, submitted). In this report, we describe experiments that test the chemoprotective effect of transgenic, drug-resistant marrow when transplanted into normal recipients. Because we anticipated that the protective effect of transplanted transgenic marrow would be limited to the hematopoietic system, conditions of MTX administration that result in selective toxicity for the
hematopoietic system were first established. Normal and DHFR transgenic lines 01, 02, 03, and 04 (Morris et al, submitted) were administered 650 rads cesium irradiation, a dose that we previously determined to result in substantial endogenous spleen colony formation as a measure of residual endogenous hematopoiesis. MTX was then administered daily at a dose of 0.25 mg/kg on days 1 through 4, 0.5 mg/kg on days 5 through 8, and 1.0 mg/kg on day 9 and thereafter. This dose schedule is 25% the dose previously shown to be lethal for normal FVB/N mice (Morris et al, submitted) and approximates the dose reported by several groups to be lethal for normal BMT recipients.25-29 We found that this dose schedule was lethal for 62% of normal animals, 100% of transgenic line 01 animals, and 75% of line 02 animals administered the sublethal dose of total body irradiation (TBI) (Fig 1A). Earlier experiments with transgenic lines 01 and 02 (both trp31 transgenics) have shown little or no transgene expression (Morris et al, submitted). As previously observed for unirradiated animals, transgenic line 04 (arg22) was resistant (100% survival) to normally lethal doses of MTX after sublethal irradiation. Transgenic line 03 (trp31) was also substantially MTX resistant (88% survival) under these conditions. Hematocrits at days 22 and 29 were reflective of the overall resistance to MTX (normal FVB/N, line 01 and line 02 were less resistant than line 03, which was less resistant than line 04), after which time the hematocrits of surviving animals returned to near normal (Fig 1B).

Histopathologic analysis of tissues taken from control normal FVB/N mice that were administered a sublethal dose of TBI showed no presence of significant lesions in the gastrointestinal tract after these animals were administered either MTX (Fig 2B) or PBS as a control (Fig 2A), even though MTX administration resulted in extremely reduced hematocrit with ensuing death (Fig 1). These results show that (1) sublethal irradiation followed by low-dose MTX administration results in substantial hematopoietic toxicity while sparing the gastrointestinal tract and (2) expression of a variant DHFR transgene protects against the toxicity of MTX administered under conditions that specifically cause marrow toxicity.
Protection of recipients from low-dose MTX toxicity by transplantation with DHFR transgenic marrow. To determine the chemoprotective effect of marrow expressing MTX-resistant DHFR activity, FVB/N mice were transplanted with $10^6$ donor marrow cells from normal FVB/N, transgenic line-03, or transgenic line-04 animals and then administered MTX according to the dose schedule established above for sublethally irradiated animals. Under these conditions, MTX administration was lethal for all animals transplanted with normal FVB/N marrow (median survival time, 28 days; Fig 3A). In contrast, all animals transplanted with either line-03 or line-04 marrow survived. Weekly hematocrit readings indicated that line-04 recipients suffered significantly less hematologic toxicity, never dropping below a mean reading of 31 and recovering by the third week, whereas hematocrit readings for line-03 marrow recipients fell to a mean of 18 and took as long as 5 weeks to recover (Fig 3B). Thus, marrow from transgenic lines 03 and 04 rescued transplant recipients from MTX doses that were lethal for animals transplanted with normal marrow, with line-

![Graph](https://via.placeholder.com/150)

**Fig 3.** MTX sensitivity of normal FVB/N mice transplanted with normal or DHFR transgenic marrow. Normal FVB/N females were subjected to irradiation and BMT as described in Materials and Methods. Recipients were infused with $10^6$ donor marrow cells procured either from normal FVB/N mice, transgenic line 03, or transgenic line 04 and then administered low-dose MTX according to the schedule described in the legend to Fig 1. Normal FVB/N animals injected with PBS were included as a control for toleration of the daily intraperitoneal injections (100% survival, not shown). (A) Kaplan-Meier plot showing the fraction of animals surviving as a function of time after BMT and the initiation of MTX administration for the groups indicated (N = 7 through 10). (B) Weekly mean hematocrit readings. Standard deviations (not shown) were less than 10% except for transgenic line 03 recipients on day 21 (4.3) and on day 28 (8.4). The experiment was terminated after the hematocrits of surviving animals returned to the normal range.

