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EXPRESSION AND FUNCTION OF MULTIDRUG RESISTANCE P-GLYCOPROTEIN IN A CULTURED NATURAL KILLER CELL-RICH POPULATION REVEALED BY MRK16 MONOCLONAL ANTIBODY AND AHC-52

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Abstract—Natural killer (NK) cells have been reported recently to be the highest in expressing multidrug resistance (MDR) P-glycoprotein among normal mature lymphoid cells. Using a cultured NK cell-rich population, we have examined the expression and function of P-glycoprotein, in particular its role in NK cell-mediated cytotoxicity, by employing two MDR-reversing agents (nicardipine and AHC-52, a nicardipine analog almost devoid of calcium channel blocking activity) and monoclonal antibody against P-glycoprotein (MRK-16). The expression of P-glycoprotein was detected by flow cytometry and polymerase chain reaction of reverse transcribed mRNA. P-glycoprotein was functional in terms of rhodamine dye excretion and its susceptibility to the MDR-reversing agents. Since the concentration of nicardipine required for 50% inhibition (IC_{50}) of rhodamine dye excretion (2 μM) was close to that of AHC-52 (5 μM), it was suggested that their inhibitory effects were not due to calcium channel blocking activity, and that AHC-52 is a selective inhibitor for P-glycoprotein. The IC_{50} of nicardipine for NK cell-mediated cytotoxicity (33 μM) was also close to that of AHC-52 (26 μM), indicating that P-glycoprotein is involved in NK cell-mediated cytotoxicity. In support of this, MRK16 inhibited NK cell-mediated cytotoxicity in a concentration-dependent manner. Both binding of target cells to NK cells and post-binding events were affected by AHC-52, suggesting that P-glycoprotein is involved in several steps in NK cell-mediated cytotoxicity.

Key words: natural killer cells; P-glycoprotein; cytotoxicity

P-glycoprotein is the product of the *MDR1* gene known to confer MDR‖ on cell lines and human tumors by pumping cytotoxic drugs out of the cells [1, 2]. Its expression has been reported recently to be associated with a cell volume-regulated chloride channel [3, 4], thereby raising the hypothesis that this protein functions both as a drug transporter and as a chloride channel [4]. P-glycoprotein is abundantly present in epithelia where the volume-regulated chloride channels are involved in electrolyte and fluid movement [5]. Furthermore, MDR-reversing agents, such as verapamil and quinine, were reported to inhibit the cell volume regulation of epithelial cells [6], suggesting the possibility that P-glycoprotein expressed on epithelia may act as the cell volume-regulated chloride channel.

Although immunohistochemical research has demonstrated almost no expression of P-glycoprotein in lymphoid tissues [5], recent flow cytometric analyses have nevertheless revealed its expression on normal human PBMC [7–9]. Since NK cells and CTLs are the highest expressers of P-glycoprotein [7, 8], a possibility exists that it is involved in a target cell killing reaction. There were reports that potassium and chloride channel blockers inhibited NK cell or CTL-mediated cytotoxicity [10, 11]. Because some of these channel blockers are also known to act as MDR-reversing agents [12], we subsequently hypothesized that P-glycoprotein might be a common target for these channel blockers. In this study, we have examined the expression and function of P-glycoprotein using a cultured NK cell-rich population, in particular its role in NK cell-mediated cytotoxicity.

MATERIALS AND METHODS

Culture preparation. Human PBMC were separated from the venous blood of healthy donors via the Ficoll–Urografin density gradient method [9]. A cultured NK cell-rich population was obtained by

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‖ Abbreviations: MDR, multidrug resistance; PBMC, peripheral blood mononuclear cells; NK, natural killer; CTL, cytotoxic T lymphocyte; IL-2, interleukin 2; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PE, phycoerythrin; Rh123, rhodamine 123; FBS, fetal bovine serum; PCR, polymerase chain reaction; cDNA, complementary DNA; β_2m, β_2-microglobulin; AHC-52, methyl 2-(*N*-benzyl-*N*-methylamino) ethyl-2,6-dimethyl-4-(2-isopropyl-pyrazolo[1,5-a]pyridine-3-yl)-1,4-dihydropyridine-3,5-dicarboxylate.

co-culturing with mitomycin C-treated Daudi cells in the presence of 100 U/mL of IL-2 (1.25×10^7 U/mg protein; Ajinomoto Co., Japan) for 8–10 days [13].

