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Effect of ziram on natural killer, lymphokine-activated killer, and cytotoxic T lymphocyte activity

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Abstract Ziram is a carbamate pesticide, which is widely used throughout the world as a fungicide in agriculture and as an accelerating agent in latex production. In the present study, we investigated the effect of ziram at 0.031–4 μM in vitro on human natural killer (NK) and lymphokine-activated killer (LAK) and murine cytotoxic T lymphocyte (CTL) activity and found that it significantly inhibited all three activities in a concentration-dependent manner. To explore the mechanism of ziram-induced inhibition of NK activity, NK-92MI cells, a human NK cell line, were used. We previously confirmed that NK-92MI cells express CD56, perforin, granzyme (Gr) A, GrB, Gr3/K, and granulysin and are highly cytotoxic to K562 cells in the chromium release assay. NK-92MI cells were treated with ziram at 0.125–4 μM for 4 or 16 h at 37°C in vitro. Then, intracellular levels of perforin, GrA, GrB, Gr3/K, and granulysin were determined by flow cytometry. It was found that ziram significantly reduced Gr3/K, granulysin, perforin, GrA, and GrB levels. The extent of the decrease differed among the proteins, and the order was as follows: Gr3/K > granulysin > perforin, GrA, and GrB. Taken together, these findings suggest the ziram-induced inhibition of NK, LAK, and CTL activities to be at least partially mediated by decreases in the intracellular levels of Gr3/K, granulysin, perforin, GrA, and GrB.

Keywords CTL · Granulysin · Granzyme · LAK · NK activity · Perforin · Ziram

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Introduction

Ziram is a carbamate pesticide, which is widely used throughout the world as a fungicide in agriculture and as an accelerating agent in latex production (Richardson 1993). We previously found that ziram induces apoptosis and necrosis in U937 cells, a monocyte cell line (Li et al. 2011). Several studies have found that ziram significantly inhibited natural killer (NK) activity (Whalen et al. 2003; Wilson et al. 2004; Taylor et al. 2005; Taylor and Whalen 2009); however, the precise mechanism underlying this inhibition is still unclear. In addition, there has been no study investigating the effect of ziram on cytotoxic T lymphocyte (CTL) and lymphokine-activated killer (LAK) activity.

It has been reported that NK, CTL, and LAK cells kill target cells through the directed release of cytolytic granules containing perforin, granulysin (Clayberger and Krensky 2003; Okada et al. 2003; Li et al. 2008), and granzymes including granzyme (Gr) A, GrB, Gr3/K, GrH, and GrM in humans (Beresford et al. 1997; Smyth and Trapani 1995; Hirata et al. 2006; Li et al. 2005, 2008). To explore the mechanism of ziram-induced inhibition of NK activity, we tried to investigate whether ziram affects intracellular levels of perforin, granulysin, and granzymes. NK-92MI is an IL-2-independent NK cell line derived from NK-92 cells by transfection with human IL-2 cDNA (Tam et al. 1999). The cell line expresses high levels of the CD56 surface marker and intracellular perforin, GrA, GrB, Gr3/K, and granulysin (Li et al. 2005, 2006, 2007, 2008) and is cytotoxic to a wide range of malignant cells, killing both K562 (Li et al. 2006, 2007, 2008) and Daudi (Tam et al. 1999) cells in chromium release assays.

In the present study, we first investigated the effect of ziram on NK, CTL, and LAK activities then used the

NK-92MI cell line to examine its effect on intracellular levels of perforin, granulysin, and GrA, GrB, and Gr3/K for exploring the mechanism of ziram-induced inhibition of NK activity.

