

Methotrexate Exacerbates Tumor Progression in a Murine Model of Chronic Myeloid Leukemia

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ABSTRACT

Expression of drug-resistant forms of dihydrofolate reductase (DHFR) in hematopoietic cells confers substantial resistance of animals to antifolate administration. In this study, we tested whether the chemoprotection conferred by expression of the tyrosine-22 variant DHFR could be used for more effective therapy of the 32Dp210 murine model of chronic myeloid leukemia (CML). 32Dp210 tumor cells were found to be sensitive to methotrexate (MTX) *in vitro*, whereas cells expressing the tyrosine-22 DHFR gene were protected from MTX at up to micromolar concentrations. MTX administered at low dose (2 mg/kg/day) did not protect normal C3H-He/J mice from 32Dp210 tumor infused intravenously, with drug toxicity limiting the administration of higher doses. Animals engrafted with transgenic tyrosine-22 DHFR marrow were protected from greater MTX doses (up to 6 mg/kg/day). However, the in-

creased doses of MTX afforded by drug-resistance gene expression surprisingly resulted in decreased survival of the transplanted tumor-bearing animals, with increased levels of tumor detected in peripheral blood. This apparent exacerbation of tumor progression by MTX was not observed in DHFR transgenic mice in which all cells and tissues contain the drug-resistance gene. This suggests that increased tumor progression in MTX-administered animals resulted from MTX sensitivity of a nonhematopoietic host component, thus allowing tumor expansion. We conclude that MTX exacerbates tumor progression in the 32Dp210 model of CML, and that based on this model alternate DHFR inhibitors combined with drug-resistant DHFR or other chemotherapeutic agent/drug-resistance gene combinations may be required for the application of drug-resistance gene expression to the treatment of CML.

Chronic myeloid leukemia (CML) is a disease of the hematopoietic stem cell, resulting in the expansion and premature circulation of relatively mature myelocytes. In the majority of cases, it is characterized by a translocation between chromosomes 22 and 9, resulting in fusion of the *bcr* and *abl* genes (Rowley, 1990). The resulting *bcr-abl* fusion oncogene has been shown to be necessary and sufficient for malignant transformation of hematopoietic cells (Daley et al., 1990). Chemotherapy for CML typically involves interferon- α , which can prolong survival but is not curative (Hehlmann et al., 2000; Hochhaus et al., 2000), although recently the Abl tyrosine kinase inhibitor STI571 has shown promise in clinical trials (Druker et al., 2001a,b). The most effective treatment for CML has been allogeneic bone marrow transplant, but is limited by donor incompatibility leading to graft rejection or graft-versus-host disease. Autologous marrow transplants for CML generally lead to relapse, either due to contamination of the graft with *bcr-abl*⁺ cells or incomplete

ablation of *bcr-abl*⁺ cells remaining in the patient (Deisseroth et al., 1994).

Antifolates such as methotrexate (MTX) inhibit the enzyme dihydrofolate reductase (DHFR), resulting in depletion of reduced folates necessary for thymidylate and purine synthesis and toxicity for actively dividing cells (Jolivet et al., 1983). Variant forms of DHFR resulting from amino acid substitutions at various positions in the coding sequence are less susceptible to MTX than wild-type DHFR (Simonsen and Levinson, 1983; Morris and McIvor, 1994; McIvor, 2002), and can render cells resistant to antifolates. Introduction of a variant DHFR gene into mouse bone marrow has been shown to confer increased antifolate resistance to transplanted mice (Williams et al., 1987; Corey et al., 1990; May et al., 1995; Spencer et al., 1996; James et al., 1997). The chemoprotection provided by drug-resistant marrow potentially allows for improved antitumor chemotherapy at greater antifolate doses (Zhao et al., 1997b). A gene therapy approach involving transduction of marrow with a drug-resistant DHFR gene may also improve the efficacy of autologous marrow trans-

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ABBREVIATIONS: CML, chronic myeloid leukemia; MTX, methotrexate; DHFR, dihydrofolate reductase; GFP, green fluorescent protein; IL-3, interleukin-3; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline.

plant for treatment of CML, by allowing post-transplant antifolate selection against untransduced normal and leukemic cells (Zhao et al., 1997a).

Many murine models of bcr-abl⁺ CML often result in syndromes more closely resembling acute leukemia or lymphoma in a subset of tumor-bearing animals (Daley et al., 1990; Voncken et al., 1995), rather than chronic-phase myeloid leukemia. One such model of CML is the 32Dp210 cell line (Carlesso et al., 1994), established by insertion and expression of human bcr-abl cDNA in the murine myeloid cell line 32D (Greenberger et al., 1983). We have observed that C3H mice infused with 32Dp210 cells exhibit rapid infiltration of myeloblastic 32Dp210 cells in a variety of tissues, in a syndrome resembling blast-phase or acute myeloid leukemia. This article focuses on the effect of MTX on progression of the 32Dp210 tumor model in normal mice and in mice protected from MTX toxicity by engraftment with marrow transgenic for the tyrosine-22 drug-resistant DHFR variant. Although in vitro assays revealed that 32Dp210 cells are susceptible to MTX toxicity, we found that administration of MTX to tumor-bearing mice exacerbated progression of 32Dp210 tumor, rather than providing improved antitumor chemotherapy. These results suggest the use of other DHFR inhibitors in combination with drug-resistant DHFR or other combinations of drugs/resistance genes for improved chemotherapy of CML.

Materials and Methods

Mammalian Cell Lines and Culture. The C3H mouse-derived, bcr-abl⁺ myeloblast cell line 32Dp210 (Carlesso et al., 1994) and all derivatives (see below) were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA). The GFP⁺ retrovirus-producer cell line PA317-LNChRG (Muldoon et al., 1997) was maintained in Dulbecco's modified Eagle's medium (Invitrogen). 32Dp210+LasBD cells (Zhao et al., 1997a) were supplemented with 20% 3T3-MoNTIL3-conditioned medium as an IL-3 source (Orchard et al., 1993). All media were supplemented with 10% newborn calf serum (Summit Biotechnology, Fort Collins, CO), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO), 50 U/ml penicillin, 50 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen). All cells were maintained in a humidified atmosphere at 37°C and 5% CO₂.

32Dp210+GFP cells were derived by transduction of 32Dp210 cells with LNChRG retrovirus (containing an enhanced GFP gene) at a multiplicity of infection of approximately 9 in the presence of 8 µg/ml polybrene (Sigma-Aldrich). Cells were exposed to virus overnight, cultured for 5 days, and GFP⁺ cells were isolated by fluorescence-activated cell sorting (FACS; see below). The FACS-isolated fluorescent cells were injected into C3H-He/J mice (see below), recovered from the spleen of a tumor-bearing animal, and re-sorted by FACS to obtain a fluorescent, tumorigenic cell population.

Cell Viability Assay. Cell viability assays were conducted using the CellTiter 96 AQueous Nonradioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 1000 cells in the appropriate medium were inoculated into flat-bottom 96-well cell culture plates (Corning Glassworks, Corning, NY). Varying amounts of methotrexate (amethopterin; Sigma-Aldrich) were added to a final volume of 100 µl. After 4 days of incubation, 20 µl of a tetrazolium indicator solution consisting of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium and phenazine methosulfate was added to each well and incubated with the cells for 2 h. Absorbance at 492 nm was then determined using a Bio-Rad 2550 enzyme immunoassay plate reader (Bio-Rad, Hercules, CA). Cell viability is expressed as a

percentage of that observed for control cells in the absence of MTX. Results are reported as the mean of three determinations.

