Cyclosporin A Enhances Susceptibility of Multi-drug Resistant Human Cancer Cells to Anti-P-glycoprotein Antibody-dependent Cytotoxicity of Monocytes, but Not of Lymphocytes

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Cyclosporin A (CsA) was previously found to bind to P-glycoprotein expressed on multidrug-resistant (MDR) cancer cells. In the present study, the effect of CsA on anti-P-glycoprotein monoclonal antibody (mAb)-dependent cell-mediated cytotoxicity (ADCC) against human MDR cells was examined. The ADCC reaction was assessed by 4-h ^{51}Cr-release assay. Highly purified lymphocytes (>99%) and monocytes (>99%) obtained from blood mononuclear cells (MNC) of healthy donors were used as effector cells. CsA decreased the cytotoxic activity of MNC against MDR cells, but enhanced their ADCC activity in the presence of anti-P-glycoprotein mAb MRK16. Lymphocyte-mediated ADCC and natural killer activity against MDR cells were also suppressed by addition of CsA. CsA induced a significant dose-dependent increase in monocyte-mediated ADCC activity. Interestingly, pretreatment of MDR cancer cells, but not of monocytes, with CsA significantly enhanced ADCC activity mediated by monocytes, but not by lymphocytes. A CsA analog (PSC833) and FK-506, but not verapamil also increased the sensitivity of MDR cells to ADCC by monocytes. CsA did not affect the binding of monocytes to MDR cells in the presence of MRK16 mAb. These results indicate that CsA may directly enhance the susceptibility of MDR cancer cells to the monocyte-mediated ADCC reaction.

Key words: Drug resistance — P-glycoprotein — ADCC — Cyclosporin A

Resistance of tumors to multiple drugs is a major problem in cancer chemotherapy. P-glycoprotein, which transports various cytotoxic drugs out of the cells, is one of the key molecules in multidrug resistance.[1, 2] P-glycoprotein is a binding protein for anti-cancer drugs and an ATPase, and is localized in the plasma membranes of MDR[4] cells.[3–5] The expression of P-glycoprotein is elevated in intrinsically drug-resistant cancers as well as in some tumors that acquired drug resistance during chemotherapy.[6, 7] Because P-glycoprotein appears to be involved in both acquired and intrinsic MDR of human cancers, the selective killing of tumor cells expressing P-glycoprotein should be effective for cancer therapy. In an attempt to develop an effective therapeutic procedure for human MDR cancers, we therefore raised mAbs against the MDR P-glycoprotein.[8] These mAbs (e.g., MRK16) induced lysis of MDR cancer cells in vitro by human effector cells,[9, 10] and caused rapid regression of established s.c. MDR tumors in nude mice, with complete regression in some animals.[11]

Much attention has recently been paid to the problem of overcoming MDR by use of various compounds such as verapamil,[12] CsA,[13, 14] a CsA analog (PSC833)[15] and FK-506,[16] which reverse MDR in vitro and in vivo when combined with antitumor agents. These drugs inhibit the efflux of antitumor agents through their binding to P-glycoprotein, resulting in greater intracellular accumulation of the antitumor agents, and so overcome drug resistance.[14–16]

Because of the possible suppression of antitumor effector activity by CsA,[17, 18] it is important from a therapeutic point of view to examine whether CsA can affect anti-P-glycoprotein antibody-dependent cell-mediated cytotoxicity against MDR cancer cells. In this study, we found that CsA enhanced anti-P-glycoprotein mAb-dependent MNC-mediated cytotoxicity against MDR human cancer cells, and that this enhancement was due to augmentation by CsA of the susceptibility of MDR cancer cells to ADCC activity mediated by monocytes, but not by lymphocytes.

MATERIALS AND METHODS

Cell lines The human ovarian tumor A2780 and its adriamycin-resistant variant, 2780^AD, were kindly supplied by Drs. R. F. Ozols and T. C. Hamilton, National Cancer Institute. The characteristics of these cell lines have been reported.[19] Human myelogenous leukemia K562 cells resistant to adriamycin (K562/ADM) were established as described previously.[20] Human KB carcinoma cells resistant to colchicine (KB-C4) were generously provided by Dr. I. Pastan, National Cancer Institute.[21] Preliminary experiments showed that 2780^AD and K562/ADM cells expressed high levels of P-glycoprotein on their surfaces, whereas KB-C4 expressed low levels as determined by indirect immunofluorescence analysis with MRK16 antibody (data not shown). Cell cultures were maintained on plastic in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum and gentamicin, designated as CRPMI 1640, 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. Bioproducts (Walkerville, MD). CsA (MW=1203) and its non-immunosuppressive derivative (PSC833, (3'-keto-Bmt^1)-(val^2)-cyclosporin A) (MW=1215) was provided by Sandoz Pharmaceutical Co., Tokyo. FK-506 (MW=822) was a gift from Fujisawa Pharmaceutical Co., Osaka. Verapamil (MW=491) was obtained from Eisai Co., Tokyo. Bovine liver catalase and bovine kidney SOD were obtained from Worthington Biochemical Co. (Freehold, NJ) and Calzyme Laboratories (San Luis, CA), respectively. N^G-Monomethyl-L-arginine acetate was purchased from Research Biochemicals (Natick, MA). The anti-P-glycoprotein mAbs MRK16 (IgG2a) and MRK17 (IgG1) were obtained as described previously.[9] The mouse-human chimeric antibody MH162 was purified as described previously.[10] MH162 mAb of the IgG1 isotype had the same binding specificity as MRK16. None of these materials contained endotoxins, as judged by *Limulus* amebocyte assay (Seikagaku Kogyo Co., Tokyo: minimum detection level 0.3 ng/ml).

