Biochemical Pharmacology, Vol. 54, pp. 143–148, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00160-3

Reduction of Intracellular pH by Inhibitors of Natural Killer Cell Activity, Nicardipine, 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), and 4,4′-Diisothiocyano-2,2′-disulfonic Acid Stilbene (DIDS)

*Takashi Yamashiro, Naoko Watanabe and Yoshiro Kobayashi**

Department of Biomolecular Science, Faculty of Science, Toho University, Chiba 274, Japan

ABSTRACT. Our previous study showed that nicardipine and its 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, were equally effective in inhibiting natural killer (NK) cell activity, perhaps through inhibition of P-glycoprotein. In this study, we confirmed this finding using a human NK-like cell line, YTN, which is highly cytotoxic to JY cells. The YTN cell-mediated cytotoxicity toward JY cells was inhibited by nicardipine and AHC-52 in a concentration-dependent manner, the concentrations required for 50% inhibition being 14 and 7 μM, respectively. We then examined by flow cytometry whether these reagents modulate the intracellular pH (pH_i), since P-glycoprotein reportedly plays a role in pH_i homeostasis, perhaps by altering chloride translocation. Both reagents reduced pH_i at concentrations similar to those required for inhibition of the cytotoxicity. In addition, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS), an inhibitor of anion exchangers, also inhibited NK cell activity, with an IC_{50} value of 160 μM, and reduced pH_i at a similar concentration, although it is not a P-glycoprotein blocker. Thus, the inhibitory activities of nicardipine, AHC-52, and DIDS toward NK cell activity paralleled their lowering activities of pH_i, suggesting the possibility that disregulation of pH_i is related to inhibition of NK cell activity. BIOCHEM PHARMACOL **54**;1:143–148, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. NK cell; nicardipine; AHC-52; DIDS; intracellular pH

NK† cells play important roles in a variety of immune reactions against tumors, viruses, and microorganisms, as do CTL [1]. There are at least two pathways responsible for this cytotoxicity: granule-exocytosis and Fas-Fas ligand pathways [2]. The granule-exocytosis pathway explains the majority of cytotoxic activity, since perforin-deficient mice were reported to be almost devoid of cytotoxic activity [2].

Overexpression of P-glycoprotein, a product of the *MDR1* gene, is known to confer MDR on cell lines and tumors, and two mechanisms have been proposed for the action of P-glycoprotein. One is that cytotoxic drugs are actively pumped out of cells by P-glycoprotein [3]. The other is that cytotoxic drugs are retained less efficiently due to a decreased plasma membrane electrical potential and/or an elevated pH_i in cells overexpressing MDR proteins [4–7]. In support of the latter mechanism, an MDR-reversing reagent was reported to reverse the "anomalous" pH_i homeostasis caused by P-glycoprotein, and to lower the elevated pH_i [5, 7]. P-Glycoprotein is not only expressed on malignant cells, but has also been detected in normal tissues, such as the adrenal cortex, the lumenal surfaces of the kidneys, liver, jejunum, and pancreas, placental trophoblasts, and endothelial cells in the brain and testis [8]. Moreover, NK cells and CTL express the highest levels of P-glycoprotein among normal mature lymphoid cells [9, 10].

We and others have reported that both NK cells and CTL express functional P-glycoprotein as defined by rhodamine retention and its inhibition by MDR-reversing reagents [11–13]. Moreover, the cell-mediated cytotoxicity is inhibited by both MDR-reversing reagents [11, 12] and a monoclonal antibody against P-glycoprotein (MRK16) [11, 13]. However, the mechanism responsible for the inhibition of cell-mediated cytotoxicity remains to be determined.

Stilbene disulfonates, such as DIDS, were reported to

* Corresponding author: Yoshiro Kobayashi, Ph.D., Department of Biomolecular Science, Toho University, Miyama, Funabashi, Chiba 274, Japan. Tel. and FAX 81-474-72-7696.