![Southern blot](https://via.placeholder.com/150)

**Fig 4.** Southern analysis of DNA extracted from BMT recipients. Marrow cells were flushed at the time the experiment shown in Fig 3 was terminated, ie, when the hematocrit of all surviving animals had returned to the normal range. DNA was extracted, digested with Bgl II, and analyzed for presence of the DHFR transgene by Southern analysis as described in Materials and Methods. DNA extracted from the marrow of recipients transplanted with normal FVB/N marrow (administered PBS) was included as a negative control (−), and transgenic line-04 tail DNA was included as a positive control (+). Samples 1 through 8 were obtained from recipient animals transplanted with transgenic line-04 marrow (see Fig 3). Position of the 4.9-kb Bgl II transgene fragment, consistent in size with tandem integrants, is indicated (Tg) as well as the position of the endogenous mouse DHFR gene (En).
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40 marrow recipients exhibiting a much more rapid hematologic recovery. These results are consistent with relative MTX resistance previously observed for transgenic lines 03 and 04 (Morris et al, submitted).

Engraftment of transgenic donor marrow was assessed by extracting DNA from the bone marrow of line-04 marrow recipients killed 30 days after BMT and analyzing for the presence of cells containing the DHFR transgene by Southern analysis (Fig 4). All animals exhibited a transgene signal quantitatively similar to that observed for a transgenic control sample, indicating that hematopoietic cells in the bone marrow were overwhelmingly derived from the donor DHFR transgenic marrow.

Resistance of DHFR transgenic marrow recipients to MTX doses lethal for normal animals. MTX dose-escalation studies were conducted to determine the degree of recipient drug-resistance brought about by transplantation with DHFR transgenic marrow. Normal FVB/N recipients were transplanted with 10⁶ normal or transgenic line-04 marrow cells and then placed on one of two MTX dose schedules (Fig 5) consisting of either two times or four times that of the initial BMT experiment described above. Both of these dose schedules were lethal for all animals receiving normal FVB/N marrow (mean survival time, 18 days at 2.0 mg/kg, 14 days at 4.0 mg/kg) and survival times for transgenic line-04 marrow recipients were significantly improved (P < .02 at both MTX doses). However, at the higher doses of MTX, survival of line-04 marrow recipients was compromised; 6 of 10 mice survived at 2.0 mg/kg MTX (Fig 5A), and only 1 of 10 mice survived at 4.0 mg/kg MTX (Fig 5B). Surprisingly, the one animal that survived the 4.0 mg/kg MTX resisted an amount of MTX previously shown (Morris et al, submitted) to be uniformly lethal for normal nonirradiated, untransplanted animals. These results provided an indication of the systemic drug-resistance capability imparted by DHFR transgenic marrow.

To further explore the resistance of DHFR transgenic marrow recipients to higher doses of MTX, animals were infused with a larger number (10⁷) of either normal or transgenic line-04 donor marrow cells and then administered MTX at a final dose of 4.0 mg/kg daily (four times the dose administered in the initial BMT experiment; Fig 3). Normal, untransplanted FVB/N animals were included as a control group for side-by-side comparison with BMT recipients. Under these conditions, animals transplanted with normal FVB/N marrow were the first to succumb (Fig 6; median survival time, 29 days) followed by normal, untransplanted FVB/N animals (median survival time, 37 days). In contrast, animals transplanted with 10⁷ transgenic line-04 marrow cells exhibited 90% survival, with hematocrits that barely fell below 40 before recovering to normal in the third week (Fig 6B). Thus, transplantation with transgenic line-04 marrow protected recipient animals not only from MTX levels that were lethal for recipients of normal marrow (ie, final dose, 1.0 mg/kg), but furthermore protected animals from doses of MTX that were lethal for normal, untransplanted animals (ie, final dose, 4.0 mg/kg), showing substantial systemic resistance brought about by expression of the drug-resistance gene exclusively in hematopoietic cells.