Isolation and culture of human effector cells (MNC, lymphocytes and monocytes) Leukocytes from peripheral blood (200 ml) of healthy donors were collected in an RS-6600 rotor of a Kubota KR-400 centrifuge, and MNC were separated from leukocytes in lymphocyte separation medium (Litton Bionetics, Kensington, MD). MNC were separated into lymphocytes and monocytes by centrifugal elutriation in a Beckman JE-5.0 elutriation system.[22] Fractions enriched in lymphocytes (>99%) and monocytes (>95%) were obtained at 3000 rpm with flow rates of 26 ml/min and 30–36 ml/min, respectively. More than 97% of the cells were viable, as judged by the trypan blue dye exclusion test. The monocyte fraction was washed twice with phosphate-buffered saline, and resuspended in CRPMI 1640 at various concentrations. 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 from their morphology and nonspecific esterase staining.

ADCC assay MNC, lymphocytes or monocytes were used as effector cells. In some experiments, lymphocytes and monocytes were incubated for the indicated periods in medium with CsA before addition of target cells for ADCC assay. The target cells were labeled with ^51Cr as described before.[9, 19] In some experiments, ^51Cr-labeled target cells were incubated in medium for the indicated periods with CsA before ADCC assay. Various numbers of effector cells (100 μl of MNC, lymphocytes or monocyte monolayer cells) in 96-well Microtest III plates were mixed with a suspension (100 μl) of 1×10^4 ^51Cr-labeled target cells that had been incubated at 37°C for 30 min with various concentrations of monoclonal or chimeric antibody. The plates were centrifuged for 3 min at 100g, 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 gamma counter. Determinations were carried out in triplicate. The percentage of specific cytolysis was calculated from the ^51Cr-releases from test samples and control samples, as follows:

$$\% \text{ specific lysis} = \frac{E-S}{M-S} \times 100$$

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.

Effector-target cell binding assay Samples of 2780^AD cells (1×10^5/well) were plated in 96-well Microtest III plates. After 24 h, the cells became confluent and nonadherent cells were removed by washing. Monolayers of confluent 2780^AD cells (target cells) were incubated in 100 μl of medium alone or with 1 μg/ml of MRK16 mAb at 37°C for 30 min. Then ^51Cr-labeled monocytes (5×10^5/100 μl) were added to the target cell monolayers with various concentrations of CsA, and the plates were centrifuged for 3 min at 300g. The cells were incubated for 30 min at room temperature and unbound cells were removed by gentle washing 5 times. Then the target cell monolayers were solubilized with 1 N NaOH (50 μl) and the radioactivity of monocytes bound to target cells was

counted in a gamma counter. Percent adherence was calculated from the ^{51}Cr-release from test samples and control samples as follows:

$$\% \text{ binding} = \frac{\text{observed cpm}}{\text{total cpm}} \times 100$$

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

RESULTS

Effect of CsA on MNC-mediated ADCC activity We examined the effect of CsA on MNC-mediated cytotoxicity against A2780 parent cells and MDR 2780^{AD} cells in the presence of MRK16 mAb. For this, blood MNC of normal donors were tested for ADCC on A2780 cells or 2780^{AD} cells at an E/T ratio of 20:1. The results in Fig. 1 show that MNC were spontaneously more cytotoxic to 2780^{AD} cells than to the parent A2780 cells. MRK16 mAb caused a significant increase in MNC-mediated cytotoxicity against MDR 2780^{AD} cells compared with that against A2780 cells. Under the same experimental conditions, CsA (60 μg/ml) caused significant decrease in NK activity of MNC against 2780^{AD} cells, but at more than 10 μg/ml it enhanced the ADCC activity with MRK16 mAb.

