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Requirement of Expression of P-glycoprotein on Human Natural Killer Leukemia Cells for Cell-Mediated Cytotoxicity

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ABSTRACT. The requirement of P-glycoprotein, a product of the multidrug resistance (*MDR*)1 gene, for natural killer (NK) cell-mediated cytotoxicity was examined by using a human NK-like cell line, YTN, which is cytotoxic toward JY cells. YTN cells express P-glycoprotein, as judged by flow cytometry and polymerase chain reaction of reverse-transcribed mRNA. YTN cell-mediated cytotoxicity was inhibited by MDR-reversing reagents as well as the $F(ab')_2$ fragment of a monoclonal antibody against P-glycoprotein. Furthermore, antisense oligonucleotides for *MDR*1 mRNA inhibited expression of P-glycoprotein as well as YTN cell-mediated cytotoxicity. Thus, this study provides firm evidence that P-glycoprotein plays an essential role in cell-mediated cytotoxicity. BIOCHEM PHARMACOL **55**;9:1385–1390, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. P-glycoprotein; NK cells; cytotoxicity; antisense oligonucleotide

P-glycoprotein, a product of the *MDR1*‖ gene, is known to confer MDR on cell lines and tumors by pumping cytotoxic drugs out of the cells [1]. P-glycoprotein not only is expressed on malignant cells, but also has been identified in normal tissues such as the adrenal cortex, lumenal surfaces of the kidneys, liver, jejunum and pancreas, placental trophoblasts, and endothelial cells in the brain and testis [2]. Moreover, NK cells and killer T cells express the highest level of P-glycoprotein among normal mature lymphoid cells [3, 4].

P-glycoprotein expressed in the brain capillary endothelium prevents chemotherapeutic agents from entering the central nervous system and thereby functions as a component of the blood–brain barrier [5]. On the other hand, P-glycoprotein is expressed abundantly in epithelia, where volume-regulated chloride channels are involved in electrolyte and fluid movement, and MDR-reversing agents, such as verapamil and quinine, are reported to inhibit the cell volume regulation of epithelial cells upon hypotonic shock [6]. Thus, the hypothesis was proposed that P-glycoprotein directly or indirectly regulates a volume-sensitive chloride channel in epithelia. However, recent work with antisense oligonucleotides and a monoclonal antibody revealed that the activity of this volume-sensitive chloride channel does not depend on endogenous expression of P-glycoprotein in a human small intestinal epithelial cell line [7]. These findings have raised a question regarding the physiological role of P-glycoprotein in epithelia.

We and others have reported that both NK cells and killer T cells express functional P-glycoprotein in terms of rhodamine dye excretion and its inhibition by MDR-reversing reagents [8–10]. Moreover, cell-mediated cytotoxicity was inhibited by both MDR-reversing reagents [8, 9] and a monoclonal antibody against P-glycoprotein (MRK16) [8, 10]. However, all of these studies involved heterogeneous cell populations, namely a cultured NK cell-rich population consisting of 50–90% of $CD16^+$ and/or $CD56^+$ cells [8], PBMC [9], and killer T cells generated in a mixed lymphocyte culture [10]. Therefore, the possibility cannot be ruled out that both the MDR-reversing reagents and MRK16 inhibited cell-mediated cytotoxicity indirectly. Moreover, there have been no widely accepted specific MDR-reversing reagents. Furthermore, the possibility cannot be excluded that MRK16 inhibited cell-mediated cytotoxicity through steric hindrance. The requirement of P-glycoprotein in cell-mediated cytotoxicity, therefore, has not been firmly established.

To determine whether P-glycoprotein is really involved in cell-mediated cytotoxicity, we employed a human NK-

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‖ *Abbreviations:* MDR, multidrug resistance; NK, natural killer; PBMC, peripheral blood mononuclear cells; FBS, fetal bovine serum; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; Rh123, rhodamine 123; and β_2m, β_2-microglobulin.

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like cell line, YTN, which is cytotoxic for an EBV-transformed cell line, such as JY, through the interaction of CD28 with B7 [11], and antisense oligonucleotides for MDR1 mRNA to specifically inhibit expression of P-glycoprotein.

