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Effects of Diazinon on the Murine Host Defense System

Yeo-Pyo Yun, Kwan-Hoi Kim, Se-Chang Lee and Jin Tae Hong*

College of Pharmacy, Chungbuk Naional University

**National Institute of Safety Research, Korea*

ABSTRACT—**Diazinon which is one of the most heavily used organophosphate pesticide in Korea, was examined for its effects on the murine host defense system. Immunotoxicological assay parameters adopted in this study were carbon clearance for macrophage function, susceptibility to tumor challenge, and pathotoxicological indicators. Subchronic exposure of pesticide to rodents resulted in the suppression of immune functions, enhancement of susceptibility to tumor challenge, and moderate histological changes of lymphoid organ without any significant alterations of clinical status.**

Keywords□**Diazinon, Carbon clearance, Susceptibility to tumor challenge, Pathotoxicology**

Considering that excessively used pesticides could be exposed to human through several processes, it is important to know as possible about the effects of pesticides and degradation products on human and animals. Toxicologists have begun to examine the immune system when the hazards and risks of a chemical are assessed, since an impaired immune function may alter susceptibility to disease.[1-6] Since Balkhovityanova's report[7] in 1968, there have been many reports that pesticides affected the immune responses of laboratory animals.[8-10] Organophosphates were also reported to alter immunological parameters, but there could not be found common pattern in their action. Their effects were diverse depending on the dose, administration route, duration and test subjects etc.

Diazinon (0,0-diethyl 0-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate) is a potent cholinesterase inhibitor that acts by interfering with the metabolism of acetylcholine, which results in accumulation of acetylcholine at neuroreceptor transmission sites. Exposure produces a broad spectrum of clinical effects indicative of massive overstimulation of the cholinergic system, including muscarinic effects, nicotinic effects, and CNS effects. These effects present clinically as feelings of headache, weakness, dizziness, blurred vision, psychosis, respiratoy difficulty, paralysis, convulsion, and coma. Typical findings are given by the memoric "SLUD", which stands for salivation, lacrimation, urination, and defecation. A small percentage of patients may fail to demonstrate miosis, a classic and diagnostic hallmark. Onset of clinical manifestation of organophosphate poisoning usually occurs within 12 hours of exposure.

Diazinon of which residues in foods are very high, is most heavily used in Korea.[11,12] But their specific toxic effects reported hitherto have been limited to the acute toxicity and neurological system, and few report has appeared far to our knowledge in relation to immunological effects.

In this study, we examined carbon clearance for macrophage function, susceptibility to tumor challenge, and pathotoxicological indicators following subchronic exposure of Diazinon.

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Reprint request: Dr. Y.P. Yun at the above address

Experimental Methods

Materials

Diazinon was kindly supplied by Korean Institute for Environmental Science. Cyclophosphamide was purchased from Sigma Chemical Co. and Pelikan drawing ink (17 black India) from Pelikan AG. All other chemicals used were of reagent

grade.

Animals and Treatment

Male Sprague Dawley rats and male ICR mice were obtained from the Experimetal Animal Bleeding Center of National Institute of Safety Research. Animals were given commercial rodent chow (Samyang Co.) and water *ad libitum*. Animals were acclimated for at least 1 week in the experimental condition prior to experimentation conducted and maintained on a 12-hr (7AM to 7PM) light-dark cycles. To minimize circadian effects, all animals were immunized, dosed and killed between 9 and 11 AM. Diazinon was dissolved in corn oil and diluted so that rats and mice were given orally a volume of 0.2 m*l* and 0.1 m*l*, respectively. Diazinon treatment was conducted at the dose of 3.5 mg/Kg and 35 mg/Kg for consecutive 10 days. Cyclophosphamide was dissolved in sterile saline immediately prior to use and injected into positive control group at the dose of 45 mg/Kg body weight for 4 days. Control group received coin oil alone.