Flow cytometry. The expression of P-glycoprotein and the cell surface markers in the cultured NK cell-rich population were analysed by flow cytometry using a FACScan system (Becton Dickinson, Mountain View, CA) as previously described [9]. Cells (5×10^5) were treated with 0.05 mL of MRK16 mAb or control mAb (IgG2a) [14] at 25 μg/mL for 30 min at 4°. Excess antibody was then removed by washing twice in PBS containing 10% human serum and 0.01% sodium azide (washing buffer), followed by adding 0.05 mL of FITC-labeled $F(ab')_2$ fragment of goat anti-mouse IgG, IgM antibodies (1:40 dilution) (Tago Inc., Burlingame, CA) and incubating the mixture for 30 min at 4°. After washing the resultant cells three times, we carried out the flow cytometric analysis. The result of staining was gated according to forward scatter and side scatter, with 5000 gated events being displayed as a histogram for P-glycoprotein for live cells. For identification of surface markers, FITC-labeled anti-Leu-11 (CD16) antibody and PE-labeled anti-Leu-19 (CD56) antibody (Becton Dickinson) were used instead of the antibodies described above.

Dye efflux assay. Dye efflux was assayed according to Chaudhary and Roninson [15]. Briefly, cells were stained with 100 ng/mL Rh123 dye (505 nm excitation wavelength, 534 nm emission wavelength; Molecular Probes, Inc., Eugene, OR) for 10 min in 5 mL RPMI 1640 containing 10% FBS, and after two washes with pre-cooled PBS, the cells were incubated for 3 hr at 37° in 10 mL of dye-free RPMI 1640 containing 10% FBS with or without MDR-reversing agents, namely nicardipine (Mediolast, Milano, Italy) and AHC-52 (Kyorin Pharmaceutical Co., Ltd, Tokyo, Japan). As a negative control, excretion of Rh123 was examined at 4°. All cells were then washed with pre-cooled PBS, followed by a FACScan analysis. The filter configuration for Rh123 was the same as that used for FITC.

Detection of MDR1 *mRNA.* By PCR using cDNA, the *MDR-* 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, respectively, according to the method described by Noonan *et al.* [16]. It should be noted here that the former two primers are derived from different exons of the *MDR1* gene, separated by a long intron, thereby preventing amplification of genomic DNA sequence. Briefly, total cellular RNA was prepared by an RNA extraction kit (Sanko, Tokyo, Japan) from a cultured NK cell-rich population. cDNA was then synthesized by a cDNA synthesis kit (Stratagene, La Jolla, CA) with 1 μg of total cellular RNA, followed by PCR with cDNA derived from 250 ng of RNA. Each cycle of PCR included 30 sec of denaturation at 94°, 1 min of primer annealing at 55°, and 2 min of extension/synthesis at 72°. The number of PCR cycles used was 30.

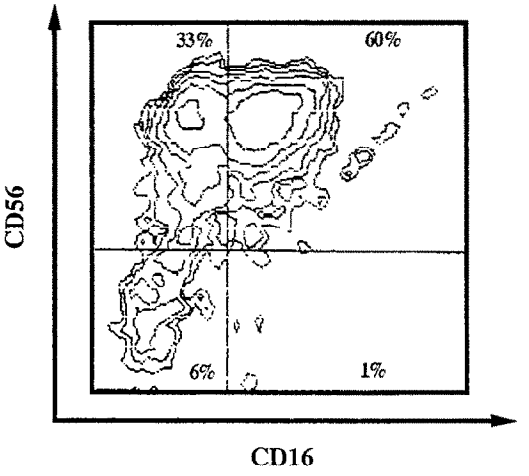

Fig. 1. Surface phenotype of a cultured NK cell-rich population. The cultured NK cell-rich population was stained with FITC-anti-Leu-11 (CD16) antibody and PE-anti-Leu-19 (CD56) antibody, followed by analysis with flow cytometry.