Materials and methods

Reagents

Alpha minimum essential medium (α-MEM) without ribonucleosides and deoxyribonucleosides, inositol, 2-mercaptoethanol (2-ME), folic acid, and glutamine, mitomycin C (MMC) were obtained from Sigma (St. Louis, MO). RPMI 1640 medium was purchased from GIBCO. Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS), and heat-inactivated at 56°C for 30 min prior to use. Sodium ^{51}Cr-chromate was obtained from Perkin Elmer (Boston, MA). Fluorescein isothiocyanate (FITC)-mouse anti-human perforin (IgG2b), GrA (IgG1), GrB (IgG1), FITC/PE-negative isotypic control antibodies, and Cytofix/Cytoperm and Perm/Wash solutions were purchased from BD PharMingen (San Diego, CA). Mouse anti-human granzyme 3/K (Gr3/K, IgG2b) monoclonal antibody and mouse IgG2b as an isotypic control were purchased from Abcom (Cambridge, UK). PE-goat anti-mouse IgG was purchased from Vector Laboratories Inc. (Burlingame, CA). Rabbit anti-human granulysin polyclonal antibody was described previously (Li et al. 2005, 2006). Ziram was purchased from Wako Pure Chemical Industries (Osaka, Japan) and prepared as stock solutions in DMSO. Human recombinant IL-2 (rIL-2) was kindly provided by Takeda chemical industries (Osaka, Japan).

Animals

The animals used in this study were specific pathogen-free (SPF) male C57BL/6 J mice aged 6 weeks. The mice were acclimatized for at least 1 week before the study. They were housed in groups (2–3/cage) in an air-conditioned SPF room with a 12-h light/dark cycle and provided with sterile food and water ad libitum. The experiments were performed in accordance with the Guidelines for Animal Experiments prepared by the Committee for the Welfare of Experimental Animals of Nippon Medical School.

Cell lines

The NK-92MI cell line was obtained from ATCC (Manassas, VA) and was maintained in α-MEM without ribonucleosides and deoxyribonucleosides with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 0.2 mM inositol, 0.1 mM 2-ME, 0.02 mM folic acid, and 10% FBS at 37°C in a 5% CO_2 incubator (Li et al. 2005, 2006, 2008). The K-562 and YAC-1 target cell lines were also obtained from ATCC and were maintained in RPMI 1640 medium containing 10% FBS.

Preparations for CTL and LAK cells

The ethics committee of the Nippon Medical School approved this study. The samples of human peripheral blood were obtained from healthy donors with consent. Human LAK cells were generated by incubating human peripheral blood lymphocytes at 1×10^6 cells/ml in medium containing 575 units/ml of rIL-2 for 72 h at 37°C in 5% CO_2 in a 24-well culture plate (Li et al. 2000, 2002). Murine CTL cells were produced by incubating C57BL/6 J mouse splenocytes (5×10^6 cells/ml) with MMC-treated YAC-1 sensitizer cells (10^5 cells/ml) at a 50:1 ratio in a culture dish for 120 h at 37°C in 5% CO_2 as described previously (Li et al. 2000, 2004). Cell lines used as targets were K-562 cells for assaying human NK and LAK activities, and YAC-1 cells for assaying murine CTL activity (Li et al. 2000, 2002, 2008).

Treatment with ziram

In Japan, the residual standards for ziram in rice and potato are 0.3 and 0.2 ppm, calculated as carbon disulfide, respectively (http://ceis.sppd.ne.jp/fs2006/factsheet/pdf/1-249.pdf). Thus, 0.031–4 μM (approximately 0.01–1.25 ppm) of ziram was applied in the present study.

The NK, CTL, and LAK cells at 1×10^5/ml were treated with ziram at 0 (0.1% DMSO), 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2, or 4 μM for 1, 2, 4, or 16 h at 37°C in a 5% CO_2 incubator and then harvested and washed twice with PBS/RPMI-1640 medium for the subsequent NK, LAK, CTL activity assays and flow cytometric analysis.

NK, LAK, and CTL activity assay

A standard microtiter ^{51}Cr-release assay was used to measure NK, LAK and CTL activities as described previously (Li et al. 2000, 2006, 2007). Briefly, target cells (100 μl at 1×10^7 cells/ml) were labeled with a sodium ^{51}Cr-chromate solution (100 μCi) for 60 min at 37°C in 5% CO_2 and washed 4 times in RPMI 1640 medium containing 10% FBS. The target cells (100 μl at 1×10^5/ml) were plated into round-bottomed 96-well microplates, and then the effector cells treated with ziram were added to the wells in triplicate at different effector/target (E/T) ratios. Following a 4-h incubation at 37°C in 5% CO_2, the microplates were centrifuged at 1,500 rpm for 5 min, and 0.1 ml of supernatant from each well was collected and measured for activity

in a gamma counter. The cytolytic activity induced by NK-92MI was calculated by averaging cpm for triplicate wells as described previously (Li et al. 2006, 2007).

