Transgenic mice that express the human multidrug-resistance gene in bone marrow enable a rapid identification of agents that reverse drug resistance

[chemosensitizers/daunomycin/taxol/(R)-verapamil/chemotherapy]

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ABSTRACT The development of preclinical models for the rapid testing of agents that circumvent multidrug resistance in cancer is a high priority of research on drug resistance. A common form of multidrug resistance in human cancer results from expression of the MDR1 gene, which encodes a M, 170,000 glycoprotein that functions as a plasma membrane energy-dependent multidrug efflux pump. We have engineered transgenic mice that express this multidrug transporter in their bone marrow and demonstrated that these animals are resistant to leukopenia by a panel of anticancer drugs including anthracyclines, vinca alkaloids, etoposide, taxol, and actinomycin D. Differential leukocyte counts indicate that both neutrophils and lymphocytes are protected. Drugs such as cisplatin, methotrexate, and 5-fluorouracil, which are not handled by the multidrug transporter, produce bone marrow suppression in both normal and transgenic mice. The resistance conferred by the MDR1 gene can be circumvented in a dose-dependent manner by simultaneous administration of agents previously shown to be inhibitors of the multidrug transporter in vitro, including verapamil isomers, quinidine, and quinine. Verapamil and quinine, both at levels suitable for human trials that produced only partial sensitization of the MDR1-transgenic mice, were fully sensitizing when used in combination. We conclude that MDR1-transgenic mice provide a rapid and reliable system to determine the bioactivity of agents that reverse multidrug resistance in animals.

Resistance to chemotherapy poses a major obstacle to the cure of human cancers that are not amenable to definitive surgical or radiation treatment. Numerous laboratory investigations have uncovered a broad spectrum cross-resistance to natural product cytotoxic compounds that do not share any obvious functional or structural similarities (1). This phenomenon is termed multidrug resistance (MDR) (2, 3). The evidence indicates that the presence of a M, 170,000 plasma membrane protein, termed P-glycoprotein, which is encoded in humans by the MDR1 gene (4, 5) is sufficient to produce MDR in cancer cells (1–5). P-glycoprotein functions as a multidrug transporter that rapidly extrudes many types of hydrophobic chemotherapeutic agents from the target cancer cell before the drugs can exert their cytotoxic effects (6, 7).

Recently, it has become evident that many drug-resistant human tumors express the MDR1 gene (8), that MDR1 RNA levels are elevated in many cancers that have not responded to chemotherapy (2, 9, 10), and, in some cases, the presence of MDR1 gene expression predicts poor results in chemotherapy with agents affected by the multidrug transporter (11, 12). A number of different agents that inhibit the activity of the multidrug transporter, such as verapamil, have been described (13) and the majority of these appear to be substrates for the transporter, which compete with anticancer drugs for transport (1, 6, 14, 15). An initial report on a limited number of patients with drug-resistant multiple myeloma suggests reversal of MDR by verapamil in a clinical trial (16). However, the inherent side effects of verapamil due to its cardiovascular activities necessitate the search for new and better resistance modifiers (17). To confirm the activity of the MDR1 gene in a preclinical in vivo model, this laboratory has engineered transgenic mice carrying the human MDR1 gene and expressing it in their bone marrow, an organ that is normally very sensitive to anticancer drugs (18). In these mice, preliminary analysis suggested that the MDR1 gene was active since, after treatment with daunomycin, there was no fall in their peripheral leukocyte count (WBC). The current studies investigate the protection of bone marrow by the human MDR1 gene from a variety of chemotherapeutic drugs presently in clinical use and also examine the use of these transgenic mice as a suitable model for testing agents, and combinations of agents, that circumvent drug resistance.

MATERIALS AND METHODS

MDR Transgenic Mice. The construction of the original plasmid carrying the full-length cDNA encoding MDR1 (19) and the injection of this cDNA under control of a chicken β-actin promoter using standard techniques (20) have been described (18). The cDNA construct (1–2 ng) was injected into fertilized mouse embryos of single-cell stage, and these transgenic embryos were implanted in foster mice. After establishing a homozygous line (MDR-39) of mice carrying the transgene, homozygous males were backcrossed to MDR-negative females of the progenitor line (C57BL/6 × SJL)F1. The resulting MDR1 heterozygous descendants were analyzed for expression of the human MDR1 gene using tail samples (6, 20) and hybridized with the MDR1-specific probe MDR5A generated in this laboratory (21). In these studies, only 6- to 8-week-old sex-matched littermates were investigated.

