The role of cyclosporin A on antibody-dependent monocyte-mediated cytotoxicity against human multidrug-resistant cancer cells

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Abstract : A P-glycoprotein (P-gp) inhibitor, cyclosporin A (CsA) was found to enhance the susceptibility of multidrug resistant (MDR) cancer cells to anti-P-gp antibody-dependent cellular cytolysis (ADCC) by monocytes, but the exact mechanism is unknown. In this study, we examined whether CsA enhanced the susceptibility of MDR cells through its inhibitory effect of P-gp function by using anti-ganglioside GM2 (GM2) monoclonal antibody (Ab), KM966, instead of anti-P-gp Ab, MRK16. Monocyte-ADCC induced by both KM966 and MRK16 against P-gp positive human MDR ovarian cancer cells was significantly augmented by addition of CsA. KM966, but not MRK16, induced monocyte-ADCC against P-gp negative human ovarian cancer cells and CsA enhanced this ADCC activity, indicating that suppressive effect of P-gp function by CsA was not essential to the enhancement of ADCC. Moreover, pretreatment of tumor cells with CsA augmented their susceptibility to monocyte-ADCC irrespective of P-gp expression. Interestingly, KM966 or MRK16 induced monocyte-ADCC against various human lung cancer cells expressing either GM2 or P-gp, but CsA did not affect these ADCC. These findings suggest that CsA may enhance the susceptibility to the monocyte-ADCC of ovarian cancer cells, but not of lung cancer cells, irrespective of its suppressive effect of P-gp function. J. Med. Invest. 44 : 185-191, 1998

Key Words : *multidrug resistance, P-glycoprotein, ganglioside GM2, ADCC, cyclosporin A*

INTRODUCTION

Multidrug resistance (MDR) is a major problem in cancer chemotherapy. P-glycoprotein (P-gp), which localizes in the plasma membranes of MDR cells (1-3) and transports various cytotoxic drugs to outside the cells, is one of the key molecules in MDR (4, 5). Recently, even a low level of P-gp expression was reported to be useful as a marker of resistance to combination chemotherapy in ovarian and small cell lung cancers (6). Thus, the selective killing of tumor cells expressing P-gp seems very important for successful cancer therapy. We reported that MRK16 (7), a monoclonal antibody (Ab) that recognizes the extracellular domain of human P-gp, caused rapid regression of established s.c. MDR tumors in nude mice (8) by Ab-dependent cellular cytotoxicity (ADCC) mediated by macrophages (9, 10).

On the other hand, cyclosporin A (CsA) (11, 12) reverses MDR *in vitro* and *in vivo* when combined with antitumor agents. CsA inhibits the efflux of antitumor agents through their binding to P-gp, resulting in intracellular accumulation of the antitumor agents and so overcomes drug resistance (12). The combined use of MRK16 with CsA synergistically reversed MDR of P-gp-positive cancer cells (13). We recently found that CsA enhanced MRK16-dependent monocyte-mediated cytotoxicity against MDR human cancer cells, and that this enhancement was due to the augmentation by CsA of the susceptibility of MDR cancer cells to monocyte-ADCC (14). But exact mechanism is unknown.

Recently, it was found that the mouse-human chimeric anti-GM2 Ab KM966 reacted to various human tumor cells including lung cancer and lysed these tumor cells through the ADCC reaction in the presence of human effector cells (15, 16). In this study, using KM966 instead of MRK16, we examined whether CsA enhanced the susceptibility of multidrug-resistant cancer cells to MRK16-dependent ADCC mediated by monocytes through its inhibitory effect of P-gp function.

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MATERIALS AND METHODS

Cell lines

The human cell lines used in this study are listed in Table 1. A2780 and its adriamycin-resistant variant, AD10 (also called 2780^{AD}), were kindly supplied by Drs. R. F. Ozols and T. C. Hamilton (National Cancer Institute) (2). H69 cells were from the American Type Culture Collection (Rockville, MD) and etoposide-resistant variant of H69 cells (H69/VP) (17) were kindly provided by Dr. N. Saijo (National Cancer Center Research Institute, Tokyo). SBC-3 cells were from Japanese Cancer Research Resources Bank. RERF-LC-MS and RERF-LC-AI cells (18) were kindly provided by Dr. M. Akiyama (Radiation Effects Research Foundation, Hiroshima). Cell cultures were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum and gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air. Cytotoxicity assays were performed when the cultured target cells were in the exponential phase of growth.