Cell staining and flow cytometric analysis

In the present study, we used the fluorescent intensity of FITC-perforin/GrA/GrB and PE-Gr3/granulysin, which represents the intracellular levels of perforin, GrA, GrB, Gr3/K, and granulysin in NK-92MI (Li et al. 2005, 2008), and the percentages of perforin, GrA, GrB, Gr3/K, and granulysin-positive cells among NK-92MI cells to evaluate the effect of ziram. The NK-92MI cells were fixed and permeabilized with the Cytofix/Cytoperm solution for 20 min at 4°C, and then intracellular perforin, GrA, and GrB were stained with FITC anti-human perforin, GrA, and GrB for 30 min at 4°C according to the manufacturer's instructions (BD PharMingen, San Diego, CA). Intracellular granulysin was stained with a rabbit anti-human granulysin polyclonal antibody after fixation/permeabilization with the Cytofix/cytoperm solution and then stained with PE-goat anti-rabbit IgG for 30 min at 4°C in the dark. Intracellular Gr3/K was stained with a mouse anti-human G3/K monoclonal antibody after fixation and permeabilization with the Cytofix/cytoperm solution and then stained with PE-goat anti-mouse IgG for 30 min at 4°C in the dark. After staining, the cells were washed twice with the cytoperm solution and once with PBS containing 1% FBS. Flow cytometric analysis was performed with a FACScan flow cytometer (Becton–Dickinson, San Jose, CA) as described previously (Li et al. 2005, 2006, 2008). NK-92MI cells were identified by their characteristic appearance on a dot plot of FSC versus SSC and electronically gated to exclude dead cells and debris (Li et al. 2008).

Statistical analyses

Statistical analyses were performed using a one-way ANOVA followed by a post hoc test, Tukey's test, with SPSS 16.0 J software for Windows. A linear correlation analysis and unpaired *t*-test were also used. The significance level for *P* values was set at <0.05.

Results

Effects of ziram on NK, CTL, and LAK activities

As shown in Fig. 1a, ziram significantly inhibited human NK activity in NK-92MI cells in vitro in a concentration- and time-dependent manner ($r_{1h} = -0.783$, $P < 0.05$, 0–4 μM; $r_{2h} = -0.727$, $P < 0.05$, 0–4 μM; $r_{4h} = -0.806$, $P < 0.05$, 0–1 μM). However, there was almost no difference between 2 and 4 μM, suggesting that the maximal effective concentration for inhibiting NK activity should be 2 μM. Based on the results of NK activity, we treated human LAK cells and murine CTL cells with ziram for 2 h to investigate the dose effect of ziram on the two effector cells. As shown

