Review

The Mechanism of Organophosphorus Pesticide-Induced Inhibition of Cytolytic Activity of Killer Cells

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The main toxicity of organophosphorus pesticides (OPs) is neurotoxicity, which is caused by the inhibition of acetylcholinesterase. OPs also affect immune responses including effects on antibody production, IL-2 production, T cell proliferation, decreasement of CD5 cells, and increasement of CD26 cells and autoantibodies. However, there have been few papers investigating the mechanism of OP-induced inhibition of cytolytic activity of killer cells. This study reviews the new mechanism of OP-induced inhibition of activities of natural killer (NK), lymphokine-activated killer (LAK) and cytotoxic T lymphocytes (CTL). NK, LAK and CTL induce cell death in tumor or virus-infected target cells by two main mechanisms. The first mechanism is direct release of cytolytic granules that contain perforin, granzymes, and granulysin by exocytosis to kill target cells, which is called the granule exocytosis pathway. The second mechanism is mediated by the Fas ligand (Fas-L)/Fas pathway. To date, it has been reported that OPs inhibit NK, LAK and CTL activities by at least the following three mechanisms: 1) OPs impair the granule exocytosis pathway of NK, LAK and CTL cells by inhibiting the activity of granzymes, and by decreasing the intracellular level of perforin, granzyme A and granulysin, which was mediated by inducing degranulation of NK cells and by inhibiting the transcript of mRNA of perforin, granzyme A and granulysin; 2) OPs impair the FasL/Fas pathway of NK, LAK and CTL cells, as investigated by using perforin-knockout mice, in which the granule exocytosis pathway of NK cells does not function and only the FasL/Fas pathway remains functional; 3) OPs induce apoptosis of immune cells. *Cellular & Molecular Immunology*. 2006;3(3):171-178.

Key Words: apoptosis, granulysin, granzyme, NK cell, organophosphorus pesticide (OP), perforin

Introduction

Organophosphorus pesticides (OPs) are potent inhibitors of serine esterases such as acetylcholinesterase and serum cholinesterase (1). The main toxicity of organophosphorus pesticides is neurotoxicity, which is caused by the inhibition of acetylcholinesterase (1, 2). It has been reported that OPs affect immune response including effects on neutrophil function (3), macrophage (4-7), antibody production (8, 9), IL-2 production (10), serum complement (11), and T cell proliferation induced by IL-2 (12), concanavalin A and phytohemagglutinin (13) in animals and humans. Thrasher et al. (14, 15) reported that higher-than-usual frequencies of allergies and sensitivities to antibiotics together with a decrease in CD5 cells and increases in CD26 cells and autoantibodies were found in patients following chlorpyrifos exposure. Increased expression of CD26 cells and decreased expression of CD5 cells are associated with autoimmunity, where an individual's immune system acts against itself, rather than against infections (16). Rodgers also reported that oral administration of malathion increased the level of anti-dsDNA antibodies in MRL-lpr mice (17). Exposure to chlorpyrifos was associated with multiple chemical sensitivity (18).

Organophosphorus pesticides inhibit NK, LAK and CTL activity

In the process of immunotoxicologic investigations on several organophosphorus compounds, we found that diisopropyl methylphosphonate (DIMP) and diethyl methylphosphonate (DEMP), the by-products generated during sarin synthesis in the Tokyo sarin disaster, significantly inhibited human and murine natural killer (NK), and murine cytotoxic T lymphocyte (CTL) activities *in vitro* (19). DIMP and DEMP have also been shown to be potent inhibitors of serine esterases, such as acetylcholinesterase and serum cholinesterase, which are similar to OPs in

