Monoclonal Antibody MRK16 Reverses the Multidrug Resistance of Multidrug-resistant Transgenic Mice

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ABSTRACT

Using multidrug-resistant (MDR)-transgenic mice, whose bone marrow cells express the human MDR1 gene at a level approximately equal to that found in many human cancers, we determined the efficacy of human-specific anti-P-glycoprotein monoclonal antibody MRK16 in overcoming multidrug resistance in an intact animal. MRK16 alone (2 mg) did not significantly affect the WBC counts of the MDR-transgenic mice, but MRK16, as well as the F(ab')2 fragments of MRK16, led to a dose-dependent circumvention of bone marrow resistance against daunomycin, doxorubicin, vincristine, vinblastine, etoposide, and taxol. This sensitizing effect could not be enhanced by combining MRK16 with low molecular weight chemosensitizing agents such as verapamil, quinine, quindine, or cyclosporin A. We also investigated the concept of specifically targeting and killing multidrug-resistant cells by using MRK16 coupled to Pseudomonas exotoxin (PE). MRK16-PE resulted in a dose-dependent killing of bone marrow cells in MDR-transgenic mice, whereas no bone marrow toxicity was observed in normal control mice. Administration of excess MRK16 prior to injection of MRK16-PE successfully blocked the effect of MRK16-PE. MOPC-PE, a non-MDR-related control monoclonal antibody conjugate, did not target and kill multidrug-resistant bone marrow cells in MDR-transgenic mice. Thus, these immunological approaches to reversing multidrug resistance appear to be both specific and effective.

INTRODUCTION

Cytotoxic chemotherapy has achieved some cures of metastatic cancers which are not amenable to elimination by surgery or radiation. However, despite initial responses, many tumors eventually fail chemotherapy, because standard chemotherapy is inadequate to deal with a large tumor burden in many kinds of cancer and dose intensification, which might lead to better results, is often hindered by inherent toxic side effects of present chemotherapeutic agents (1).

A common form of MDR¹ in human cancer results from expression of the MDR1 gene, which encodes a plasma membrane energy-dependent multidrug efflux pump (2-4), termed P-glycoprotein. Pharmacological intervention aimed at inhibiting this multidrug transporter should improve the activity of existing chemotherapy against human cancer. Numerous agents which interfere with the activity of P-glycoprotein, such as verapamil, have been described (5), and the majority of these appear to be substrates for the transporter, which compete with anticancer drugs for their transport (6). However, inherent and potentially toxic side effects of prototypical resistance-modifiers limit the clinical usefulness of these drugs (7), and the development of new and better agents has been delayed by the difficulty of introducing valid animal models (8). Using recombinant DNA technology, a transgenic mouse has been engineered whose bone marrow cells express physiological amounts of human P-glycoprotein, protecting it from the myelosuppressive effect of chemotherapy (4, 8, 9). Measurements of peripheral WBC counts in these transgenic animals provide a rapid and reliable system to assess bioactivity of agents that reverse MDR (4, 8, 10).

Investigations using monoclonal antibody MRK16, which is directed against an external epitope of human P-glycoprotein (5, 11), have indicated that MRK16 alone may inhibit cell growth of MDR cell lines in vitro (12). In addition, studies in nude mice suggest that concomitant administration of MRK16 can prevent development of tumors resulting from s.c. inoculation of drug-resistant ovarian cancer cells and that subsequent injection of MRK16 can slow growth or even sometimes induce regression of established s.c. tumors (13). The present studies determine the efficacy of MRK16 in overcoming multidrug resistance in a well characterized MDR-transgenic mouse system and evaluate MRK16-Pseudomonas exotoxin immunocoujugates as an alternate immunological approach to kill multidrug-resistant cells in an intact animal.

MATERIALS AND METHODS

MDR-Transgenic Mice. The development and characterization of transgenic mice expressing the human MDR1 gene in their bone marrow has been reported elsewhere (9, 10). A plasmid carrying the full length MDR1 complementary DNA under control of a chicken β-actin promoter was injected into fertilized C57BL/6 × SJL F₁ mouse embryos, and these transgenic embryos were implanted in foster mice. After establishment of a homozygous line (MDR⁻), males were backcrossed to MDR-negative C57BL/6 × SJL F₁ females, to generate heterozygous descendants. The amount of MDR1 mRNA in bone marrow cells of MDR-heterozygous mice is comparable to that detected in the MDR cell line KB-8-5 (9). This level of MDR expression corresponds to a 3-18-fold resistance, depending on the drug used (14). Immunocytochemical analysis demonstrated that virtually all of these bone marrow cells contain P-glycoprotein at their membrane surface (15). In these studies, only 6-8-week-old sex-matched littermates were investigated.