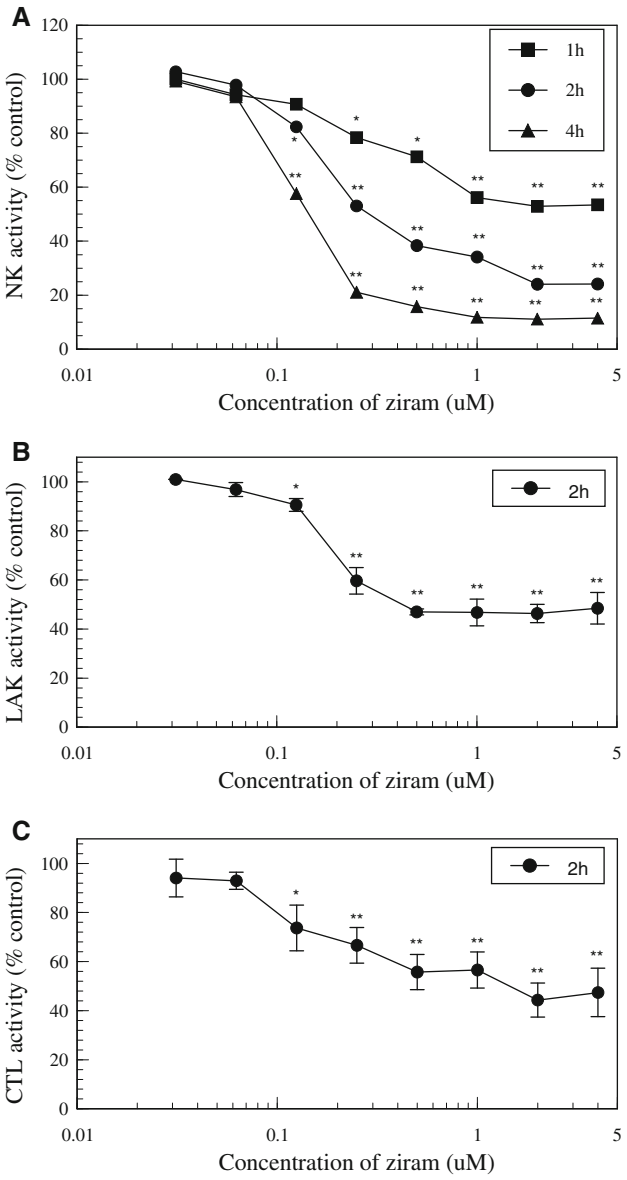

Fig. 1 Effect of ziram on human NK (**a**) and LAK (**b**) activities and murine CTL activity (**c**). **a** Concentration- and time-dependent inhibition of human NK activity ($n = 5$), $r_{1h} = -0.783$ ($P < 0.05$, 0–4 μM), $r_{2h} = -0.727$ ($P < 0.05$, 0–4 μM), $r_{4h} = -0.806$ ($P < 0.05$, 0–1 μM); **b** Concentration-dependent inhibition of human LAK activity by ziram (Mean ± SE, $n = 5$), $r = -0.745$ ($P < 0.05$, 0–2 μM); **c** Concentration-dependent inhibition of murine CTL activity by ziram (Mean ± SE, $n = 5$), $r = -0.715$ ($P < 0.05$, 0–4 μM). NK cells were treated with ziram for 1, 2, or 4 h, respectively, and LAK and CTL cells were treated with ziram for 2 h only. One-way ANOVAs indicated that the concentration of ziram significantly affected NK, LAK, and CTL activity (all $P < 0.01$). *$P < 0.05$, **$P < 0.01$, significantly different from 0 μM by Tukey's test

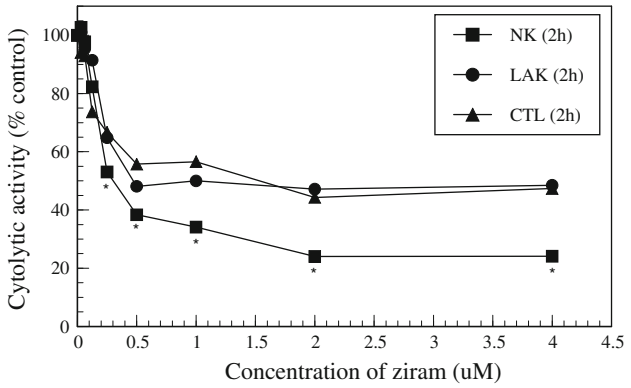

Fig. 2 Difference in sensitivity to ziram among human NK, human LAK, and murine CTL activity. NK, LAK, and CTL cells were treated with ziram for 2 h, respectively. *$P < 0.05$, significantly different from LAK and CTL activity by unpaired t test

in Fig. 1b and c, ziram also significantly decreased human LAK and murine CTL activities in vitro in a concentration-dependent manner (LAK: $r = -0.745$, $P < 0.05$, 0–2 μM; CTL: $r = -0.715$, $P < 0.05$, 0–4 μM). Similarly, there was almost no difference between 2 and 4 μM, suggesting that the maximal effective concentration for LAK and CTL activity was also 2 μM. The degree of decrease in these activities differed among the effector cells investigated and the order was as follows: human NK > human LAK, murine CTL (Fig. 2).

Effect of ziram on the intracellular levels of perforin, GrA, GrB, Gr3/K, and granulysin in NK-92MI

As shown in Fig. 3a, ziram induced a marked shift in the peak of PE-anti-Gr3/K to the left, which means that the fluorescent intensity became weaker after the treatment. Both the fluorescent intensity of PE-anti-Gr3/K and the percentage of Gr3/K-positive cells significantly decreased in a concentration-dependent manner (Fig. 3b, c).