Animals and Bone Marrow Transplant. C3H-He/J and FVB/N mice were obtained at 6 to 8 weeks of age from the National Institutes of Health Facility at Frederick, MD. The tyrosine-22 DHFR transgenic FVB/N mice (line 11) used for these experiments were previous described (James et al., 1997). F1 offspring of C3H × FVB/N matings were designated C3F-F1 mice; line 11 DHFR transgenic FVB/N mice were used in mating pairs with C3H-He/J mice to generate tyrosine-22 DHFR transgenic C3F-F1 mice. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited conventional facility according to institutional guidelines.

For bone marrow transplant, transgenic tyrosine-22 DHFR marrow was obtained by flushing marrow from femurs and tibiae of euthanized transgenic C3F-F1 mice. Transgenic marrow cells (5–10 × 10⁶) were injected by tail vein into lethally irradiated (800 rads cesium) nontransgenic C3F-F1 mice. Animals were allowed to engraft for 2 months before subsequent administration of tumor or MTX.

Tumor Administration and Therapy. 32Dp210 or 32Dp210+GFP tumor cells were injected in 0.5 ml of Dulbecco's phosphate-buffered saline (PBS; Celox Laboratories, Inc., St. Paul, MN) via tail vein into C3H-He/J or C3F-F1 mice. Starting 1 day after tumor injection, PBS or MTX was administered daily by intraperitoneal injection. Animal weight and health were monitored daily, and moribund animals were euthanized by CO₂ asphyxiation. At regular intervals, peripheral blood from either the periorbital vein or saphenous vein was collected into heparinized micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) for hematocrit determination. Statistical comparison of survival between different groups was conducted by using the Kaplan-Meier product limit method (Kaplan and Meier, 1958) and calculating the log-rank statistic (Peto and Peto, 1972).

Flow Cytometric Analysis. Flow cytometry was used to follow in vivo growth of 32Dp210+GFP tumor. Blood samples for flow cytometry were transferred into tubes containing an equal volume of heparin sodium salt solution (ICN Biomedicals, Aurora, OH). The red cells were lysed and nucleated cells were fixed either using an ammonium chloride solution (8.99 g NH₄Cl, 1 g KHCO₃, and 37 mg of tetrasodium EDTA per liter; Sigma-Aldrich) and 0.5% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA), respectively, or by using Optilyse B solution (Immunotech, Marseille, France). Samples were analyzed using the fluorescein isothiocyanate/GFP channel on a BD Biosciences FACSCalibur (BD Biosciences, San Jose, CA) within 96 h of preparation to detect GFP⁺ tumor cells. The background level of fluorescence intensity for flow cytometry was determined using a sample prepared from a negative control mouse that had not received tumor or drugs, whereas a sample of 32Dp210+GFP cells was used as a positive control. Flow cytometric data were analyzed using FlowJo 3.2 software (Tree Star, Inc., San Carlos, CA) to determine the percentage of cells in each sample that exhibited GFP fluorescence greater than background.

Histology. Spleen, liver, kidney, femur, sternum, heart, lung, ileum, and Peyer's patch tissue samples were collected from moribund animals. The percentage of spleen weight was determined by dividing the spleen weight by the animal's weight at death and multiplying by 100. Tissue samples were fixed in 10% buffered formalin phosphate solution (Fisher Scientific) and femur and sternum samples were decalcified in formic acid (Fisher Scientific) overnight. Samples were then embedded in paraffin, sectioned, mounted, and stained with hematoxylin (Fisher Scientific) and eosin (Surgipath, Grays Lake, IL). Histopathological analysis was carried out without prior knowledge of sample identity.

Results

MTX Sensitivity of 32Dp210 Leukemia in Vitro. The purpose of this study was to determine the effect of MTX

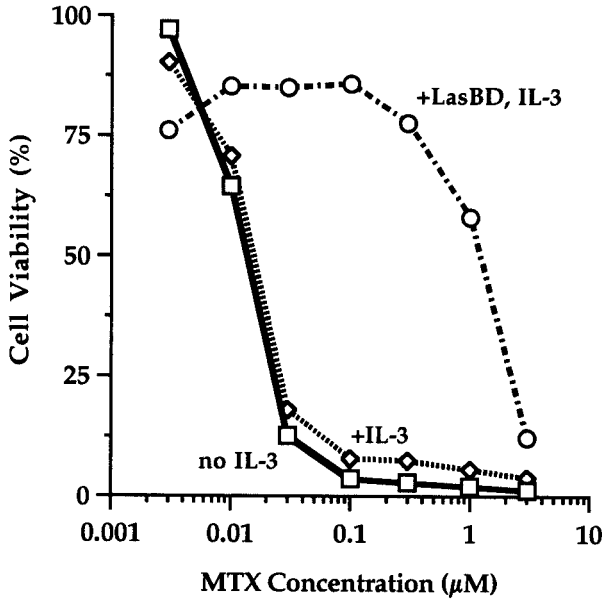
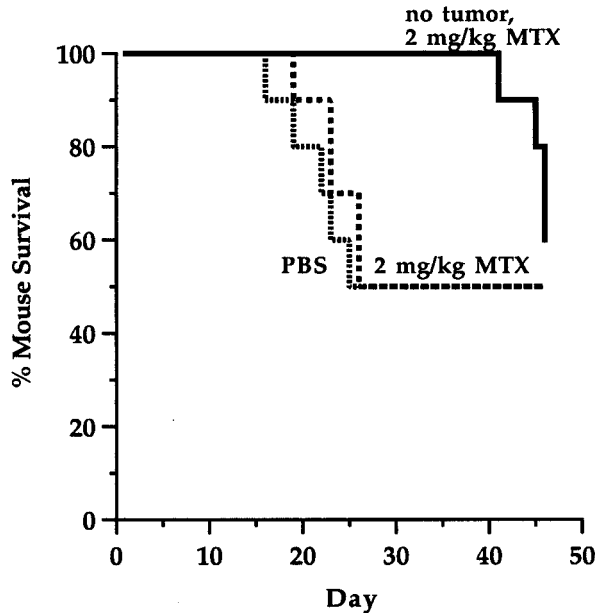


Fig. 1. MTX concentration response for growth of 32Dp210 and 32Dp210+LasBD cells in vitro. Cell growth was tested in a 4-day growth assay at various MTX concentrations. 32Dp210+LasBD cells were supplemented with IL-3 as required for growth (+LasBD, IL-3). 32Dp210 cells were incubated with or without IL-3 in the indicated experimental groups (+IL-3 or no IL-3), to control for any effect of IL-3 on cell response to MTX. Cell viability is expressed as a percentage of that observed for control cells of the same culture in the absence of MTX. Experiments were performed in triplicate as described under *Materials and Methods*. Standard deviations for both 32Dp210 groups were less than 6% at all MTX concentrations tested and were less than 15% at all points for the 32Dp210+LasBD group.

on tumor progression in the 32Dp210 murine model of CML. We first determined the effect of MTX on 32Dp210 cell viability in vitro. 32Dp210 tumor cells containing the human bcr-abl oncogene (Carlesso et al., 1994) were exposed to varying amounts of MTX, as described under *Materials and Methods*. The 32Dp210+LasBD cell line, generated by transducing 32Dp210 cells with LasBD (a retroviral vector expressing a tyrosine-22 variant DHFR gene plus an antisense sequence directed against the bcr-abl breakpoint; Zhao et al., 1997a) was also assayed (Fig. 1). IL-3 was included in the medium for 32Dp210+LasBD cells, because the bcr-abl antisense sequence restores IL-3 dependence of these cells (Zhao et al., 1997a). The 32Dp210 cell line exhibited greatly decreased cell viability at MTX concentrations of 30 nM or greater, whereas 32Dp210+LasBD cells containing the MTX-resistant DHFR gene retained >50% cell viability at MTX concentrations up to 1 µM. 32Dp210 cells are thus susceptible to MTX toxicity at doses from which cells containing a MTX-resistance gene are protected.