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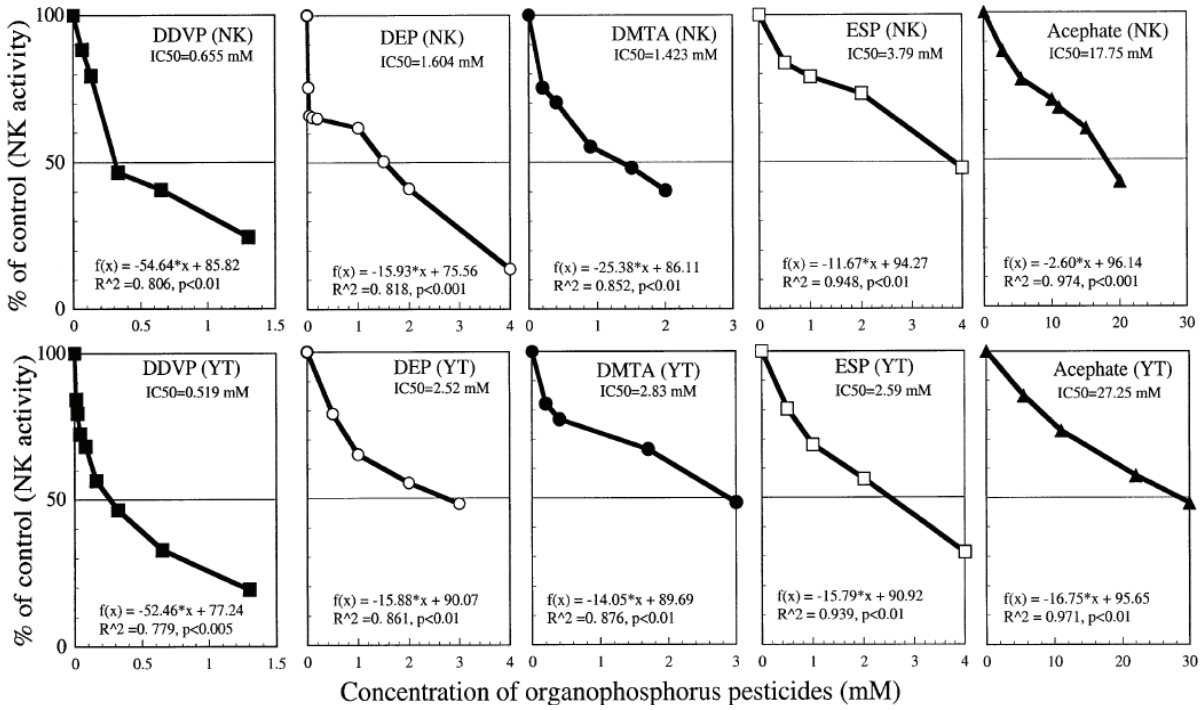

Figure 1. Effect of OPs on human NK activity and YT cell activity *in vitro.* IC50: inhibitory concentration of 50% NK activity. YT cell is a human NK cell line. Cited from Li et al., Toxicology. 2002;172:181-190 (21).

toxicity (20). Thus, we speculate that OPs may also inhibit NK and CTL activities like DIMP and DEMP. In order to clarify whether OPs also affected NK and CTL activities, we first investigated five OPs, which are dimethyl 2,2-dichlorovinyl phosphate (DDVP), dimethyl 2,2,2-trichloro-hydroxyethylphosphonate (DEP), dimethoate (DMTA), acephate and S-2-ethylsulfinyl-1-methylethyl O,O-dimethyl phosphorothioate (ESP) on human NK activity. We found that all five OPs significantly decreased human NK activity in a dose-dependent manner, and the strength of inhibition differed among the five OPs. The order was DDVP > DMTA ≥ DEP ≥ ESP > acephate (21, Figure 1). Then we investigated the effect of DDVP on murine splenic NK, lymphokine-activated killer (LAK) and CTL activities and human LAK and CTL activities. DDVP significantly decreased human NK (21-23) and LAK (21), and murine NK, LAK and CTL activities *in vitro* (21) and *in vivo* (24) in a dose-dependent manner, and the degree of decrease in these activities differed among the effector cells investigated. The order was as follows: human NK > murine NK > murine CTL > murine LAK > human LAK (21).