Reagents

Fetal bovine serum was purchased from M.A. Bio-products (Walkerville, MD). CsA was provided from Sandoz Pharmaceutical Co., Tokyo. The anti-P-gp Ab MRK16 (7) and the mouse-human chimeric anti-GM2 Ab KM966 (16) were purified as described previously. None of these materials contained endotoxins, as judged by *Limulus* amebocyte assay (Seikagaku Kogyo Co., Tokyo : minimum detection level 0.1 ng/ml).

Analysis by flow microfluorometry

Tumor cells were harvested and resuspended in PBS supplemented with 10% human pooled AB serum to prevent nonspecific antibody binding. After incubation for 30 min at 4°C, the cells were washed once and incubated for 30 min at 4°C in PBS containing MRK16, KM966 (10 μg/ml) or mouse control serum (Tago, Inc., Burlingame, CA) (10 μg/ml). The cells were then washed with PBS, and fluorescein-conjugated goat anti-mouse IgG (H+L) (Immunotech S.A., Marseille, France) or goat anti-human IgG Fc (Organon Teknika, West Chester, PA) were added as a second Ab. After 30 min-incubation at 4°C, they were washed again and the fluorescence intensity was measured with a FACScan (Becton Dickinson, Mountain View, CA) (19).

Isolation and culture of human monocytes

Leukocytes from peripheral blood (200 ml) of healthy donors were collected in an RS-6600 rotor of a Kubota KR-400 centrifuge, and mononuclear cells (MNC) were separated from leukocytes in lymphocyte separation medium (Litton Bionetics). Monocytes were separated from MNC by centrifugal elutriation in a Beckman JE-5.0 elutriation system (20). Fraction enriched in monocytes (>95%) was obtained at 3000 rpm and flow rates of 30-36 ml/min. More than 97% of the cells were viable, as judged by the trypan blue dye exclusion test. The monocyte fraction was washed twice with PBS, and resuspended in medium. These cells were plated for 1 h in 96-well Microtest III plates (Falcon, Oxford, CA), and then nonadherent cells were removed by washing with medium. At this point the purity of the monocytes was >99% as judged by their morphology and nonspecific esterase staining.

ADCC assay

The target cells were labeled with ^{51}Cr as described before (14). In some experiments, ^{51}Cr-labeled target cells were incubated in medium for 2 h with CsA before ADCC assay. Purified monocytes (2×10^5/100 μl) in 96-well Microtest III plates were mixed with a suspension (100 μl) of 1×10^4 ^{51}Cr-labeled target cells with or without various concentrations of MRK16 or KM966 in the presence or absence of CsA. The plates were centrifuged for 3 min at $100\times g$, and then incubated at 37°C for 4 h in a humidified 5% CO_2 atmosphere. After centrifugation, the radioactivity in 100 μl of supernatant was counted in a γ-counter. Determinations were carried out in triplicate. The percentage of specific cytolysis was calculated from the ^{51}Cr-releases from test samples and control samples, as follows :

$$\%\ \text{specific lysis} = (E-S)\ /\ (M-S)\times100$$

where E is the release in the test sample (cpm in the supernatant from target cells incubated with effector cells and test antibody), S is the spontaneous release (cpm in the supernatant from target cells incubated with medium alone), and M is the maximum release (cpm released from target cells lysed with 1 N HCl). In a preliminary experiment there was no significant difference in the spontaneous release between CsA-treated target cells and untreated cells.

Statistical analysis

The statistical significance of differences between groups was analyzed by Student's two-tailed *t* test. In all determinations, differences were considered significant at $P<0.05$.

RESULTS

Characteristics of tumor cell lines used in this study

We summarize the reactivity of various human cancer cell lines to anti-P-gp Ab MRK16 and anti-GM2 Ab KM966 in Table 1. Staining patterns of cells with MRK16 and KM966 are also given in Fig.1. Adriamycin-resistant ovarian cancer cells (AD10) and etoposide-resistant lung cancer cells (H69/VP) expressed P-gp and three of five lung cancer cell lines expressed GM2 on their cell surfaces. In addition, two ovarian cancer cell lines expressed GM2 irrespective of adriamycin-resistance.

Effect of CsA on anti-GM2 Ab-dependent ADCC against MDR ovarian cancer cells

We first examined whether the interaction of P-gp to MRK16 Ab was necessary for augmentation by CsA of the susceptibility of MDR cancer cells to monocyte-ADCC.