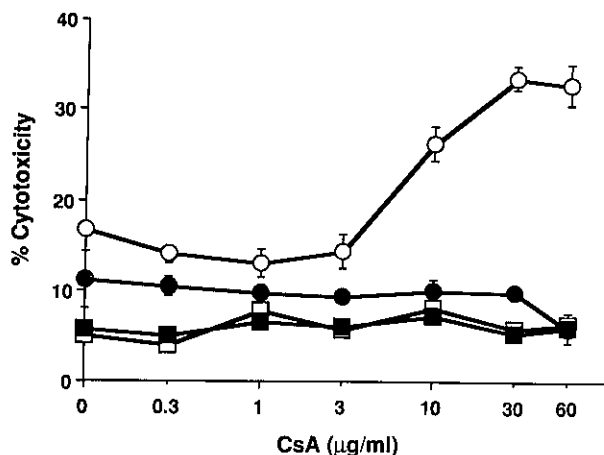Mouse mAb (MRK16) was previously found to induce lysis of MDR ovarian cancer (2780^{AD}) cells by human blood effector cells (lymphocytes and monocytes).[10] To compare the effects of CsA on generation of ADCC activities by two different effector cells, we tested the ADCC of highly purified lymphocytes and monocytes isolated from blood MNC on 2780^{AD} cells at an E/T ratio of 20:1. As shown in Fig. 2, CsA suppressed lymphocyte-mediated ADCC and NK activity at concentrations of more than 0.3 μg/ml and more than 30 μg/ml, respectively. In contrast, it induced a significant dose-dependent increase in monocyte-mediated ADCC activity against 2780^{AD} cells. Moreover, MRK16 mAb enhanced monocyte-mediated ADCC activity in a dose-dependent manner (Fig. 3). Under these conditions, neither control

Fig. 1. Effect of CsA on MNC-mediated ADCC activity. MNC were incubated with ^{51}Cr-labeled A2780 (squares) or 2780^{AD} (circles) cells with the indicated concentrations of CsA in the presence (open symbols) or absence (closed symbols) of MRK16 mAb (0.5 μg/ml). After 4 h, the reactions were terminated and % cytotoxicity was measured as described in "Materials and Methods." The E/T ratio was 20. Bars show SDs of means. Data are representative of four separate experiments.
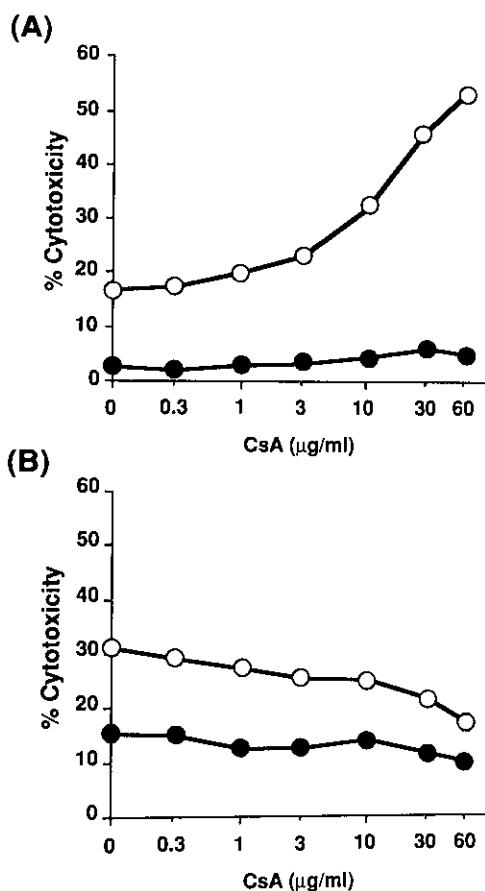

Fig. 2. Effect of CsA on monocyte- or lymphocyte-mediated ADCC activity against MDR cells. Monocytes (A) and lymphocytes (B) were incubated with ^{51}Cr-labeled 2780^{AD} cells with the indicated concentrations of CsA in the presence (○) or absence (●) of MRK16 mAb (0.5 μg/ml). After 4 h, the reactions were terminated and % cytotoxicity was measured as described in "Materials and Methods." The E/T ratio was 20. The SDs were consistently less than 10% of the means. Data are representative of three separate experiments.

mouse IgG2a nor MRK16 F(ab')^2 enhanced monocyte-mediated cytotoxicity irrespective of the presence of CsA (data not shown).

In a parallel experiment, we examined the effect of CsA on spontaneous monocyte-mediated cytotoxicity against 2780^{AD} cells at an E/T ratio of 20:1 in the absence of MRK16 mAb. Monocyte-mediated cytotoxicities in the presence or absence of CsA (30 μg/ml) were 2.7±1.8 and 3.5±2.1 (mean±SD of 16 different experiments), with no significant difference between them.

