Jpn. J. Cancer Res. 81, 1155–1161, November 1990

Monoclonal Anti-P-glycoprotein Antibody-dependent Killing of Multidrug-resistant Tumor Cells by Human Mononuclear Cells

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Mouse monoclonal antibodies (MRK16 and MRK17) against human multidrug-resistant cancer cell lines were tested for antibody-dependent cytotoxicity mediated by human blood mononuclear cells, using a 4-h ^{51}Cr release assay. MRK16 (IgG_{2a} isotype) was shown to be more effective than MRK17 (IgG_1 isotype). Moreover, when four pairs of drug-resistant and their parent sensitive human cancer cells were tested for antibody-dependent cell-mediated cytolysis (ADCC) using MRK16, only the drug-resistant cell lines were susceptible to ADCC reaction. When highly purified lymphocytes (>99%) and monocytes (>97%) were isolated from blood mononuclear cells by centrifugal elutriation and adherence, MRK16 promoted both lymphocyte- and monocyte-mediated tumor cell killing, whereas MRK17 induced only a lymphocyte-mediated ADCC reaction. These results suggest that MRK16 of IgG_{2a} subtype may be a useful therapeutic agent in eradication of drug-resistant cancer cells expressing P-glycoprotein through ADCC reaction.

Key words: Multidrug resistance — P-glycoprotein — Monoclonal antibody — Human mononuclear cells

Resistance of tumors against multiple drugs is a major problem in cancer chemotherapy. It has already been reported that P-glycoprotein, which transports various cytotoxic drugs outside the cells, is one of the key molecules in multidrug resistance.[1,2] P-glycoprotein has been proven to be a binding protein for anti-cancer drugs,[3,4] to be an ATPase,[5,6] and to be localized at the plasma membrane of resistant cells.[7,8] Transfection of cloned mdr1 sequences conferred multidrug resistance on sensitive cells.[9–11] The amount of P-glycoprotein expression in tumor samples 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.[12–14] Because P-glycoprotein appears to be involved in both acquired and intrinsic multidrug resistance in human cancers, the selective killing of the tumor cells expressing P-glycoprotein would be very important for successful cancer therapy.

Herlyn and Koprowski reported that, when anti-human cancer monoclonal antibodies (MoAbs)[4] were administered, the macrophages invaded human tumors inoculated in athymic mice, and delayed the development of the tumors.[15] These effective MoAbs induced antibody-dependent macrophage-mediated cytolysis (ADMMC) against the human cancer cells *in vitro*.[15,16] Natural killer cells (NK cells) are also useful effector cells in tumor rejection associated with antibody.[16–22]

We have already reported that the MoAbs MRK16 (IgG_{2a}) and MRK17 (IgG_1), recognizing P-glycoprotein of human cancer cells, effectively prevented tumor development in athymic mice inoculated subcutaneously with the drug-resistant human ovarian cancer cells 2780AD.[23] We found that these MoAbs could induce antibody-dependent cell-mediated cytolysis (ADCC) using mouse spleen cells as effector cells.[23] However, it remained to be determined whether these MoAbs of mouse origin could induce ADCC with human peripheral effector cells, and if so, what kinds of effector cells are involved. In order to solve these questions, we examined the ADCC activity with these MoAbs using mononuclear cells of normal human volunteers.

MATERIALS AND METHODS

Cells and culture conditions Human ovarian tumor A2780 and its adriamycin-resistant variant, 2780AD, were kindly supplied by Drs. R. F. Ozols and T. C. Hamilton, National Cancer Institute. Human myelogenous leukemia K562 cells resistant to adriamycin (K562/ADM) were established in our laboratory as described.[12] The human KB carcinoma cell line, KB3-1,

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[4] The abbreviations used are: MoAb, monoclonal antibody; IL-2, interleukin-2; MDR, multidrug resistance; MNC, mononuclear cell(s); ADCC, antibody-dependent cell-mediated cytolysis.

and its colchicine-resistant variant, KB-C4, were generously provided by Dr. I. Pastan, National Cancer Institute.[13] The acute lymphoblastic leukemia cell line (CCRF-CEM) and its vinblastin-resistant subline (CEM-VLB_{100}) were provided by Dr. W. Beck, St. Jude Children's Research Hospital.[14] The characteristics of these cell lines have already been summarized in detail.[24] The cells were cultured as described previously.[25]