Histopathologic analysis of gastrointestinal tissue (ileum) and marrow was conducted on samples collected from MTX administered animals at the time of moribundity. All animals transplanted with normal marrow exhibited severe atrophy of gastrointestinal tissue (Fig 7A) and marrow hypoplasia (Fig 7B) near the time of death. Normal, untransplanted FVB/N animals succumbing earlier (ie, before day 30) also exhibited gastrointestinal atrophy (Fig 7C) and marrow hypoplasia (Fig 7D), but these tissues were not nearly as affected in normal, untransplanted animals that succumbed later (ie, after day 30). These latter animals were characterized by apparently normal gastrointestinal tissue (Fig 7E) and normal to hyperplastic bone marrow (Fig 7F), even though their mean hematocrit was extremely low (Fig 6B), suggesting that regenerating erythroid cells failed to reach the circulation. Transgenic line-04 recipient tissues were free of gastrointestinal pathology (Fig 7G), and exhibited narrowed hyperplasia (Fig 7H), again indicative of a recovering hematopoietic system.

![Figure 5](image_url)  
**Fig 5. Effect of increased MTX doses on survival of BMT recipients.** Normal FVB/N animals were exposed to 950 rad cesium and then transplanted with either 10⁶ normal FVB/N marrow cells or 10⁷ transgenic line-04 marrow cells. Animals were then administered MTX daily according to one of two dose schedules: (A) Two times the dose used in the experiments described above in Figs 1 and 3; 0.5 mg/kg on days 1 through 4, 1.0 mg/kg on days 5 through 8, and 2.0 mg/kg on day 9 and thereafter (N = 8 to 9). (B) Four times the dose used in the experiments described in Figs 1 and 3; 1.0 mg/kg on days 1 through 4, 2.0 mg/kg on days 5 through 8, and 4.0 mg/kg on day 9 and thereafter (N = 9). Kaplan-Meier plots are shown to exhibit the fraction of animals surviving versus time after marrow transplant. Normal FVB/N animals injected with PBS were included as a control for toleration of the daily Intraperitoneal injections (not shown).
A surprising implication of the transplant experiment depicted in Fig 6A was the possibility of protection from MTX-associated gastrointestinal toxicity by expression of drug-resistant DHFR activity in hematopoietic cells alone. To verify the protection from MTX toxicity afforded by transplantation with transgenic line-04 marrow, the experiment depicted in Fig 6A was repeated, this time killing animals from all three groups at regular intervals for histopathologic analysis of the gastrointestinal tract. Although no differences in inflammatory cell infiltration, Peyer’s patches or fre-
quency of mitotic cells were observed among the three experimental groups, substantial differences in villus length were observed (Fig. 6C). Villus length increased dramatically in normal FVB/N animals at day 29, followed immediately by a substantial decline. Villus length was not increased in normal FVB/N marrow transplant recipients, but rather fell off slightly at day 29. In contrast, villus length was increased markedly by day 29 in transgenic line 04 marrow recipients and remained high on days 33 and 38. Importantly, villus length was significantly increased \( (P < .001) \) for transgenic line 04 recipients in comparison with normal marrow transplant recipients on days 29 and 33 and in comparison with normal animals on days 33 and 38, precisely the times during which animals in the latter two groups succumbed to MTX toxicity (Fig. 6A). These results provide a quantitative demonstration of the protection of normal animals from MTX-associated toxicity by transplantation with DHFR transgenic marrow.

**DISCUSSION**

Protection of recipient mice from MTX toxicity was demonstrated by transplantation with transgenic marrow expressing drug-resistant DHFR activity. Protection from lower doses of MTX specifically associated with hematopoietic toxicity was first demonstrated after transplantation with a limited number (10⁴) of drug-resistant donor marrow cells. Additionally, however, transplantation with greater numbers of marrow cells (10⁶) conferred resistance to MTX at higher doses demonstrated to be lethal for untransplanted, unmanipulated animals. Normal animals can thus be protected from lethal, systemic MTX toxicity by transplantation with donor marrow cells expressing a drug-resistant DHFR.

MTX-resistance brought about by introduction of genes encoding variant, drug-resistant DHFRs has been studied previously in animals and extensively in cell culture systems. Drug-resistance of murine, canine and human hematopoietic progenitors has been reported after retroviral-mediated transduction and expression of arg22 or ser31 variant DHFRs. In animal systems, transgenic mice expressing variant DHFR activity which exhibit substantial systemic MTX-resistance have been reported (Morris et al submitted). Retroviral transduction studies have also been conducted to introduce and express the arg22 DHFR gene in murine and canine marrow transplant systems. In the murine system, improved survival of primary recipients through protection from MTX toxicity was reported, as well as transplatability of drug-resistance function to secondary recipients, demonstrating presence and expression of the drug-resistance gene in stem cells. These latter studies established that transplantation of donor marrow cells expressing MTX-resistant DHFR activity protected or at least improved survival of recipients administered MTX at lower doses lethal for animals transplanted with normal, unmanipulated marrow.