Zabrodskii and Germanchuk (25) also reported that DDVP at 0.2LD50 and 0.8LD50 by subcutaneous injection significantly inhibited NK activity and antibody-dependent cell cytotoxicity in Wistar rats. Rodgers et al. reported that O,O,S-trimethyl phosphorothioate, an impurity in technical formulations of malathion, inhibited human NK activity *in vitro* (26) and murine and/or human CTL activity *in vivo* and/or *in vitro* (27, 28). Table 1 summarizes the immunotoxicity of organophosphorus pesticides in humans/animals.

However, there have been few reports on the mechanisms of OP-induced inhibition of NK, LAK and CTL activities. We review the mechanisms of OP-induced inhibition of cytolytic activity of killer cells in the present study.

OPs impair the granule exocytosis pathway of killer cells

It has been reported that NK, LAK and CTL cells induce tumor or virus infected target cell death by two main mechanisms (29-31). The first mechanism is the direct release of cytolytic granules that contain the pore-forming protein perforin, several serine proteases termed granzymes (32), and granulysin (33) by exocytosis to kill target cells. The second mechanism is mediated by the Fas ligand (FasL)/Fas pathway (24, 31, 34). Human NK, LAK and CTL cells have so far been demonstrated to express five granzymes. Granzyme A (GrA) is expressed in NK, PHA- or CD3-stimulated T cells, γδT cells, and has a trypsin-like specificity, which cleaves on the carboxyl side of basic residues such as arginine and lysine (35, 36). GrB is expressed in NK, PHA- or CD3-stimulated T cells, γδT cells, and cleaves on the carboxyl side of aspartic acid residues (36, 37). Gr3/K is expressed in T cells and IL-2 or ConA-stimulated T cells, NK cells and PBL, and is trypsin-like (cleavage after basic residues) (38, 39). GrH is expressed in IL-2- or PHA-stimulated PBL and CTL, and prefers cleavage after

Table 1. Summary of immunotoxicity of organophosphorus pesticides (OP)

Targets (Cells)	Parameters	Effects	Human/animal	References
Neutrophils	Neutrophil function	↓	Human	3
Macrophages	Productions of estalase and neutral proteases, phagocytic capability and size of Mϕ	↑↑↑↑	Mouse	4-7
B cells	Ab production (IgG/IgM)	↓↓	Mouse	8, 9
	B cell response to LPS	→	Mouse	9
	B cell population (CD19)	→→	Human	14, 15
	Autoantibodies	↑↑↑	Human/Mouse	14, 15, 17
T cells	IL-2 dependent proliferation	↓	Mouse	12
	IL-2 production	↓	Rat	10
	Response to Con A or PHA	↓	Rat	13
	T cell subset, CD4, CD5, CD26	CD4↓→ CD5↓↓ CD26↑↑	Human	14, 15
	CTL activity	↓↓↓↓↓	Human/Mouse	19, 21, 26-28
NK cells	NK activity	↓↓↓↓↓↓↓	Human/Mouse	19, 21-26
	Granzyme activity	↓	Human	21
	Expressions of perforin, granulysin, GrA	↓↓	Human	22, 23
LAK cells	LAK activity	↓↓	Human	21, 24
Others	FasL/Fas pathway	↓	Mice	24
	Apoptosis	↑↑↑↑ (Positive)	Human/mice	46-48
	Complement activity	↓	Human	11
	Multiple chemical sensitivity	↑	Human	18

↓: Inhibition/decrease; ↑: Increase/activation (Induction); →: No effect. Numbers of the arrow show the number of references.

hydrophobic residues such as phenylalanine (40). GrM is expressed in NK, γδT cells, and cleaves on the carboxyl side of methionine, leucine or norleucine (36, 41).