MATERIALS AND METHODS

Cells and Reagents

YT cells were originally established from a patient with an acute lymphoblastic lymphoma and a thymoma by Dr. J. Yodoi (Kyoto University) [12], and a subclone of YT, named YTN, was provided by Dr. K. Oshimi (Juntendo University). The EBV-transformed B lymphoblastoid cell line, JY, was provided by Dr. H. Yagita (Juntendo University). Vinblastine-resistant CEM and parental CEM were provided by Dr. W. T. Beck. These cells were cultured in RPMI 1640 medium containing 10% (FBS) (Life Technologies), 0.3 g/L of L-glutamine, and 60 mg/L of kanamycin sulfate.

Flow Cytometry

The expression of P-glycoprotein and cell surface markers in YTN cells was analyzed by flow cytometry using a FACScan system (Becton Dickinson) as previously described [13]. Dead cells were stained simultaneously with propidium iodide, and the data for dead cells were eliminated with proper gatings. $F(ab')_2$ fragments of the MRK16 mAb and normal mouse IgG were prepared by the standard method with pepsin, followed by purification on protein A Sepharose (Sigma). The purity was checked by SDS–PAGE.

Dye Efflux Assay

Dye efflux was assayed according to Chaudhary and Roninson [14]. Briefly, cells were stained with 100 ng/mL of Rh123 dye (Molecular Probes, Inc.) for 10 min, and after two washes with pre-cooled PBS, the cells were incubated for 4.5 hr at 37° in dye-free RPMI 1640 containing 10% FBS, with or without an MDR-reversing agent. As a negative control, excretion of Rh123 was examined at 4°. All cells were then washed with pre-cooled PBS, followed by FACScan analysis. The filter configuration for Rh123 was the same as that used for FITC.

PCR of Reverse-Transcribed mRNA

By PCR involving cDNA, the MDR1- or β_2m-specific sequence was amplified using the sense-strand primer CCCATCATTGCAATAGCAGG (residues 2596–2615), and the antisense-strand primer GTTCAAACTTCTGCTCCTGA (residues 2733–2752), which yield a 167-bp product, or using the sense-strand primer GAAAAAGATCAGTATGCCTG (residues 1552–1571), and the antisense-strand primer ATCTTCAAACCTCCATGATG (residues 2253–2262 and 3508–3517), which yield a 105-bp product [8, 15]. The reaction mixture containing cDNA, 20 mM of TRIS–HCl (pH 8.4), 50 mM of KCl, 0.2 mM of dNTPs, and $MgCl_2$ (1 mM for MDR1; 2 mM for β_2m) was heated at 91° for 5 min and then at 59° for 5 min, followed by the addition of Taq DNA polymerase and mineral oil. Amplification was performed sequentially at 72° for 2 min, at 94° for 30 sec, and at 59° for 1 min, for 30 cycles.

Assay for YTN Cell-Mediated Cytotoxicity

YTN cell-mediated cytotoxicity was measured according to the standard method for cell-mediated cytotoxicity, using ^{51}Cr-labeled JY cells as target cells. Effector cells were mixed with target cells in appropriate ratios, followed by incubation for 4 hr. DNA fragmentation associated with YTN cell-mediated cytotoxicity was also measured using [^{3}H]TdR-labeled JY cells as target cells according to a method described previously [16].

Antisense Study

The antisense and sense phosphorothioate oligonucleotides corresponding to positions −2 to +13 (relative to the translation initiation site) of the human *MDR1* coding region [17, 18] were synthesized by means of standard phosphoramidite chemistry and purified by high pressure liquid chromatography. The scrambled antisense oligonucleotides were also prepared in the same way as described above. YTN cells were washed three times with PBS, suspended in RPMI 1640 medium containing 1% FBS treated at 65° for 15 min to inactivate nuclease, and then seeded on 24-well culture plates at 1×10^5 cells/mL/well. During culture for 3 days, oligonucleotides were added to the culture medium at a final concentration of 80 μg/mL on day 1, and 40 μg/mL on days 2 and 3. All experiments involving sense or antisense oligonucleotides were repeated three times, and the results obtained were essentially reproducible.

Statistics

Student's *t*-test was performed to assess the statistical differences between groups.