Isolation and cultures of human peripheral blood lymphocytes and monocytes Leukocyte concentrate was obtained from peripheral blood (200 ml) of healthy donors by centrifugation at 1,200 rpm for 10 min using a Kubota KR-400 centrifuge with an FS-6600 rotor, and mononuclear cells were separated from the leukocyte concentrate in lymphocyte separation medium (LSM, Litton Bionetics, Kensington, MD) by centrifugation at 1,800 rpm for 20 min. Lymphocytes and monocytes were separated from the mononuclear cell samples by centrifugal elutriation with a Hitachi SRR6Y elutriation rotor.[26-28] Lymphocyte-rich and monocyte-rich fractions were obtained by centrifugation at 2,000 rpm, with flow rates of 15 ml/min and 20 ml/min, respectively. These fractions were washed twice with phosphate-buffered saline, and resuspended in culture medium. More than 97% of the cells in each fraction were viable, as judged by the trypan-blue dye exclusion test. More than 99% of the cells in the lymphocyte-rich fractions were lymphocytes. Cells in the monocyte-rich fractions (containing >90% monocytes) were plated in 96-well Microtest plates for 1 h and then the non-adherent cells were removed by washing twice with medium to form monocytic monolayers. At this point, the purity of monocytes was over 99%, as assessed by the examination of cell morphology and nonspecific esterase staining. These cells were used as effector cells for ADCC assay.

Anti-P-glycoprotein MoAbs Anti-P-glycoprotein MoAbs, MRK16 (IgG_{2a}) and MRK17 (IgG_1), were obtained as described previously.[29] Large amounts of MoAbs were prepared from the ascites fluid of mice bearing hybridoma cells. The MoAbs used in this experiment were purified by precipitation with ammonium sulfate and by DEAE-Sephacel (Pharmacia) chromatography as described.[30] Protein measurement was done by the method of Smith *et al.*[31]

ADCC assay ADCC activity was assessed by a ^{51}Cr-releasing assay. Briefly, ^{51}Cr-labeled tumor cells (2×10^5/ml) were incubated in culture medium containing anti-P-glycoprotein MoAb for 30 min at 37°C. After incubation, 100 μl of the mixture containing target cells (2×10^4) and MoAb was added to each well containing mononuclear cells, lymphocytes or monocytes at various ratios of effector to target cells. After a 6-h incubation at 37°C, the supernatant (100 μl per well) was harvested by brief centrifugation at 500*g*, and the radioactivity in the supernatant was counted in a gamma counter. Percentage cytolysis was calculated as follows:

$$\%\ \text{Cytolysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100.$$

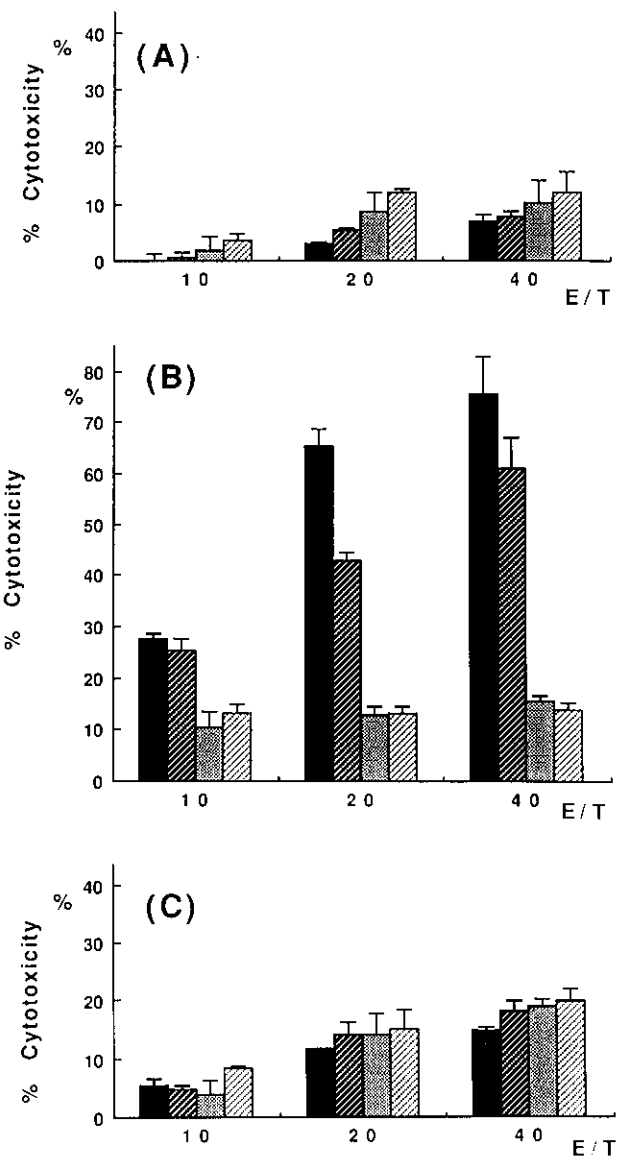

Fig. 1. ADCC by human MNC against A2780, 2780AD and Daudi cells. Mononuclear cells ($1-4\times10^5$ per well) from normal volunteers were tested for cytolytic activities against A2780 (A), 2780AD (B) and Daudi (C) cells by 6-h ^{51}Cr-release assay as described in "Materials and Methods." The concentration of MoAb was 10^{-2} μg/ml (MRK16 ■ and MRK17 ▨). Normal mouse IgG (▩) and minus IgG (▨) were used as controls.

The spontaneous release which occurred with different target cells was less than 15% of total release.

Statistical analysis The statistical significance of differences between test groups was analyzed by the use of Student's two-tailed *t* test.

RESULTS

ADCC with human mononuclear cells (MNC) against multidrug-resistant (MDR) human ovarian tumor cells In order to test whether MRK16 or MRK17 enhanced antitumor activity with human peripheral MNC, we examined the ADCC activity of human MNC from normal volunteers. The results of tumor cell lysis, with or without MoAb, are shown in Fig. 1. Anti-P-glycoprotein MoAb increased the cytolytic activity against 2780AD cells, and it appeared that MRK16 was more effective than MRK17. Control IgG did not enhance the lysis. However, the lysis of parent human ovarian cancer cells (A2780) and Daudi cells was not enhanced by these MoAbs. These results indicate that MRK16 and MRK17 specifically induced the lysis of MDR cancer cells.

Effector cell analysis Lymphocytes and monocytes separated from mononuclear cells were examined for ability to induce the lysis of MDR human cancer cells. The results are shown in Table I. Lymphocytes enhanced the lysis of 2780AD with MRK16 (from 9.4±4.0% to 32.2±5.3%). The lysis, however, was not enhanced with MRK17 (from 9.4±4.0% to 10.1±5.3%). Monocytes increased the lysis of 2780AD with both MRK16 (from 9.3±1.6% to 40.1±3.7%) and MRK17 (from 9.3±1.6% to 32.2±3.5%). However, neither of the effector cells induced ADCC against A2780 with MRK16 or MRK17. Similar results were obtained with two other volunteers (data not shown). These results indicated that MRK16 and MRK17 induced ADCC activities especially against 2780AD, and MRK16, whose subclass is IgG_{2a}, was more effective as an anticancer agent than MRK17.

Dependence of tumor lysis on MoAb concentration Using human lymphocytes and monocytes as effector cells, ADCC against 2780AD and A2780 was examined at several concentrations of MRK16. As shown in Fig. 2, the percent lysis of 2780AD cells was concentration-

Table I. Effector Cell Analysis of ADCC Induced by MRK16 and MRK17

Target cells	Effector cells	% Cytolysis		
		MoAb (−)	MRK16	MRK17
A2780	Lymphocytes	0.0±0.8	1.6±1.4	0.8±1.6
	Monocytes	2.4±1.7	3.0±2.2	3.3±2.3
2780AD	Lymphocytes	9.4±4.0	32.2±5.3	10.1±5.3
	Monocytes	9.3±1.6	40.1±3.7	32.2±3.5

Human lymphocytes and monocytes were separated by a counter-flow cell elutriater from human MNC. Cytolytic activities were tested by a 6-h ^{51}Cr-release assay at an effector-target ratio of 40. The concentration of MoAb was 10^{-2} μg/ml. The percent cytolysis was calculated as described in "Materials and Methods."