OPs are potent inhibitors of serine esterases, such as acetylcholinesterase and serum cholinesterase (1, 2), and granzymes are also serine esterases (proteases) (32, 35, 37, 38, 40, 41). Thus, we speculate that the decrease in NK, LAK and CTL activities by OPs may be mediated by the inhibition of serine proteases (granzymes), which are released from NK and CTL granules by exocytosis when target cells conjugate with the effector cells. To explore the underlying mechanism of decrease in cytolytic activity of killer cells, we investigated the effects of DDVP on the enzymatic activity of human granzymes, and found that DDVP significantly inhibited the enzymatic activity of human GrA, Gr3, GrH, GrM in a dose-dependent manner. The IC50 (inhibitory concentration of 50% granzyme activity) values were 0.05 mM for GrA and Gr3, 0.03 mM for GrH and 0.05 mM for GrM (Figure 2). In order to support our hypothesis that OPs inhibiting cytolytic activity of killer cells is mediated by the inhibition of granzymes (serine proteases), we investigated the effect of 4-(2-aminoethyl) benzenesulfonyl fluoride-HCl (*p*-ABSF), an inhibitor of serine proteases, on NK, LAK and CTL activities. *p*-ABSF significantly decreased human and murine NK and LAK, and murine CTL line activities in a dose-dependent manner, and the degree of decrease in those activities also differed among the effector cells. The order was human NK > murine NK > murine CTL line > murine LAK > human LAK. This order coincides with the results obtained with DDVP, suggesting that DDVP and *p*-ABSF have a common inhibiting mechanism on NK, LAK and CTL activities. In addition, the decreases in NK, LAK and CTL activities by *p*-ABSF + DDVP were greater than that by either *p*-ABSF alone or DDVP alone in the same concentration, suggesting that DDVP and *p*-ABSF have an additive inhibitory effect on NK, CTL and LAK activities. Taken together, the above-mentioned findings indicate that organophosphorus pesticides significantly decrease NK, LAK and CTL activities *in vitro via* granzyme inhibition (21).

In order to investigate whether OPs also affect the expression of granzyme, granulysin and perforin, we treated a human NK cell line, NK-92 cells, with DDVP *in vitro* and then analyzed the expressions of granzyme, granulysin and perforin by flow cytometry and RT-PCR. We found that DDVP significantly decreased the expression of perforin (Figure 3), granzyme A and granulysin in NK-92CI and NK-92MI cells in a dose-dependent manner (22, 23). DDVP also has a modest but significant inhibitory effect on the transcription of mRNA of perforin, granzyme A and granulysin. Moreover, we found that the decreases in perforin, granzyme A and granulysin in the granules of NK-92CI cells parallel a similar pattern by immunocytochemical analysis, which strongly suggests a possibility of degranulation (22).

Taken together, DDVP inhibits the enzymatic activity of granzymes (21), and expression of granzymes, granulysin and perforin in human NK cells, as well as induction of degranulation of NK cells (22, 23, Figure 4).

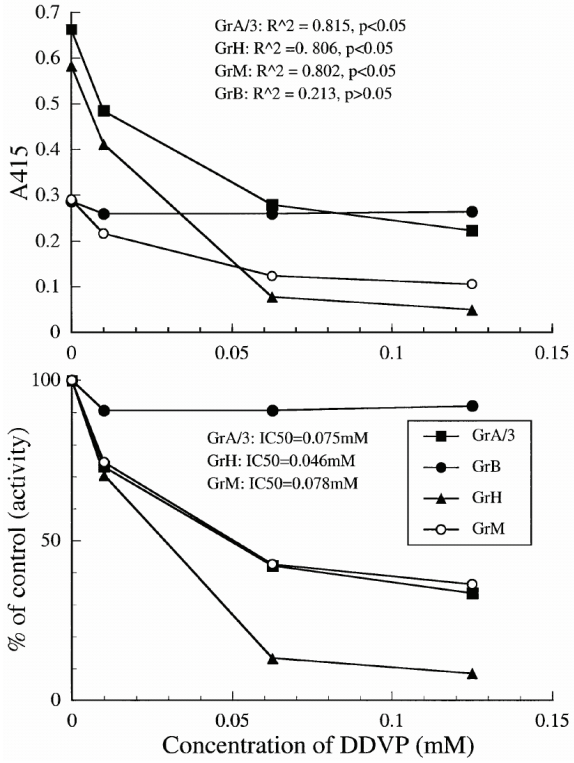

Figure 2. Inhibitory effect of DDVP on activity of human granzymes A, B, 3, H, M. IC50: inhibitory concentration of 50% activity of granzymes. Cited from Li et al., Toxicology. 2002; 172:181-190 (21).