RESULTS

Expression and Function of P-glycoprotein on YTN Cells

In this study we used a subline of a human NK-like cell line, YT [12], named YTN. YTN cells were shown, by flow cytometric analysis, to be $CD16^-56^+$ (see Fig. 6a for CD56 expression; data not shown for CD16 expression). YTN cells were then stained with MRK16 mAb, which is specific to the extracellular domain of P-glycoprotein [19]. As shown in Fig. 1c, the YTN cells expressed P-glycoprotein, although the extent of its expression was weaker than that

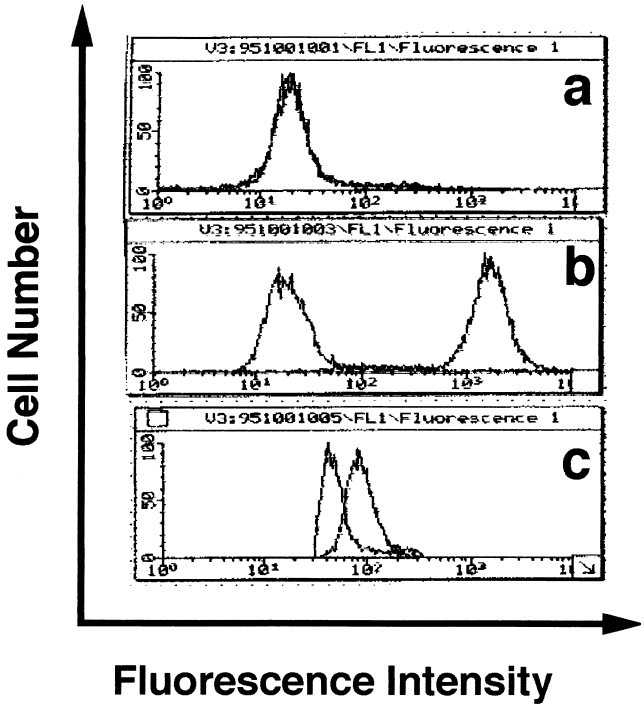

FIG. 1. Flow cytometric analysis of P-glycoprotein on YTN cells. Expression of P-glycoprotein was examined by flow cytometry using MRK16 mAb or control Ab. The experimental profile is on the right-hand side, while the control profile is on the left-hand side except for (a). (a) Parental CEM cells. (b) Vinblastine-resistant CEM cells. (c) YTN cells.

in the vinblastine-resistant CEM cells (Fig. 1b). However, parental CEM cells were not stained with MRK16 mAb (Fig. 1a).

The expression of MDR1 mRNA was also examined in YTN cells by PCR of reverse-transcribed mRNA (Fig. 2). The MDR1-specific 167-bp product was detected in lane 3 (vinblastine-resistant CEM) and to a lesser extent in lane 1

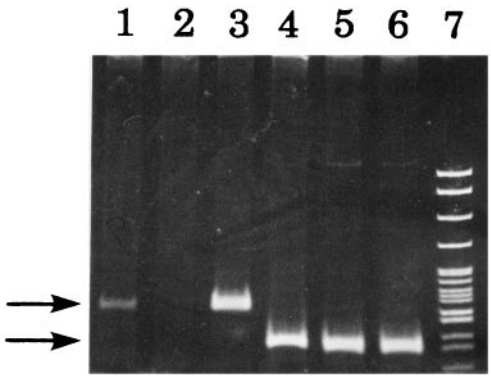

FIG. 2. Expression of P-glycoprotein mRNA. MDR1 mRNA and β_2m mRNA were examined in YTN cells (lanes 1 and 4), parental CEM cells (lanes 2 and 5), and vinblastine-resistant CEM cells (lanes 3 and 6), by reverse transcriptase-polymerase chain reaction, and MDR1 mRNA was not detected in the parental CEM cells (lane 2). The 167-bp band (indicated by the top arrow) and the 105-bp band (indicated by the bottom arrow) are the PCR products of MDR1 mRNA and β_2m mRNA, respectively. Lane 7 shows molecular weight standards.