Assay for NK cell-mediated cytotoxicity. NK cell-mediated cytotoxicity was measured according to the standard method using ^{51}Cr-labeled K562 cells as target cells. Usually effector cells were mixed with target cells at a 1:1 or 2:1 ratio, followed by incubation for 4 hr.

Assay for conjugate formation between NK cells and target cells. Conjugate formation was assayed according to the method described by Sidell *et al.* [10]. Briefly, effector cells were mixed with target cells at a 1:1 ratio, and the mixture was incubated at 37° for 1 hr. The tubes were then centrifuged, after which the conjugates were gently suspended. The percentage of conjugates was enumerated by counting the proportion of effector cells bound to target cells under a microscope.

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

RESULTS

Expression of P-glycoprotein. We and others have reported, using unseparated PBMC, that P-glycoprotein is expressed on human NK cells ($CD16^+$and/or $CD56^+$ cells), and that it is functional in terms of excretion of Rh123 and sensitivity of the excretion to MDR-reversing agents such as verapamil [7–9]. Here we used a cultured NK cell-rich population obtained by co-culturing human PBMC with Daudi cells in the presence of IL-2, since this method was reported to produce a relatively homogeneous NK cell-rich population [13]. The population thus obtained consisted of around 90% $CD16^+$ and/or $CD56^+$ cells, as assessed by flow cytometric analysis (Fig. 1).

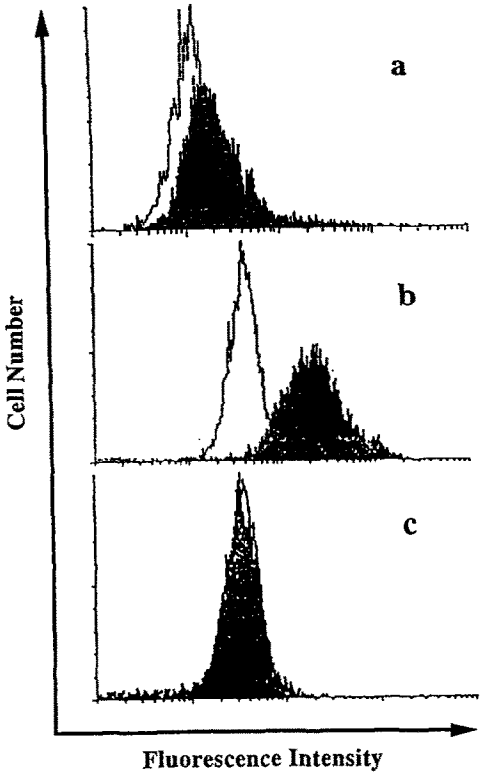

Fig. 2. Flow cytometric analysis of P-glycoprotein on a cultured NK cell-rich population. Expression of P-glycoprotein was examined by flow cytometry using MRK16 mAb or control mAb. The experimental profile is shaded, while the control profile is outlined. (a) A cultured NK cell-rich population. (b) K562/ADM. (c) Parental K562.

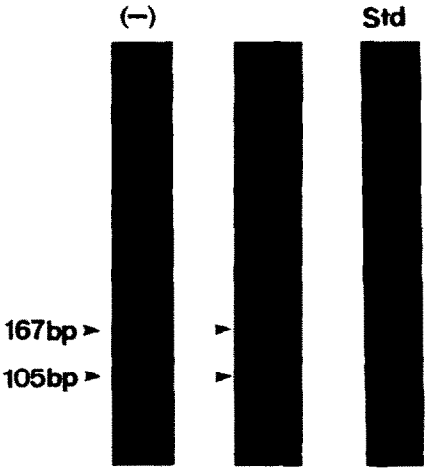

Fig. 3. Expression of P-glycoprotein mRNA in a cultured NK cell-rich population. *MDR1* mRNA and β_2m mRNA was detected in this population by PCR of reverse-transcribed mRNA. The bands with 105 bp and 167 bp indicate the PCR products of β_2m mRNA and *MDR1* mRNA, respectively. The blank tube in which no cDNA was added is denoted by (−). Std, molecular weight standards.