MTX Does Not Improve Survival of Normal 32Dp210 Tumor-Bearing Mice. The effect of MTX on 32Dp210 tumor progression in C3H-He/J mice was assayed. Animals received 10^5 or 10^6 32Dp210 tumor cells on day 0, followed by daily injections of PBS or 2 mg/kg MTX. MTX toxicity was also determined in control mice receiving no tumor. MTX (2 mg/kg/day) did not improve survival of tumor-bearing animals compared with animals receiving PBS (Fig. 2, A and B). Additionally, daily administration of 2 mg/kg MTX was eventually toxic for normal animals after day 40. Surprisingly, this dose of MTX significantly decreased survival of tumor-bearing mice receiving 10^6 32Dp210 cells compared with tumor-bearing mice administered PBS ($p < 0.02$) or tumor-free, MTX-administered control mice ($p < 0.002$). Hematocrit and weight did not

A. 10^5 tumor cells



B. 10^6 tumor cells

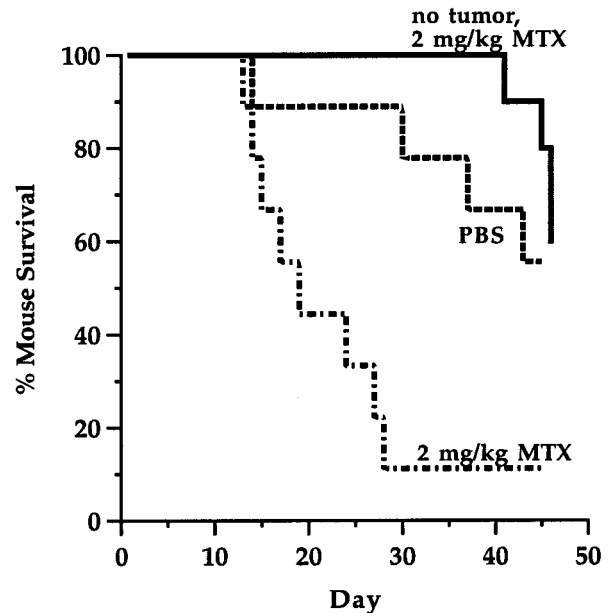
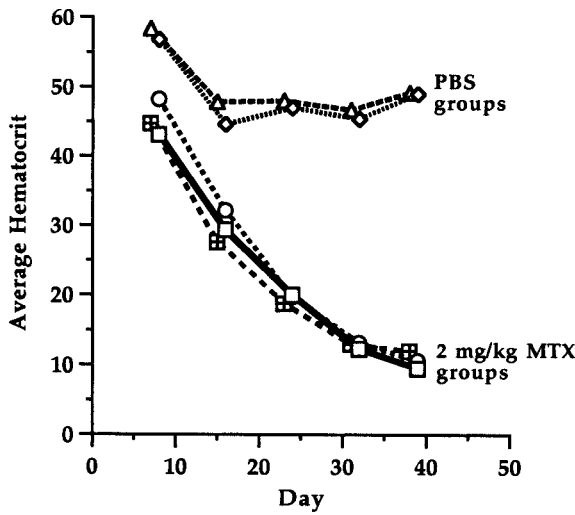
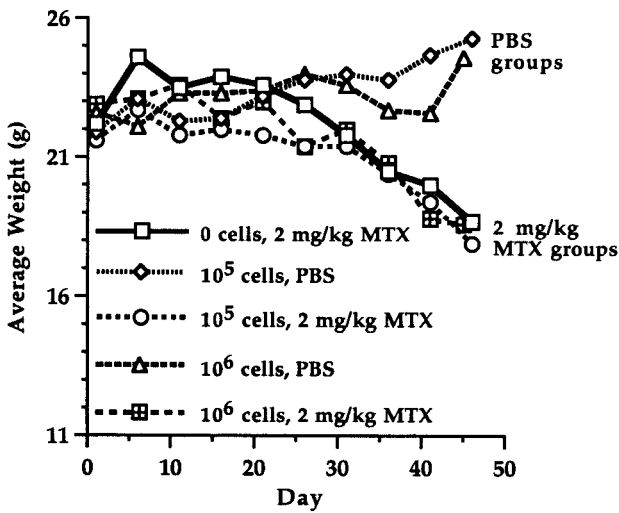


Fig. 2. Effect of MTX on survival of tumor-bearing C3H-He/J mice. Kaplan-Meier plots are shown for mice that received 10^5 (A) or 10^6 (B) 32Dp210 tumor cells by intravenous injection on day 0. Also shown in both diagrams is a control group that received no tumor ($n = 10$ for each group). MTX (2 mg/kg) or PBS was administered daily by intraperitoneal injection.

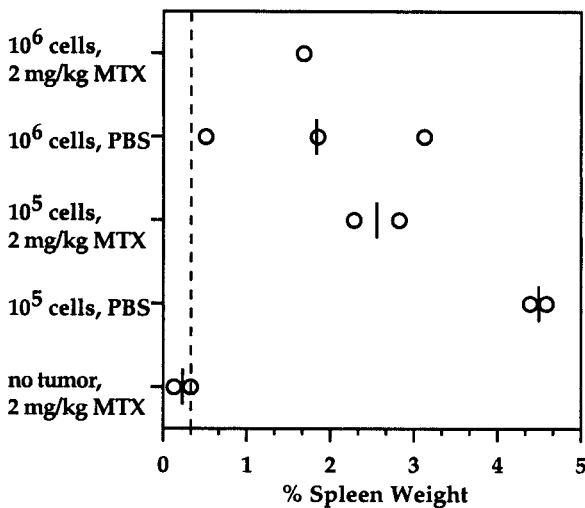
A. Hematocrit



B. Weight



C. % Spleen Weight



differ substantially between tumor-bearing animals and animals without tumor (Fig. 3, A and B), suggesting that animals receiving 10^6 tumor cells and MTX were not dying from MTX toxicity. The gastrointestinal atrophy normally associated with MTX toxicity was not observed in mice administered 10^6 tumor cells and 2 mg/kg/day MTX, further suggesting that decreased survival of these animals was associated with hastened tumor progression rather than enhanced MTX toxicity. Splens of tumor-bearing animals were greatly increased in size compared with normal animals (Fig. 3C), consistent with the splenomegaly observed in human CML. Administration of 2 mg/kg/day MTX did not restore normal spleen size.

Histological analysis, previously undescribed for this tumor, revealed massive accumulations of leukemic cells in the spleen (Fig. 4, A and B), as well as leukemic infiltrates in multiple tissues, including liver (Fig. 4C), lung (Fig. 4D), bone marrow, and peristernal skeletal muscle (data not shown). Massive necrosis of sternal bone marrow, accompanied by leukemic infiltrates in the peristernal muscle, was common. The leukemic cell population included a large number of blast cells with a large polygonal to ovoid, vesicular nucleus and one or more large eosinophilic nucleoli. The cells had abundant lightly basophilic cytoplasm and an occasional cell had small round eosinophilic cytoplasmic granules. This blast cell was the predominant cell in skeletal muscle and other nonhematopoietic sites. Cells in bone marrow and spleen tended to have more pleomorphic, often band-like nuclei that were suggestive of early myeloid stages, confirming resemblance of the 32Dp210 tumor model to an acute or blast-phase myeloid leukemia. Similar leukemic infiltration was found in tissues of all tumor-bearing animals whether or not MTX was administered (data not shown). Thus, MTX did not improve survival of tumor-bearing mice, and in fact decreased survival was observed in mice receiving 10^6 tumor cells.

