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Inhibition of Multidrug-resistant Human Tumor Growth in Athymic Mice by Anti-P-glycoprotein Monoclonal Antibodies

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In an effort to devise an effective treatment for human drug-resistant cancers, we have developed monoclonal antibodies, MRK16 and 17, reactive to the multidrug transporter protein, P-glycoprotein. The monoclonal antibodies given intravenously effectively prevented tumor development in athymic mice inoculated subcutaneously with drug-resistant human ovarian cancer cells 2780^{AD}. Treatment with MRK16 induced rapid regression of established subcutaneous tumors and apparent cures of some animals. Complement-dependent cytotoxicity (MRK16) and antibody-dependent cell-mediated cytolysis (MRK16 and 17) were observed with these antibodies. These monoclonal antibodies may have potential as treatment tools against multidrug resistant human tumors possessing the P-glycoprotein.

Key word: Multidrug resistance — Monoclonal antibody — P-glycoprotein — Human tumor — Athymic mice

Resistance of tumors to multiple drugs is a major problem for cancer chemotherapy. Resistance to multiple agents such as doxorubicin (adriamycin), vinca alkaloids, and actinomycin D can be acquired by tumor cells after treatment with a single drug.[1,2] The gene responsible for multidrug resistance, termed *mdr*, encodes a membrane glycoprotein (P-glycoprotein) that acts as a pump to transport various cytotoxic drugs out of the cell.[3] The P-glycoprotein has been shown to bind anticancer drugs[4,5] and to be an ATPase[6,7] localized at the plasma membrane of resistant cells.[8,9] Transfection of cloned *mdr* sequences confers multidrug resistance on sensitive cells.[10–12]

The amount of P-glycoprotein expression has been measured in tumor samples and was found to be elevated in intrinsically drug-resistant cancers of the colon, kidney, and adrenal as well as in some tumors that had acquired drug resistance after chemotherapy.[13–15] As P-glycoprotein appears to be involved in both acquired multidrug resistance and intrinsic drug resistance in human cancer, the selective killing of tumor cells expressing P-glycoprotein could be very important for cancer therapy. Recently, we have described two mouse monoclonal antibodies, MRK16 and MRK17, that react with P-glycoprotein of human tumor cells.[16] In the present study, we have evaluated the therapeutic potential of these antibodies in the treatment of multidrug-resistant human ovarian cancer (2780^{AD}) xenografts in athymic nude mice. 2780^{AD} cells are cross-resistant *in vitro* to various other drugs such as vinca alkaloids, actinomycin D, and colchicine and the effect of these antitumor agents can be potentiated by the calcium channel blocker verapamil.[17,18] High molecular weight (Mr 170,000–180,000) P-glycoprotein has been demonstrated to be overexpressed in the 2780^{AD} cells.[16,19]

Two experiments were designed to evaluate the effect of MRK16 and MRK17 on the growth of 2780^{AD} cells (10^7/mouse) inoculated sc in athymic mice. In the first experiment (Fig. 1A), animals received intravenous injection of various amounts of MRK16 or MRK17 on days 2 and 7 after tumor inoculation. Subcutaneous tumors never developed in mice treated with 10 μg or more per animal of MRK16 and 30 μg or more per animal of MRK17 antibodies. When the mice were treated with 1 μg per animal of MRK16 or 1 or 10 μg per animal of MRK17, the development of tumors was substantially retarded as compared to control mice where palpable tumors developed by day 20.

In the second experiment (Fig. 1B), therapy with antibodies was started after tumors became palpable (150–400 mm^3 in volume). One group of animals received 6 mg/kg of adriamycin intravenously 3 times. Adriamycin did not induce significant inhibition of 2780^{AD} tumor growth. In contrast, adriamycin did significantly inhibit *in vivo* growth of the parent tumor A2780 [76% inhibition was observed on day 45 (data not shown)]. A second group of animals received 1 mg of MRK16 intravenously on days 20, 23, and 26 and the average tumor volume was measured. One or two days after the first MRK16 injection, the size of the tumor masses remarkably decreased in all five mice. After the third MRK16 injection, the average tumor mass was substantially