Organophosphorus pesticides impair the FasL/ Fas pathway of killer cells

There has been only one paper investigating whether OPs affect the FasL/Fas pathway of killer cells using perforin-knockout (PKO) mice (24). It has been reported that the granule exocytosis pathway in PKO mice does not function against Fas antigen-negative target cells (24, 34, 42) and that the NK, CTL and LAK of PKO mice kill targets only by FasL/Fas pathway (24, 42). Thus, the authors used PKO mice to investigate the effect of DDVP on FasL/Fas pathway by determining the NK, CTL and LAK activities in PKO mice.

In this study, it was found that DDVP significantly decreased the NK, CTL and LAK activities of PKO mice in a dose-dependent manner, and that the CTL and LAK activities of PKO mice were significantly blocked by anti-FasL antibody, suggesting that DDVP and anti-FasL antibody have the same or a similar mechanism of inhibiting LAK and CTL activities. Moreover, DDVP decreases the expression of Fas antigen on YAC-1 cells (a target cell in NK activity assay), and the expression of FasL on LAK cells in a dose-dependent manner, respectively (Figure 5). Taken together, these findings indicate that the DDVP-induced inhibition of NK, LAK and CTL activities in PKO mice is mediated by the impairment of the FasL/Fas pathway (24).

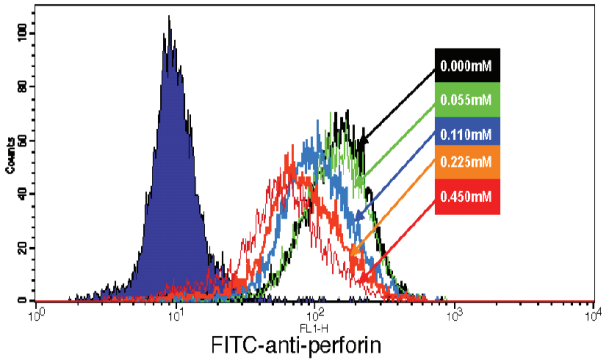

Figure 3. Effect of DDVP on the expression of perforin in human NK-92CI after 15 h *in vitro* treatment. The X axis shows the fluorescent intensity of FITC-anti-perforin, which represents the intracellular level of perforin, the Y axis shows the counts of NK cells. The solid histogram shows the control stained with FITC-mouse IgG2b (isotype control) and the blank histograms show the results stained with FITC-mouse anti-human perforin after treatment with DDVP at 0 (black), 0.055 (green), 0.110 (blue), 0.225 (orange) and 0.452 (red) mM from the right to the left, respectively. Cited from Li et al., Toxicology. 2005;213:107-116 (22).

OPs induce apoptosis of immune cells

It has been reported that OPs induced apoptosis in rat primary cortical neurons (43), in SH-SY5Y human neuroblastoma cells (44), and in murine preimplantation embryos (45). On the other hand, there are 4 reports that OPs induce apoptosis of immune cells (46-49). Saleh et al. (46, 47) have shown that paraoxon (POX: the bioactive metabolite of parathion) and parathion cause apoptotic cell death in a murine EL4 T-lymphocytic leukemia cell line through activation of caspase-3. In this study, pretreatment of EL4 cells with the caspase-9-specific inhibitor zLEHD-fmk attenuated POX-induced apoptosis in a dose-dependent manner, whereas the caspase-8 inhibitor zIETD-fmk had no effect. Furthermore, activation of caspase-9, -8, and -3 in response to POX treatment was completely inhibited in the presence of zLEHD-fmk, implicating the involvement of caspase 9-dependent mitochondrial pathways in POX-stimulated apoptosis. Indeed, under both *in vitro* and *in vivo* conditions, POX triggered a dose- and time-dependent translocation of cytochrome c from mitochondria into the cytosol. Investigation of the mechanism of cytochrome c release revealed that POX disrupted mitochondrial transmembrane potential. Neither this effect nor cytochrome c release was dependent on caspase activation, since the general inhibitor of the caspase family zVAD-fmk did not influence both processes. Finally, POX treatment also resulted in a time-dependent up-regulation and translocation of the proapoptotic molecule Bax to mitochondria. Inhibition of this event by zVAD-fmk suggests that the activation and translocation of Bax to mitochondria is subsequent to activation of the caspase cascades. The results indicate that