Test Conditions. MRK16 (lots 910131C and 910522C) and MRK16 F(ab')₂ (lot 910405C) were provided courtesy of Hoechst Japan Ltd. (Kawagoe City, Japan). The manufacturer had purified MRK16 by Protein A affinity chromatography and prepared F(ab')₂ fragments by pepsin digestion. MOPC21 is a mouse myeloma antibody (IgG1) with no known reactivity with mouse or human tissues and was purchased from Sigma Chemical Company (St. Louis, MO). Cyclosporin A, verapamil, and etoposide were gifts of Sandoz AG (Basel, Switzerland), BASF Bioresearch Corp. (Cambridge, MA), and Bristol-Myers Squibb Company (Syraucuse, NY), respectively. Taxol was from the Developmental Therapeutics Program, National Cancer Institute. All other drugs were purchased from Sigma. For injection, drugs were dissolved in sterile water (1 mg/ml), except for taxol and etoposide, which were dissolved in 50% dimethylsulfoxide-10% ethanol-40% water and 30% dimethylsulfoxide-10% ethanol-50% water, respectively. The drugs

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3 The abbreviations used are: MDR, multidrug resistance (resistant); PBS, phosphate-buffered saline; PE, Pseudomonas exotoxin.

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were administered by a single i.p. injection into the lower right quadrant of the abdominal cavity. Drug concentrations were adjusted so that a maximum volume of 400 µl was injected per experiment. Each experiment included a minimum of 2 animals/group and was repeated at least once (minimum n = 4). Statistically significant difference from the control was achieved when a reduction of ≥ 50% was noted under these conditions. In protocols involving MRK16, the monoclonal antibody was dissolved in 0.01 M PBS containing 2.5% glycine, 3% sucrose, and 0.1% NaCl as additives, in accordance with the manufacturer’s instructions, and was given 6 h prior to application of chemotherapeutic agents and/or other chemosensitizers. Peripheral blood was collected by peri-orbital bleeding with heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) and was diluted 1/20 (v/v) in 3% acetic acid solution, for RBC lysis. The refractile viable WBC were counted on days 0 (before treatment), 3, 5, and 7, in an Ultra-plane Neubauer’s hemocytometer (Hausszer Scientific, Pittsburgh, PA). The differential WBC count 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.

Construction and Testing of Immunoconjugates. PE, purified from the culture medium of Pseudomonas aeruginosa, was purchased from Swiss Serum (Berne, Switzerland). PE, at 5 mg/ml in 0.2 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, was mixed with a 20-fold molar excess of succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate and incubated at room temperature for 30 min. Protein was separated from the unreacted cross-linker on a PD10 column. Monoclonal antibodies MRK16 or MOPC-21 (Sigma), at 5 mg/ml, were mixed with a 20-fold molar excess of 2-iminothiolane hydrochloride, in 0.2 M sodium phosphate buffer (pH 8.0) containing 1 mM EDTA, and were incubated at 37°C for 1 h. The derivatized antibody was separated from the reactants on a PD10 column. Derivatized antibody and PE were then mixed and incubated at room temperature for 20 h (16). The resulting immunotoxin, coupled by a thioether bond, was then purified by successive chromatography on Mono Q and TSK250 columns (17). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18) served to assess purity of the eluates. The major immunotoxin peak consisted of a 1:1 conjugate of antibody and PE, and yields were approximately 14% of the starting materials.

Inhibition of protein synthesis was used to measure the activity of immunotoxin on cell lines (16, 17). MDR-KB-V1 cells were seeded at 1 x 10^5 cells/ml, in 96-well flat-bottomed microplates, 24 h prior to addition of immunotoxin. Immunotoxins were diluted with 0.2% human serum albumin in PBS and incubated at 37°C for 24 h. Cells were then assayed for incorporation of [³H]leucine (specific activity, 1408 Ci/nmol; New England Nuclear), as described earlier (16, 17). The mean values of triplicate wells were expressed as percentage of controls that did not receive immunotoxin. For animal studies, immunotoxins were diluted in PBS containing 0.2% human serum albumin, adjusted to a final volume of 200 µl, and injected i.p. as described above.