Test Conditions. Cisplatin and etoposide were a gift of Bristol-Myers Squibb (Syracuse, NY). Verapamil and (R)-verapamil were provided by courtesy of BASF Bioresearch (Cambridge, MA). Taxol was from the Developmental Therapeutics Branch, National Cancer Institute (Bethesda, MD). All other drugs were purchased from Sigma. The drugs were

The abbreviations used are:

Abbreviations: MDR, multidrug resistance; WBC, leukocyte count.

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administered by a single intraperitoneal injection in the lower right quadrant of the abdominal cavity. Drug concentrations were adjusted so that a maximum vol of 400 µl was injected per experiment. Experiments included a minimum of three animals per group and were repeated at least once. The results were highly reproducible with <10% variation.

**Measurements.** Peripheral blood was collected by periorbital bleeding with heparin-treated microhematocrit capillary tubes (Fisher) and diluted 1:20 (vol/vol) in 3% acetic acid solution for erythrocyte lysis. The refractile, viable leukocytes were counted on an ultraplane Neubauer's hemocytometer (Hauser Scientific, Pittsburgh, PA). The hematocrit was measured by centrifuging (12,000 × g; 5 min) a heparin-treated microcapillary tube and expressing the value as a ratio of the length of the erythrocyte column to that of the whole blood column. The differential WBC and an estimate of the number of platelets were obtained by using air-dried whole blood smears, which were exposed for 5 min to modified Wright's stain (Accustain; Sigma), neutralized for 5 min in phosphate buffer (pH 7.2), and washed for 10 min in distilled water. Subsequently, the blood film was analyzed under an oil immersion lens.

**RESULTS**

**Effects of MDR Drugs on Peripheral WBCs.** Previously it was shown that MDR transgenic mice were resistant to the bone marrow suppressive effects of daunomycin (18). To determine whether the marrow was protected against other agents, bone marrow toxicity of chemotherapeutic agents for normal and MDR transgenic mice was assessed. Drugs were administered i.p. in a single dose on day 0. Values are expressed as the ratio of WBCs on day 3 to those on day 0. Experiments included a minimum of three animals per group and were repeated at least once. Dosage was selected to ensure a significant (>50%) reduction of WBCs in normal mice without causing general toxicity. DOX, doxorubicin; VBL, vincristine; TAX, taxol; DAU, daunomycin; ETO, etoposide; ActD, actinomycin D; 5FU, 5-fluorouracil; MTX, methotrexate.

**FIG. 1.** Bone marrow toxicity of chemotherapeutic agents for normal and MDR transgenic mice. Drugs were administered i.p. in a single dose on day 0. Values are expressed as the ratio of WBCs on day 3 to those on day 0. Experiments included a minimum of three animals per group and were repeated at least once. Dosage was selected to ensure a significant (>50%) reduction of WBCs in normal mice without causing general toxicity. DOX, doxorubicin; VBL, vincristine; TAX, taxol; DAU, daunomycin; ETO, etoposide; ActD, actinomycin D; 5FU, 5-fluorouracil; MTX, methotrexate.

**FIG. 2.** Time-dependent effects of taxol and/or chemosensitizers on the WBC of mice. (A) Normal mice. (B) MDR transgenic mice, taxol alone or combined with quinine. (C) MDR transgenic mice, taxol alone or combined with quinine. (D) MDR transgenic mice, taxol alone or combined with verapamil. Experiments were conducted as described in Materials and Methods and in the legend to Fig. 1. Quinine at 40 mg/kg; quinidine at 150 mg/kg; verapamil at 30 mg/kg; taxol at 4 mg/kg; taxol at 6 mg/kg; taxol at 10 mg/kg.
To determine which cells in the bone marrow were protected in the MDR mice, animals were challenged with daunomycin (10 mg/kg) and differential WBC performed on day 0 (before treatment) and on day 5. In the control mice, there was a fall in the total WBC, which was due to a fall in both neutrophils and lymphocytes. There was no significant change in the hematocrit or in the number of platelets as estimated from a blood smear, and none of the animals exhibited bleeding during the experiments. Basophil and eosinophil counts were too low to evaluate the effects of chemotherapy. In the MDR mice there was no change in the granulocyte or lymphocyte counts, indicating that both major types of leukocytes were protected (Table 1).