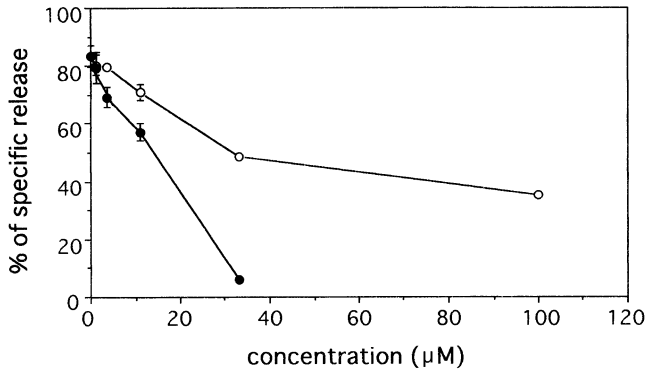

FIG. 3. Effects of nicardipine and AHC-52 on YTN cell-mediated cytotoxicity as measured by ^{51}Cr release. The cytotoxic activity of YTN cells was assayed at the effector to target ratio of 2 in the presence of nicardipine (●) or AHC-52 (○) according to the general method. Values are means ± SEM, N = 3. Total radioactivity was 11,690 cpm, and the spontaneous release was 6.3 ± 0.6%.

(YTN), but not in lane 2 (parental CEM), whereas the level of the β_2m-specific 105-bp product was almost the same in YTN, parental CEM, and vinblastine-resistant CEM (lanes 4–6).

To confirm that the P-glycoprotein expressed on YTN cells is functional, we examined the excretion of Rh123 from the cells and its sensitivity to two MDR-reversing reagents, namely, nicardipine (a calcium channel blocker) and a structural analog, methyl 2-(*N*-benzyl-*N*-methylamino)ethyl-2,6-dimethyl-4-(2-isopropyl-pyrazolo [1,5-*a*]pyridine-3-yl)-1,4-dihydro-pyridine-3,5-dicarboxylate (AHC-52), which is devoid of calcium channel blocking activity [20, 21]. Rh123 excretion from YTN cells was inhibited by AHC-52 and nicardipine in a concentration-dependent manner; the concentrations required for 50% inhibition (IC_{50}) were determined to be 3.2 ± 0.5 and 2.0 ± 0.3 μM, respectively (means ± SEM of three independent determinations).

Inhibition of YTN Cell-Mediated Cytotoxicity by MDR-Reversing Agents and MRK16 mAb

We next examined whether the YTN cell-mediated cytotoxicity was inhibited by these MDR-reversing agents. Both nicardipine and AHC-52 inhibited YTN cell-mediated cytotoxicity in a concentration-dependent manner; the IC_{50} values, which were defined as the concentrations at which ^{51}Cr release at an effector/target ratio of 2 was reduced to that at an effector/target ratio of 1, were determined to be 6.8 ± 1.7 and 13.7 ± 3.8 μM (means ± SEM of three independent determinations) (Fig. 3). The inhibition of the cytotoxicity with 11, 33, and 100 μM (for AHC-52) and 11 and 33 μM (for nicardipine) was statistically significant ($P < 0.01$, 0.001, and 0.001 for AHC-52; $P < 0.001$ and 0.001 for nicardipine, respectively). We observed that DNA fragmentation associated with YTN cell-mediated cytotoxicity was also inhibited by these

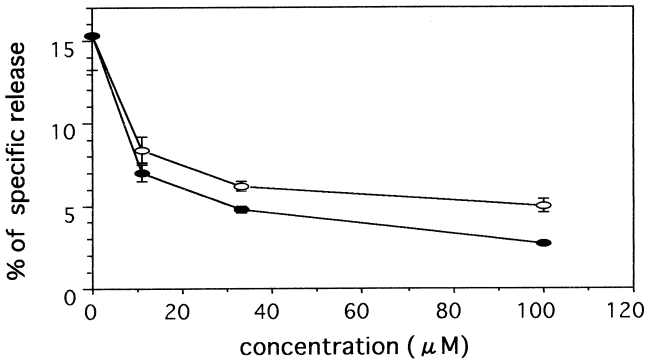

FIG. 4. Effects of nicardipine and AHC-52 on YTN cell-mediated cytotoxicity as measured by [^{3}H]TdR release. The cytotoxic activity of YTN cells was assayed at the effector to target ratio of 2 in the presence of nicardipine (●) or AHC-52 (○), according to the method described under Materials and Methods. Values are means ± SEM, N = 3. Total radioactivity was 14,730 cpm, and the spontaneous release was 4.3 ± 0.3%.

agents in a concentration-dependent manner; the IC_{50} values, which were defined in the same way as for ^{51}Cr release, were determined to be 23 ± 4.2 μM (for AHC-52) and 7.9 ± 2.2 μM (for nicardipine), respectively (means ± SEM of three independent determinations) (Fig. 4). The inhibition of the cytotoxicity with 11, 33, and 100 μM was statistically significant ($P < 0.01$, 0.005, and 0.002 for AHC-52; $P < 0.005$, 0.002, and 0.001 for nicardipine, respectively).