The cultured NK cell-rich population was stained with MRK16 mAb, which is specific to the extracellular domain of P-glycoprotein [14], and FITC-labeled second antibody. As shown in Fig. 2a, the cell population expressed P-glycoprotein, although the staining intensity was rather weak. It should be noted, however, that the staining of the cell population with MRK16 mAb was specific, because control mAb did not stain the cell population. We obtained reproducible results using the cultured NK cell-rich population from several other donors (data not shown). As other controls, panels b and c of Fig. 2 also show staining patterns of Adriamycin®-resistant K562 cells (K562/ADM) [14] and parental K562 cells with MRK16 mAb. The former expressed P-glycoprotein strongly, while the latter expressed virtually no P-glycoprotein.

The expression of *MDR1* mRNA was also examined in this population by PCR of reverse-transcribed mRNA (Fig. 3), although the level of expression was not quantitatively measured. Both β_2m mRNA and *MDR1* mRNA were detected in this population. It should be noted that the two primers used here to detect *MDR1* mRNA are derived from different exons of the *MDR1* gene, separated by a long intron, thereby preventing amplification of genomic DNA sequence.

*Excretion of Rh*123 *and its sensitivity to MDR-reversing agents.* To confirm that P-glycoprotein expressed on the cultured NK cell-rich population is also functional, we examined the excretion of Rh123 from the cells and its sensitivity to two MDR-reversing agents, namely nicardipine (also known as a calcium channel blocker) and AHC-52 (a nicardipine analog almost devoid of calcium channel blocking activity) [12, 17]. As shown in Fig. 4, Rh123 excretion from the cultured NK cell-rich population was inhibited by AHC-52 in a concentration-dependent manner, and the concentration required for 50% inhibition (IC_{50}) was determined to be 5 μM (the mean value of three independent determinations). Although data are not shown, the IC_{50} of nicardipine was 2 μM (the mean value of two independent determinations).

We also examined whether parental K562 cells and K562/ADM cells expressed functional P-glycoprotein. Although K562 cells hardly excreted Rh123, K562/ADM cells strongly excreted Rh123, which was inhibited by a variety of MDR-reversing agents (data not shown). These findings further confirmed the data in Figs 2 and 3.

Effects of MDR-reversing agents on NK cell-mediated cytotoxicity. Next we examined whether NK cell-mediated cytotoxicity was also inhibited by these MDR-reversing agents. Both nicardipine and AHC-52 inhibited NK cell-mediated cytotoxicity in a concentration-dependent manner. Figure 5 shows the inhibition by AHC-52, and the IC_{50} was determined to be 26 μM (the mean value of four independent determinations). We found that the IC_{50}