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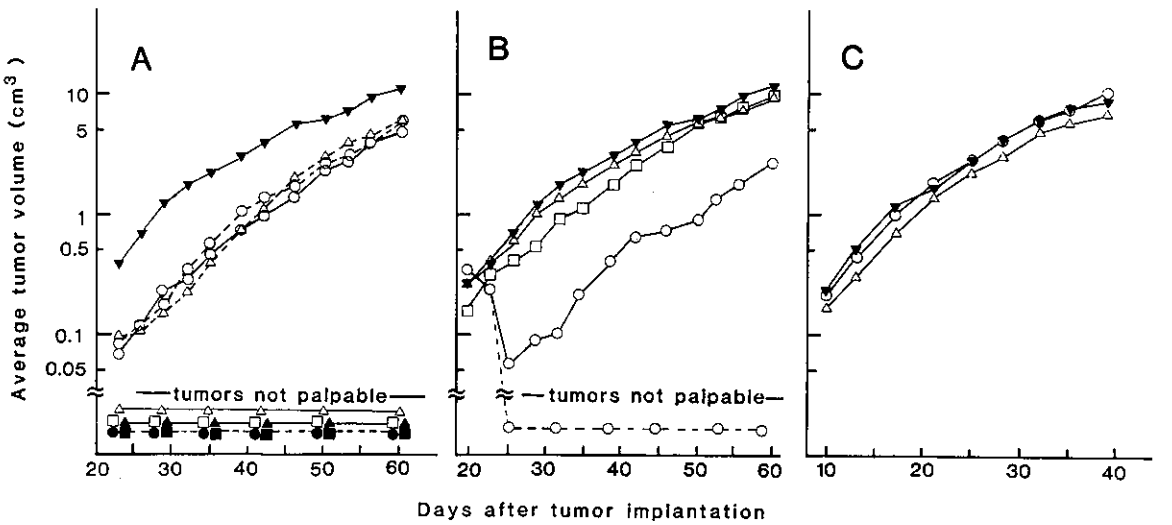

Fig. 1. *In vivo* effects of MRK16, MRK17, and adriamycin on the growth of drug-resistant tumor xenografts. A multidrug-resistant human ovarian carcinoma cell line 2780^{AD}, and its parent drug-sensitive cell line A2780[17,18] were kindly provided by R. F. Ozols and T. C. Hamilton (National Cancer Institute), and were prepared and maintained as described.[16] Balb/c *nu/nu* mice were obtained from Charles River, Japan. Mouse monoclonal anti-P-glycoprotein antibodies, MRK16 (IgG_{2a}) and MRK17 (IgG_1), were prepared and purified as described.[6,7] All of the animals were given injections of 10^7 2780^{AD} cells sc on day 0. In (A), on days 2 and 7 after tumor cell inoculation, groups of animals (5 nude mice per group) were treated iv with 0.001 (○), 0.01 (△), 0.03 (□), 0.1 (●), 0.3 (▲), or 1 mg (■) of MRK16 (——; solid line) or MRK17 (– – –; broken line) antibody. Untreated animals (▼) served as controls. In (B), mice were inoculated sc with 10^7 2780^{AD} cells as above. When the tumors became palpable on day 20 (150–400 mm^3 in volume; as determined from the formula: $V=1/2\ ab^2$, where a is the longest diameter and b is the shortest diameter of the tumor), mice were randomized into 4 groups (5 mice/group). One group was untreated (▼), a second group was treated with 1 mg of MRK16 on days 20, 23, and 26 (○), a third group was treated with 1 mg of MRK17 on days 20, 23 and 26 (△), and the fourth group was treated with 6 mg/kg of adriamycin on days 20, 24, and 28, an optimal dose and schedule for adriamycin treatment (□). Tumor growth was measured every 3 to 4 days until day 60. MRK16 treatment resulted in rapid regression of subcutaneous tumors. Thus, only one of five animals continued to bear a palpable tumor after day 23. An additional animal showed a recurrent tumor between days 42–60. The average tumor volume of these two mice is shown (○- - -○). The remaining three animals were tumor-free at day 60, as is shown in the figure (○- - -○). In (C), drug-sensitive parent A2780 cells (10^7 cells per mouse) were inoculated sc and MRK16 (○) and MRK17 (▲) were given at 1 mg per animal on days 2 and 7 after tumor inoculation. As in A and B, (▼) represents the growth of untreated control tumors. In the above experiments, standard deviation (SD) was within 40% of each point.

reduced and in four out of five mice, the tumor mass had completely disappeared. Among these four mice, a palpable tumor recurred in one animal on day 42; the other 3 mice remained tumor-free up to day 60. A third group of animals received 1 mg of MRK17 intravenously on days 20, 23, and 26. No significant inhibition of tumor growth was observed.