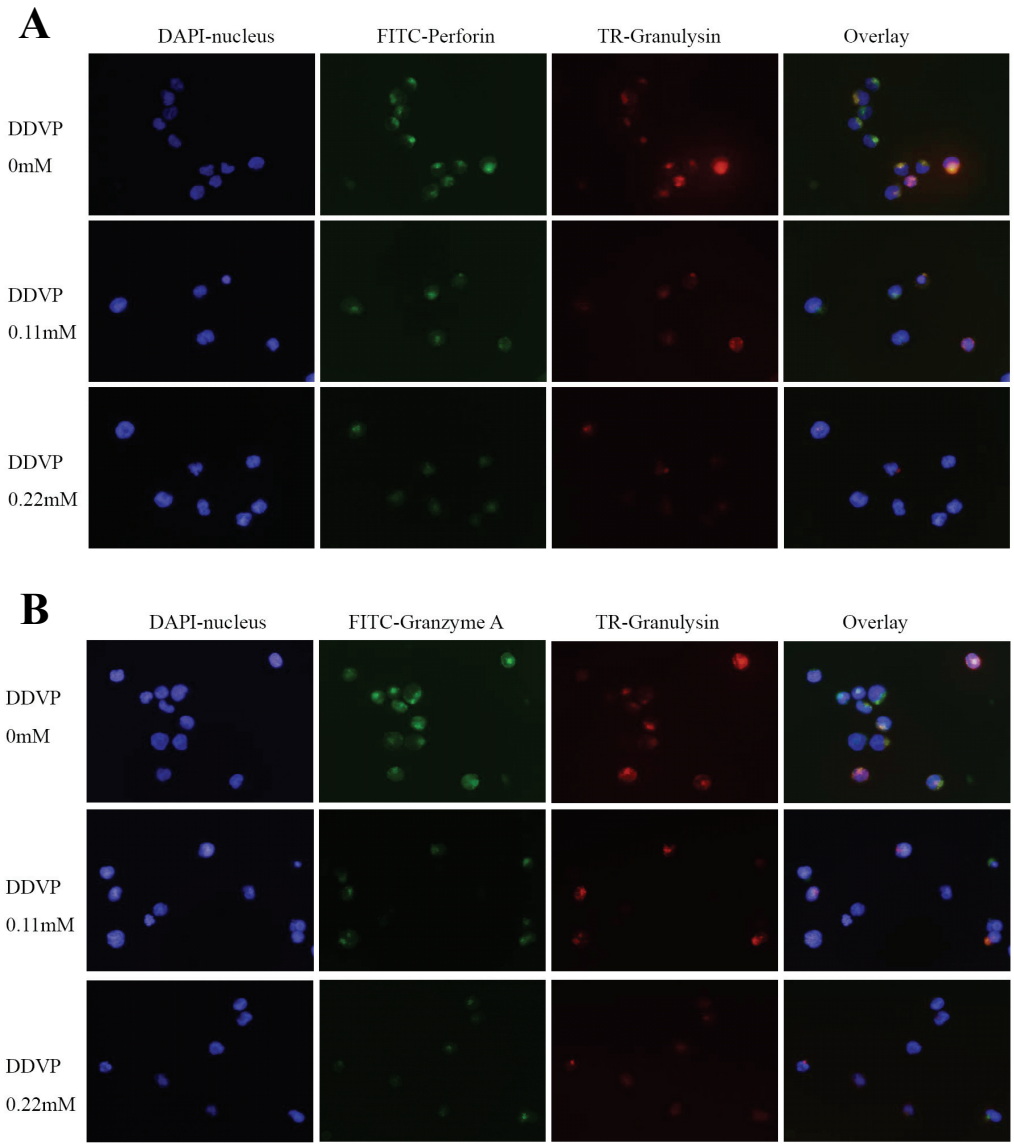

Figure 4. Effect of DDVP at 0.11 and 0.22 mM on intracellular perforin/granulysin (A) and granzyme A/granulysin (B) in NK-92CI cells after 15 h *in vitro* treatment. The NK-92CI cells were fixed/permeablized with cytofix/cytoperm solution, and then double-staining of perforin/granulysin and granzyme A/granulysin was performed. The intracellular perforin and granzyme A were stained with FITC-anti-human perforin and granzyme A, respectively. Intracellular granulysin was first stained with rabbit anti-human granulusin polyclonal antibody, then stained with TR-goat anti-rabbit IgG. Cited from Li et al., Toxicology. 2005;213:107-116 (22).

POX induces apoptosis in EL4 cells through a direct effect on mitochondria by disrupting its transmembrane potential, causing the release of cytochrome c into the cytosol and subsequent activation of caspase-9 (46).

In order to explore the mechanism of OP-induced immunotoxicity, we also investigated whether OPs induced apoptosis in human immune cells, and examined the underlying mechanism. We treated human immune cells, a human monocyte like cell line (U937), with chlorpyrifos, an OP, and found that chlorpyrifos induced cell death of U937 in a dose- and time-dependent manner, as shown by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide) and