RESULTS

There have been reports suggesting a cytotoxic effect of MRK16 alone in MDR cell cultures, as well as in athymic mice bearing s.c. MDR tumors (12, 13, 19). We now wished to determine its efficacy in an intact animal expressing the human MDR1 gene in bone marrow cells, at a level approximately equal to that found in drug-resistant tumors in people (20). The resulting multidrug resistance of the bone marrow is readily assessed by measuring the total WBC count of a transgenic mouse prior to and several days after administration of cytotoxic drugs (9, 21). Fig. 1 demonstrates that a dose of MRK16 as high as 2 mg/20-g animal did not affect the WBC count of MDR-transgenic mice. This result may suggest a selective immune cell-mediated toxicity of MRK16 against cells (13).

We next evaluated the potential of MRK16 as a chemosensitizer in this novel model. The data depicted in Fig. 2 indicate that the transgenic mice were resistant to the bone marrow-suppressive activity of 10 mg/kg daunomycin, whereas the same dose in normal control mice (9, 10, 21) lowered the WBC count by approximately 70% of the pretreatment value. However, injection of MRK16 6 h prior to administration of 10 mg/kg daunomycin led to a dose-dependent circvention of multidrug resistance, and a dose of 2 mg MRK16 with daunomycin reduced the WBC count by ≥ 50% (Fig. 1). Table 1 summarizes our results with MRK16 plus chemotherapeutic agents from the MDR family of drugs. It is evident that MRK16 reversed resistance against anthracyclines such as daunomycin and doxorubicin, Vinca alkaloids such as vincristine and vinblastine, epipodophyllotoxins such as etoposide, and taxol. In addition, MRK16 F(ab')2 fragments displayed a similar ability to overcome multidrug resistance in vivo, compared to the intact MRK16 antibody (Table 1). This is of potential clinical interest, since F(ab')2 fragments are less immunogenic and may penetrate tumors better than intact antibodies.

However, despite these encouraging results, we observed that the efficacy of MRK16 as a chemosensitizer was inferior to that of chemical compounds, such as verapamil, cyclosporin A, di-hydropyridines, quinine, and quinidine, which had previously been investigated in this transgenic bioassay (8, 10, 15, 21). These drugs induced a fall in the WBC count of MDR-transgenic mice that was equivalent to the drop seen in non-MDR mice given chemotherapy alone. Thus, we explored other options to improve the potency of MRK16-enhanced toxicity to MDR cells.

First, we combined MRK16 with different prototypical chemosensitizers. Previous studies had determined that low
doses of drugs such as verapamil and quinine (10) or others, which produced only partial sensitization of the MDR-transgenic mice, were fully sensitizing when used in combination. Fig. 2 shows that the combination of verapamil and cyclosporin A in conjunction with daunomycin chemotherapy was highly effective in MDR-transgenic mice (Fig. 2A). However, MRK16 combined with either verapamil (Fig. 2B), cyclosporin A (Fig. 2C), quinine (Fig. 2D), or quinidine (data not shown) did not exceed significantly the sensitization against daunomycin produced by any drug alone.