In Fig. 4, one-way ANOVAs indicated that the concentration of ziram significantly affected the intracellular level of the five anti-cancer proteins (all $P < 0.05$). However, the extent of the effect differed among the proteins. GrA decreased with 0.5 μM; then, no further effects are obtained with the higher concentrations although there were significant differences between 0.5, 2, or 4 μM and 0 μM. Similarly, perforin decreased with 1 μM, and then it was stable with the following higher concentrations; there were significant differences between 1, 2, or 4 μM and 0 μM. There was a significant difference only between 4 and 0 μM in GrB. On the other hand, ziram significantly reduced the intracellular levels of Gr3/K and granulysin in NK-92MI cells in a concentration-dependent manner. Taken together, the extent of the decrease differed among the proteins, and

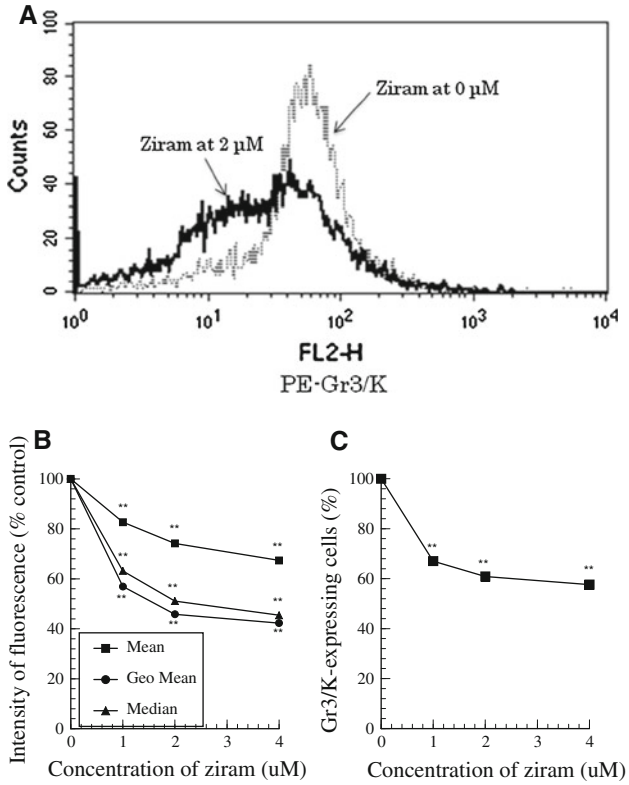

Fig. 3 Effect of ziram treatment for 16 h on the intracellular level of Gr3/K (**a**, **b**) and the percentage of Gr3/K-expressing cells (**c**) in NK-92MI. All experiments were repeated at least 3 times with similar results. One-way ANOVAs indicated that the concentration of ziram significantly affected the intracellular level of Gr3/K and the percentage of Gr3/K-expressing cells in NK-92MI ($P < 0.01$). **$P < 0.01$, significantly different from 0 μM by Tukey's test

the order was as follows: Gr3/K > granulysin > perforin, GrA, and GrB. Figure 5 further showed that ziram significantly reduced the intracellular levels of Gr3/K and granulysin in NK-92MI cells in a concentration-dependent manner at lower concentrations, suggesting that ziram has a similar effect on Gr3/K and granulysin at lower concentrations.

Discussion

The present study demonstrated that ziram significantly inhibited NK, CTL, and LAK activities in a concentration-dependent manner, that the degree of inhibition differed among the effector cells investigated, and that human NK cells are more sensitive to ziram than human LAK cells and murine CTL cells. We previously found that human NK cells were more sensitive to organophosphorus pesticides than human LAK cells and murine CTL cells (Li et al. 2002; Li and Kawada 2006), suggesting that NK cells are more susceptible to the effect of chemicals. Although