† *Abbreviations:* AHC-52, 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; carboxyl-SNARF-1 AM, carboxy-SNARF-1 acetoxymetyl ester; CTL, cytotoxic T lymphocytes; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; MDR, multidrug resistance; and NK, natural killer.

Received 26 November 1996; accepted 3 February 1997.

inhibit the CTL effector function, presumably through blocking transmembrane chloride flux [14]. Since chloride channels and Cl^-/HCO_3 exchangers play critical roles in pH_i homeostasis, we wanted to examine the possibility that pH_i is altered by MDR-reversing reagents and DIDS. In this study, we found that a human NK leukemia cell line, YTN, which is highly cytotoxic toward several target cells such as JY cells through interaction between CD28 and B7 [15], expresses functional P-glycoprotein, and, therefore, we used it as a model for exploring the possibility described above.

MATERIALS AND METHODS

Cells and Reagents

YT cells were established originally from a patient with an acute lymphoblastic lymphoma and a thymoma by Dr. J. Yodoi (Kyoto University) [16], and a subclone of YT, named YTN, was provided by Dr. K. Oshimi (Juntendo University). The EVB-transformed B lymphoblastoid cell line, JY, was provided by Dr. H. Yagita (Juntendo University). These cells and K562 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (GIBCO, Grand Island, NY, U.S.A.), 0.3 g/L of L-glutamine, and 60 mg/L of kanamycin sulfate. Nicardipine and DIDS were purchased from Mediolast (Milano, Italy), and Sigma (St. Louis, MO, U.S.A.), respectively. A nicardipine analog, AHC-52, was provided by Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan).

Flow Cytometry

The expression of P-glycoprotein and cell surface markers in YTN, JY, and K562 cells was analyzed by flow cytometry using a FACScan system (Becton Dickinson, Mountain View, CA, U.S.A.) as previously described [17]. YTN cells were positive for P-glycoprotein and CD28, as shown by flow cytometric analysis with MRK16 and a monoclonal anti-human CD28 antibody (YLEM, Rome, Italy) (data not shown). JY cells were positive for CD80, whereas K562 cells were only marginally positive for it, as revealed by flow cytometric analysis with a monoclonal anti-human CD80 antibody (Ancell Co., Bayport, MN, U.S.A.) (data not shown). For pH_i measurement, YTN cells were loaded for 2 hr with 10 μM carboxyl-SNARF-1 AM (Molecular Probe Inc., Eugene, OR, U.S.A.) at a cell density of 10^6 cells/mL in RPMI 1640 medium containing 10 mM HEPES (pH 7.2). The cells were then centrifuged and suspended in Na^+ buffer composed of 137 mM NaCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.9 mM $CaCl_2$, and 5 mM glucose, followed by analysis with a flow cytometer with excitation at 488 nm, the emission being measured at 585 (FL2) and 640 nm (FL3).

Assay of 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 [15]. Effector cells were mixed with target cells in appropriate ratios, followed by incubation for 4 hr.

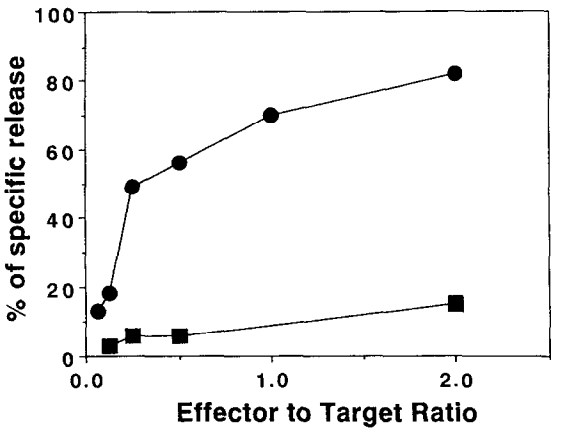

FIG. 1. Cytotoxic activity of YTN cells. The cytotoxic activity of YTN cells toward JY cells or K562 cells was assayed in duplicate according to the general method [15]. YTN cells were mixed with 2 × 10^4 of ^{51}Cr-labeled JY cells (●) or K562 cells (■) at various effector to target ratios, followed by incubation for 4 hr. The results are expressed as percent of specific release, which was defined as (experimental release − spontaneous release)/(total radioactivity − spontaneous release) × 100. Experiments were carried out three times, and the representative result is shown.