MTX Exacerbates 32Dp210 Tumor Progression at Higher Doses of Drug in Mice Engrafted with DHFR Transgenic Marrow. To determine the effect of increased MTX doses on 32Dp210 tumor progression in mice, it was necessary to render recipient animals drug-resistant by expressing a MTX-resistance gene in the marrow. We have previously demonstrated that this can be achieved by transplantation of marrow from DHFR transgenic mice established on an FVB/N inbred strain background. For this study we transplanted marrow from transgenic tyrosine-22 DHFR

Fig. 3. MTX toxicity and splenomegaly in tumor-bearing C3H-He/J mice. Animals in each of the groups depicted in Fig. 2 were analyzed for hematocrit (A), weight (B), and spleen weight (C). Hematocrits (A) were determined from peripheral blood samples collected at regular intervals, as described under *Materials and Methods*. Each point is the mean hematocrit for surviving mice from the group at that time point. Standard deviations were less than 10% for all groups. Animal weights (B) were determined daily. Each data point is the mean weight for surviving animals of the group, plotted at 5-day intervals. Standard deviations were within 3 g at each time point. Spleen weights (C) were measured from harvested spleens of moribund animals, and values were expressed as a percentage of the weight of the mouse at death, as described under *Materials and Methods*. The values shown do not include all moribund animals from each group, because tissues from some animals were not available at the time of death. The mean spleen weight for each group is also shown (indicated by solid vertical line). Spleen weight for untreated control mice receiving no tumor was approximately 0.3% of the total weight of the animal (indicated by dashed line).

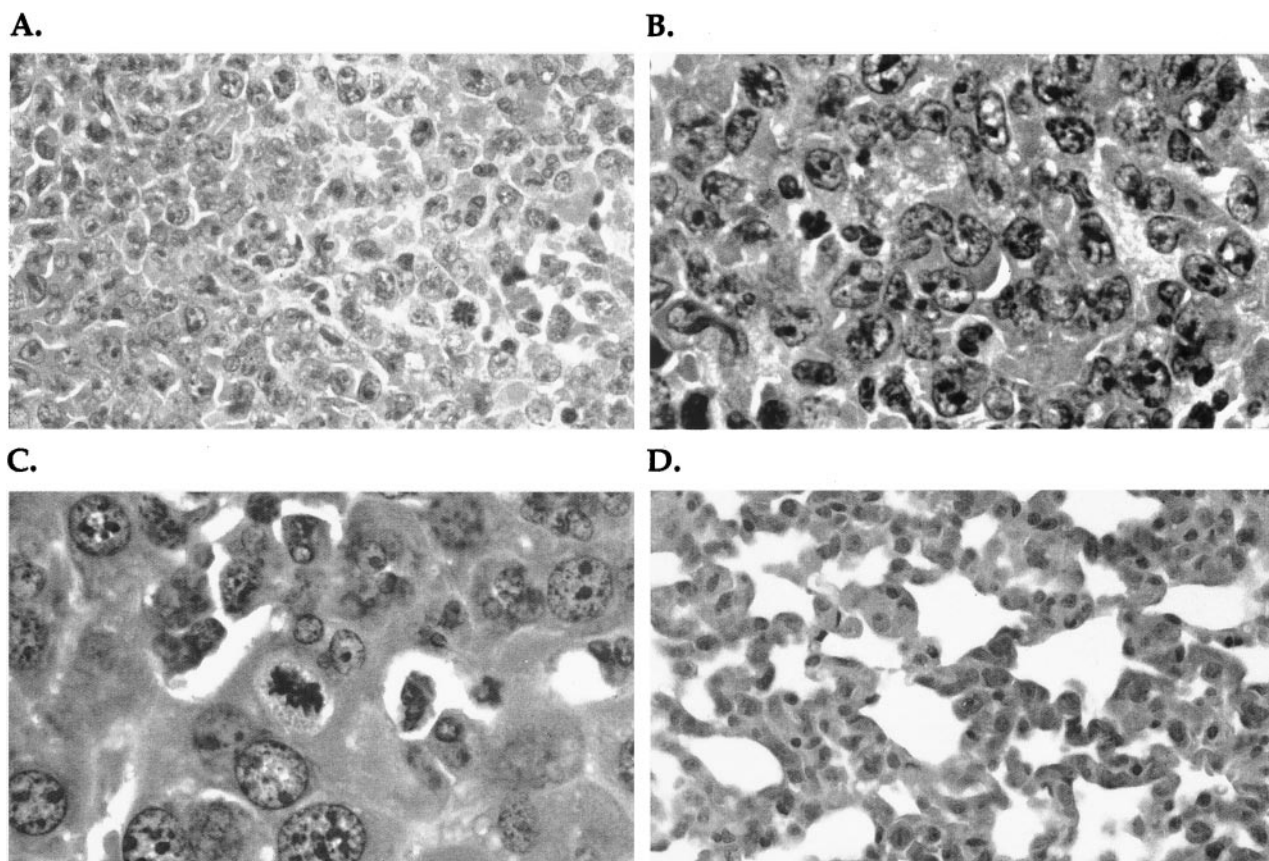


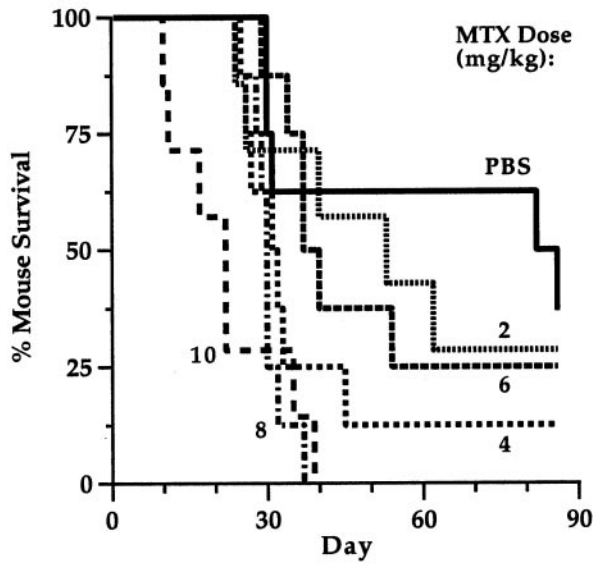
Fig. 4. Histopathological analysis of 32Dp210 tumor in C3H-He/J mice. Representative tissue samples are shown for tumor-bearing animals administered PBS daily by intraperitoneal injection. All sections were prepared and stained with hematoxylin and eosin as described under *Materials and Methods*. A, spleen with diffuse leukemic infiltrate. One leukemic cell is in mitosis (200 \times). B, spleen, showing dense infiltrate of leukemic cells. Some cells (center) have elongated, band-like nuclei (400 \times). C, liver. Leukemic cell in metaphase, showing eosinophilic cytoplasmic granules (400 \times). D, lung. Leukemic infiltrate in alveolar septa (200 \times).