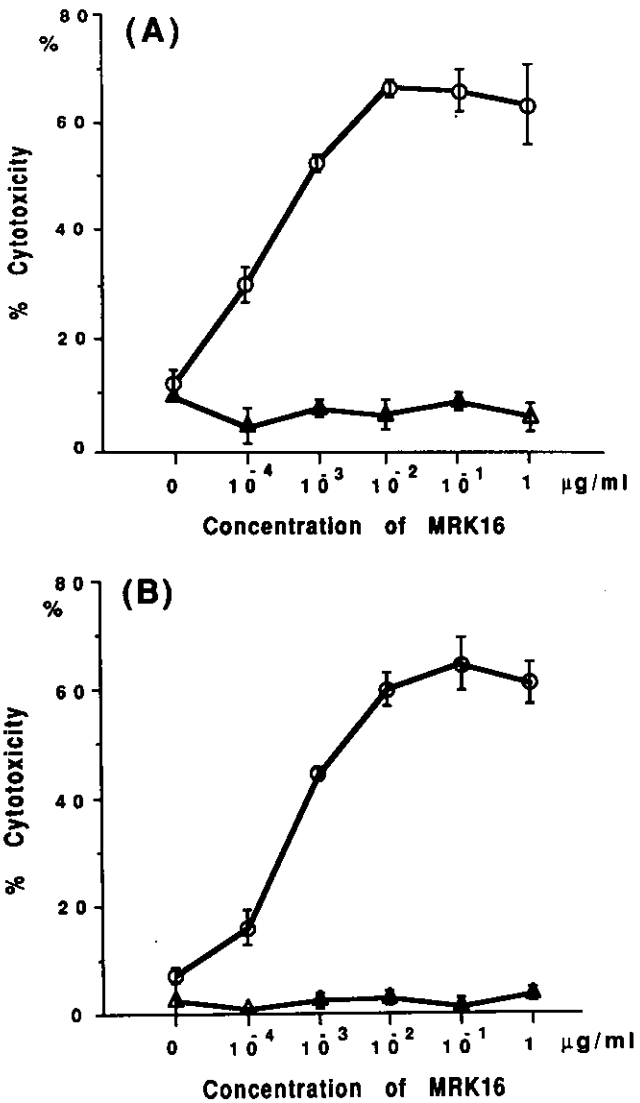

Fig. 2. Dependence of tumor lysis on MoAb concentrations. Separated human lymphocytes (A) and monocytes (B) were cultured with ^{51}Cr-labeled A2780 (△) and 2780AD (○), in the presence of various concentrations of MRK16 (IgG_{2a}). After 6 h, the reactions were terminated and the lysis was quantified as described in "Materials and Methods." The effector-target ratio was 40.