RESULTS

YTN Cell-Mediated Cytotoxicity, and its Inhibition by Nicardipine, AHC-52, and DIDS

YT cells were reported to be cytotoxic toward EB virus-transformed B cells, such as JY cells, but not K562 cells [15]. We confirmed this using YTN cells, a subline of YT cells, as effector cells. As shown in Fig. 1, YTN cells were highly cytotoxic toward JY cells in an effector to target ratio-dependent manner, showing specific release of around 80% at the effector to target ratio of 2. In contrast, they were only marginally cytotoxic toward K562 cells, showing specific release of around 15% at the effector to target ratio of 2.

YTN cells were found to express functional P-glycoprotein, as shown by flow cytometric analysis with MRK16 and the inhibition of rhodamine release by two MDR-reversing reagents, nicardipine and AHC-52, the IC_{50} values being 2.0 ± 0.3 and 3.2 ± 0.5 μM, respectively. These values were in good agreement with those for a cultured NK cell-rich population [11].

We then examined whether these reagents inhibited the YTN cell-mediated cytotoxicity. As shown in Fig. 2, both nicardipine and AHC-52 inhibited the YTN cell-mediated cytotoxicity in a concentration-dependent manner; the IC_{50} values at which ^{51}Cr release at the given effector to target ratio (for instance, 2) was reduced to that at half of the effector to target ratio (in this case, 1) were 13.7 ± 3.8 and 6.8 ± 1.7 μM (mean ± SEM of three independent

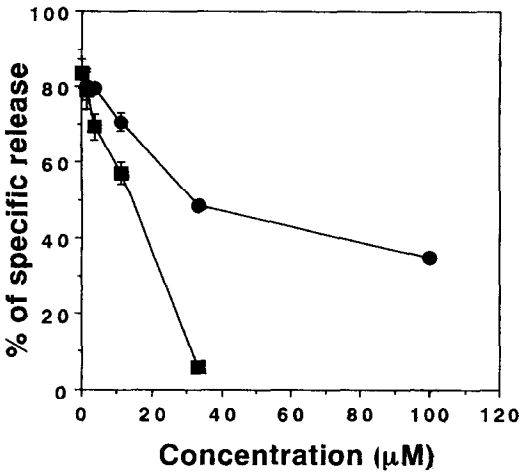

FIG. 2. Effects of nicardipine and AHC-52 on YTN cell-mediated cytotoxicity. The cytotoxic activity of YTN cells toward JY cells was assayed at the effector to target ratio of 2 in the presence of nicardipine (●) or AHC-52 (■) according to the method described in the legend of Fig. 1. Values are expressed as means ± SD. Experiments were carried out three times, and the representative result is shown.

determinations), respectively. Such definition of IC_{50} has proven useful in comparing IC_{50} values of NK cells from different donors [11]. These values were also in good agreement with those for a cultured NK cell-rich population [11].

