IMMUNOMODULATION OF HUMAN NATURAL KILLER CELL CYTOTOXIC FUNCTION BY TRIAZINE AND CARBAMATE PESTICIDES

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Introduction

Triazine (atrazine) and carbamates (maneb, metiram, zineb and ziram) are currently used agricultural pesticides and are known endocrine disruptors[1]. Atrazine is the most heavily used agricultural pesticide in North America[2,3]. It is widely used in controlling weeds in numerous crops including corn, sorghum, sugarcane, soybeans, wheat, pineapple and various grasses[3,4]. Atrazine has been detected in ground and surface water throughout the United States[2,5,6]. Human exposure pathways for this chemical include occupational exposure through both inhalation and dermal absorption during its manufacture, its formulation and its application by spraying. Atrazine is readily absorbed through gastrointestinal tract[2]. When a single dose of 0.53 mg atrazine was administered to rats by gavage, 20% of the dose was excreted in the feces within 72 hours. The other 80% absorbed across the lining of the gastrointestinal tract into the bloodstream[2]. Maneb, metiram and zineb are ethylenebis (dithiocarbamate) fungicides and are used in the U.S to treat variety of crops[8-11]. These compounds can be metabolized to ethylene thiourea, which is classified as a carcinogen[8]. Residues of zineb have been reported in hay and in wheat (if zineb application occurred later in crop development)[11,12]. Ziram is another dithiocarbamate fungicide used to treat a variety of crops[12,13]. It is also used as an additive in rubber products including latex gloves. Ziram was shown to be a sensitizing agent in a modified local lymph node assay[14] and thus potentially may be a factor in allergic reaction and/or skin irritation in response to latex gloves[15].

The effects of these compounds on human immune function for the most part have not been examined. Human natural killer (NK) lymphocytes play a central role in immune defense against virus infection and formation of primary tumors[16]. NK cells are able to lyse appropriate target cells without prior sensitization, putting them at the forefront of lymphocyte defenses against tumor and virally infected cells[17]. Thus, any agent that interferes with the ability of NK cells to lyse their targets could increase the risk of tumor incidence and/or viral infections. The present study investigated the effects of atrazine, maneb, metiram, zineb and ziram on the ability of human natural killer cells to lyse tumor cells. The compounds were tested in both purified NK cells as well as a cell preparation that contained both T and NK lymphocytes (T/NK cells). Lymphocytes were exposed to the compounds for periods of time ranging from 1 hour to 6 days. Effects of the different compounds on tumor lysis were compared. Additionally, differences in the effect of a given compound on purified NK cells versus T/NK cells were investigated. These data allow for comparison of toxic potential of the 5 compounds. Further, the results indicate that several of these compounds have immunomodulatory effects on both T and NK lymphocyte function.

Material and Methods

Blood Sampling and Isolation of NK Cells: Peripheral blood from healthy adult (male and female), volunteer donors was used for this study. Heparin-treated whole blood or blood bank buffy coats (American Red Cross, Portland, OR) were applied to Ficoll-Hypaque (1.077 g/mL) (Sigma, St. Louis, MO) and centrifuged at 500 g for 30 min to remove red blood cells. Mononuclear cells were collected from the Ficoll-Hypaque and washed twice (250 g, 10 min.) with phosphate buffered saline (PBS). In order to remove platelets, the cells were suspended in a small volume (2.5 - 5 mL) of PBS and layered on to 2.5-5 ml of bovine calf serum (BCS) and centrifuged at 200 g for 5 min. The pellet was resuspended in PBS and the process was repeated. The cells were then suspended in complete medium which consisted of RPMI-1640 supplemented with 10% heat-inactivated BCS, 2 mM L-glutamine and 50 U penicillin G with 50 µg streptomycin/ml. Non adherent cells (30-40% $CD16^+$, 60-70% $CD3^+$) were prepared by incubating the cells in glass Petri dishes (150 X 15 mm) at 37 °C and air/CO_2 , 19:1 for 1 h[17].