These results indicate that 2780^{AD} cells are resistant to adriamycin *in vivo* as well as *in vitro*, and that the monoclonal anti-P-glycoprotein antibodies inhibited the growth of these drug-resistant tumor cells *in vivo*. The action of the MRK16 antibody was readily demonstrable even when administered after the tumors were well established and growing, resulting in total disappearance of tumors and cure of several mice. These monoclonal antibodies had no effect on the *in vivo* growth of drug-sensitive parent tumor A2780 cells (Fig. 1C), suggesting that the effect of these antibodies was specific for the drug-resistant tumor cells, which overexpress the antigen P-glycoprotein.

Several mechanisms may be involved in the *in vivo* growth inhibition of the drug-resistant tumor cell xenografts by anti-P-glycoprotein antibodies. Previously, it was reported that MRK17 itself had a moderate cytostatic effect on the *in vitro* growth of drug-resistant cells, while MRK16 had little effect on the *in vitro* growth of resistant cells.[16] As the *in vivo* growth inhibition was more remarkable in treatment with MRK16 than with MRK17 (Fig. 1), it is likely that the major mechanism of action of the *in vivo* growth inhibition is not a direct cytostatic effect of the antibodies, but it could be related to immune responses.

Complement-dependent cytotoxicity (CDC) for 2780^{AD} cells was clearly demonstrated *in vitro* using MRK16 (mouse IgG_{2a}) and rabbit complement (Fig. 2 A), while it was not detected using MRK17 (mouse IgG_1). Antibody-dependent cell-mediated cytolysis (ADCC) was also studied *in vitro* (Fig. 2B). Cell-mediated cytotoxicity was more clearly demonstrated using MRK16 than MRK17. Growth inhibition of human tumor cells *in vivo* by mouse monoclonal antibodies has been reported in some other tumor xenograft models.[20,21] Mouse IgG_{2a} monoclonal antibodies were more efficient in tumor cell killing by the antibody-dependent cell-mediated mechanism compared with other mouse IgG subclasses.[22] These previous reports are compatible with our present observations that MRK16 (IgG_{2a}) was more effective in treatment of established tumors than was MRK17 (IgG_1) (Fig. 1).

The finding that MRK16 treatment selectively inhibits the growth of multidrug-resistant cells *in vivo* raises the possibility that the present immunotherapy could be used to kill multidrug-resistant human cancer cells in patients. The expression of the P-glycoprotein has now been measured in several human cancers[13–15] and many cancers have been found to contain elevated amounts of P-glycoprotein.[13–15] It is possible that the level of P-glycoprotein on the surface of some human cancers might be sufficient to serve as a target for MRK16 immunotherapy. However, a number of substantial obstacles still remain to be overcome before such treatment can be applied in patients. One obvious difficulty arises from the fact that the P-glycoprotein is constitutively expressed in several normal human organs and tissues including adrenal, kidney, liver, and colon.[23,24] Presumably, the function of P-glycoprotein in these tissues is to transport normal metabolites or cytotoxic compounds into the blood, urine, bile or lumen of the colon.[25] MRK16 and 17 are reactive to multidrug-resistant human tumors but they are not reactive to multidrug-resistant mouse