In addition, we confirmed a previous report that DIDS inhibits CTL-mediated cytotoxicity [14] using YTN cells as effector cells, as shown in Fig. 3. In this case, the effector to target ratio was 0.25, and the IC_{50} was determined to be 159 ± 52 μM (mean ± SEM of three independent determinations). DIDS, even at 1 mM, did not inhibit

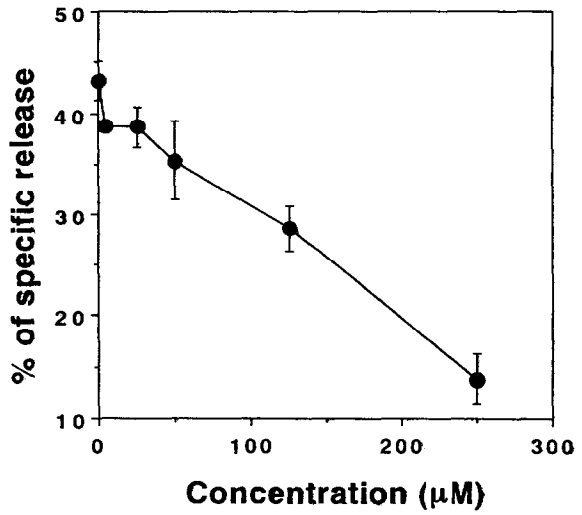

FIG. 3. Effect of DIDS on YTN cell-mediated cytotoxicity. The cytotoxic activity of YTN cells toward JY cells was assayed at the effector to target ratio of 0.25 in the presence of DIDS according to the method described in the legend of Fig. 1. Values are expressed as means ± SD. Experiments were carried out three times, and the representative result is shown.

rhodamine release (Fig. 4) and, therefore, was assumed to be a non-MDR-reversing reagent.

pH_i Measurement by Flow Cytometry

We then examined by flow cytometry whether nicardipine, AHC-52, and DIDS alter pH_i. When pH_i was equilibrated with various extracellular pHs (6.2, 7.2, and 8.8) by nigericin, SNARF-1-loaded YTN cells shifted from the lower right of the diagonal to the upper left, as shown in Fig. 5 (A–C). If we insert a single line extending outward from the origin to facilitate discrimination of the pattern, cells to the right of this line are more acidic, whereas cells to the left are more alkaline. When the ratio of the number of cells to the left of this line to that to the right of this line was adopted as the relative indicator of pH_i, the ratios of 0.07, 1.86, and 2.43 corresponded to extracellular (equal to intracellular in the presence of nigericin) pHs of 6.2, 7.2, and 8.8, respectively. It should be noted here that each dot does not necessarily denote a single event, and thus the ratio calculated above may be different from the "ratio" visually estimated.

We then examined whether inhibitors of NK activity, nicardipine, AHC-52, and DIDS, lowered pH_i. As shown in Fig. 6, nicardipine reduced pH_i in a concentration-dependent manner. The ratios of 0.94, 1.35, and 1.63 corresponded to 33 μM nicardipine (Fig. 6A), 11 μM nicardipine (Fig. 6B), and DMSO 1% (solvent control) (Fig. 6C), respectively. This was also true for AHC-52 [the ratios of 0.29, 0.98, and 2.08 corresponded to 33 μM AHC-52 (Fig. 7A), 11 μM AHC-52 (Fig. 7B), and DMSO 1% (solvent control) (Fig. 7C), respectively] and for DIDS [the ratios of 0.59, 1.76, and 2.59 corresponded to 250 μM DIDS (Fig. 8A), 125 μM DIDS (Fig. 8B), and DMSO 1% (solvent control) (Fig. 8C)].

DISCUSSION

In this study, YTN cells were found to express functional P-glycoprotein, as shown by flow cytometric analysis with MRK16 and the inhibition of release of Rh123 by nicardipine and AHC-52. Therefore, we assumed that YTN cells were a good model for exploring the role of P-glycoprotein in cell-mediated cytotoxicity. Here we present data showing that nicardipine, AHC-52, and DIDS commonly inhibited the YTN cell-mediated cytotoxicity and lowered the pH_i in YTN cells. Although the concentration–response relations were difficult to compare, these reagents exerted their actions at similar concentrations in both cases, supporting the possibility that lowering of pH_i may be associated with the inhibition of NK activity by these reagents.