LDH (lactate dehydrogenase) assays and PI (propidium iodide) uptake. Then, we investigated whether chlorpyrifos-induced cell death consisted of apoptosis, as determined by analysis of Annexin-V staining and the intracellular level of active caspase-3 by flow cytometry, and DNA fragmentation analysis. We found that chlorpyrifos induced apoptosis in U937 in a time- and dose-dependent manner, as shown by Annexin-V staining. DNA fragmentation was detected when cells were treated with chlorpyrifos (Figure 6). Chlorpyrifos also induced an increase of intracellular active caspase-3 in U937 cells in a dose-dependent manner, and a caspase-3 inhibitor, Z-DEVD-FMK, significantly inhibited the

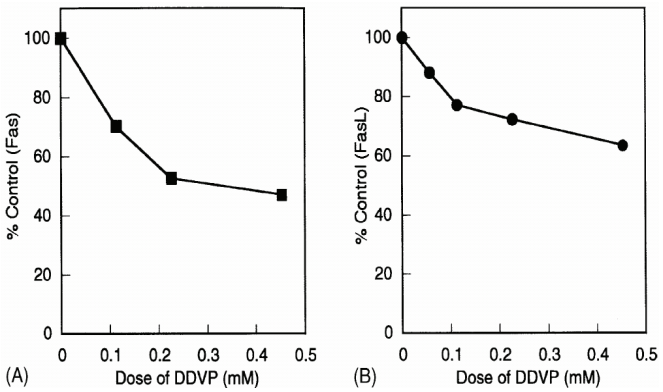

Figure 5. Effects of DDVP on the expression of Fas antigen on the surface of YAC-1 cells (A) and on the expression of FasL on the surface of LAK cells (B). Cited from Li et al., Toxicology. 2004;204:41-50 (24).

chlorpyrifos-induced apoptosis. These findings indicate that chlorpyrifos can induce apoptosis in U937 cells (48). Das et al. (49) also reported that OPs such as monocrotophos, profenofos, chlorpyrifos and acephate significantly induced apoptosis and necrosis in cultured human peripheral blood lymphocytes *in vitro* using DNA diffusion assay.