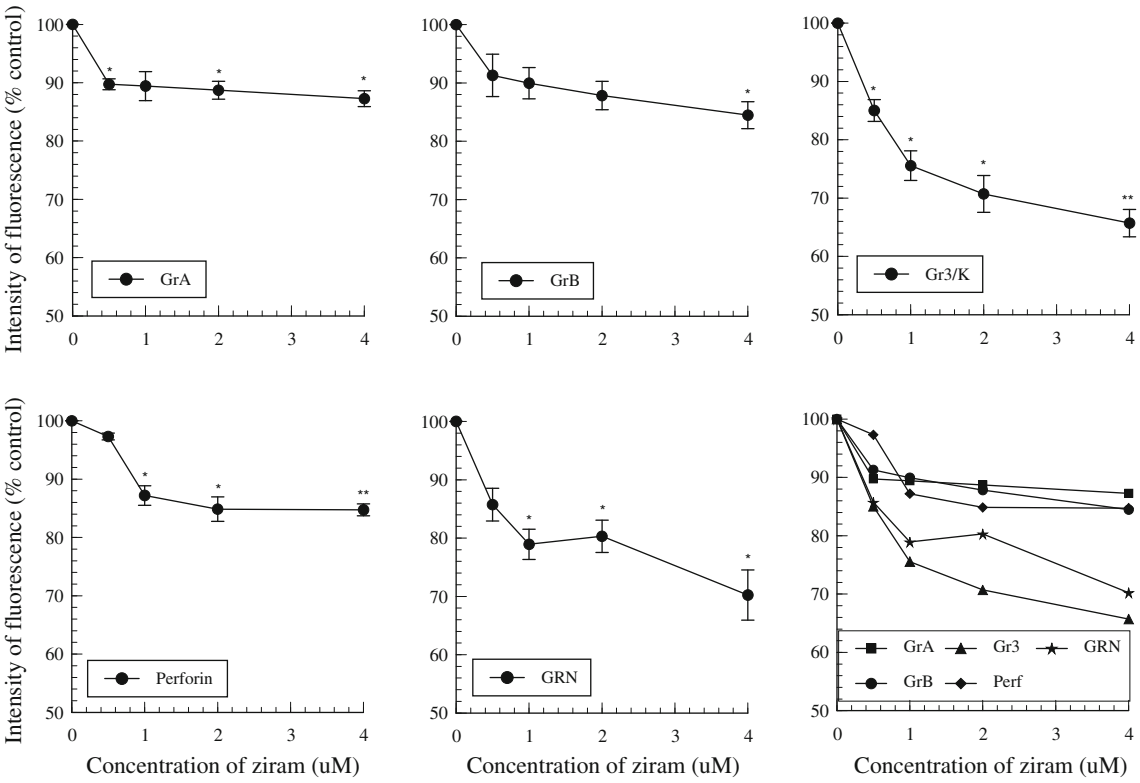

Fig. 4 Effect of ziram treatment for 4 h on the intracellular levels of GrA, GrB, Gr3/K, perforin, and granulysin (GRN) in NK-92MI (Mean ± SD, $n = 3$). The last figure shows the differences in sensitivity to ziram among the five anti-cancer proteins. One-way ANOVAs indicated that the concentration of ziram significantly affected the intracellular level of the five anti-cancer proteins (all $P < 0.05$). *$P < 0.05$, **$P < 0.01$, significantly different from 0 μM by Tukey's test

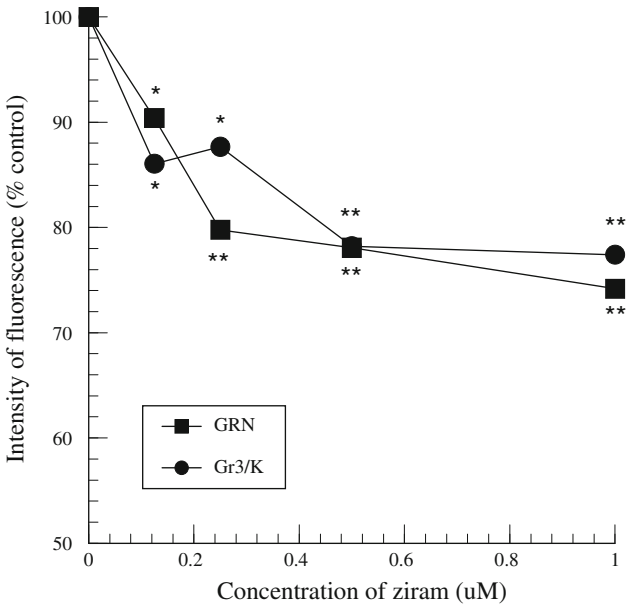

Fig. 5 Effect of ziram treatment for 4 h at lower concentrations on the intracellular levels of Gr3/K and granulysin in NK-92MI. All experiments were repeated at least 3 times with similar results. One-way ANOVAs indicated that the concentration of ziram significantly affected intracellular levels of Gr3/K and granulysin (all $P < 0.05$). *$P < 0.05$, **$P < 0.01$, significantly different from 0 μM by Tukey's test

several studies have reported that ziram inhibits NK activity (Whalen et al. 2003, Wilson et al. 2004, Taylor et al. 2005, Taylor and Whalen 2009), the present study is the first to investigate the effect of ziram on CTL and LAK activities. However, the mechanism underlying the inhibition of NK activity is still unclear.