C3F-F1 mice into lethally irradiated normal C3F-F1 recipient mice (see *Materials and Methods*). To better track tumor progression in the animals, 32Dp210 cells were transduced with a retroviral vector encoding an enhanced GFP as described under *Materials and Methods*. After engraftment of the transgenic marrow, mice were given 10^5 32Dp210+GFP tumor cells and then administered MTX at varying doses. As previously observed, MTX did not improve survival of tumor-bearing animals (Fig. 5A), but rather appeared to decrease survival, particularly for animals receiving 4 mg/kg/day MTX (median survival time of 86 days for mice receiving PBS compared with 32 days for mice receiving 4 mg/kg/day MTX). This decrease in survival was statistically significant based on survival data through day 80, nearly 2 months after the majority of deaths occurred in the MTX-treated group ($p < 0.05$ for tumor-bearing mice receiving 4 mg/kg/day MTX compared with mice receiving PBS), but not statistically significant after day 81 ($p = 0.07$). MTX doses up to 6 mg/kg/day were not lethal for tumor-free transgenic-marrow recipients, whereas higher MTX doses resulted in the death of tumor-free control animals (data not shown). These results are consistent with our previous characterization of MTX dose response in tumor-free FVB/N mice (May et al., 1996). Average hematocrits of tumor-bearing animals receiving 6 mg/kg/day MTX or less remained above 35 (Fig. 5B), indicating that these mice were not suffering from MTX-mediated hematological toxicity. Additionally, histological analysis revealed

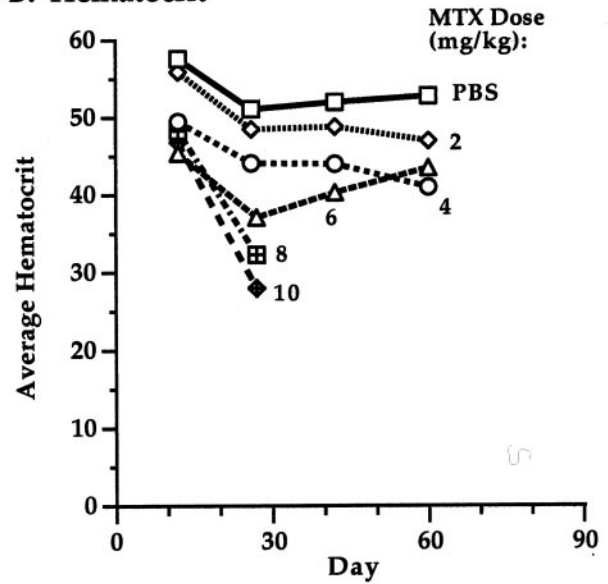
normal intestinal structure in tumor-bearing animals administered 2 or 4 mg/kg/day MTX (Fig. 5, C and D), whereas severe intestinal necrosis was observed in animals administered 8 or 10 mg/kg/day MTX (Fig. 5, E and F). Increased MTX-mediated gastrointestinal toxicity was thus not observed in tumor-bearing animals, and taken together with the hematocrit data this indicates that the decreased survival in tumor-bearing animals observed at some MTX doses was not caused by enhanced MTX toxicity.

Tumor load in each animal was measured by flow cytometry to detect fluorescent GFP⁺ tumor cells in peripheral blood samples collected on day 26 or 27 of the experiment (Fig. 6A). This time point corresponded approximately to the beginning of animal mortality for those receiving ≤ 8 mg/kg/day MTX. PBS-administered animals contained only background levels of GFP⁺ cells in their peripheral blood. In contrast, the groups administered ≤ 8 mg/kg/day MTX included a substantial number of animals exhibiting detectable ($>1\%$) GFP⁺ tumor in the peripheral blood, whereas animals receiving 10 mg/kg/day MTX succumbed to drug toxicity before tumor progression was observed. MTX administration (particularly a dose of 4 mg/kg/day) thus resulted in increased numbers of circulating 32Dp210+GFP cells in the peripheral blood of tumor-bearing animals. The lack of substantial amounts of GFP⁺ tumor cells in samples from the PBS group at this time point is not anomalous, because we have observed that 32Dp210+GFP tumor cells are generally