Using the inbred FVB/N, DHFR-transgenic system established in this laboratory (Morris et al, submitted), we confirmed that transplantation with transgenic marrow expressing arg22 DHFR activity protected animals from MTX at low doses nevertheless lethal for animals transplanted with normal marrow (Fig. 3). We also found that marrow expressing trp31 variant DHFR activity protected transplant recipients from lethal, low-dose MTX toxicity, although not as effectively as the arg22 DHFR transgenic marrow. However, the most surprising result from the experiments reported here was that transplantation with sufficient DHFR transgenic marrow cells protected recipients from MTX at higher doses which caused not only hematopoietic toxicity, but also severe gut necrosis in animals transplanted with normal marrow. Gastrointestinal protection from MTX toxicity was qualitatively verified, demonstrating a significant increase in villus length for recipient animals transplanted with arg22 (transgenic line 04) DHFR transgenic marrow. Exclusive expression of MTX-resistant DHFR activity in hematopoietic cells thus brought about substantial systemic drug-resistance reminiscent of that observed in transgenic mice which express the drug-resistance gene in many tissues (Morris et al, submitted). These results suggest a key role for hematopoietic cells in mediating the sensitivity or resistance of animals to MTX, acting as a site of MTX mediated pathology with possible tissue-to-tissue interactions. Whether this effect is direct or indirect (e.g. rapid recovery of hematologic function leading to normal food intake, allowing repair of gut lesions) is yet to be investigated.

MTX-associated disruption of hematopoietic cells and gastrointestinal tissue has been well-documented both in animal model systems as well as in human clinical studies. We found that mice receiving either sublethal doses of irradiation or higher doses of irradiation followed by BMT exhibited marked marrow hypoplasia when administered low doses of MTX, but were free of significant intestinal lesions. Furthermore, we found that normal FVB/N animals administered higher doses of MTX (final dose, 4.0 mg/kg) exhibited gastrointestinal atrophy if succumbing between days 20 and 30, but after day 30 animals succumbed without severe gastrointestinal lesions. The bone marrow of these mice also appeared normal. The most outstanding characteristic of normal FVB/N mice dying after 30 days was an extremely low (<10) hematocrit in spite of an abundance of red blood cell precursors in the marrow, suggesting that there may have been a maturation defect or that bone marrow regeneration occurred too late to compensate for the anemia. These results suggest anemia as the primary cause of death in these animals. However, regardless of the sites and timing of MTX toxicity, MTX-resistant DHFR expression in hematopoietic cells clearly protected recipient animals from lethal toxicity.

Results from this study and other previous studies have established that expression of MTX-resistant DHFR activity in the marrow can protect animals from low-dose MTX-associated hematopoietic toxicity. This in itself could potentially be useful in human applications, augmenting the usefulness of MTX as a chemotherapeutic agent and selecting for the outgrowth of genetically engineered cells. However, our observations of increased systemic resistance of animals to MTX brought about by exclusive expression of MTX-resistant DHFR activity in the marrow implies a much broader potential for this approach toward improved chemotherapeutic application of MTX as an antitumor agent. Chemoprotection by MTX-resistant DHFR gene transfer might not be limited to special circumstances that render hematopoietic tissues particularly sensitive to MTX such as extreme cytoreduction and BMT, but may be expanded to
include MTX-sensitive tumors not usually treated by TBI plus BMT and for which MTX toxicity to normal tissues limits its utility. Future experiments should define the extent to which increased dose tolerance can be achieved by expression of arg22 or other DHFR variants in hematopoietic cells, thereby optimizing this system and eventually applying it toward improved antitumor chemotherapy.

NOTE ADDED IN PROOF

The paper referred to as "Morris et al, submitted" in the text has been accepted for publication: Morris JA, May C, Kim HS, Ismail R, Wagner JE, Gunther R, McIvor RS: Comparative methotrexate resistance of transgenic mice expressing two distinct dihydrofolate reductase variants. Transgensics (in press)

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REFERENCES

Protection of mice from lethal doses of methotrexate by transplantation with transgenic marrow expressing drug-resistant dihydrofolate reductase activity

C May, R Gunther and RS McIvor