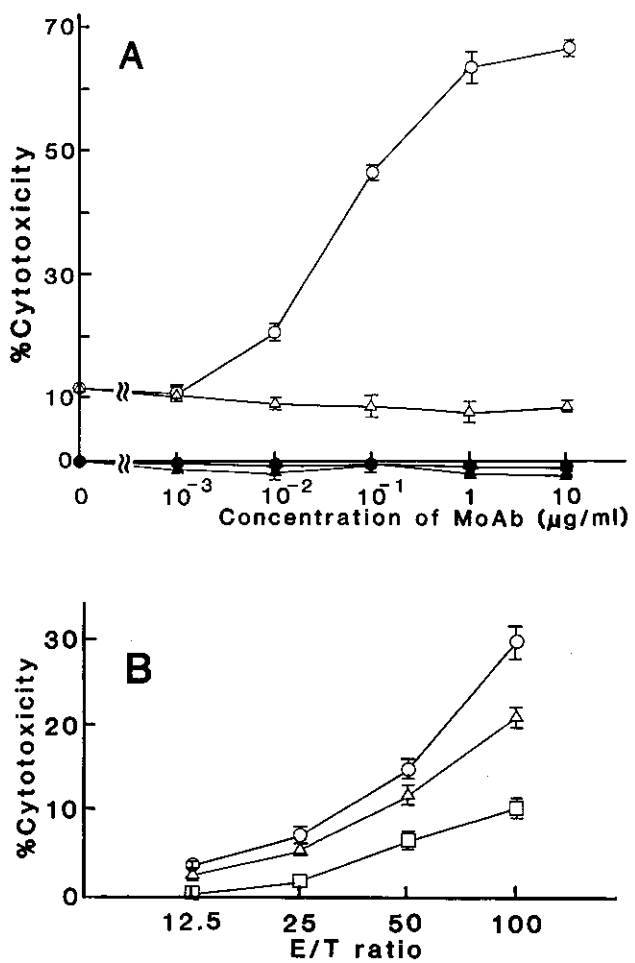

Fig. 2. (A) Complement-dependent cytotoxicity with MRK16 or MRK17 for $A2780^{AD}$ cells. Target cells were labeled with ^{51}Cr as described.[31] Cell suspension (100 μl) containing 10^4 labeled target cells was incubated at 37°C for 30 min with MRK16 and MRK17 in a 96-well microculture plate. Then the mixtures were further incubated at 37°C for 45 min with (○) or without (●) complement for MRK16 and with (△) or without (▲) complement for MRK17, respectively. A 100 μl aliquot of a 1:20 dilution of selected rabbit serum (Cederlane Lab., Canada) was used as a source of complement. After the reaction, the radioactivity in 100 μl of supernatant was counted in a gamma counter. Determination was carried out in triplicate and the bar represents SD. The percent specific cytolysis was calculated from ^{51}Cr release of test samples and control samples, as follows: % specific release = $[(E-S)/(M-S)] \times 100$, where E=experimental release (cpm in supernatant from target cells incubated with complement and experimental antibody), S=spontaneous release (cpm in supernatant from target cells incubated with medium alone), and M=maximum release (cpm released from target cells lysed with 1% Triton X-100). (B) Antibody-dependent cell-mediated cytolysis with MRK16 or MRK17 for $A2780^{AD}$ cells. Spleen cells of Balb/c mice, which had been given daily ip injections of 1KE OK432 for 1 week to activate the killer activity of spleen cells, were used as the effector cell source.[32] Cell suspension (100 μl) containing 10^4 labeled target $A2780^{AD}$ cells was incubated at 37°C for 30 min with 1 μg/ml MRK16 (○), 1 μg/ml MRK17 (△), or without MoAb (□) in a 96-well microculture plate. Then 100 μl of cell suspension containing effector spleen cells (effector-to-target ratio 100, 50, 25, 12.5, and 0) was added to each well. The plate was incubated at 37°C for 12 h in a humidified 5 % CO_2 atmosphere. After centrifugation, the radioactivity in 100 μl of supernatant was counted in a gamma counter. Determination was carried out in triplicate and the bar represents SD. The percentage of specific cytolysis was calculated as described in the legend to Fig. 2A.

tumors.[16] So it would be difficult to detect undesirable side effects with these antibodies in the present animal experiments, and actually we could not observe body weight loss in mice after the treatment with these antibodies. However, in humans, undesirable side effects of the anti-P-glycoprotein antibody might occur with respect to the normal function of the organs mentioned above. Further toxicological studies are needed before clinical application. A second major obstacle is that the mouse antibodies, as foreign proteins, may evoke counteracting immune reactions that may destroy their effectiveness and may also cause allergic reactions in the patients.[26, 27] We are now constructing chimeric antibodies in which the antigen-recognizing variable regions of MRK16 are joined with the constant regions of human antibodies.[28] These antibodies may be less likely to produce an immune response in patients than the all-mouse monoclonals.

Further problems in clinical application of these antibodies could be: 1) ADCC and CDC in patients may not be so strong as observed in the present animal experiments. 2) Human tumor cells are heterogeneous and they do not always carry P-glycoprotein. In this respect, we need a combination therapy with both anti-P-glycoprotein monoclonal antibody and antitumor agents. Experiments along this line are in progress at the animal level. 3) Antigenic modulation in patients.

While some problems still exist in the use of immunotherapy with MRK16 and 17 to kill multidrug-resistant human cancer cells,[29, 30] the present preclinical studies demonstrate that such treatment can be effective, and suggest that this therapeutic approach should be investigated further.

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