Table 1. Characteristics of human cancer cell lines used in this study

Cell line	Origin	Surface antigen		Enhancement of ADCC by CsA	
		P-gp	GM2	MRK16	KM966
AD10 ($A2780^{AD}$)	Ovarian cancer	+	+	+	+
A2780	Ovarian cancer	−	+	−	+
H69	Lung cancer (Small cell)	−	+	−	−
H69/VP	Lung cancer (Small cell)	+	−	−	−
SBC-3	Lung cancer (Small cell)	−	+	−	−
RERF-LC-MS	Lung cancer (Adeno)	−	+	−	−
RERF-LC-AI	Lung cancer (Squamous cell)	−	−	−	−

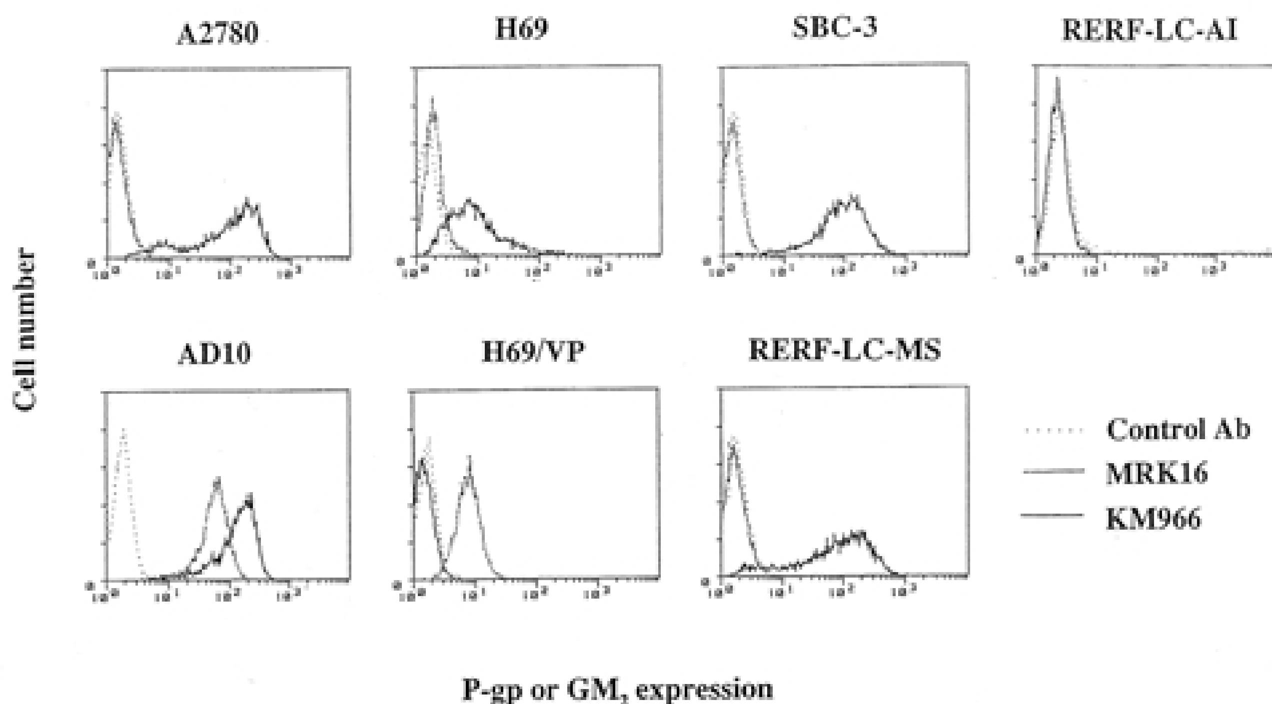

Fig.1. P-gp and GM2 expression on various human cancer cell lines used in this study. Tumor cells were stained with MRK16 (dotted line), KM966 (solid line), or control Ab (dashed line). P-gp and GM2 expression was examined as described in "Materials and Methods."

For this, we used anti-GM2 Ab (KM966) to induce monocyte-ADCC against AD10 cells. Monocytes were not spontaneously cytotoxic against AD10 cells, and CsA did not enhance this cytotoxicity in the absence of Ab. In addition, neither control mouse IgG, control human IgG, nor MRK16 F(ab')2 enhanced monocyte-mediated cytotoxicity irrespective of the presence of CsA (data not shown). Both MRK16 and KM966 caused significant increase in the monocyte-mediated cytotoxicity against AD10 cells (Fig 2 A, B), which was significantly enhanced by addition of CsA.