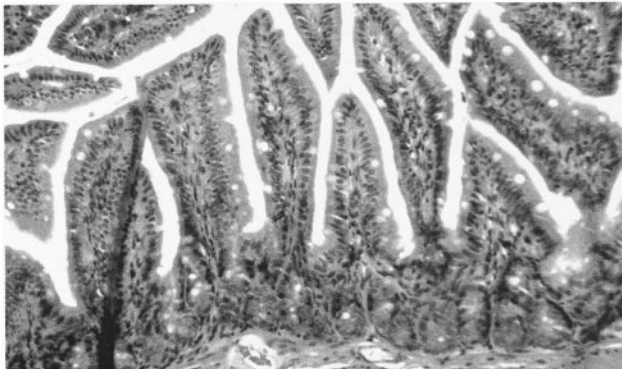
A. Mouse survival



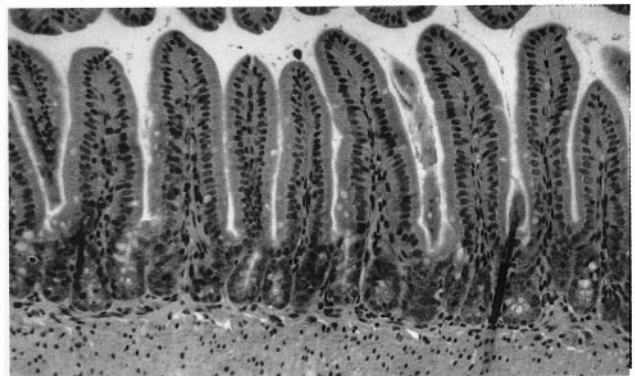
B. Hematocrit



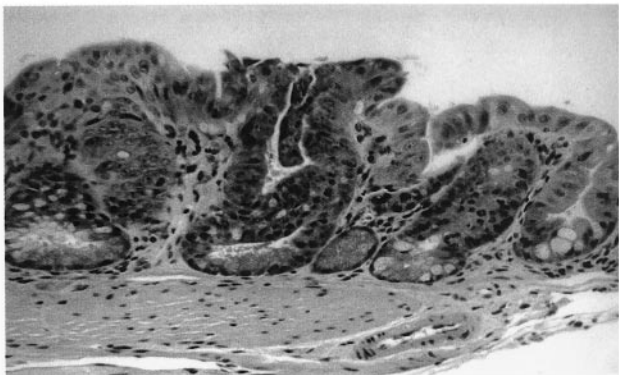
C. 2 mg/kg MTX



D. 4 mg/kg MTX



E. 8 mg/kg MTX



F. 10 mg/kg MTX

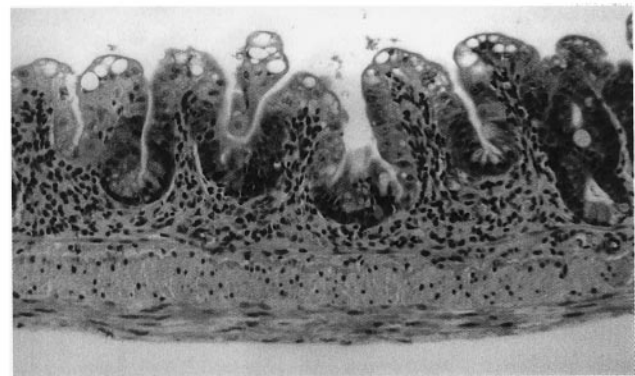
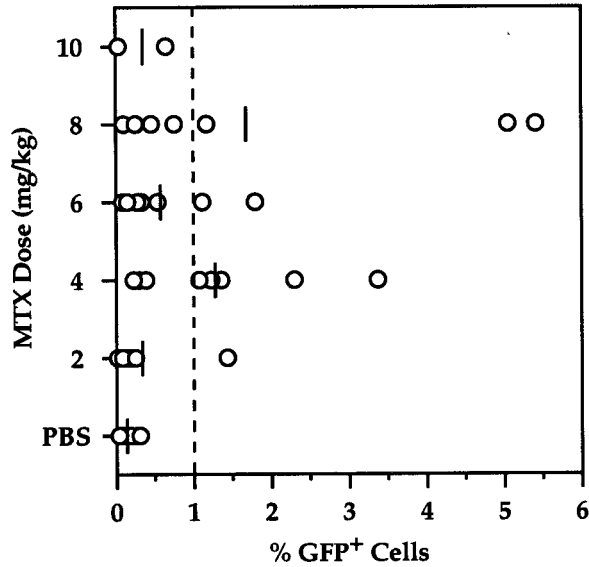


Fig. 5. Effect of MTX on tumor-bearing C3F-F1 mice transplanted with transgenic tyrosine-22 DHFR marrow. A, Kaplan-Meier plot of animal survival. Mice received 10^5 32Dp210+GFP tumor cells by intravenous injection on day 0 ($n = 8$ for each group). PBS or MTX at various doses was administered daily by intraperitoneal injection. B, average hematocrits from samples collected at biweekly intervals. Each point is the mean hematocrit for surviving mice from the indicated group at that time point. Standard deviations were less than 10 at each point. Numbers refer to the MTX dose (mg/kg) administered. C and D, histopathological analysis of small intestines of mice receiving 2 or 4 mg/kg/day MTX. The normal mucosa shows tall villi lined by tall columnar epithelium (125 \times). E and F, histopathological analysis of small intestines of mice receiving 8 or 10 mg/kg/day MTX. Necrosis of epithelial cells has resulted in severe mucosal atrophy with loss of villi. Attempted epithelial regeneration is evidenced by cuboidalization and large size of luminal epithelial cells (125 \times). At 10 mg/kg/day (F) the lamina propria contains an infiltrate of mononuclear inflammatory cells. All sections were prepared and stained with hematoxylin and eosin as described under *Materials and Methods*.

A. GFP⁺ Cells

B. % Spleen Weight

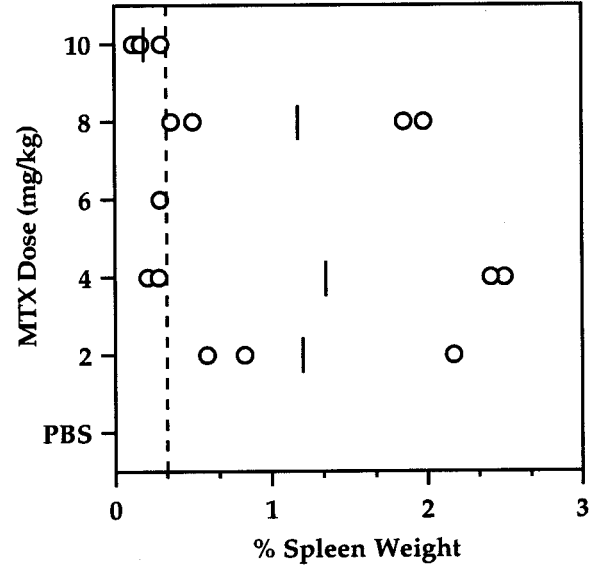


Fig. 6. Effect of MTX on tumor progression in C3F-F1 mice transplanted with transgenic tyrosine-22 DHFR marrow. A, 32Dp210+GFP tumor cells detected in peripheral blood samples collected from mice at day 26 or 27 of drug treatment. Blood samples were treated, and GFP⁺ tumor cells were quantitated by flow cytometry as described under *Materials and Methods*. Values shown are the GFP⁺ cells expressed as a percentage of the total number of cells analyzed (approximately 5000 cells total for each sample) from individual mice. The mean value for each group is also shown (indicated by solid vertical line). A GFP⁺ cell percentage of 1% or greater (indicated by dashed line) was deemed substantial, based on background levels observed in control samples lacking GFP⁺ cells. B, spleen weights from moribund animals expressed as a percentage of total animal weight. The values shown do not include all moribund animals from each group, because tissues from some animals were not available. The mean spleen weight for each group is also shown (indicated by solid vertical line). Spleen weight for untreated control mice receiving no tumor was approximately 0.3% of the total animal weight (indicated by dashed line).

only detectable by flow cytometry at levels >1% within a week or less of subsequent mortality. Spleen measurements of moribund animals indicated that MTX did not restore normal spleen size to tumor-bearing animals (Fig. 6B), except at 10 mg/kg/day, where animals succumbed to drug toxicity with no tumor progression observed. Together, these data indicate that MTX exacerbated tumor progression even at higher doses of drug (4–6 mg/kg/day) against which animals are protected by prior transplantation with transgenic tyrosine-22 DHFR marrow.

MTX Does Not Exacerbate 32Dp210 Tumor Progression in DHFR Transgenic Mice. Mice transplanted with DHFR transgenic marrow preconditioned by lethal total body irradiation subsequently become engrafted with predominantly donor-derived drug-resistant hematopoietic cells (May et al., 1995; James et al., 1997). Therefore, we hypothesized that MTX exacerbation of 32Dp210 tumorigenicity resulted from MTX sensitivity of some nonhematopoietic host tissue function. To test this hypothesis, 4 mg/kg MTX was administered daily to transgenic tyrosine-22 DHFR C3F-F1 mice (containing the drug-resistant DHFR gene in *all* cells) after injection of 10^6 32Dp210+GFP cells (Fig. 7). Tumor-bearing C3F-F1 mice lacking the tyrosine-22 DHFR transgene were administered PBS or a reduced dose of MTX (2 mg/kg/day). In this experiment, decreased survival was not observed in MTX-administered transgenic animals compared with normal animals administered PBS, although neither did MTX significantly improve survival of tumor-bearing transgenic animals. Flow cytometry of peripheral blood samples collected on day 16 or 17 (corresponding to the onset of animal mortality in this experiment) indicated that administration of 2 mg/kg/day MTX resulted in increased levels of

GFP⁺ tumor cells in normal mice. In contrast, GFP⁺ tumor cells were undetectable at this time point in DHFR⁺ transgenic mice administered 4 mg/kg/day MTX (Fig. 8A). Hematocrits were decreased at this time point in normal animals receiving 2 mg/kg/day MTX, but were unaffected by 4 mg/kg/day MTX administration in the transgenic group, indicating