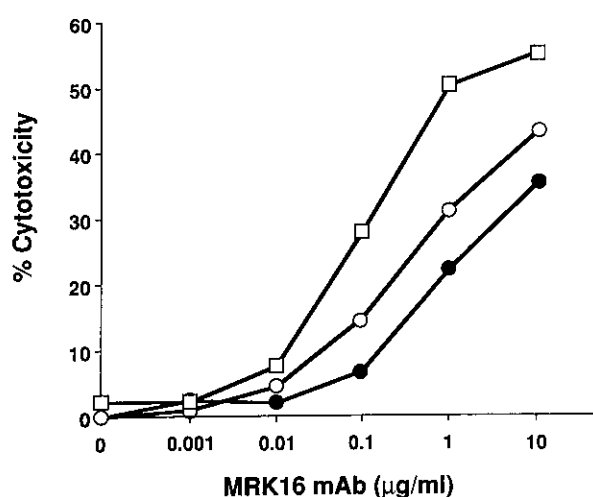Fig. 3. Dose-dependent effect of MRK16 mAb on monocyte-mediated ADCC activity. Monocytes were incubated with ^{51}Cr-labeled 2780^{AD} cells with the indicated concentrations of MRK16 mAb in the absence (●) or presence of 10 μg/ml (○) or 30 μg/ml (□) of CsA. After 4 h, the reactions were terminated and % cytotoxicity was measured as described in "Materials and Methods." The E/T ratio was 20. SDs were consistently less than 10% of the means. Data are representative of three separate experiments.

Effect of pretreatment of target or effector cells with CsA on ADCC To determine whether CsA could modify target cells or effector cells, MDR target (2780^{AD}) cells were incubated for 2 h in medium with various concentrations of CsA, and then washed. More than 99% of MDR cells treated with CsA (30 μg/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 their sensitivity to the cytotoxic activity of lymphocytes, irrespective of the presence of MRK16 mAb (Table I). Similarly, CsA did not affect spontaneous monocyte-mediated cytotoxicity against 2780^{AD} cells in the absence of MRK16 mAb. However, under these experimental conditions, more than 10 μg/ml of CsA significantly increased the sensitivity of 2780^{AD} cells to ADCC by monocytes.

In a parallel experiment, effector cells (monocytes or lymphocytes) were incubated with 30 μg/ml of CsA for various periods, and then washed. As shown in Table II, pretreatment of monocytes with CsA did not affect their ADCC activity. On the other hand, pretreatment of lymphocytes with CsA for more than 2 h significantly suppressed their NK and ADCC activities against 2780^{AD} cells. We also examined the effect of the E/T ratio on monocyte-mediated ADCC activity against CsA-treated 2780^{AD} cells. 2780^{AD} cells that had been incubated for 2 h with CsA (30 μg/ml) were incubated with monocytes at various E/T ratios in the presence of MRK16 mAb. Control mouse mAb (IgG2a) did not induce monocyte-mediated cytotoxicity against CsA-treated or untreated 2780^{AD} cells at any E/T ratio. But as shown in Fig. 4, MRK16-dependent monocyte-mediated cytotoxicity against CsA-treated 2780^{AD} cells was higher than that against untreated 2780^{AD} cells at E/T ratios of more than 10.

Table I. Effect of Pretreatment of Target Cells with CsA on ADCC Activity Mediated by Monocytes or Lymphocytes[a]

Effector cells	Addition of MRK16 mAb (0.5 μg/ml)	Pretreatment of 2780^{AD} cells with CsA (μg/ml)			
		0	3	10	30
Monocyte	−	1.9±0.7[b]	1.5±0.4	3.3±0.6	3.5±0.7
	+	37.8±0.1	38.8±1.1	45.7±1.7	48.2±1.1
Lymphocyte	−	24.3±2.7	23.6±3.4	23.5±3.0	23.1±3.0
	+	49.6±2.0	47.5±3.1	47.8±1.3	48.2±1.4

a) ^{51}Cr-labeled 2780^{AD} cells were incubated with the indicated concentrations of CsA at 37°C for 2 h, then washed. The resultant cells were incubated with monocytes or lymphocytes in the presence or absence of MRK16 mAb (0.5 μg/ml). After 4 h, the reactions were terminated and the cytotoxicity was quantified as described in "Materials and Methods." The E/T ratio was 20.

b) Mean±SD for triplicate cultures. Data are representative of three separate experiments.