Macrophage Function

The carbon clearance test for *in vivo* phagocytosis is based on the work of Biozzi *et al.*[13] Phagocytic activity was determined 2 days after the last pesticide administration. For the preparation of suspension of carbon ink, carbon ink was diluted 1/6 with 1% gelatin and kept in a stoppered tube at 37℃ during experminent. Injection was executed *via* the lateral tail vein at the dose of 0.01 m*l* of colloidal carbon solution per gram of mouse. This corresponded to approximately 16 mg carbon per 100g body weight of mouse. At the interval of 10 min, 20 min, 30 min, and 40 min, 20 m*l* of blood sample was obtained from the retro-orbital pluxus. The collected blood samples were expelled into each vial containing 1 m*l* sodium carbonate and then measured absorbance against water blank at 600 nm. From these results, phagocytic indices and corrected phagocytic indices were calculated.

Host Susceptibility to Tumor Challenge

The sarcoma-180 tumor cell was supplied by Tokyo Cancer Research Center and has been maintained in ascite form in this laboratiry by serial i.p. passage in ICR mice. Control and pesticide exposed ICR mice were inoculated s.c. in the right inguinial area with 4×10^4 cells 2 days after the last pesticide administration. This tumor dose had been previously titrated *in vivo* and produced a 20~30% progressive tumor incidence in control group. Mice were examined once a week for 60 days to assess the development of tumors.

The Number of Circulating Leukocyte

Blood was collected from the retro-orbital plexus, on 1st, 2nd, 4th and 7th day after the sample treatment. Collected blood was about 80 μ*l* and was mixed with 320 μ*l* of citrate saline. The number of nucleated cells were counted in hemacytometer chamber with microscope. Turk's solution was used for staining leukocytes and lysis of unnucleated cells. Triple counting per sample was carried out and the mean value of results was calculated. The number was compared with that obtained from control mice.

Clinico-chemical Values

Blood samples were collected *via* a cardiac puncture and allowed to clot for 30 min at room temperature. Centrifuging the specimen, the obtained serum was stored at −20℃ until analyzed for clinico-chemical values. Clinico-chemical values (serum proteins, serum enzymes, cholesterol, triglyceride, BUN and glucose) were determined using Photometer 4020.

Histopathology

Animals were sacrificed with ether for biopsy. Liver, spleen and thymus were removed and fixed in 10% buffered formalin. Samples were dehydrated with automatic tissue processor and embedded in paraffins. Sections were cut by microtome and stained with hematoxylin and eosin, and examined hitologically.

Statistical Analysis

All data were examined for their statistical significances with the Student's t-test.

Table 1. Body and relative organ weights in mice administered Diazinon

Treatment	Dose(mg/kg)	Body wt.(g)	Liver/body(%)	Spleen/body(%)	Thymus/body(%)
Control	–	27.4± 0.7	5.28± 0.03	0.51± 0.03	0.26± 0.03
Diazinon	3.5	27.3± 0.6	5.34± 0.05	0.52± 0.04	0.25± 0.03
	35	27.2± 0.5	5.43± 0.07[a]	0.51± 0.02	0.20± 0.02[b]
Cyclophosphamide	45	25.8± 0.8	6.27± 0.03[c]	0.22± 0.03[c]	0.09± 0.02[c]

* Body and relative organ weights were determined 3 days after the last treatment
Data are presented as mean± SE. n=7 per group
[a] Significantly different from control group at $p<0.1$
[b] Significantly different from control group at $p<0.05$
[c] Significantly different from control group at $p<0.01$

Table 2. Effect of Diazinon on the number of circulating leukocytes in mice

Treatment	Dose(mg/kg)	1st day	2nd day	4th day	7th day
Control	–	10800± 200	10300± 300	10200± 400	12200± 300
Diazinon	3.5	9000± 300	10500± 300	10000± 400	11900± 400
	35	7800± 200	9700± 400	9400± 400	10500± 500
Cyclophosphamide	45	3000± 200[a]	2900± 300[a]	4700± 300[a]	8600± 300[b]

Data are presented as mean± SE. cells/mmi; n=12 per group
[a] Significantly different from control group at $p<0.1$
[b] Significantly different from control group at $p<0.05$

Results

While none of the Diazinon treated animals died or revealed overt toxicity during experimental period, Diazinon exposure resulted in suppression in immunity. There was no significant alteration in body and relative organ weights in mice administered Diazinon although positive control showed decreased immunoorgan/body weight ratio (Table 1).