It has been reported that NK, CTL, and LAK cells kill target cells through the directed release of cytolytic granules containing perforin, granulysin, and granzymes including GrA, GrB, and Gr3/K (Kägi et al. 1994a; Clayberger and Krensky 2003; Okada et al. 2003, Li et al. 2005, 2008), a process known as granule exocytosis. Cytotoxicity mediated by T cells, LAK cells, and NK cells is greatly impaired in perforin-deficient mice (Kägi et al. 1994b; Li et al. 2004). GrA plays a critical role in triggering apoptosis in target cells either directly or via the activation of cellular caspases and also cleaves IL-1β, the nucleosome assembly protein called putative HLA-associated protein II, TAF-Iβ, histones, and lamins (Beresford et al. 1997; Zhang et al. 2001). GrB and Gr3/K were isolated from the rat NK tumor cell line RNK-16 (Shi et al. 1992) and induce caspase-independent apoptosis through the disruption of mitochondrial potential and DNA fragmentation with a similar kinetics to GrA-induced apoptosis (Shi et al. 1992; MacDonald et al.

1999). Granulysin, a lytic molecule expressed by human CTL and NK cells, is active against tumor cells, and a variety of microbes can easily enter target cells in the absence of perforin and eventually induce apoptosis (Clayberger and Krensky 2003; Okada et al. 2003). Granulysin is well associated with the diverse activities of NK and CTL cells in physiological and pathological settings and could be a useful serum marker to evaluate the overall status of host cellular immunity (Ogawa et al. 2003). Thus, to explore the mechanism of ziram-induced inhibition of NK activity, we investigated whether ziram affects the intracellular levels of perforin, granulysin, GrA, GrB, and Gr3/K in NK cells.

As expected, ziram significantly reduced the intracellular levels of perforin, GrA, GrB, Gr3/K, and granulysin in NK-92MI cells, especially in Gr3/K and granulysin, which showed a concentration-dependent manner. The degree of the decrease differed among the proteins, with Gr3/K and granulysin more sensitive to ziram than perforin, GrA and GrB. This is the first report exploring the mechanism of ziram-induced inhibition of NK activity in terms of the granule exocytosis pathway. These findings suggest the ziram-induced inhibition of NK activity to be at least partially mediated by decreases in the intracellular levels of perforin, GrA, GrB, Gr3/K, and granulysin. On the other hand, Taylor and Whalen (2009) reported that ziram affected tumor-cell-binding capacity, cell-surface marker CD16 expression, and ATP levels in human NK cells. However, the results also indicate that ziram-induced losses of lytic function cannot be fully explained by changes in binding, cell-surface protein expression, or ATP levels, suggesting other mechanisms to be involved in the ziram-induced inhibition of NK activity.

Human exposure to ziram may occur by coming into contact with latex rubber, ingesting treated crops, or via inhalation (Taylor and Whalen 2009). In Japan, the residual standards for ziram in rice and potato are 0.3 and 0.2 ppm, calculated as carbon disulfide, respectively (http://ceis.sppd.ne.jp/fs2006/factsheet/pdf/1-249.pdf), suggesting the potential of human exposure from crops. In the present study, we used 0.031–4 μM (approximately 0.01–1.25 ppm) of ziram in vitro, which are reasonable related to the possible human exposure.

Conclusions

The present findings suggest that ziram significantly inhibited NK, CTL, and LAK activity, and the inhibition of NK activity may be at least partially mediated by decreases in the intracellular levels of GrA, GrB, Gr3/K, perforin, and granulysin in NK cells. In addition, they also suggest that determining the effect of chemicals on the levels of perforin, GrA, GrB, Gr3/K, and granulysin in NK cells by flow cytometry can be an alternative to measuring NK activity in a chromium release assay.

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Conflict of interest None.

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