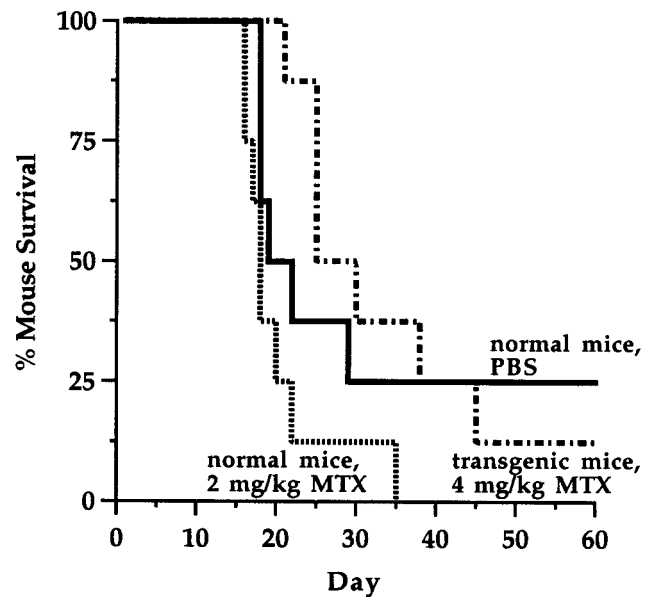
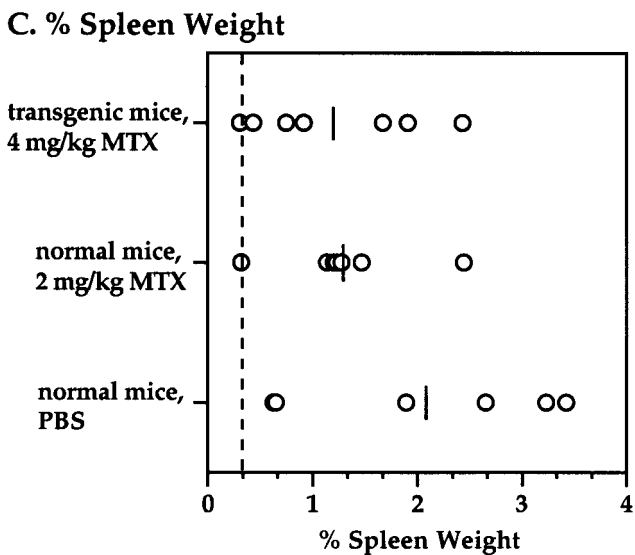
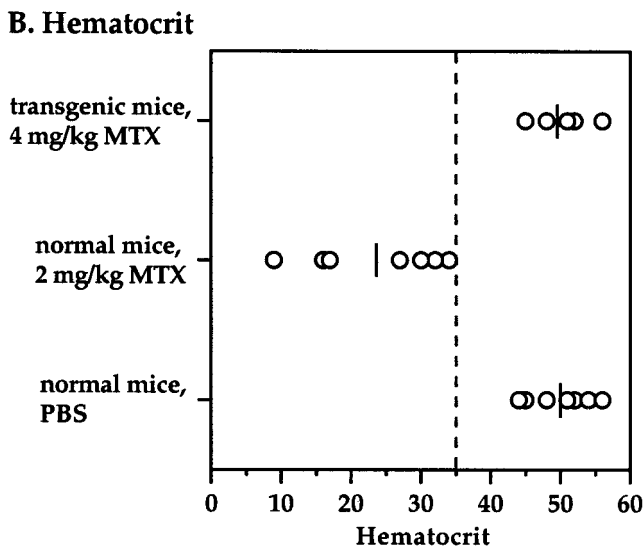
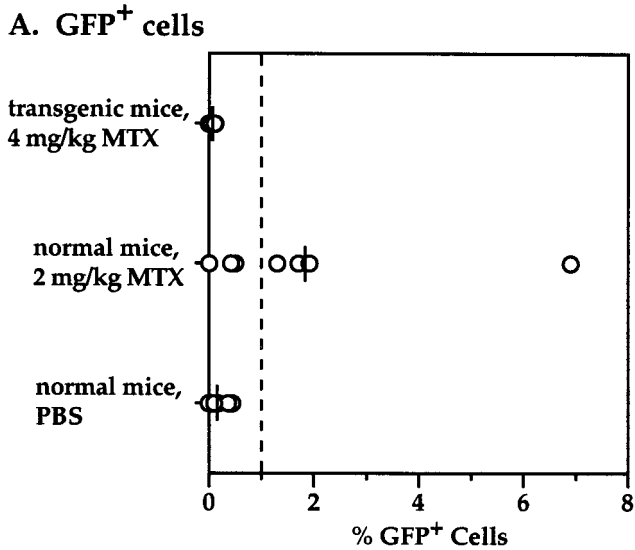


Fig. 7. Effect of MTX on survival of tumor-bearing tyrosine-22 DHFR transgenic C3F-F1 mice. Mice received 10^6 32Dp210+GFP tumor cells by intravenous injection on day 0 ($n = 8$ for each group). Normal C3F-F1 mice received either PBS or 2 mg/kg MTX, and transgenic C3F-F1 mice received 4 mg/kg MTX administered daily by intraperitoneal injection.



that transgenic animals were not suffering from hematological toxicity (Fig. 8B). These results are in direct distinction from the results obtained in animals transplanted with DHFR transgenic marrow, in which a similar chemoprotective effect was observed (Fig. 5B; hematocrit), but in which GFP⁺ tumor cells were detected in the peripheral blood of MTX-administered animals (Fig. 6A). Measurements from moribund animals indicated that MTX did not restore normal spleen size to tumor-bearing animals (Fig. 8C). Overall, the results from this experiment indicate that 4 mg/kg/day MTX did not exacerbate 32Dp210+GFP tumor progression in transgenic tyrosine-22 DHFR C3F-F1 mice, in contrast to our previous results obtained at the same MTX dose in normal C3F-F1 mice engrafted with transgenic tyrosine-22 DHFR marrow. Tumor exacerbation in DHFR transgenic-marrow transplant recipients was therefore most likely the result of some MTX-sensitive nonhematopoietic host component, because tumor exacerbation was not observed in mice containing the MTX-resistant tyrosine-22 DHFR gene in all tissues.

Discussion

The effectiveness of MTX as an antitumor agent was assayed to evaluate a drug-resistance gene therapy approach in the 32Dp210 murine model of chronic myeloid leukemia. MTX was found to be toxic for 32Dp210 cells in vitro at doses against which cells containing a MTX-resistance gene were protected. In vivo studies in normal animals revealed that MTX administration did not increase survival of tumor-bearing animals. MTX administration at higher doses afforded by engraftment with DHFR transgenic marrow did not reduce 32Dp210 tumorigenicity in normal transplanted animals, but rather exacerbated tumor progression, resulting in earlier death and earlier appearance of tumor cells in peripheral blood. This exacerbation effect was not evident in tumor-bearing transgenic tyrosine-22 DHFR mice that received MTX, suggesting that the promotion of tumor progression in bone marrow transplant recipients was due to MTX sensitivity of a nontransgenic host component. This possibility does not rule out immune suppression in the transgenic bone marrow transplant recipients, which could occur through disruption of a nontransgenic host component required for immune cell maturation.