Finally, we evaluated the novel concept of selectively targeting and killing cancer cells with toxins directed via antibodies or receptor-binding proteins (22). Therefore, we conjugated MRK16 to PE, using a conjugation method that produces a very stable thioether bond (17, 22), and tested its usefulness in MDR cell lines. Fig. 3 demonstrates that MDR-KB-V1 cells were highly sensitive to MRK16-PE (50% inhibitory concentration = 0.12 pmol/ml) and that this effect was specific, since it could be blocked by preincubation with excess MRK16 before addition of MRK16-PE. These experiments indicate that, in a cytotoxicity assay based on tritiated leucine uptake, MRK16 coupled to PE by a thioether bond leads to results similar to those previously reported when MRK16 was conjugated to PE by a disulfide bond and cytotoxicity was evaluated by a clonogenic assay (23). Having established that MRK16-PE was active against cultured cells, we wished to assess the effect of MRK16-PE in mice. We, therefore, determined the 50% lethal dose of MRK16-PE in MDR-negative normal mice, which was 1.5 μg; the 50% lethal dose in MDR-transgenic mice reached 4 μg. Although statistically not significant, this appears to indicate that more antigen is available for binding, thus preventing nonspecific liver toxicity due to PE (22). In Fig. 4, we have plotted the changes in WBC count observed after MRK16-PE administration. In normal mice (Fig. 4A) no decrease in WBC could be detected. However, in MDR-transgenic mice (Fig. 4B) MRK16-PE induced a dose-dependent reduction of WBC of nearly 70%. Fig. 4C and D, shows control experiments. Injection of excess MRK16 6 h prior to MRK16-PE effectively eliminated the marrow toxicity (Fig. 4C). MOPC21 is a monoclonal antibody that does not react with human or mouse cells, and MOPC-PE, a non-MDR-related conjugate, did not decrease the WBC of MDR-transgenic mice (Fig. 4D). These

Table 1 Reversal of multidrug resistance by MRK16 and MRK 16 F(ab')¡ in MDR-transgenic mice

| Monoclonal antibodies were administered 6 h prior to injection of chemotherapeutic agents, and WBC counts were performed on days 0 (before treatment), 3, 5, and 7. Values are expressed as percentage of WBC remaining on day 5, compared to original value. |
|---|---|---|---|---|
| WBC remaining (%) | MRK16 | MRK 16 F(ab')¡ |
| Daunomycin (10 mg/kg) | 100 | 63 | 56 | 48 | 55 |
| Doxorubicin (10 mg/kg) | 100 | 60 | 53 | 46 | 49 |
| Vinblastine (5 mg/kg) | 100 | 55 | 46 | 43 | ND* |
| Vinblastine (10 mg/kg) | 100 | 55 | 54 | 49 | ND |
| Etoposide (10 mg/kg) | 100 | 65 | 56 | 51 | ND |
| Taxol (10 mg/kg) | 100 | 82 | 75 | 74 | 82 |

* ND, not determined.
two results show that MRK16-PE is specifically cytotoxic to P-glycoprotein-expressing bone marrow cells in vivo.

Table 2 summarizes the differential WBC counts of MDR-transgenic mice, to reveal cell type-specific toxicities of PE conjugates. In addition to the differences in total WBC count induced by MOPC-PE or MRK16-PE (see Fig. 4, B and D), the relative distribution of peripheral WBC appeared also to be affected (Table 2). MOPC-PE (0.75 µg) significantly altered neither number nor cell type of leukocytes present in blood 5 days after injection of conjugate. Dose escalation to 2 µg led to changes that mainly consisted of an absolute and relative increase in the number of neutrophils, which may be interpreted as the sign of a nonspecific stress reaction. However, MRK16-PE seemed to display a preferential effect on certain cell types of the differential WBC count, as indicated by absolute numbers. At 1 µg, the total WBC count was reduced by nearly 50%, but distribution of cells still remained within the range of that of untreated mice. Higher doses, up to 4 µg, further decreased the absolute number of both major types of leukocytes, i.e., neutrophils and lymphocytes, but resulted in a relative dominance of lymphocytes. These observations appear to indicate that MRK16-PE acts on MDR bone marrow cells, rather than on peripheral leukocytes, because specific targeting and killing of precursors of the myeloid lineage in bone marrow could result in this preferential decline of neutrophils after 5 days, due to their shorter half-lives, compared to cells of the lymphatic lineage. Nevertheless, from these findings, it cannot be excluded that an immune mechanism, such as peripheral destruction in the spleen, would lead to the same results.

![Graph showing effects of MRK16-PE on MDR cell line KB-V1](image1)

**Fig. 3.** Effects of MRK16-PE on MDR cell line KB-V1. Experiments were conducted as explained in "Materials and Methods." Cytotoxicity was measured in a tritiated leucine uptake assay, and values are expressed as percentage of controls that did not receive treatment. □, MRK16-PE; ▪, MRK16 (excess) plus MRK16-PE.