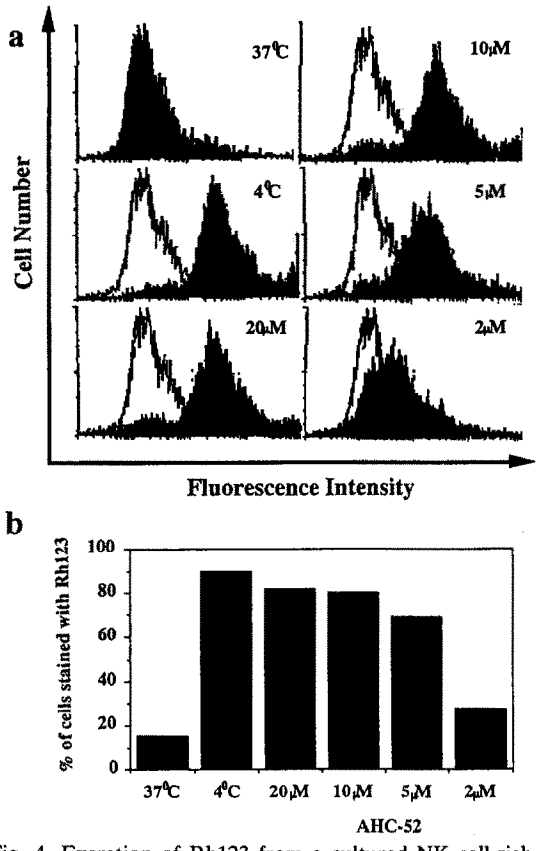

Fig. 4. Excretion of Rh123 from a cultured NK cell-rich population and concentration-dependent inhibition by AHC-52. (a) The function of P-glycoprotein expressed in this population was examined by flow cytometric analysis of the excretion of Rh123 at 37° in the presence or absence of various concentrations of AHC-52. As a negative control, the excretion of Rh123 at 4° was examined. The experimental profiles are shaded, while the control profile at 37° is outlined. (b) The results in (a) expressed as a percent of cells stained with Rh123.

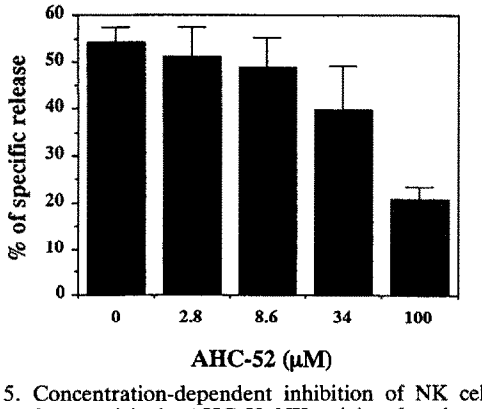

Fig. 5. Concentration-dependent inhibition of NK cell-mediated cytotoxicity by AHC-52. NK activity of a cultured NK cell-rich population was assayed in triplicate in the absence (solvent control; 1% DMSO) or presence of various concentrations of AHC-52 at an effector/target ratio of 2. Each bar represents the standard error of the mean.

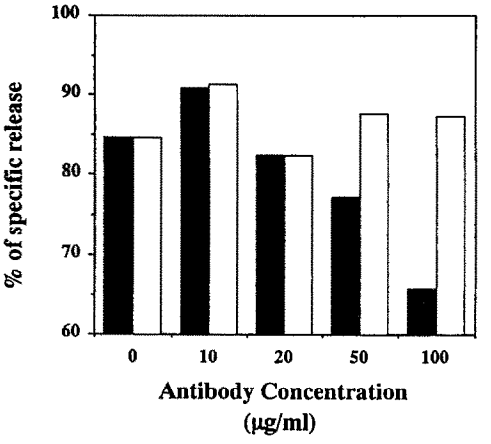

Fig. 6. Concentration-dependent inhibition of NK cell-mediated cytotoxicity by $F(ab')_2$ of MRK16 mAb. NK activity of a cultured NK cell-rich population was assayed in triplicate in the presence of either $F(ab')_2$ of MRK16 mAb (filled bars) or $F(ab')_2$ of control IgG (open bars) at an effector/target ratio of 2. The standard error of each value was within 5% (not shown).

of AHC-52 for cytotoxicity was similar to that of nicardipine (26 vs 33 μM), suggesting that P-glycoprotein is the target of these MDR-reversing agents. We also observed a similar inhibition of NK cell-mediated cytotoxicity by these agents at a different effector/target ratio (data not shown).