Currently, allogeneic marrow transplant is the only curative treatment for CML. Unfortunately, availability of suitable donor material is an issue, and graft-versus-host disease

Fig. 8. Effect of MTX on tumor progression in normal and tyrosine-22 DHFR transgenic mice. A, 32Dp210+GFP tumor cells detected in peripheral blood samples collected from mice at day 16 or 17 of drug treatment. Values shown are the GFP⁺ cells expressed as a percentage of the total number of cells analyzed (7,000–10,000 cells total for each sample) from individual mice. The mean value for each group is also shown (indicated by solid vertical line). A GFP⁺ cell percentage of 1% or greater (indicated by dashed line) was deemed substantial, based on background levels detected in control samples lacking GFP⁺ cells. B, hematocrits of blood samples taken at day 16 or 17 of drug treatment. Each point is the hematocrit for an individual mouse. The mean hematocrit for each group is also shown (indicated by solid vertical line). A hematocrit level of 35 or greater was deemed normal (indicated by dashed line), based on samples from untreated control animals. C, spleen weights from moribund animals, expressed as a percentage of the total animal weight. The values shown include all moribund animals from each group. The mean spleen weight for each group is also shown (indicated by solid vertical line). Spleen weight for untreated control mice receiving no tumor was approximately 0.3% of the total animal weight (indicated by dashed line).

has been observed in up to 68% of CML patients transplanted with human leukocyte antigen-matched sibling donor material (Weisdorf et al., 1991). Chemotherapeutic approaches for CML have been only partially successful, consisting primarily of interferon- α , which can induce hematological remission and prolong survival but is not curative (Hehlmann et al., 2000; Hochhaus et al., 2000). However, STI571, a selective inhibitor of Abl tyrosine kinase, has proved effective in inhibiting growth of bcr-abl⁺ cells (Druker et al., 1996), and recent clinical trials have shown promise for the treatment of CML (Druker et al., 2001a,b). MTX has been effective in treatment of a variety of malignancies, including choriocarcinoma, breast cancer, non-Hodgkin's lymphoma, and acute lymphoid leukemia (Jolivet et al., 1983; Bertino, 1993). MTX is commonly used to control graft-versus-host disease after allogeneic marrow transplant (Simpson, 2000). MTX is not typically used as an antitumor agent for treatment of CML, although MTX coadministration has been shown to improve the efficacy of interferon- α in delaying CML progression to blast crisis (Kanda et al., 1999).

Numerous studies have reported the protection of mice from toxic doses of antifolate (MTX or trimetrexate) by expression of drug-resistant DHFR in hematopoietic cells (Williams et al., 1987; Corey et al., 1990; Li et al., 1994; May et al., 1995; Spencer et al., 1996; James et al., 1997). Administration of higher doses of antifolate in animals thus protected could allow for more effective treatment of tumors known to be sensitive to antifolates (Zhao et al., 1997b). Drug-resistant DHFR expression could also be used to allow antifolate administration at higher doses, which may be effective against tumors that are not usually sensitive to antifolates at lower doses, such as CML.

Autologous marrow transplants for treatment of CML commonly result in relapse either due to bcr-abl⁺ tumor cells contaminating the graft, or incomplete elimination of tumor cells in the host during the preparative regimen for bone marrow transplant (Deisseroth et al., 1994). Introduction of a drug-resistance gene into autologous marrow before transplant could allow for post-transplant drug administration to selectively ablate leukemic cells lacking the drug-resistance gene. One problem with this approach is the possibility of introducing the drug-resistance gene into tumor cells contaminating the donor marrow. This problem could be addressed for CML by including antisense sequences directed against the fusion region of the bcr-abl message, thus restoring a more normal phenotype to transduced bcr-abl⁺ cells in the graft. This strategy was previously examined using the 32Dp210 model of CML, demonstrating a 3 log-fold reduction in tumorigenicity for tumor cells transduced with LasBD, a retroviral vector containing the tyrosine-22 DHFR gene in addition to antisense sequences directed against the bcr-abl breakpoint (Zhao et al., 1997a).

This article examines the effectiveness of expressing the variant tyrosine-22 DHFR gene in mouse marrow for improved MTX chemotherapy in the 32Dp210 model of CML. As with previous studies involving antifolate administration in other mouse strains transplanted with either transgenic or retrovirally transduced marrow containing a variant DHFR gene (Williams et al., 1987; Corey et al., 1990; Li et al., 1994; Zhao et al., 1994, 1997b; May et al., 1995, 1996; Spencer et al., 1996; James et al., 1997, 2000), we found that the tyrosine-22 DHFR gene conferred increased resistance of

C3F-F1 mice to MTX, allowing for MTX administration at higher doses. However, the increased MTX administration afforded by engraftment with drug-resistant transgenic marrow did not improve animal survival in the 32Dp210 model of CML, but rather accelerated progression of the tumor. The tyrosine-22 DHFR transgenic mouse system has also been assayed for improved chemotherapy of FMC, a mammary adenocarcinoma established in this laboratory from FVB/N mice. MTX administration was found to be relatively ineffective at inhibiting FMC tumor progression in animals transplanted with drug-resistant marrow, but MTX did not exacerbate FMC tumor progression in those animals (J. L. Frandsen, B. Weigel, C. L. Sweeney, M. D. Diers, R. Gunther, and R. S. McIvor, manuscript in preparation). Therefore, the accelerated progression of 32Dp210 tumor by MTX is not characteristic of all tumors in this transgenic system.

The fact that MTX did not increase 32Dp210 tumor progression in DHFR transgenic animals (i.e., animals in which *all* tissues contain the DHFR gene) provides some insight into the mechanism by which MTX exacerbates tumorigenicity in normal and bone marrow transplant recipient animals. The results are consistent with the presence of some MTX-sensitive nonhematopoietic host function that provides an appropriate setting for tumor growth upon MTX administration. MTX administration could thus create a niche, or some space, in this drug-sensitive tissue, which allows the tumor to become established, grow, and expand. Alternately, given the apparent role of the immune response in controlling human CML (Molldrem et al., 2000; Wu et al., 2000), tumor exacerbation in this model may be due to some degree of immune suppression by MTX. However, the lack of MTX-mediated acceleration of tumor progression in wholly transgenic animals argues against a marrow-derived, MTX-sensitive host component. This interpretation would imply that transgenic immune cells (e.g., T or B cells) retain some sensitivity to MTX despite the presence and expression of the drug-resistant DHFR gene.

Whatever the mechanism by which MTX exacerbates tumorigenicity, it is apparent that the combination of DHFR gene transfer with increased doses of MTX is an ineffective approach in preclinical studies with the 32Dp210 model of CML. Some modification of this strategy will be necessary to achieve an efficacious response. It is possible that the application of DHFR gene transfer for CML in this model could be improved by the use of other DHFR inhibitors besides MTX. In particular, the lipophilic antifolate trimetrexate is also a potent inhibitor of DHFR, but does not rely on the reduced folate carrier for transport into cells and does not require polyglutamylation for inhibition of DHFR (Lin and Bertino, 1987). Trimetrexate is a more specific inhibitor of DHFR than MTX, because MTX-polyglutamates also directly inhibit thymidylate synthase as well as glycinamide ribonucleotide and aminoimidazole carboxamide ribonucleotide transformylases involved in de novo purine synthesis (Takimoto, 1996). Additionally, transport of exogenous nucleosides can potentially rescue cells from MTX toxicity (Nelson and Drake, 1984; Sur et al., 1993). Use of a nucleoside transport inhibitor can restore MTX toxicity to cells expressing wild-type DHFR while maintaining differential toxicity relative to cells expressing drug-resistant DHFR (Warlick et al., 2000), and may allow for increased effectiveness of antifolates for chemotherapy in the 32Dp210 murine model of CML. Trimetrex-

ate in combination with nucleoside transport inhibition has been used effectively for in vivo selection of murine hematopoietic stem cells (Allay et al., 1998). Alternatively, the introduction and expression of other drug-resistance genes, such as the multidrug resistance gene 1 (Aran et al., 1999) or O⁶-methylguanine-DNA-methyltransferase gene (Reese et al., 1996) could be used to provide chemoprotection to recipient animals and may prove beneficial for improved tumor chemotherapy. These approaches are currently under investigation for ultimate application in the treatment of CML in combination with antisense sequences directed against the bcr-abl oncogene, as previously described (Zhao et al., 1997a).

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