In conclusion, the above findings indicate that OPs inhibited NK, LAK and CTL activities mediated by at least the following three mechanisms:

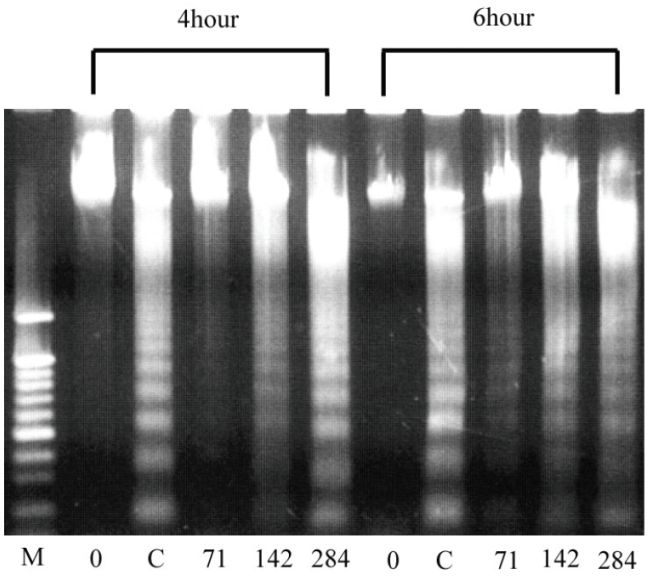

Figure 6. Chlorpyrifos-induced DNA fragmentation in U937 cells were determined by agarose gel electrophoresis. M, marker of the DNA ladder; C, a positive control, camptotecin at 6 μM. The concentrations of chlorpyrifos were 0, 71, 142 and 284 μM. Data shown are representative of three similar experiments. Cited from Nakadai et al., Toxicology. 2006 May 9; [Epub ahead of print], in press (48).

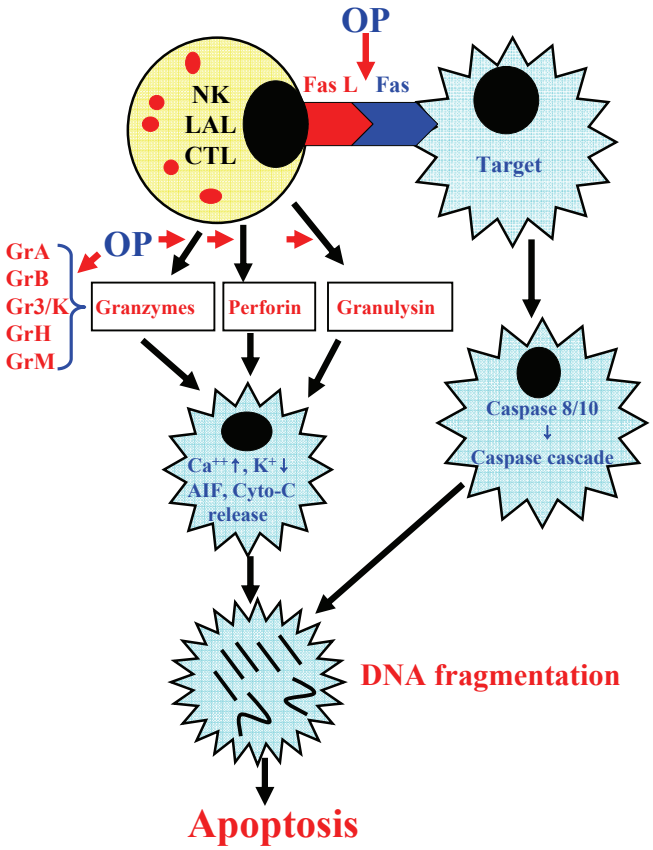

Figure 7. Organophosphorus pesticides impair the granule exocytosis pathway (perforin/granzyme A/granulysin pathway) and the FasL/Fas pathway of NK, LAK and CTL cells. OP: organophosphorus pesticides. →, inhibition.

1. OPs impair the granule exocytosis pathway of NK, LAK and CTL cells (Figure 7);
2. OPs impair the FasL/Fas pathway of NK, LAK and CTL cells (Figure 7);
3. OPs induce apoptosis of immune cells.

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