Table II. Effect of Pretreatment of Monocytes or Lymphocytes with CsA on Their Cytotoxicity[a]

Target cells	Addition of MRK16 mAb	Time of pretreatment with CsA (30 μg/ml)					
		Monocyte			Lymphocyte		
		0	2 h	4 h	0	2 h	4 h
A2780	−	2.1±0.7[b]	4.0±2.2	1.1±1.1	10.1±2.2	7.9±1.3	7.0±2.1
	+	1.3±0.9	0.7±0.3	0.0±0.9	11.3±0.1	5.3±2.1	3.7±1.8
2780[AD]	−	5.2±2.5	8.5±2.2	5.5±0.7	21.6±0.9	12.5±2.9	12.9±0.7
	+	34.9±1.7	33.2±4.6	31.3±3.7	47.3±3.2	24.9±0.1	21.2±3.7

a) Monocytes or lymphocytes were incubated with medium or 30 μg/ml of CsA at 37°C for the indicated period, then washed. The resultant cells were incubated with ^{51}Cr-labeled A2780 or 2780[AD] cells in the presence or absence of 0.5 μg/ml of MRK16 mAb. After 4 h, the reactions were terminated and the cytotoxicity was quantified as described in "Materials and Methods." The E/T ratio was 20.

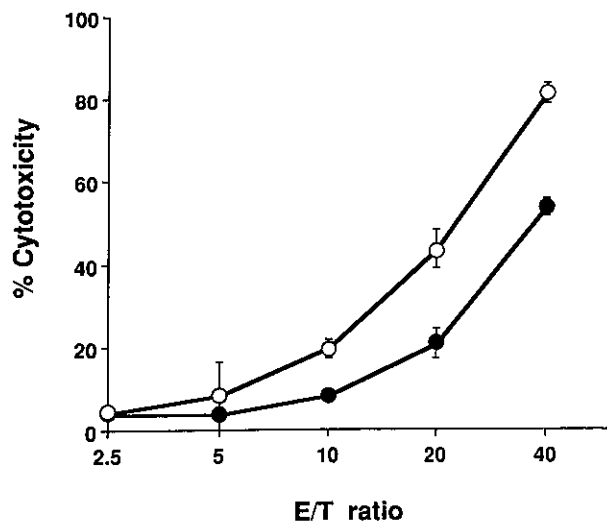b) Mean±SD for triplicate cultures. Data are representative of three separate experiments.

Fig. 4. Effect of E/T ratio on ADCC activity against CsA-treated 2780[AD] cells. ^{51}Cr-labeled 2780[AD] cells were incubated for 2 h at 37°C in medium without (●) or with (○) 30 μg/ml of CsA. Then the cells were washed and incubated with monocytes at the indicated E/T ratios in the presence of 0.5 μg/ml of MRK16 mAb. After 4 h, the reactions were terminated and % cytotoxicity was measured as described in "Materials and Methods." Bars show SDs of means. Data are representative of four separate experiments.
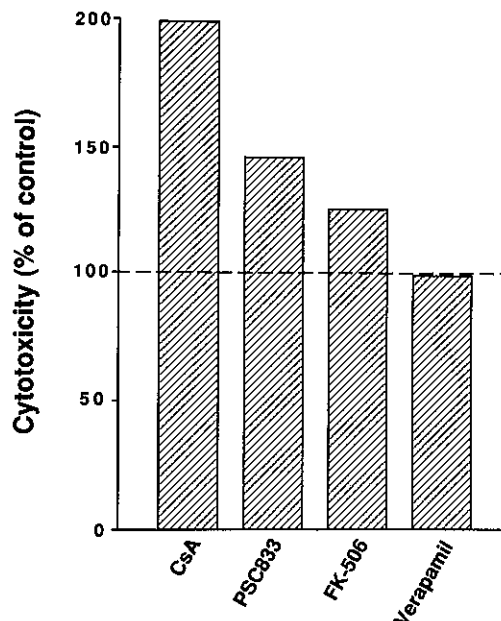

Fig. 5. Effect of various chemosensitizing agents on the susceptibility of MDR cells to ADCC mediated by monocytes. Monocytes and ^{51}Cr-labeled 2780[AD] cells at a ratio of 20:1 were incubated in medium with or without CsA (30 μg/ml), PSC833 (30 μg/ml), FK-506 (30 μM) or verapamil (30 μM) in the presence or absence of MRK16 mAb (0.5 μg/ml). Four hours later their ADCC activities were measured as described in "Materials and Methods." Data are means of at least two different experiments. SDs were consistently less than 10% of the means. Percent of control was calculated as follows: 100×(value for ADCC with the indicated agent)/ (value for ADCC in medium alone).