K562 cells expressed virtually no P-glycoprotein as assessed by flow cytometric analysis using MRK16 mAb (Fig. 2c), and therefore, it seems very likely that the MDR-reversing agents affected P-glycoprotein on NK cells but not K562 cells. To confirm this further, pulse treatment of the cultured NK cell-rich population and/or target cells with AHC-52 was performed. When NK cells were treated with 100 μM AHC-52 for 1 hr, percent specific release of ^{51}Cr was reduced from 79.2 to 62.6% ($P < 0.002$). In contrast, when K562 cells were treated with AHC-52, percent specific release of ^{51}Cr was somewhat augmented (79.2 vs 84.1%; $P < 0.10$). These results clearly demonstrate that this agent inhibits NK cells. Note that the treatment of both effector and target cells with AHC-52 showed greater inhibition of percent specific release of ^{51}Cr than that of either effector or target cells (54.8 vs 62.6%; $P < 0.05$; 54.8 vs 84.1%; $P < 0.001$).

Inhibition of NK cell-mediated cytotoxicity by $F(ab')_2$ of MRK16 mAb. Although it was suggested from the above findings that the inhibitory effects of nicardipine and AHC-52 were not due to calcium channel blocking activity, and that AHC-52 is a selective inhibitor for P-glycoprotein, the possibility still remained that P-glycoprotein is not involved in this inhibition. Therefore, we examined whether $F(ab')_2$ of MRK16 mAb inhibited cytotoxicity. As shown in Fig. 6, $F(ab')_2$ of MRK16 mAb inhibited cytotoxicity in a concentration-dependent manner, whereas control $F(ab')_2$ did not.

Effect of MDR-reversing agents on conjugate

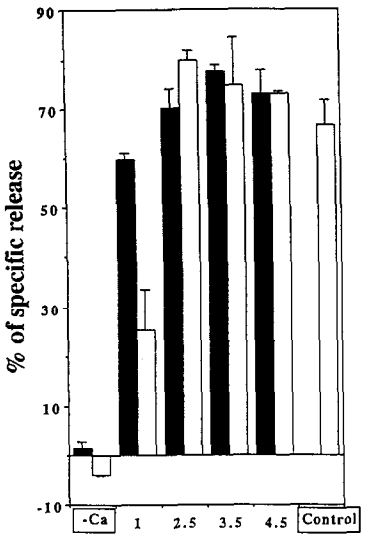

Fig. 7. Effect of AHC-52 on post-binding events of NK cell-mediated cytotoxicity. After incubation of a cultured NK cell-rich population in the presence of 2.5 mM EGTA and 2.5 mM $MgCl_2$, the cells were allowed to bind for 60 min at 37°, after which $CaCl_2$ was added to a final concentration of 5 mM to initiate the post-binding events. The cells were then incubated at 37° for an additional 4 hr, after which ^{51}Cr release was determined. The results are expressed as means ± SD (N = 3). AHC-52 (33 μM) (filled bars) or DMSO (1%) (open bars) was added at the time indicated. "-Ca" denotes the result obtained in the absence of calcium. "Control" denotes the result obtained in the presence of calcium without addition of AHC-52 or DMSO.

formation. Since the initial event for NK cell-mediated cytotoxicity is conjugate formation between NK cells and target cells, we next examined whether AHC-52 inhibited the process, according to the method described by Sidell *et al.* [10]. AHC-52 (33 μM) reduced the percentage of conjugated cells from 49.3 ± 2 to 44.1 ± 5.2% (N = 6; $P < 0.05$), when the cultured NK cell-rich population was mixed with the same number of K562 cells.

Effect of AHC-52 on post-binding events of NK cell-mediated cytotoxicity. To determine whether AHC-52 affects post-binding events, we performed the calcium pulse experiment, which is based on the findings that binding requires magnesium, but not calcium, and that post-binding events can be initiated by the addition of calcium [10]. Figure 7 demonstrates that NK cell-mediated cytotoxicity did not occur in the absence of calcium. Only when AHC-52 was added just after binding, was significant inhibition observed, in comparison with the solvent (DMSO) control ($P < 0.005$).