Highly purified NK cells were obtained using a rosetting procedure. Buffy coats were mixed with 1 mL of RosetteSep™ Human NK cell enrichment antibody cocktail (StemCell Technologies, Vancouver, British Columbia, Canada) per 30 mL of buffy coat. The mixture was incubated for 1 h at room temperature with periodic mixing. Following the incubation the 4 mL of the mixture was layered onto 4 mL of Ficoll-Hypaque (1.077 g/mL) (Sigma), and centrifuged at 1200g for 20 min. The cell layer was collected and washed twice with PBS and stored in complete media at 1 million cells/mL. The resulting cell preparation was >95% $CD16^+$, 0% $CD3^+$ by fluorescence microscopy[18].

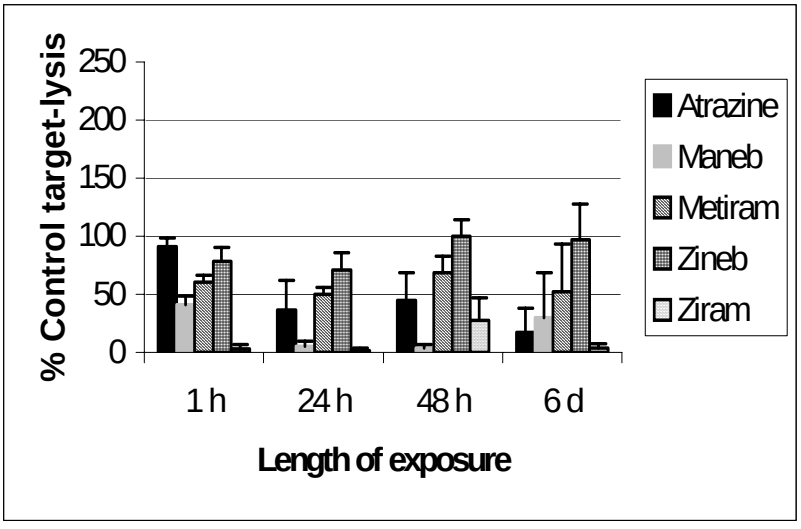
Chemical Preparation: All compounds were obtained from Wako Chemicals, JAPAN. Dimethylsulfoxide (DMSO) was obtained from Sigma Chemical Co. St. Louis, MO, USA). Neat standards were resuspended in DMSO. The chemicals were diluted in gelatin media (0.5% gelatin replaced the calf serum in complete medium) to achieve the desired concentrations, so that the final concentration of DMSO did not exceed 0.01%.

Cell Viability and Cytotoxicity: Cell viability was determined by trypan blue exclusion. Prior to assay, the NK cells were separated by centrifugation from complete medium (RPMI 1640) and transferred to gelatin media. Cell numbers and viability were assessed at the beginning and end of the assays. Cell numbers and their viability did not vary among experimental conditions. NK cytotoxicity was measured using a ^{51}Cr release assay[16]. The target cell in all cytotoxicity assays was the NK-susceptible K562 (human chronic myelogenous leukemia) cell line. An appropriate number of K562 cells were incubated with ^{51}Cr in 1 ml of BCS for 1.5 hr at 37 °C in air/CO_2 (19:1). The cells were then washed twice with gelatin medium. T/NK (effector) cells ($5.0x10^5$/100 µL for 50:1 ratio) were added to the wells of round-bottom microtitre plates. The effectors were diluted to 25:1 ratio ($2.5x10^5$/100 µL), and 12.5:1 ratio ($1.25x10^5$/100 µL); each ratio was tested in triplicate. For highly purified NK cells the ratios used were 12.5:1, 6.25:1, and 3:1.Targets were added ($1x10^4$/100µL) to each well, and the plate was centrifuged at 150 g for 3 min. and incubated for 2 hr at 37 °C (air/CO_2 ,19:1). After incubation 0.1 ml aliquot of the supernatant was collected and counted for radioactivity for 60 sec in a Packard COBRA gamma counter (Packard Instrument Co., Meriden, CT, USA) gamma radiation counter. Specific lysis was calculated as follows: 100x[(test c.p.m - spontaneous c.p.m.)/maximum c.p.m.- spontaneous c.p.m.)]. Maximum release was produced by adding 100 µL of 10% Triton X-100. Statistical analysis of the data was carried out utilizing ANOVA and Student's t-test.