Effect of CsA on monocyte-ADCC against P-gp-negative ovarian cancer cells

We next examined whether the inhibition of P-gp function by CsA is correlated to the enhancement by CsA of monocyte-ADCC against MDR cells. For this, we used A2780 cells, which were P-gp-negative parent cell line of AD10 cells, as target cells. Monocytes were not spontaneously cytotoxic to A2780 cells. The cytotoxicity was not augmented by addition of MRK16 nor CsA (data not shown). KM966 induced monocyte-ADCC against A2780 cells and CsA significantly augmented this ADCC (Fig.3).

Effect of pretreatment of target cells with CsA on ADCC

We previously reported (14) that CsA modified MDR cancer cells to become susceptible to MRK16-dependent monocyte-ADCC. To determine whether CsA could affect the susceptibility of target cells to KM966-dependent ADCC, we pretreated target cells with CsA for 2 h. More than 99% of target cells treated with CsA (30 mg/ml) were viable by the trypan blue dye exclusion test, and there was no significant difference in proliferation between CsA-treated and untreated cells (data not shown). Pretreatment of the target cells with CsA did not affect spontaneous monocyte-mediated cytotoxicity against AD10 and A2780 cells in the absence of Ab. In the same

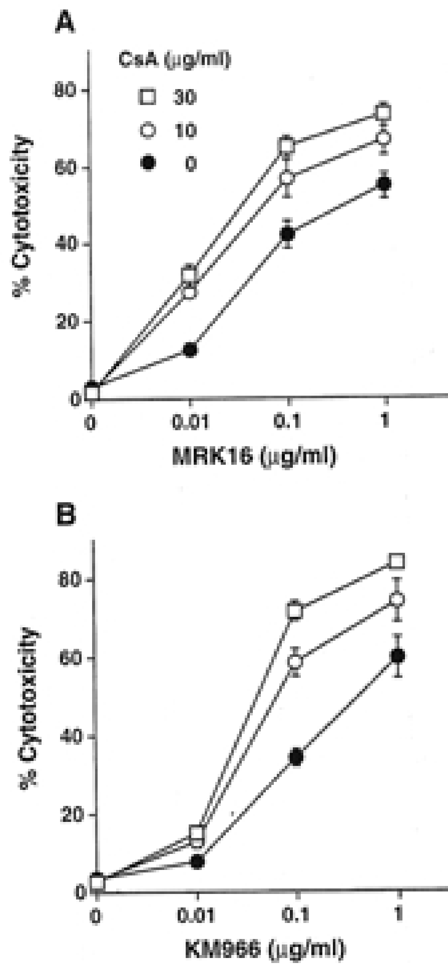

Fig.2. Effect of CsA on anti-GM2 Ab-dependent ADCC against MDR ovarian cancer cells. ^{51}Cr-labeled AD10 cells were incubated with monocytes with MRK16 or KM966 in the presence or absence of CsA. E/T ratio was 20. Percent cytotoxicity was determined as described in "Materials and Methods." Bars show SDs of means for triplicate cultures. Data are representative of three separate experiments.

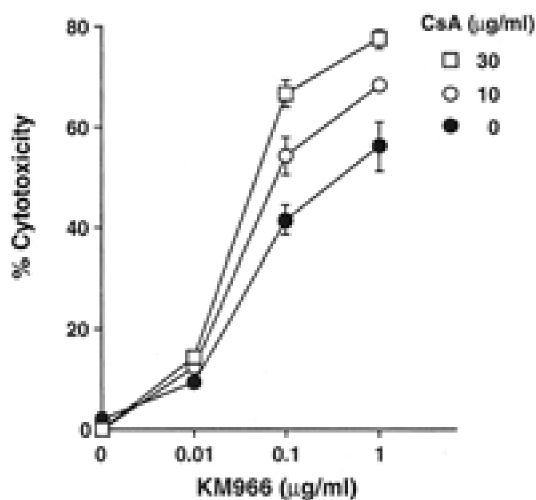

Fig.3. Effect of CsA on monocyte-ADCC against P-gp-negative ovarian cancer cells. ^{51}Cr-labeled A2780 cells were incubated with monocytes with MRK16 or KM966 in the presence or absence of CsA. E/T ratio was 20. Percent cytotoxicity was determined as described in "Materials and Methods." Bars show SDs of means for triplicate cultures. Data are representative of three separate experiments.

experimental conditions, CsA significantly increased the sensitivity of both AD10 and A2780 cells to ADCC by monocytes in the presence of KM966 (Fig.4 A, B).