Flow cytometric measurement of pH_i was reported previously to demonstrate intracellular acidification in association with the induction of apoptosis upon withdrawal of interleukin 2 [18]. When pH_i is measured spectrofluorometrically, only the average pH_i in an entire population can be estimated, so if there is pH_i heterogeneity in a given

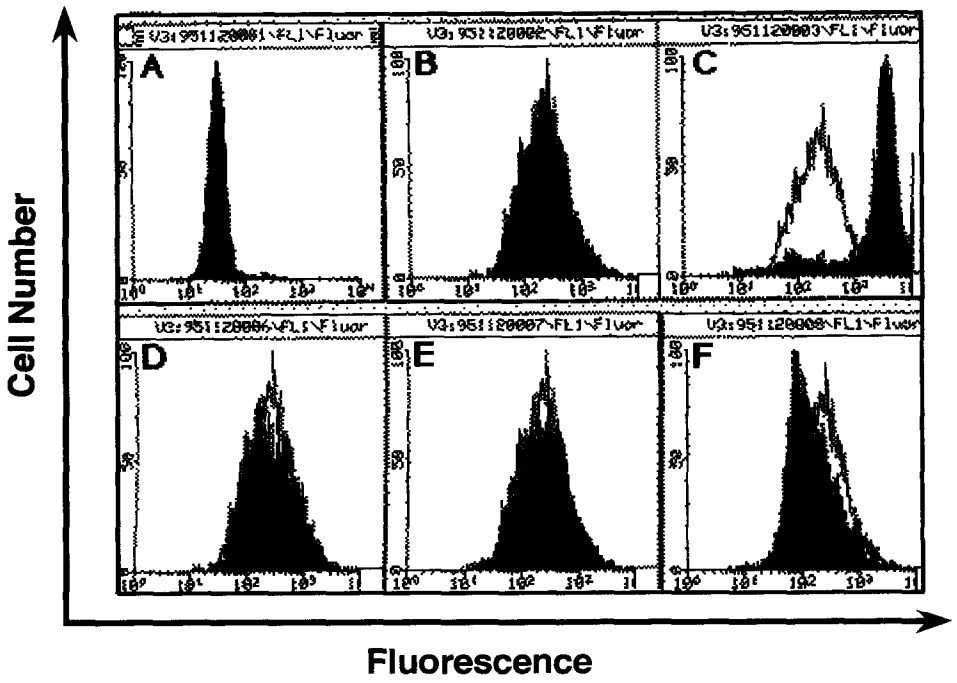

FIG. 4. Release of Rh123 from YTN cells and lack of effect by DIDS. The function of P-glycoprotein expressed in YTN cells was examined by flow cytometric analysis of the release of Rh123 at 37° in the presence or absence of various concentrations of DIDS, as previously described [11]. As a negative control, the release of Rh123 at 4° was examined. The experimental profiles are shaded, while the control profile at 37° is outlined. (A) Unstained, (B) 37°, (C) 4°, (D) 1 mM DIDS, (E) 0.5 mM DIDS, and (F) 1% DMSO.

population, this method has serious drawbacks. In contrast, the flow cytometric analysis performed in this study should be superior to conventional spectrofluorometry in delineating the altered pH_i in a subpopulation, although there was no apparent pH_i heterogeneity in our samples. Since our FACScan system cannot be used to calculate the mean ratios between FL2 and FL3, we adopted the ratio of the number of cells to the left of a line extending outward from the origin to that to the right of this line as the relative indicator of pH_i. This ratio was reduced from 1.63 to 0.94 by 33 μM nicardipine, from 2.08 to 0.29 by 33 μM AHC-52, and from 2.59 to 0.59 by 250 μM DIDS. Although we examined the alteration of pH_i by 100 μM nicardipine and 100 μM AHC-52, the cells were stained with carboxyl-SNARF-1 AM differently from the other cells, providing no meaningful data. It should be noted here that the ratios thus calculated should not be converted to pH_i, because the ratios are not the same as the mean ratios between FL2 and FL3. DIDS was reported to inhibit Cl^-/HCO_3^- exchange without immediately lowering pH_i [19], but in our study YTN cells were pretreated with DIDS for 2 hr. Although the reason for such a discrepancy is not known at present, long exposure to DIDS may lower pH_i.