Furthermore, $F(ab')_2$ of MRK16 mAb inhibited the cytotoxicity in a concentration-dependent manner, whereas the control $F(ab')_2$ did not demonstrate such inhibition, as shown in Fig. 5. The inhibition of the cytotoxicity with 25, 50, and 100 μg/mL of $F(ab')_2$ of MRK16 mAb was statistically significant ($P < 0.05$, 0.005, and 0.001, respectively), whereas the inhibition with 10 μg/mL of $F(ab')_2$ of MRK16 mAb was not statistically significant.

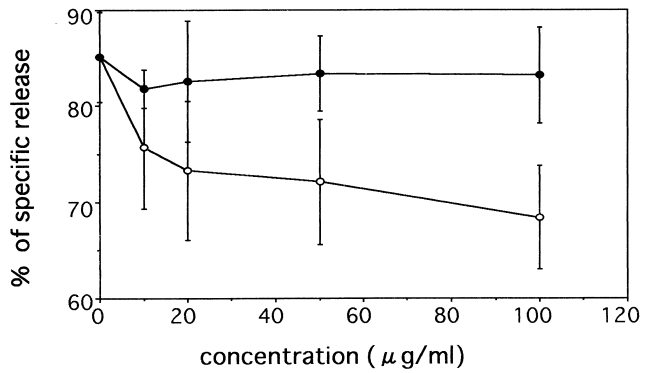

FIG. 5. Concentration-dependent inhibition of YTN cell-mediated cytotoxicity by the $F(ab')_2$ fragment of MRK16 mAb. YTN cell-mediated cytotoxicity was assayed in sets of six in the presence of either the $F(ab')_2$ fragment of MRK16 mAb (○) or the $F(ab')_2$ fragment of control IgG (●), at an effector/target ratio of 2. Each bar represents the SEM. Total radioactivity was 12,570 cpm, and the spontaneous release was 11.6 ± 1.0%.

Effects of Antisense Oligonucleotides Corresponding to mRNA of P-glycoprotein on YTN Cell-Mediated Cytotoxicity

We next examined the effects of antisense oligonucleotides on the expression of P-glycoprotein. The cells treated with antisense oligonucleotides for MDR1 mRNA (Fig. 6f) expressed a reduced level of P-glycoprotein on their surface in comparison with those treated with sense oligonucleotides (Fig. 6e). In contrast, treatment with either antisense or sense oligonucleotides did not affect the expression of CD56 on YTN cells (Figs. 6a–c), indicating that the antisense oligonucleotides specifically inhibited the P-glycoprotein expression.

As shown in Fig. 7, the cells treated with antisense oligonucleotides exhibited approximately half as much cytotoxic activity as those treated with sense oligonucleotides, although the cells treated with sense oligonucleotides exhibited reduced cytotoxicity as compared with untreated cells. Of note was that the inhibition of the cytotoxicity by the antisense oligonucleotides was much more significant than that by the sense oligonucleotides. The difference between the inhibition by the antisense oligonucleotides and that by the sense oligonucleotides was statistically significant at effector/target ratios of 1, 2, 5, and 10 ($P < 0.02$, 0.01, 0.01, and 0.001, respectively).

DISCUSSION

In this study, we examined whether P-glycoprotein is required for NK cell-mediated cytotoxicity, employing a human NK-like cell line, YTN, which is cytotoxic for an EBV-transformed cell line, such as JY, through the interaction of CD28 with B7. YTN cell-mediated cytotoxicity was inhibited by MDR-reversing agents and the $F(ab')_2$ fragment of MRK16, and by treatment with antisense oligonucleotides for MDR1 mRNA. Although P-glycoprotein is expressed in normal tissues such as epithelia, the physiological role has not been established except for that in the blood–brain barrier, where P-glycoprotein is presumed to play a role as a drug transporter. This study provides firm evidence that P-glycoprotein plays an essential role in cell-mediated cytotoxicity, which is presumably unrelated to a drug transporter.