The bone marrow of the MDR mice was found to be completely resistant to the action of several different cytotoxic drugs that had a profound effect on normal bone marrow. One example is shown in Fig. 2, where the effect of increasing amounts of taxol (4, 6, and 10 mg/kg) on the WBC of normal mice is depicted. There was a clear dose-response relationship, with all concentrations producing a marked decrease in the WBC, which reached a nadir on day 5 and then began to increase (Fig. 2A). In the MDR mice, even the highest dose of 10 mg/kg had no effect.

### Effect of MDR Reversing Agents

The clear-cut difference in the response of normal and transgenic mice made it possible to test the effect of standard drugs that reverse MDR, such as verapamil, quinine, and quindine. None of these agents administered by themselves had any effect on the WBC of normal mice (Fig. 2A) or of transgenic mice (data not shown). However, when administered with a cytotoxic agent such as taxol (Fig. 2B–D), the chemosensitizing agents had a large effect on the WBC. Fig. 2 (B–D) shows that quinine (40 mg/kg), quindine (150 mg/kg), and verapamil (30 mg/kg) all caused a profound fall in the WBC of the transgenic mice when given with taxol. Furthermore, these agents acted in a dose-dependent manner (Fig. 3). In the examples shown in Fig. 3, groups of transgenic animals received a single dose of daunomycin (10 mg/kg) and increasing amounts of verapamil (Fig. 3A), quinidine (Fig. 3B), or quinine (Fig. 3C). At the highest doses of reversing agent, the fall in WBC induced by the daunomycin was equivalent to the drop seen in non-MDR mice given daunomycin alone.

(R)-verapamil is a verapamil stereoisomer that can be given in large amounts in vivo because it has less cardiovascular activity than the (L)-isomer (22) but exhibits similar potency to overcome MDR compared to racemic verapamil in vitro (23). It is currently in phase 1 clinical trials as a MDR-reversing agent of potential clinical interest. Some of the effects of (R)-verapamil with or without daunomycin are shown in Fig. 4. By itself, (R)-verapamil even at 150 mg/kg had no effect on the WBC (Fig. 4A). With daunomycin it did have a small but significant effect on the WBC in non-MDR mice (Fig. 4B). The basis of this effect is currently unknown. However, in the MDR mice its effects are much more dramatic. Daunomycin at 8 mg/kg had no effect by itself (Fig. 4C), but with (R)-verapamil the WBC fell by 80% (Fig. 4D).

Because the reversing agents currently in use all have specific pharmacological actions of their own, as well as a common action on the multidrug transporter, it has been suggested that they can be used in combination at lower dose levels to overcome drug resistance without producing undesirable side effects (24). Since intrinsic cardiovascular effects of prototype reversing agents such as verapamil proved to be a limitation in experimental therapeutic trials to inactivate MDR (16, 17), we chose to combine verapamil with the antimalarial quinine (25) rather than its optical isomer quindine, which has potent anti-arrhythmic effects. To examine the efficacy of this approach, MDR transgenic animals were tested with daunomycin (8 mg/kg) with either verapamil (0.5 mg/kg) or quinine (20 mg/kg) alone (Fig. 5A) or together (Fig.

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**Fig. 3.** Dose-dependent reversal of bone marrow protection against daunomycin effected by the human MDR1 gene. (A) Daunomycin (DAU) (10 mg/kg) combined with verapamil. (B) Daunomycin (10 mg/kg) combined with quinine. (C) Daunomycin (10 mg/kg) combined with quindine. Experiments were conducted as described in Materials and Methods and in the legend to Fig. 1.

MDR drugs, the MDR mice were treated with appropriate amounts of seven different commonly used antinecancer drugs belonging to the MDR group. As shown in Fig. 1, there was no fall in the WBC of transgenic animals after treatment with doxorubicin, vinblastine, vincristine, taxol, daunomycin, etoposide, or actinomycin D, whereas the WBC fell by >50% in the normal animals. Three non-MDR drugs were also studied at concentrations that lowered the WBC in normal mice. There was no difference in the response of the MDR and non-MDR animals to 5-fluorouracil, methotrexate, or cisplatin (Fig. 1). These experiments show that the MDR transgenic mice are specifically resistant to drugs in the MDR family and not to other cytotoxic agents that suppress bone marrow.
DISCUSSION