Results and Discussion

Highly purified NK cells were exposed to atrazine, maneb, metiram, zineb, or ziram for 1h, 24h, 48h, and 6 days. The concentrations of a given compound used at each exposure time did not affect cell viability. Figure 1 shows the percent of control (NK) lysis of tumor cells from replicate experiments where pure NK cells were exposed to the compounds for 1h, 24 h, 48 h, or 6d.

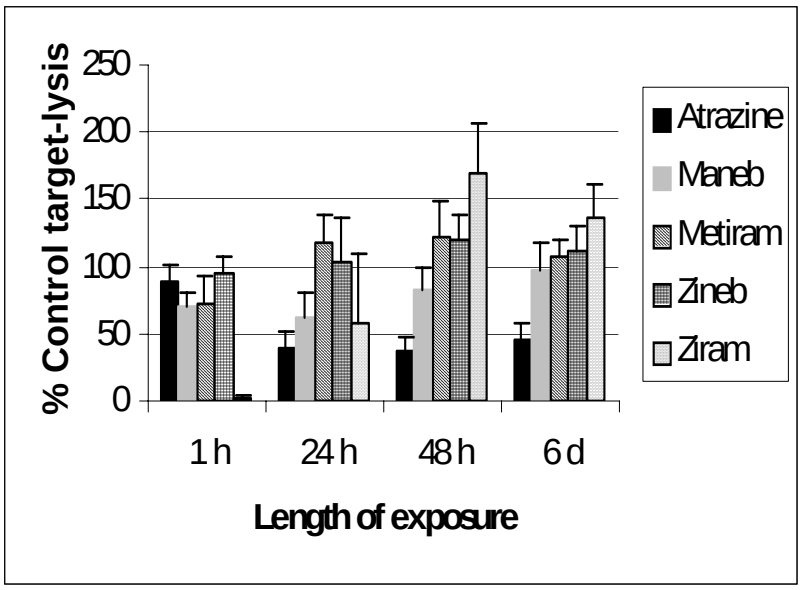
FIGURE 1



After a 1 h exposure to 10 μM of the compounds tumor lysis was inhibited by three of the compounds: maneb, 59±8%; metiram, 40±6%; ziram, 97±4% (12:1 ratio) ($p<0.00001$). A 24h exposure (10 μM for all compounds except ziram) decreased lysis: atrazine, 63±25%; maneb, 95±4%; metiram, 50±6%; ziram (2.5 μM), 99±2% at the 12:1 ratio ($p<0.0001$). A 48 h exposure to 10 μM atrazine, maneb, metiram, zineb decreased lytic function: atrazine, 55±24%; maneb, 95±3%; metiram, 31±15% ($p<0.0001$). The 48 h exposure to ziram was at 1 μM and thus the percent inhibition is somewhat less (72±19%, $p<0.000001$) than at 24h. A 6 d exposure to 10 μM atrazine, maneb, metiram or zineb and 1μM ziram decreased lysis: atrazine, 83±21%; maneb, 70±39%; metiram, 52±41%; ziram 96±4% ($p<0.00001$ for all except metiram ($p<0.01$)).

T/NK cells were exposed to the compounds as described above for the purified NK cells. As with the purified NK cells the concentrations of a given compound used at each exposure time did not affect cell viability. Figure 2 shows the percent of control (T/NK) lysis of tumor cells when T/NK cells were exposed for the same length of time at the same concentrations of the compounds as used for the purified NK cells (replicate experiments).