YTN cells express P-glycoprotein, as evidenced by flow cytometry with MRK16 and RT-PCR (Figs. 1 and 2). P-glycoprotein expressed on YTN cells is functional in terms of rhodamine dye excretion and its inhibition by MDR-reversing agents. These findings are compatible with previous reports that NK cells express functional P-glycoprotein [3, 4]. YTN cells are cytotoxic to JY cells (this study) and to a lesser extent K562 cells (data not shown), as reported previously [11]. Thus, YTN cells are one of the best types of effector cells for studying the role of P-glycoprotein in NK cell-mediated cytotoxicity.

YTN cell-mediated cytotoxicity was inhibited by the MDR-reversing agents nicardipine and its analog, AHC-52,

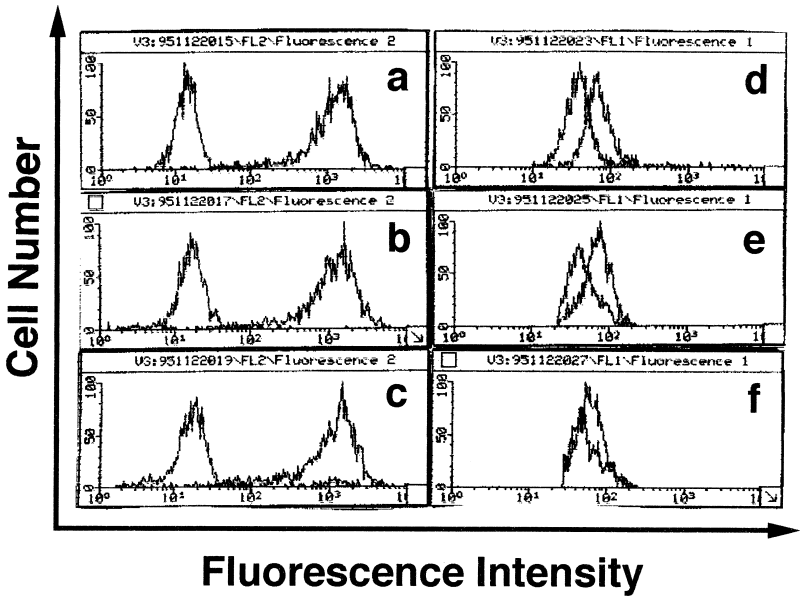

FIG. 6. Effect of antisense oligonucleotides on expression of P-glycoprotein on YTN cells. YTN cells were cultured in the absence or presence of sense or antisense oligonucleotides for MDR1 mRNA, as described under Materials and Methods, followed by staining with phycoerythrin-labeled anti CD56 mAb (Becton Dickinson) (a, b, c) or MRK16 mAb (d, e, f). The experimental profile is on the right-hand side, while the control profile is on the left-hand side. (a, d) Control, (b, e) sense oligonucleotides, and (c, f) antisense oligonucleotides.

with IC_{50} values of 6.8 ± 1.7 and 13.7 ± 3.8 μM, respectively. Although no specific inhibitors for studying the function of P-glycoprotein have been reported, in this study we used AHC-52, which is structurally related to nifedipine and nicardipine. In terms of the potency for blocking calcium channels, AHC-52 is 500 times less potent than nifedipine, which is 10 times less potent than nicardipine [21, 22]. Therefore, AHC-52 should be 5000 times less potent than nicardipine in this regard, but it was only half as potent as nicardipine both as an MDR-reversing agent and as an inhibitor of cell-mediated cytotoxicity, indicating that in cell-mediated cytotoxicity these two inhibitors exert their effects as MDR-reversing agents. The reason why the IC_{50} values of MDR-reversing agents for YTN cell-mediated cytotoxicity were several-fold larger than those for Rh123 excretion is not known at present. These two MDR-reversing agents also inhibited DNA fragmentation associated with YTN cell-mediated cytotoxicity with IC_{50} values similar to those for ^{51}Cr release.