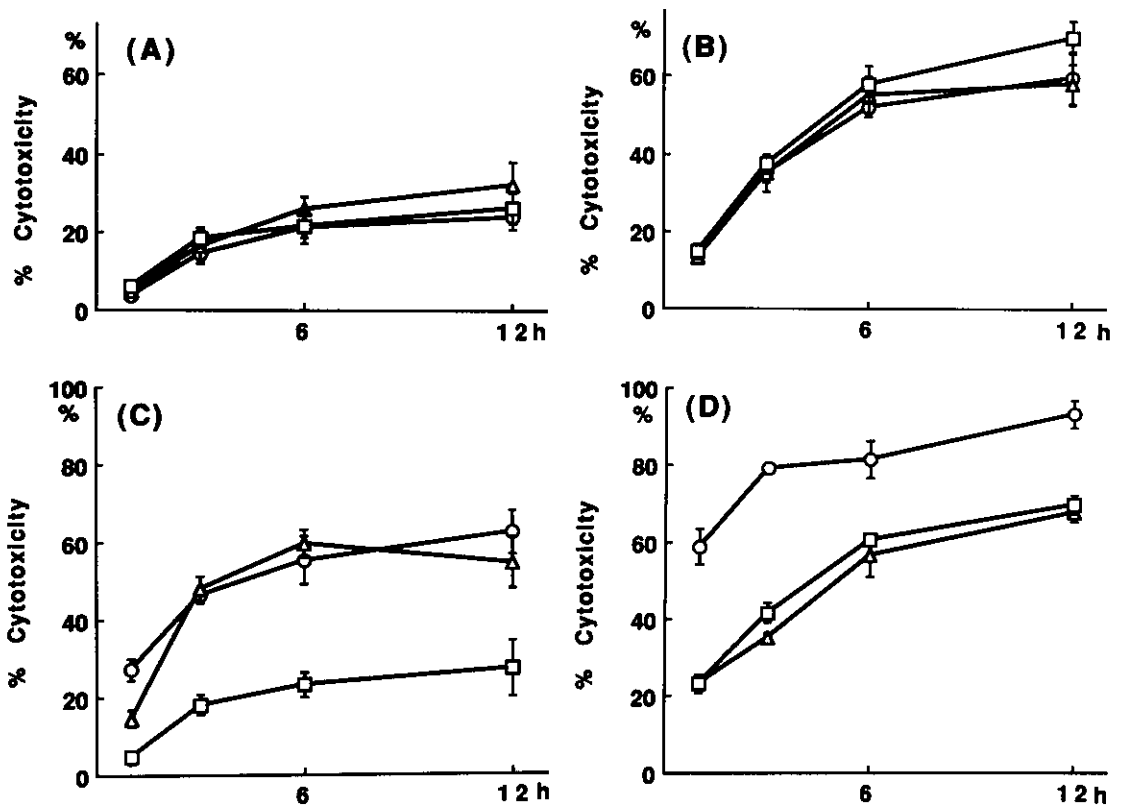

Fig. 3. Kinetics of ADCC against 2780AD and A2780 with MRK16 and MRK17. Monocytes (A)(C) and lymphocytes (B)(D) were cultured in microtest plates III, and ^{51}Cr-labeled target cells A2780 (A)(B) and 2780AD (C)(D) were added to the wells in the presence of MRK16 (10^{-2} μg/ml) (○) or MRK17 (10^{-2} μg/ml) (△) or the absence of MRK16 and MRK17 (□). At the indicated time intervals the cultures were terminated and lysis was quantified as described in "Materials and Methods." The effector-target ratio was 40.

dependent and increased with increasing concentration of MRK16. The maximum lysis occurred at MRK16 concentrations above 10^{-2} μg/ml. However, the cytotoxicity to A2780 was not increased with increasing concentrations of MRK16, indicating the specificity of the antibody.

Kinetics of ADCC As shown in Fig. 3, cytolysis of A2780 and 2780AD with and without anti-P-glycoprotein MoAb almost reached a plateau at 6 h. The lysis of A2780 with monocytes (in A) and lymphocytes (in B) was not influenced by MRK16 or MRK17 at any incubation time. The lysis of 2780AD was elevated with MRK16 and MRK17, and the extent of lysis was similar with both antibodies using human monocytes as effector cells (in C). However, with human lymphocytes as effector cells, MRK17 had no effect on the lysis of 2780AD cells at any incubation time (in D).

The lymphocytes and monocytes of the other two volunteers also enhanced the lysis of 2780AD similarly (data not shown). From these results, we chose a 6-h incubation time for the following experiments.

Spectrum of target cells In this experiment, we used the four pairs of resistant human cancer cells and parent-sensitive cells. The overexpression of P-glycoprotein of these resistant cancer cells, 2780AD, K562/ADM, KB-C4 and CEM-VLB$_{100}$, has been reported previously.[28] As K562 and K562/ADM cancer cells were sensitive to lymphocytes, the effector-target ratio of 20 was used in this experiment instead of the ratio of 40 used previously.

As shown in Fig. 4, the lysis of resistant cells was enhanced by MRK16. The percent lysis of 2780AD was elevated from 30.4±4.6% to 86.0±2.6%. Those of K562/ADM, KB-C4 and CEM-VLB$_{100}$ were elevated from 59.6±4.2% to 78.9±4.4%, from 3.2±0.7% to 28.3±3.2%, and from 20.6±3.4% to 42.2±6.0%, respectively. However, the lysis of their parent-sensitive tumors and Daudi cells was not affected by MRK16. The lymphocytes of the other two volunteers increased