Effect of CsA on monocyte-ADCC against various lung cancer cells

In the previous study (14), we found that CsA augmented the sensitivity to monocyte-ADCC of various tumor cell lines such as K 562/ADM (adriamycin-resistant erythroleukemia) and KB-C4 (colchicine-resistant epidermoid carcinoma) cells as well as AD10 cells. Etoposide-resistant small cell lung carcinoma cells H69/VP expressed P-gp as shown in Fig.1. We next examined effect of CsA on MRK 16-dependent monocyte-mediated cytotoxicity against H69/VP cells. Interestingly, while optimal dose of MRK16 (1 μg/ml) significantly induced ADCC activity against P-gp positive H69/VP cells, this ADCC activity was not augmented by addition of CsA (up to 30 μg/ml) (Fig.5 A).

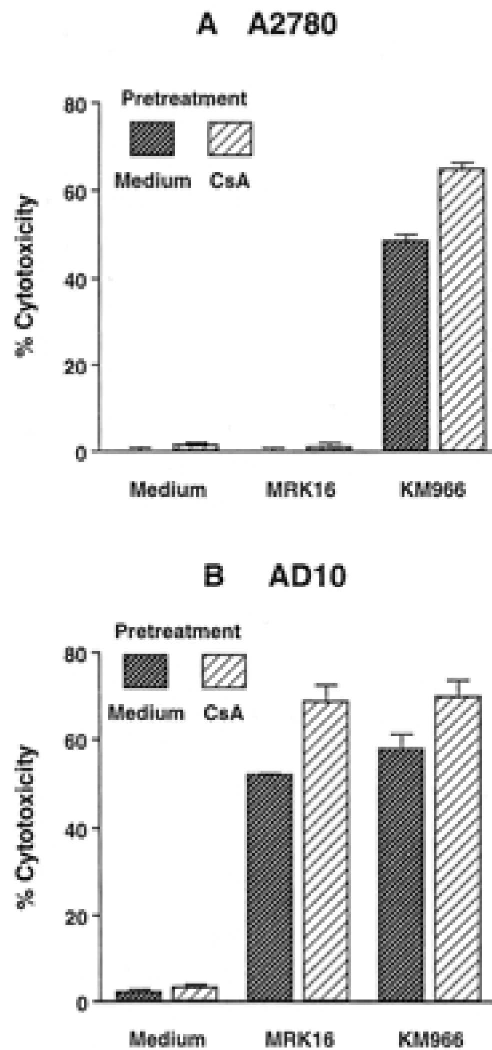

Fig.4. Effect of pretreatment of target cells with CsA on ADCC. ^{51}Cr-labeled target cells were incubated in medium with or without CsA (30 μg/ml) for 2 h at 37°C, then washed. The resultant cells were incubated with monocytes in the presence or absence of MRK16 (1 μg/ml) or KM966 (1 μg/ml). E/T ratio was 20. Percent cytotoxicity was determined as described in "Materials and Methods." Bars show SDs of means for triplacate cultures. Data are representative of two separate experiments.

We also examined the effect of CsA in the presence of suboptimal dose of MRK16 (0.1 μg/ml), but CsA did not enhance ADCC against H69/VP cells (Fig.5B). Moreover, KM966 did not induce monocyte-ADCC against H 69/VP cells, irrespective of the presence of CsA (data not shown).

We next examined the effect of CsA on monocyte-ADCC against various human lung cancer cells in the presence of anti-GM2 Ab, KM966. For this, in addition to A2780 and AD10 cells as a control, we used four human lung cancer cell lines with different levels of GM2 expression on cell surface. Fig.6 shows that KM966 induced ADCC against A2780 and AD10 cells, which was significantly augmented by CsA. In contrast, KM966 induced no significant ADCC against RERF-LC-AI cells which had no GM2, and CsA did not affect the cytotoxicity. KM966 induced monocyte-ADCC against GM2-positive lung cancer cells (H69, SBC-3, and RERF-LC-MS), These ADCC were not enhanced by addition of CsA. Moreover, MRK16 did not induce monocyte-ADCC against these lung cancer cell lines, irrespective of the presence of CsA (data not shown).