Effects of PSC833, FK-506 and verapamil on monocyte-mediated ADCC We compared the abilities of CsA and other compounds (PSC833, FK-506 and verapamil) capable of reversing MDR to augment the susceptibility of MDR target cells to ADCC by monocytes. The results are shown in Fig. 5. In the absence of MRK16 mAb there was no significant difference in monocyte-mediated ADCC irrespective of the presence of chemosensitizing agents. Like CsA (30 μg/ml), PSC833 (30 μg/ml) and

FK-506 (30 μM) caused a significant increase in the sensitivity of the cells to ADCC by monocytes ($P<0.05$), PSC833 being more effective than FK-506. Under the

Table III. Augmentation by CsA of the Sensitivities of Various MDR Cancer Cells to Monocyte ADCC

| Target cells | Addition of MRK16 mAb (0.5 μg/ml) | % Cytotoxicity[a] | | | |
| | | Monocyte | | Lymphocyte | |
		Medium	CsA	Medium	CsA
A2780	—	$1.0 \pm 2.4^{b)}$	0.1 ± 1.3	4.5 ± 1.8	2.6 ± 3.4
	+	0.5 ± 0.7	1.2 ± 0.6	4.8 ± 1.8	3.2 ± 0.4
2780[AD]	—	1.3 ± 1.5	5.0 ± 0.6	34.1 ± 1.7	19.6 ± 2.2
	+	34.0 ± 4.6	56.9 ± 3.2	40.4 ± 0.9	21.1 ± 1.0
K562/ADM	—	4.4 ± 1.0	4.8 ± 0.9	44.4 ± 4.0	12.7 ± 2.9
	+	40.8 ± 3.4	78.3 ± 1.6	68.5 ± 3.7	21.4 ± 3.0
KB-C4	—	2.5 ± 0.7	3.6 ± 0.7	4.4 ± 2.1	4.4 ± 1.1
	+	13.3 ± 0.5	23.5 ± 3.0	6.9 ± 1.6	5.7 ± 1.7

a) ^{51}Cr-labeled target cells were incubated with monocytes or lymphocytes in medium with or without 30 μg/ml of CsA, in the presence or absence of 0.5 μg/ml of MRK16 mAb. After 4 h, the reactions were terminated and % cytotoxicity was measured as described in "Materials and Methods." The E/T ratio was 20.
b) Mean ± SD for triplicate cultures. Data are representative of two separate experiments.

same experimental conditions, verapamil did not affect their sensitivity at all.

Augmentation by CsA of the sensitivities of various MDR cancer cells to monocyte ADCC For this study, we used three human MDR cancer cells (2780[AD], K562/ADM and KB-C4) expressing different levels of P-glyco-protein on their surfaces. The results are summarized in Table III. MRK16 mAb induced greater ADCC activity mediated by lymphocytes or monocytes against highly P-glycoprotein-expressing (2780[AD] and K562/ADM) cells than that against the low-expressing KB-C4 cells. Under these experimental conditions, CsA augmented monocyte-mediated ADCC activities against these cells, but suppressed lymphocyte-mediated ADCC activity against K562/ADM and KB-C4 cells.

Effects of different anti-P-glycoprotein mAbs on augmentation by CsA of ADCC sensitivity We examined the effect of CsA using two mouse antibodies (MRK16 and MRK17)[11] and one mouse-human chimeric antibody (MH162) that recognize P-glycoprotein.[19] Like MRK16, MRK17 and MH162 induced ADCC activity and these effects were augmented by CsA (30 μg/ml) (Table IV). Moreover, the lymphocyte-mediated ADCC activity induced by MH162 was also suppressed by CsA (30 μg/ml).

In a parallel experiment, we examined whether macrophage products were responsible for the enhancement by CsA of the sensitivity of MDR cancer cells to monocyte ADCC. Supernatants obtained from 4-h cultures of monocytes and CsA-treated 2780[AD] cells with MRK16 mAb were not cytotoxic to 2780[AD] cells (data not shown). Addition of N^G-monomethyl-L-arginine acetate (1.2 mM), an inhibitor of nitric oxide production, did not affect monocyte-mediated ADCC against 2780[AD] cancer

Table IV. Effects of Various Anti-glycoprotein mAbs on Augmentation by CsA of ADCC Sensitivity

| Monoclonal antibody (0.5 μg/ml) | % Cytotoxicity[a] | | | |
| | Monocyte | | Lymphocyte | |
	− CsA	+ CsA	− CsA	+ CsA
Medium	$3.6 \pm 0.4^{b)}$	4.8 ± 0.6	27.4 ± 2.2	23.6 ± 3.0
MRK16	29.1 ± 1.6	47.2 ± 2.9	42.1 ± 4.1	30.7 ± 2.4
MRK17	16.2 ± 0.5	34.1 ± 0.8	ND[c]	ND
MH162	29.5 ± 1.7	46.2 ± 3.7	67.2 ± 3.5	51.4 ± 3.6

a) ^{51}Cr-labeled 2780[AD] cells were incubated with monocytes or lymphocytes in medium with or without 30 μg/ml of CsA in the presence or absence of 0.5 μg/ml of anti-P-glycoprotein mAb. After 4 h, the reactions were terminated and % cytotoxicity was measured as described in "Materials and Methods." The E/T ratio was 20.
b) Mean ± SD for triplicate cultures. Data are representative of three separate experiments.
c) ND, not done.

cells (data not shown). Moreover, in the presence of MRK16 mAb, recombinant human monokines (IL-1α, IL-1β, IL-6 and TNF-α) were not cytotoxic to MDR 2780[AD] cells that had been treated with CsA (data not shown).