FIGURE 2



Lysis was inhibited by exposure to maneb and ziram: maneb, 30±11%; ziram, 98±2% ($p<0.001$) (50:1 ratio). A 24 h exposure to 10 μM atrazine, maneb, metiram, zineb, and 2.5 μM Ziram inhibited lysis: atrazine, 61±13% ($p<0.0001$); maneb, 38±18% ($p<0.0005$), ziram, 41±51% ($p<0.05$). When T/NK cells were exposed to 10 μM atrazine, maneb, metiram, zineb or 1 μM ziram for 48 h there was inhibition of cytolytic function only with atrazine, 63±10% ($p<0.00001$). Following a 6 d exposure to 10 μM atrazine, maneb, metiram or zineb and 1μM ziram for a representative experiment again only atrazine inhibited tumor-cell lysis (54±12%) ($p<0.00001$).

The results of these studies indicated that atrazine, maneb, metiram and ziram were able to decrease the cytotoxic function of purified human NK lymphocytes. However, if T cells were present in the lymphocytes preparation the negative effect of maneb, metiram, and ziram on lytic activity was ameliorated. As T cells are capable of altering NK cytotoxicity by secretion of several cytokines, it is possible that this decreased inhibition is due to the secretion of a stimulatory cytokine that reverses/blocks the negative effect of the compound on NK cytotoxic function. Further, based on these studies the relative

toxic potential of this group of compounds was: ziram>maneb>atrazine>metiram>zineb. The presence of T-lymphocytes greatly diminished the effects of the carbamates (maneb, metiram and ziram) on NK cytotoxic function, but not that of the triazine (atrazine). One further implication of the data was that the carbamates may disrupt immune function by a mechanism that is distinct from that of triazine.

References

1. Database of endocrine disruptors. 1998. Research Institute, Tokyo Metropolitan. Japan EPA. 73 pp.
2. Eisler, R. 1989. Biological Report 85, 18.
3. Capel, P. D. and Larson, S.J. 2001. Environ. Sci. Technol. 35, 648-657.
4. Reed 1982. Atrazine. (Unpubl. mimeo.) Available from U. S. Food and Drug Administration, Bureau of Foods, Washington, DC. 6 pp.
5. Kettles, M. A., Browning, S. R., Prince, T. S. and Horstam, S. W. 1997. Environ. Health Perspect. 104:1324-1331.
6. Schaffer, K. and Miller, G. 2002. Kentucky Non-point Source Conference September 23, 2002, 80-81.
7. Buchner, RP, Olson, WH, Adaskaveg, JE, Lindow, SE. 2001. Acta Horticulturae 544, 369-378.
8. Steenland, K, Cedillo,L, Tucker, J, Hines, C, Sorensen, K, Deddens, J, Cruz, V. 1997. Environ. Health Perspect. 105,1126-1130.
9. Shtenberg, AI, Kirlich, AE, Orlova, NV. 1969. Vop. Pitan. 28, 66-72.
10. Morato, GS., Lemos, T., Takahashi, RN. 1989. Neurotoxicology and Teratology 11, 421-425.
11. Kovaleva, ES and Talanov, GA. 1982. Khim. Sel'sk. Khoz. 9, 59-61.
12. Zazimko, MI, Zakhryapina, TD, Pogosov, EK. 1969. Khim. Sel. Khoz. 7, 516.
13. Caldas, ED, Conceicao, MH, Miranda, MC, de Souza, LCKR, Lima, JF. 2001. J. Agricult. And Food Chem. 49, 4521-4525.
14. De Jong, WH, Tentij, M., Spiekstra, SW, Vandebriel, RJ, Van Loveren H. 2002. Toxicology 176, 123-134.
15. Nettis, E, Assennato, G, Ferrannini, A, Tursi, A. 2002. Clin. Exp Allergy 32, 441-447.
16. Lotzova, E. 1993. Nat. Immun. 12, 177-193.
17. Whalen, MM, Williams, TB, Green, SA, and Loganathan, BG. (2002). Environmental Research 88, 199-209.
18. Whalen, MM and Loganathan, BG. 2001. Toxicol. Appl. Pharmacol. 171,141-148.