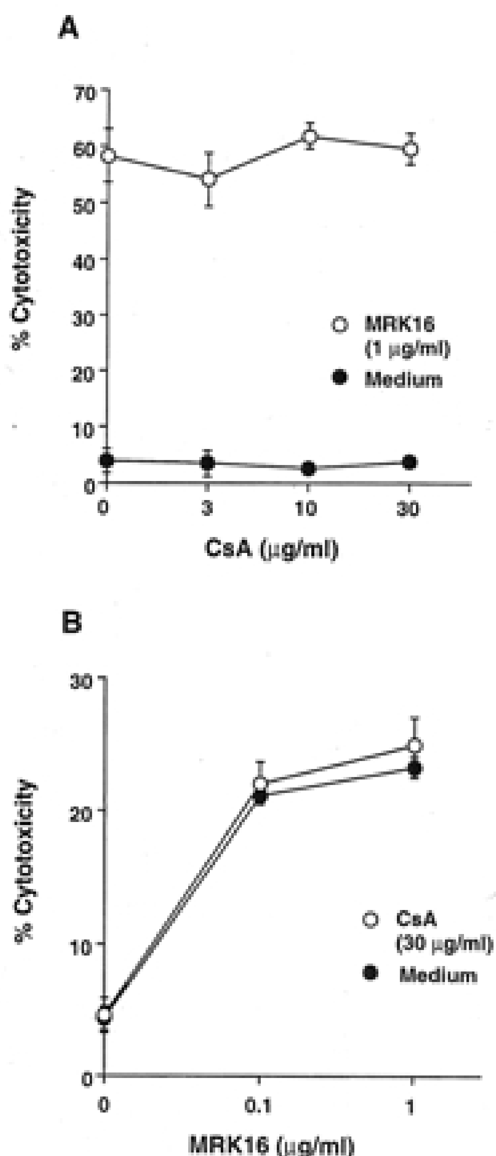

Fig.5. Effect of CsA on monocyte-ADCC against MDR lung cancer cells. ^{51}Cr-labeled H69/VP cells were incubated with monocytes with MRK16 in the presence or absence of CsA. E/T ratio was 20. Percent cytotoxicity was determined as described in "Materials and Methods." Bars show SDs of means for triplicate cultures. Data are representative of three separate experiments.

DISCUSSION

We previously reported (14) that CsA enhanced the susceptibility of P-gp-positive MDR cancer cells to MRK16-dependent ADCC by monocytes. This study was conducted to clarify whether CsA enhanced the susceptibility of MDR cancer cells through its inhibitory effect on P-gp function.

Here, we found that CsA augmented the susceptibility to monocyte-ADCC of ovarian cancer cells irrespective of the expression of P-gp, indicating that suppressive effect on P-gp function by CsA was not essential to enhance ADCC of MDR cancer cells. One possible mechanism presented in a previous report (14) to explain the enhanced ADCC by CsA was that CsA might enhance the susceptibility of MDR cancer cells through the increased intracellular accumulation of cytotoxic products, resulting in secondary membrane damage and cell death. Because P-gp might mediate efflux of not only antitumor agents but also waste and cytotoxic products (5, 21). But this possibility was ruled out by the findings in the present study.

Monocytes are known to produce complement when they are activated with stimuli (22). CsA also enhanced complement-mediated cytolysis against MDR ovarian cancer cells in the presence of MRK16 as described previously (14), indicating that CsA enhanced membrane damage. Moreover, because augmented ADCC by CsA of MDR cancer cells was partially inhibited by catalase (inhibitor of hydrogen peroxide), we presume that complement and hydrogen peroxide might be effector molecules from monocytes in ADCC reaction and that CsA might enhance the susceptibility of MDR cancer cells to such molecules. On the other hand, CsA is known to bind to phospholipid vesicles (23), interferes with the incorporation of fatty acids into membrane phospholipids (24) and depolarizes the cytoplasmic membrane (25). Therefore, CsA may modify plasma membrane of cancer cells coated with Ab to become susceptible to complement and hydrogen peroxide from monocytes.