DISCUSSION

We have demonstrated that the cultured NK cell-rich population expresses the functional P-glycoprotein (Figs 2a, 3 and 4), and that NK cell-mediated cytotoxicity is inhibited by two MDR-reversing agents (nicardipine and AHC-52; Fig. 5) and MRK16 mAb (Fig. 6) in a concentration-dependent manner.

The cell population used in this study contained around 90% of $CD16^+$ and/or $CD56^+$ cells (Fig. 1), confirming that it was a relatively homogeneous NK cell-rich population. Furthermore, NK activity of the population was 10- to 20-fold higher than that in unseparated PBMC (data not shown), in good agreement with enrichment of NK cells. Both flow cytometric analysis with MRK-16 mAb and RT-PCR clearly demonstrated the expression of P-glycoprotein in the cultured NK cell-rich population.

Although no specific inhibitors to P-glycoprotein are known to exist, it should be realized that AHC-52 is structurally similar to nifedipine and nicardipine. On the other hand, regarding their potency for blocking calcium channels, AHC-52 is 500 times less potent than nifedipine, which is 10 times less potent than nicardipine [17, 18]. Therefore, AHC-52 should be 5000 times less potent than nicardipine in this regard, but it was only 3 times less potent than nicardipine as an MDR-reversing agent, suggesting that AHC-52 is a selective inhibitor for P-glycoprotein. Thus, the similar IC_{50} values of nicardipine and AHC-52 for NK cell-mediated cytotoxicity indicated that P-glycoprotein is involved in NK cell-mediated cytotoxicity. Although the reason why the IC_{50} values of MDR-reversing agents for NK cell-mediated cytotoxicity were several-fold larger than those for Rh123 excretion is not known, it may be caused by the different affinities of Rh123 and the unknown substrate in NK cells to P-glycoprotein. Chong *et al.* [19] recently reported that NK activity in unseparated PBMC is inhibited by several MDR-reversing agents. They also used the MDR-reversing agent Ro11-2933, with a low calcium channel blocking activity, and found that its inhibitory activity for NK cell-mediated cytotoxicity is stronger than that of verapamil, although these two compounds are not structurally related.

This study demonstrated concentration-dependent inhibition of NK cell-mediated cytotoxicity by $F(ab')_2$ of MRK16 mAb, providing strong evidence for involvement of P-glycoprotein in NK cell-mediated cytotoxicity. Gupta *et al.* [20] recently reported, using CTL generated in a mixed lymphocyte culture, that MRK16 mAb inhibits killer activity in a concentration-dependent manner. Therefore, these two studies do support the idea that P-glycoprotein is involved in NK cell- and CTL-mediated cytotoxicity.

In our study, both the binding step and the post-binding step were affected by the MDR-reversing agent AHC-52. This was in good agreement with the data reported by Sidell *et al.* [10], but not those reported by Chong *et al.* [19]. It may be the result of a difference in the purity of the NK cells used, namely >90% of large granular lymphocytes for Sidell *et al.*, around 90% of NK cells for our study, and unseparated PBMC for Chong *et al.*, or from a difference in the activation stage of NK cells used, namely activated NK cells for our study and resting NK cells for Sidell *et al.* and Chong *et al.*, although the reason for this discrepancy is not known. The post-binding step includes signal transduction to

initiate the lytic reaction and exocytic release of granule proteins, such as perforin. It is possible that the volume-regulated chloride channel activity associated with P-glycoprotein [3, 4] is involved in the exocytic release [11]. However, there was a report describing the inhibitory effect of an MDR-reversing agent, namely verapamil (*R*+) without calcium channel blocking activity, on mitogen-stimulated IL-2 production of T cells [21], and therefore we cannot exclude the possibility that P-glycoprotein is also involved in such a signal transduction pathway.

In conclusion, our study clearly demonstrated the functional significance of P-glycoprotein in NK cell-mediated cytotoxicity. Future work should clarify which events in the post-binding step are regulated by P-glycoprotein.

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