Function of the MDR Transgene. These data show that the bone marrow of transgenic mice expressing a human MDR1 cDNA is resistant to doxorubicin, daunomycin, vincristine, taxol, etoposide, and actinomycin D. These drugs have very different structures but have been defined by tissue culture studies to belong to the family of drugs transported by the multidrug transporter. The data presented here plus the finding that the bone marrow of the MDR mice was not resistant to 5-fluorouracil, methotrexate, or cisplatin clearly shows that expression of a functional DNA element encoding a single multidrug transport protein is sufficient to cause resistance to drugs with widely different structures in animals. Because many drug-resistant cancers such as renal cell carcinoma express the MDR1 gene at levels comparable to those found in the marrow of the MDR transgenic mice (18), it is reasonable to ascribe their resistance to certain chemotherapeutic drugs to the ability of the product of this gene to pump the cytotoxic drugs out of the drug-resistant cancer cells. In addition, these transgenic mice can serve as a simple test system to determine whether a drug that appears on the basis of tissue culture samples to be useful in overcoming MDR will act in a similar manner in an animal.

Advantages of Using MDR Mice to Test Reversing Agents. A wide variety of drugs that are themselves not cytotoxic have been found to overcome MDR by competitively or noncompetitively inhibiting the multidrug transporter (13). Unfortunately, the reversing agents currently in clinical use are potent pharmacologic agents that have undesirable side effects at levels that must be given to substantially reverse MDR. Agents with fewer side effects remain to be developed. To determine whether an agent is capable of reversing drug resistance in vitro by using cultured cells is relatively easy; this is done by comparing its activity on a matched set of drug-sensitive and drug-resistant cell lines. However, to determine whether a reversing agent has the appropriate bioactivity and useful pharmacokinetic properties in an animal is more difficult. Until the development of this transgenic model for testing agents that reverse drug resistance, it has been necessary to show that these agents enhance the antitumor activity of a cytotoxic drug against a drug-resistant

![Graph](image-url)
tumor. These assays require many animals, take weeks to months to perform, and are not entirely reproducible because of variability in the growth of tumor cells.

In the current study, we have used both taxol and daunomycin as chemotherapeutic agents because they have known bone marrow suppressive effects (26, 27). The finding that peripheral WBCs drop gradually over several days with nadirs on day 5 (data not shown) supports the published data indicating that they are suppressing bone marrow activity rather than having a direct toxic effect on peripheral WBCs. This assay allows a rapid, quantitative estimate of the activity of reversing agents by directly comparing the WBC on days 0 and 5. Because the assay is highly reproducible, only a few animals are needed to evaluate each dose of drug. In addition, several different doses of a cytotoxic drug (Figs. 2 and 4) or a reversing agent can be given in the same experiment (Figs. 3 and 5). Furthermore, combinations of reversing agents can be tested together (Fig. 5). Because different reversing agents may bind to serum proteins (28, 29) or to different tissue sites, a functional assay of their activity in animals is necessary before taking such drugs into clinical studies.

The transgenic animal model also allows this rapid determination of the bioactivity of reversing agents without the need for expensive and time-consuming pharmacokinetic measurements. Because the toxicity of even very high doses of a drug such as taxol is blocked by bone marrow expression of the MDR1 gene in the MDR transgenic mice (Fig. 2B vs. Fig. 2A), it seems unlikely that the human MDR1 gene has a primary effect on pharmacokinetics in these animals. Similarly, the reversing effect of drugs such as verapamil cannot be attributed to altered pharmacodynamics of the chemotherapeutic drug, since chemosensitization occurs even for low doses of daunomycin (Fig. 4D) or taxol (data not shown) in the MDR animals. Since this assay simply asks if the drug works as a reversing agent in an animal, without requiring complex pharmacologic testing, it will speed the screening process and shorten the development time of MDR-reversing agents for use in human trials.

It has not escaped our attention that these MDR transgenic animals prove that bone marrow cells expressing a drug-resistance gene have a selective advantage in the presence of anticancer drugs. This is the kind of selective system that is needed for the development of gene therapy in which an unselected gene can be introduced into bone marrow, or any other organ, by selecting for expression of the drug-resistance gene. Thus, the MDR transgenic animals should serve as a useful model not only for development of anticancer therapies but for establishing new strategies for gene therapy.

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