Macrophages were previously found to kill tumor cells through oxygen-dependent mechanisms.[23, 24] Therefore, we examined whether augmentation by CsA of the MDR cell sensitivity to monocyte-ADCC was due to enhanced susceptibility of the cells to oxidative radicals produced by monocytes. For this, we examined the inhibitory effects of scavengers of superoxide and H_2O_2 on monocyte-ADCC against 2780[AD] cells with or without CsA

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treatment. Pretreatment of monocytes with SOD (300 U/ml) did not affect augmentation by CsA of the sensitivity to monocyte-mediated ADCC. Although catalase (3000 U/ml) inhibited approximately 20% of monocyte-mediated ADCC against MDR cells without CsA treatment, it decreased the augmented sensitivity of CsA-treated MDR cells to monocyte-ADCC only to the level of ADCC by monocytes without CsA treatment (data not shown).

Effect of CsA on binding affinity of effector cells We investigated the mechanism of the enhancement by CsA of monocyte-mediated ADCC. First, we examined the effect of CsA on the binding of monocytes to target cells, which is the first step of the ADCC reaction. Monocytes bound spontaneously to a 2780^{AD} cell monolayer, and this binding was markedly increased by MRK16 mAb. CsA did not affect the binding, irrespective of the presence of MRK16 mAb (Fig. 6). In a parallel experiment, we also analyzed flow microfluorometry with MRK16 mAb. 2780^{AD} cells were incubated for 4 h in medium with or without CsA (30 μg/ml), and their abilities to bind MRK16 mAb (10 μg/ml) were examined by indirect immunofluorescence analysis with FACScan. There was no difference in the binding capacity of MRK16 mAb between untreated and CsA-treated cells (data not shown).
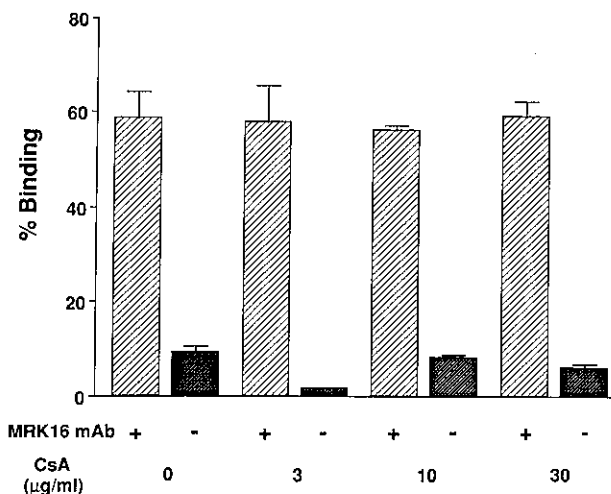

Fig. 6. Effect of CsA on the binding of monocytes to target cells. 2780^{AD} cell monolayers were incubated with or without 1 μg/ml of MRK16 mAb for 30 min at room temperature. Then ^{51}Cr-labeled monocytes were added to the 2780^{AD} cells monolayers and centrifuged at 300g. After 30 min, unbound monocytes were removed by gentle washing with PBS and % binding was measured as described in "Materials and Methods." Bars show SDs of means. Data are representative of three separate experiments.

DISCUSSION

In the present study, we found that CsA, a CsA analog (PSC833) and FK-506 enhance the susceptibility of various MDR human cancer cells to anti-P-glycoprotein mAb-dependent cytotoxicity by monocytes, but not by lymphocytes.

When monocytes were pulsed with CsA, their ADCC was not inhibited. This finding is consistent with previous reports[25] showing that CsA does not affect various functions of monocytes such as chemotaxis, monokine production and accessory function. Our previous study showed that human blood NK (CD16$^+$) cells were the main cells capable of killing MDR cancer cells in the presence of anti-P-glycoprotein mAb,[10] suggesting that CsA may inhibit lymphocyte-ADCC through the suppression of NK cell function. When lymphocytes were pretreated for more than 2 h with CsA before ADCC assay, their subsequent NK and ADCC activities were significantly decreased (Table II). This finding is in agreement with a few reports,[17, 18] but most studies[25-28] have indicated that CsA does not significantly influence NK cells in humans and rodents. The reason for this difference between our findings and those of others is unknown, but several factors such as the target cells, mAb used and the experimental conditions for NK and ADCC assays may influence results on the effect of CsA on NK cell function.