![Graph showing WBC counts of MDR-transgenic mice](image2)

**Fig. 4.** Effects of *Pseudomonas* exotoxin conjugates on the WBC of mice. A, MRK16-PE in normal C57BL/6 × SJL mice; B, MRK16-PE in MDR-transgenic C57BL/6 × SJL mice; C, MRK16 (1 mg) 6 h prior to MRK16-PE (1 µg) in MDR-transgenic C57BL/6 × SJL mice; D, MOPC-PE in MDR-transgenic C57BL/6 × SJL mice. Experiments were conducted as explained in "Materials and Methods." Values are shown as time courses of WBC counts, expressed as percentage of pretreatment values. Minimum n = 4 per data point. Statistical significance is achieved when a reduction of ≥ 50% is noted.
DISCUSSION

Our studies demonstrate that MRK16 alone does not significantly affect bone marrow cells of transgenic mice expressing physiological amounts of the human MDR1 gene, as determined from measurements of peripheral WBC (Fig. 1). However, it is obvious that MRK16 and MRK16 F(ab')2 fragments can serve as chemosensitizers in conjunction with various chemotherapeutic agents (Table 1). Previous clinical trials had determined that inherent and potentially dangerous side effects of conventional chemosensitizers, such as verapamil, limit their use in humans and that reduced dosage can achieve only transient responses. Hence, new, better, and less toxic chemosensitizers need to be developed (7, 15). Given the low toxicity of the immunological approaches to reverse multidrug resistance described in this paper, further efforts at immunological circumvention of multidrug resistance may be warranted, perhaps aimed at the development of monoclonal antibodies with increased effectiveness at inhibiting the multidrug transporter.

Typically, reversal of multidrug resistance has been accomplished by exposing drug-resistant cells to alternate substrates for the multidrug transporter, which, in themselves, are not cytotoxic. In tissue culture, it has been shown that a wide variety of chemosensitizers circumvent drug resistance by inhibiting the transport of the cytotoxic agent. Thus, elucidation of this mechanism has led to a rational approach for the reversal of drug resistance and the possible treatment of drug-resistant cancers (4). Because chemosensitizers currently in use have specific pharmacological actions of their own, as well as a common action on the multidrug transporter, it appears feasible to use them in combination at lower dose levels, to overcome drug resistance without producing undesirable side effects. In fact, we showed previously that verapamil and quinine together give a much greater effect than either drug alone in MDR-transgenic mice (10), and in this paper we demonstrate similar enhancement with the combination of verapamil and cyclosporin A (Fig. 2A).

MRK16 recognizes external epitopes of P-glycoprotein. Results presented here and elsewhere (12, 24) showing inhibition of P-glycoprotein function by MRK16 suggest that the external epitopes recognized by MRK16 are implicated in the formation of the putative pore-forming structure of P-glycoprotein (6). Since drug binding site(s) are believed to be on the cytoplasmic side of the transporter (3, 5, 6) or in the plasma membrane (25), it appeared worth addressing the question of whether combinations of MRK16 with conventional chemosensitizers could act synergistically. Unfortunately, in an intact animal, the combination of MRK16 with various other reversing agents (Fig. 2, B–D) did not evoke a more pronounced chemosensitization than either drug alone. This result may suggest that multidrug transporters to which MRK16 is bound cannot be further inhibited by substrate-like competitive inhibitors, implying that the antibody may be interfering with transporter-inhibitor interactions, perhaps by inducing allosteric changes in P-glycoprotein or by sensitizing cells to drugs indirectly.

Our studies also showed that MRK16-PE was effective in vivo (Fig. 4). This anti-P-glycoprotein-toxin conjugate specifically and with high efficiency killed multidrug-resistant cells in vitro (23) (this work) and in an intact animal. For the time being, several potential uses for MRK16-PE may be anticipated. It may be exploited to purge cell populations, such as bone marrow preparations, of multidrug-resistant cells. Although use in an adjuvant setting for the treatment of drug-resistant metastatic disease might be tempting, it will be important to evaluate effects of MRK16-PE on normal tissues such as brain capillary endothelial cells and the rare bone marrow cells that express P-glycoprotein on their surfaces (26–28). These studies suggest that immunological approaches to the circumvention of multidrug resistance, and the killing of multidrug-resistant cells, are feasible and effective. Further efforts at developing more efficient reagents and exploring potential toxicities seem warranted.

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