Intracellular pH is maintained through the concerted

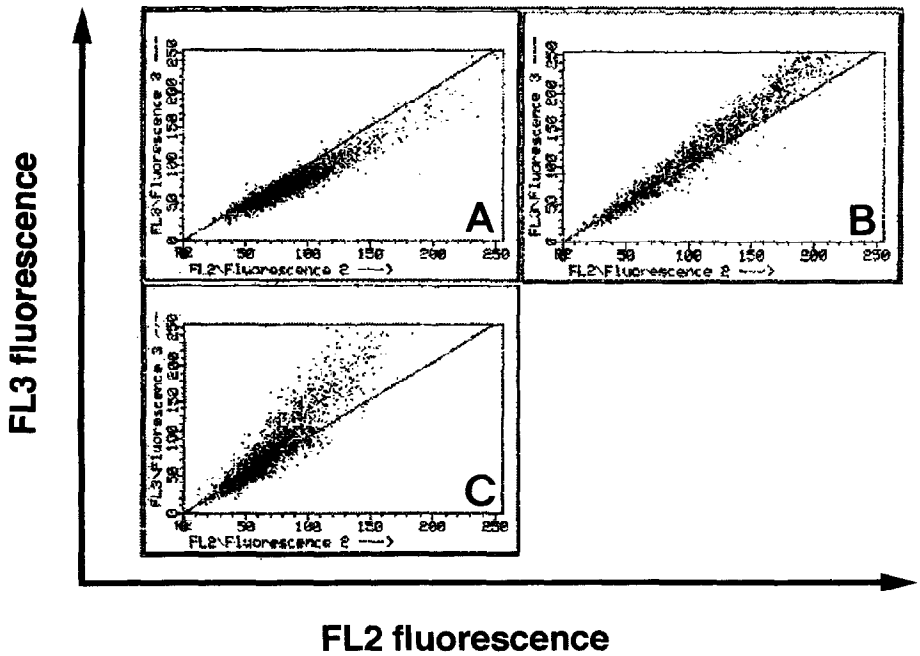

FIG. 5. Flow cytometric analysis of pH_i of SNARF-1-loaded YTN cells. SNARF-1-loaded YTN cells were washed with K^+ buffer comprised of 140 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 5 mM glucose, and 25 mM HEPES (A: pH 6.2; B: 7.2; C: 8.8), and then plated on 24-well plates. After treatment with 10 μM nigericin for 2 hr, the cells were analyzed with a flow cytometer.