CsA significantly augmented MRK16 mAb-dependent MNC-mediated cytotoxicity against MDR cancer cells expressing P-glycoprotein. This enhancement was due to enhanced susceptibility of the target tumor cells to monocyte-mediated ADCC, because pretreatment of MDR cancer cells with CsA caused a dose-dependent increase in ADCC mediated by monocytes, but not by lymphocytes. Interestingly, pretreatment of MDR cells with CsA did not affect their sensitivity to NK activity or the NK cell-mediated ADCC reaction (Table I). These findings could be explained by differences in the binding ability and cytotoxic molecules of effector cells in the ADCC reaction. The former possibility was ruled out in the present study by the finding that CsA did not increase the binding of target MDR cancer cells to MRK16 mAb or that between monocytes and target MDR cancer cells in the presence of MRK16 mAb (Fig. 6). The latter possibility was supported by the facts that cytolysin[29] and H$_2$O$_2$[24] were reported to be key molecules of the ADCC reactions mediated by lymphocytes and monocytes, respectively, although the exact killing mechanism(s) in ADCC reactions is still unknown. We found that SOD and N^G-monomethyl-L-arginine acetate did not affect augmentation by CsA of monocyte-mediated ADCC activity against 2780^{AD} cells, suggesting that the reaction did not involve superoxide or nitric oxide. Interestingly, cat-

alase, which inactivates H_2O_2, only partially reduced the enhanced susceptibility of MDR cells to monocyte-mediated ADCC. Neutrophils also mediate the ADCC reaction through production of reactive oxygen species.[30] Indeed, we found that CsA enhanced neutrophil-mediated ADCC activity against MDR 2780[AD] cells in a dose-dependent manner (data not shown). This finding suggests that the increased susceptibility of MDR cancer cells to ADCC by monocytes and neutrophils was due in part, but not completely, to their increased susceptibility to oxidative attack by H_2O_2 produced by these effector cells. In addition, various monokines have been shown to be cytotoxic to certain tumor cells, but TNF-α (1000 U/ml), IL-1 α (100 U/ml) and IL-1 β (100 U/ml), which are known to be cytotoxic effector molecules of macrophages,[31, 32] were not cytotoxic to 2780[AD] cells, irrespective of the presence of CsA (data not shown).

CsA is known to bind to phosholipid vesicles,[33] interfere with the incorporation of fatty acids into membrane phospholipids[34] and depolarize the cytoplasmic membrane.[35] In the present study, CsA at concentrations of up to 30 μg/ml was not cytotoxic to MDR 2780[AD] cells, as assessed by measuring viability and proliferation of the cells. These findings suggest that CsA may enhance membrane damage of MDR cells coated with anti-P-glycoprotein mAb by killing molecules from monocytes, not from lymphocytes. On the other hand, it has been found to cause competitive inhibition of the binding of antitumor agents to P-glycoprotein, and so to decrease the efflux of antitumor agents.[15] Thus, because P-glycoprotein may mediate efflux of not only antitumor agents but also waste and cytotoxic products,[2, 36] CsA may also increase the intracellular accumulation of such cytotoxic products, resulting in secondary membrane damage and cell death. Further investigations are necessary to clarify the exact mechanism(s) of enhancement of ADCC by CsA.

The minimal concentration of CsA capable of enhancing ADCC reaction was at least 10 μg/ml. However, as shown in Table II, pretreatment of MDR cells for 2 h with CsA (10 μg/ml) was enough to enhance significantly monocyte-mediated ADCC. These findings, together with a report by Verweij et al.,[37] showing that the peak blood level of CsA reached 13.1 μg/ml, indicate that it may be possible to obtain a blood level of CsA that enhances ADCC.

Like, CsA, other compounds including a CsA analog (PSC833), FK-506 and verapamil have also been reported to reverse multidrug resistance in vitro and in vivo, when combined with antitumor drugs.[12-16] All of these drugs inhibit the transport of antitumor agents by P-glycoprotein.[14-16] Concentrations of these chemosensitizing agents used in the present study were previously found to be sufficient to reverse multidrug resistance in vitro.[15, 16] Interestingly, like CsA, PSC833 and FK-506 increased the susceptibility of MDR tumor (2780[AD]) cells to ADCC mediated by monocytes, but verapamil did not affect monocyte-mediated cytotoxicity against the cells (Fig. 5). Recently, we found that the combined use of MRK16 mAb with CsA, but not with verapamil, synergistically reversed the drug resistance of MDR cells in the presence of vincristine or adriamycin in vitro.[38] Further studies on the mechanisms of the enhancement by CsA of monocyte-mediated ADCC against MDR cancer cells may provide a rationale to develop an effective therapeutic modality for overcoming MDR cancer in humans.

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