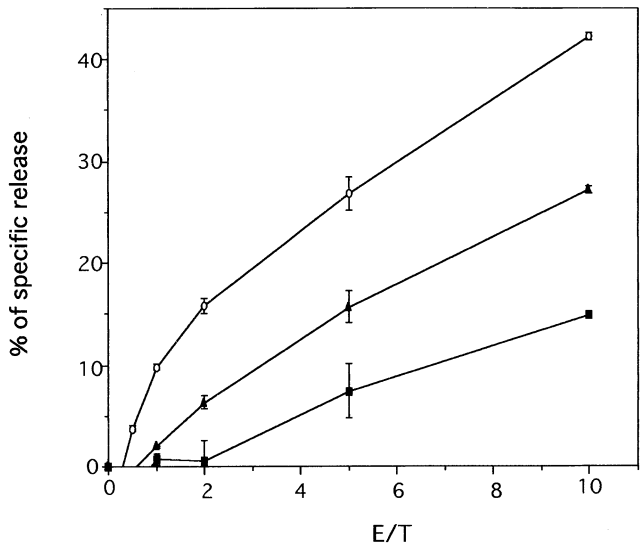

FIG. 7. Effect of antisense oligonucleotides on YTN cell-mediated cytotoxicity as measured by ^{51}Cr release. YTN cells were cultured in the absence or presence of sense or antisense oligonucleotides for MDR1 mRNA, as described under Materials and Methods, followed by assaying of the cell-mediated cytotoxicity in triplicate at various effector/target ratios. Key: (○) control, (▲) sense oligonucleotides, and (■) antisense oligonucleotides. Each bar represents the standard error of the mean. Total radioactivity was 6540 cpm, and the spontaneous release was 7.9 ± 0.7%.

YTN cell-mediated cytotoxicity was also inhibited by the $F(ab')_2$ fragment of MRK16, which was statistically significant. Since JY cells do not express P-glycoprotein (data not shown), MRK16 presumably exerted its inhibitory effect through interaction with P-glycoprotein on YTN cells.

Treatment with antisense oligonucleotides for MDR1 mRNA specifically inhibited expression of P-glycoprotein on YTN cells, although the inhibition was incomplete. It should be noted that the inhibitory effect of the antisense oligonucleotides on the expression of P-glycoprotein was confirmed by three independent experiments. In contrast, the antisense oligonucleotides for MDR1 mRNA did not affect the expression of CD56 on YTN cells. Of note was that the antisense oligonucleotides for MDR1 mRNA reduced the cytotoxic activity of YTN cells toward JY cells much more significantly than the sense oligonucleotides. The sense oligonucleotides themselves affected the cytotoxic activity of YTN cells, which is presumably due to a nonspecific effect of a high concentration of sense oligonucleotides (Fig. 7). It should be noted here that cytotoxic activity was reduced under the particular culture condition for antisense experiments. In addition, scrambled antisense oligonucleotides did not reduce the cytotoxic activity of YTN cells as did antisense oligonucleotides, confirming the specificity of action of oligonucleotides (our unpublished results).

What role might P-glycoprotein play in NK cell-mediated cytotoxicity? NK cells are known to utilize two

different mechanisms for cytotoxicity: granule-exocytosis and Fas–Fas ligand interaction [23]. The former requires extracellular calcium, whereas the latter does not require extracellular calcium for the induction of cytotoxic activity. Since YTN cell-mediated cytotoxicity was blocked completely by a calcium chelator (data not shown), the mechanism of granule-exocytosis is responsible for the cell-mediated cytotoxicity of YTN cells. Therefore, we presume that P-glycoprotein may be involved in steps including the signal transduction pathway leading to exocytosis, the regulation of activity of granzymes and perforin in granules, and the exocytotic process. One model suggests that P-glycoprotein acts as an ATP-dependent drug efflux pump [24], whereas another model suggests that P-glycoprotein is involved in intracellular pH homeostasis [25]. It is possible that P-glycoprotein causes the secretion of a substance(s) crucial to cytotoxicity such as ATP [23, 26]. Since both YTN cells and JY cells contain granzyme A activity as well as granzyme B activity (Jinnai T, Nakano A and Kobayashi Y, unpublished data), we need methods other than enzyme release to evaluate the role of P-glycoprotein in the exocytotic process. Further studies are now in progress to elucidate the role of P-glycoprotein in NK cell-mediated cytotoxicity.

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