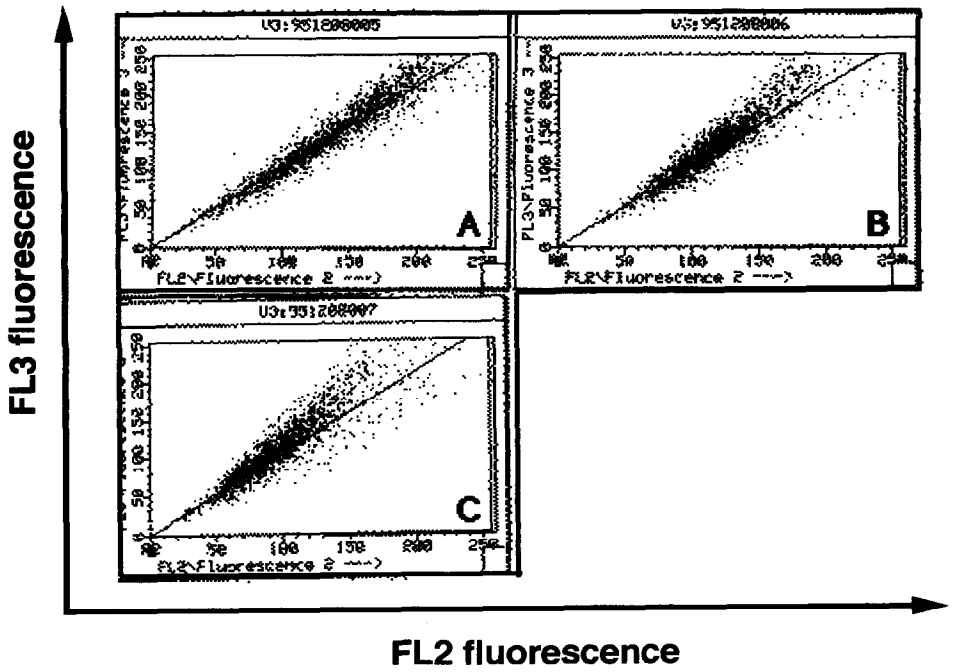

FIG. 6. Flow cytometric analysis of pH_i of YTN cells treated with nicardipine. After treatment of YTN cells with 33 μM (A) and 11 μM (B) nicardipine, and 1% DMSO (C) for 2 hr, the cells were loaded with SNARF-1 as described under Materials and Methods. The cells were then analyzed with a flow cytometer. A single line was drawn extending outward from the origin to facilitate discrimination of the pattern.

actions of Na^+/H^+ antiporters, Cl^-/HCO_3^- exchangers, and other channels and/or transporters that exist within the plasma membrane of eucaryotic cells. P-Glycoprotein has also been reported to be involved in pH_i homeostasis [4–7], and some cells expressing high amounts of P-glycoprotein exhibit alkaline pH, which is reversed by P-glycoprotein modulators [5]. Although more extensive study is required to reveal the characteristics of pH_i homeostasis in YTN cells, our study suggests that lowering of pH_i is related to the inhibition of NK activity by nicardipine and AHC-52.

Since the YTN cell-mediated cytotoxicity required extracellular calcium (Yamashiro T and Kobayashi Y, unpublished data), granule-exocytosis may constitute a major mechanism for the cytotoxicity. However, because YTN cells spontaneously secrete significant amounts of granzymes A and B (our unpublished data), it is difficult to show that P-glycoprotein-mediated regulation of pH_i is critical for granule-exocytosis. The development of an assay system for granule-exocytosis independent from enzyme secretion is required for future study.