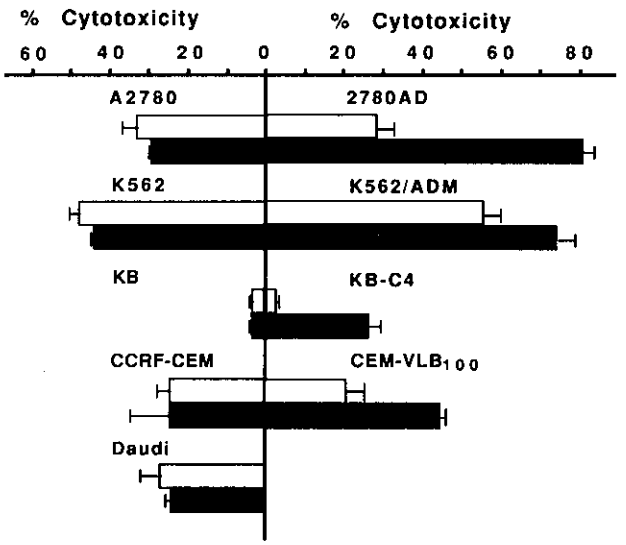

Fig. 4. Spectrum of target cells. Four pairs of sensitive cells and their resistant cells were used as target cells. Target cells with (■) or without (□) 10^{-2} μg/ml of MRK16 were added to wells which contained human lymphocytes. This mixture was incubated for 6 h, and 100 μl of the supernatant was γ-counted. The effector-target ratio was 20. The percent cytolysis was calculated as described in "Materials and Methods."

the cytolysis of these resistant human cancer cells with MRK16 similarly to that observed with the volunteers shown in Fig. 4 (data not shown).

DISCUSSION

Development of drug resistance in malignant cells is responsible for many therapeutic failures in clinical oncology. P-glycoprotein was recently found to be expressed on the surface of cancer cells that were resistant to multiple drugs.[24] The monoclonal anti-P-glycoprotein antibody responded to the drug-resistant cancer cells, but not to the parent sensitive cancer cells *in vitro*.[24]

ADCC reaction against various cancer cells is thought to be mediated by cells bearing Fc receptors, such as macrophages, lymphocytes and natural killer cells.[15, 18–22] In this study, two types of anti-P-glycoprotein MoAb (MRK16 and MRK17) were used to induce ADCC against four pairs of MDR human cancer cells and their parent sensitive cells using human mononuclear cells (lymphocytes and monocytes) as effector cells. Without these MoAbs, lymphocyte- and monocyte-mediated cytotoxicity was observed against both the MDR cancer cells and their parent cells. We observed that human mononuclear cells in the presence of these MoAbs could mediate ADCC reaction against the MDR cancer cells expressing P-glycoprotein, but not against their parent cells. Interestingly, the present findings showed that MRK16 of IgG_{2a} subtype could promote both human lymphocyte- and monocyte-mediated tumor cell killing, whereas MRK17 of IgG_1 subtype induced only monocyte-mediated cytotoxicity. These different capabilities of MRK16 and 17 in the ADCC reaction seem to be deeply related to the different therapeutic effects of these antibodies reported previously, where we showed that MRK-16 was more effective than MRK17 in inducing regression and cure of an established drug-resistant human tumor (2789AD) growing subcutaneously in athymic nude mice.[23]

Several cytokines such as interferon γ and interleukin (IL)-2 were recently found to potentiate ADCC reaction through the increase of Fc receptor function.[32, 33] It has already been reported that ADCC mediated by human peripheral blood lymphocytes was increased after pre-incubation with IL-2 *in vitro*.[32, 34] Combined therapy with IL-2, LAK cells and anti-tumor monoclonal antibodies is more effective than the application of MoAb alone against metastatic cancer in murine models.[35] Thus, combined therapy with an anti-P-glycoprotein MoAb and such cytokines may be beneficial for the destruction of drug-resistant cancer cells.

In this study, we found that anti-P-glycoprotein MoAb (MRK16) was also effective in inducing the ADCC reaction against four resistant variants of human cancers (2780AD, K562/ADM, KB-C4 and CEM-VLB_{100}) expressing P-glycoprotein. Thus, these findings suggest that anti-P-glycoprotein MoAb may be a useful therapeutic agent in overcoming the drug resistance of cancer cells expressing P-glycoprotein.

Administration of anti-cancer MoAb of mouse origin to cancer patients was reported to reduce the growth of their metastatic cancers.[36–38] Our anti-P-glycoprotein MoAb might be a clinically useful therapeutic agent against P-glycoprotein-positive MDR cancers. However, great care is necessary before clinical application of these MoAbs, since P-glycoprotein is constitutively expressed in several normal human organs such as adrenal, kidney, liver, colon and brain.[39–42] At the present stage, however it is clear that these MoAbs are of considerable interest as candidate therapeutic agents against drug-resistant human cancers.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture; the Ministry of Health and Welfare; and the Cell Science Research Foundation, Japan. We thank N. Aihara for her help in preparing the manuscript.

(Received April 27, 1990/Accepted August 6, 1990)

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