We reported (14) that CsA augmented the susceptibility of various human MDR cancer cell lines such as AD10, K562/ADM, and KB-C4 cells to monocyte-ADCC. But it was not the same in the case of human lung cancer cell lines irrespective of the expression of P-gp. CsA, however, efficiently reversed vincristine-resistance of P-gp-positive lung cancer cells (H69/VP) (data not shown). Moreover, Glissont, *et al*. also reported (26) that CsA enhanced accumulation of etoposide in MDR H69 cells (VPR-2) to overcome their etoposide resistance, indicating that CsA could inhibit drug transport by P-gp expressed on human lung cancer cells. Because complement-mediated cytolysis against H69/VP cells in the presence of MRK16 was not augmented by addition of CsA (data not shown), plasma

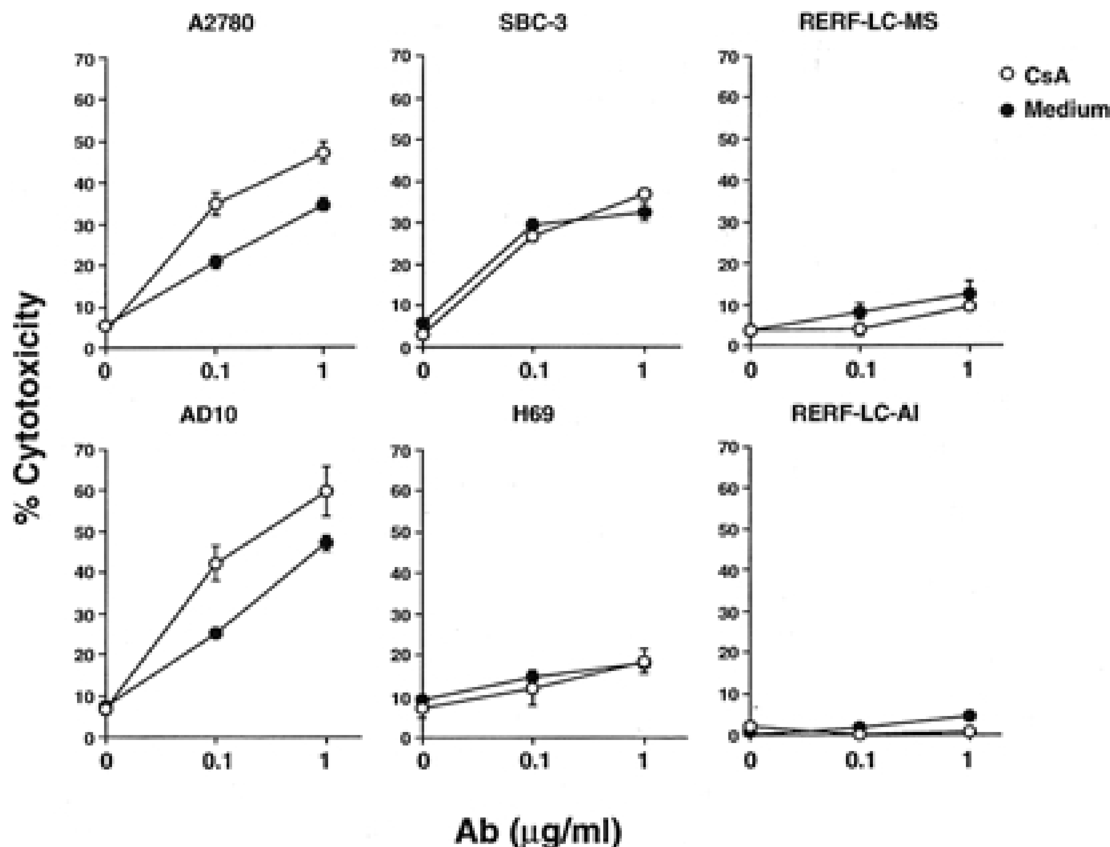

Fig.6. Effect of CsA on monocyte-ADCC against various lung cancer cells. ^{51}Cr-labeled target cells were incubated with monocytes with MRK16 or KM966 in the presence or absence of CsA. E/T ratio was 20. Percent cytotoxicity was determined as described in "Materials and Methods." Bars show SDs of means for triplicate cultures. Data are representative of three separate experiments.

membrane of lung cancer cells may not be the same as that of others (e.g., ovarian cancer cells) on sensitivity to CsA. However, the reason why CsA did not affect the susceptibility of lung cancer cells to monocyte-ADCC is still unknown. Further examinations to clarify the mechanism of insufficient effect of CsA on ADCC against lung cancer may be useful for developing novel therapeutic strategy to lung cancer.

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