While cyclophosphamide treated group showed severe leukopenia, pesticide treated groups revealed more varied and less marked alterations. Generally the number of circulating leukocytes in the pesticide treated group were lower than those of control group as shown in Table 2. Corrected phagocytic indices were significantly decreased in the pesticide treated group at higher dosed group (Table 3).

Diazinon exposure resulted in a dose-dependent enhancement of susceptibility to sarcoma-180 tumor (Table 4). An increased frequency of tumor development occurred in diazinon treated group compared to control group. The incidence of progressive tumor increased from 30% in control group to 60% in Diazinon treated group.

Table 5 shows the enzyme activities in serum

Table 3. Effect of Diazinon on the carbon clearance activity in mice

Treatment	Dose (mg/kg)	Phagocytic index (K)	Corrected phagocytic index
Control	–	0.017± 0.004	3.72± 0.07
Diazinon	3.5	0.015± 0.002	3.66± 0.15
	35	0.012± 0.004	3.30± 0.09[a]
Cyclo-phosphamide	45	0.016± 0.003	3.22± 0.15[a]

* Phagocytic index (K) is the slope of the logarithm of blood concentration against time
** Corrected phagocytic index is a constant obtained from a formula relating the cube root of K to the ratio of body weight to weight of liver and spleen
[a] Significantly different from control group at $p<0.05$

Table 4. Tumor development following injection of sarcoma-180 in Diazinon treated mice

Treatment	Dose (mg/kg)	No. of mice tested	No. of tumor developed	Percentage (%)
Control	–	10	3	30
Diazinon	3.5	8	4	50
	35	10	6	60
Cyclophos-phamide	45	8	7	87.5

* Mice were challenged with 4×10^4 sarcoma-180 cells by S.C in the right inguinal area
** Percentage is tumors per number of tested animal ratio

of the rats administered Diazinon. Examination of serum enzyme activities indicated that ALT, AST and alkaline phosphatase were not affected by Diazinon exposure in this experimental condition. Table 6 shows the effects of Diazinon on serum lipids. The results indicated that serum cholesterol levels were increased but serum triglyceride was decreased in the group exposed to Diazinon. BUN and blood glucose levels were found quite normal. In the sample treated group, serum globulin levels were elevated and serum albumin was decreased (Table 7).

Table 5. Enzyme activities in serum of the rats administered Diazinon

Treatment	Dose(mg/kg)	ALT(GPT)/(U/*L*)	AST(GOT)/(U/*L*)	Alkaline phosphatase(U/*L*)
Control	–	22.4± 1.1	60.1± 3.8	85.3± 6.5
Diazinon	3.5	19.8± 1.7	59.5± 7.8	88.4± 9.7
	35	19.5± 2.1	59.7± 5.6	97.5± 7.9
Cyclophosphamide	45	15.3± 2.4[a]	37.6± 1.8[b]	46.3± 1.7[c]

* Data are presented as mean± SE cells/mmi; n=7 per group
[a] Significantly different from control group at $p<0.05$
[b] Significantly different from control group at $p<0.01$

Table 6. Clinico-chemical values in the rats administered Diazinon

Treatment	Dose (mg/kg)	Cholesterol (mg/d*l*)	Triglyceride (mg/d*l*)	BUN (mg/d*l*)	Glucose (mg/d*l*)
Control	–	47.8± 1.8	84.7± 8.1	24.5± 1.7	72.4± 2.3
Diazinon	3.5	52.6± 2.5	57.3± 4.1[a]	20.2± 1.5	67.3± 9.7
	35	54.3± 1.3	40.1± 2.9[c]	21.5± 0.7	74.7± 4.5
Cyclophosphamide	45	65.6± 9.8[b]	51.8± 1.8[b]	22.3± 1.2	71.2± 3.3