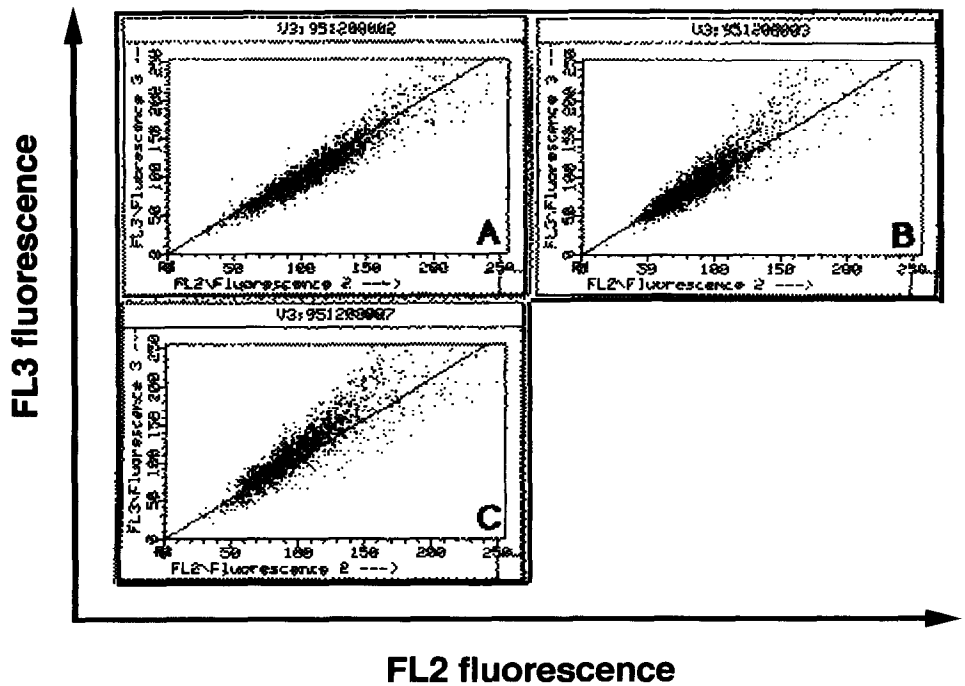

FIG. 7. Flow cytometric analysis of pH_i of YTN cells treated with AHC-52. After treatment of YTN cells with 33 μM (A) and 11 μM (B) AHC-52, and 1% DMSO (C) for 2 hr, the cells were loaded with SNARF-1 as described under Materials and Methods. The cells were then analyzed with a flow cytometer. A single line was drawn extending outward from the origin to facilitate discrimination of the pattern.

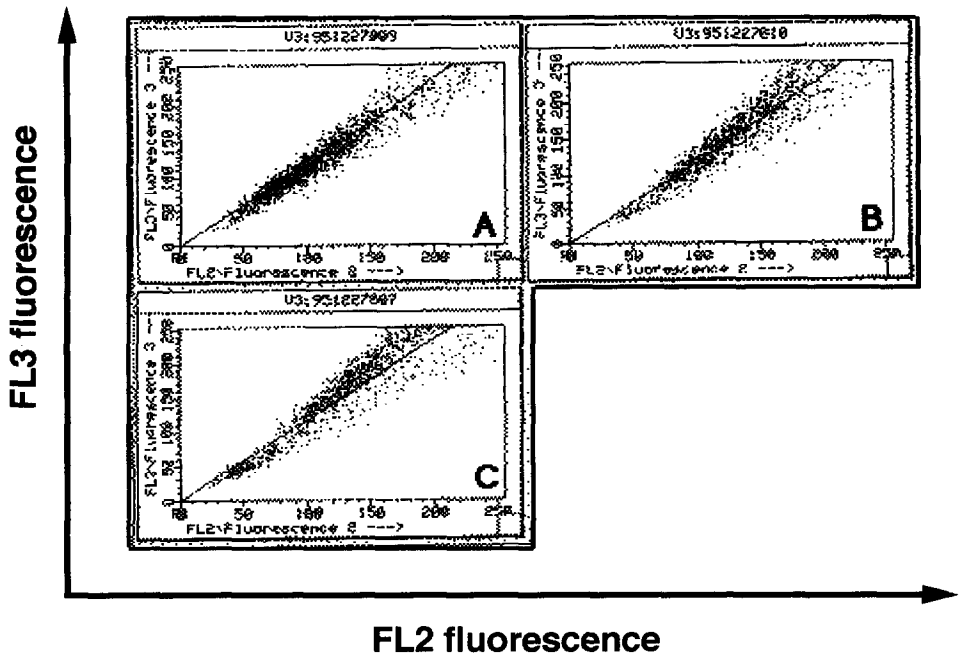

FIG. 8. Flow cytometric analysis of pH_i of YTN cells treated with DIDS. After treatment of YTN cells with 250 μM (A) and 125 μM (B) DIDS, and 1% DMSO (C) for 2 hr, the cells were loaded with SNARF-1 as described under Materials and Methods. The cells were then analyzed with a flow cytometer. A single line was drawn extending outward from the origin to facilitate discrimination of the pattern.

This work was supported, in part, by a Grant-in-Aid from the Uehara Memorial Foundation.

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