* Data are presented as mean± SE cells/mmi; n=7 per group
[a] Significantly different from control group at $p<0.1$
[b] Significantly different from control group at $p<0.05$
[c] Significantly different from control group at $p<0.01$

Table 7. Effect of Diazinon on the serum proteins of the rats

Treatment	Dose (mg/kg)	Total protein (g/d*l*)	Albumin (g/d*l*)	Globulin (g/d*l*)	A/G ratio
Control	–	6.77± 0.11	3.51± 0.11	3.25± 0.03	1.07± 0.03
Diazinon	3.5	7.79± 0.46[b]	3.52± 0.22	4.26± 0.23[c]	0.82± 0.01[c]
	35	7.59± 0.09[c]	3.09± 0.14[c]	4.39± 0.24[c]	0.68± 0.06[c]
Cyclophosphamide	45	6.62± 0.38	2.46± 0.27[c]	4.16± 0.25[c]	0.58± 0.07[c]

* Data are presented as mean± SE cells/mmi; n=7 per group
[a] Significantly different from control group at $p<0.1$
[b] Significantly different from control group at $p<0.05$
[c] Significantly different from control group at $p<0.01$

Table 8. Histological changes in the rats administered Diazinon

Treatment	Dose (mg/kg)	Thymus		No. of lymphocyte in spleen white pulp
		Atrophy	Congestion	
Control	−	−	−	−
Diazinon	3.5	−	+	−
	35	+	+	D
Cyclophosphamide	45	++	+	D

+, slight to moderate atrophy or congestion; ++, severe atrophy; −, no effect; D, slight to moderate decrease in the number of lymphocyte in spleen white pulp.

On the gross examination, thymus was shown a mild atrophy at higher dosage levels in sample treated group (Table 8). Thymus congestion was also observed in the pesticide treated group. Other organs did not exhibit any gross abnormality. Microscopic examination of spleen presented a mild decrease in the number of lymphocyte in spleen white pulp at higher dosage levels in sample treated group. Especially cyclophosphamide treated group presented necrosis and depletion of the lymphocytes in spleen white pulp. Microscopic examination of thymus revealed no significant lesions in sample treated group. However, cyclophosphamide presented a severe atrophy of cortex and depletion of thymocytes. Histological examination revealed no significant lesions of liver in sample treated group.

Discussion

The effects of organophosphate pesticide, diazinon, on the immune functions and host susceptibility to tumor challenge were examined following subchronic exposure to nontoxic levels in rats and mice. While none of animals exposed to pesticide died or revealed overt toxicity during experiment, the treated group showed suppression in immunity.

There have been many reports on the immunosuppressive effects of pesticides.[6,14] For example, some organophosphate pesticides−malathion, methyl parathion and dichlorovos−have been shown to suppress humoral immune responses in laboratory animals.[15,16] While the cyclophosphamide treated group induced severe leukopeia, the Diazinon treated group showed less marked alterations. Generally, the number of circulating leukocytes in the Diazinon treated group was lower than those of control group.

At first, decrement in the relative spleen and thymus weights suggested that pesticide exposure might alter immune function. Subsequent hitological examination revealed the decrement in the number of lymphocyte in the spleen white pulp, thymus congestion and atrophy. These results may support the immunosuppressive potential of Diazinon and were compatible with effects on the humoral immuity.

In the pesticide treated group, serum globulin levels were elevated in spite of the suppression of humoral response, and serum albumin is slightly decreased. It suggest that the pesticide exposure might alter the liver function. This condition might be compared to the early phase of acute inflamation or necrotic process, in which slight elevation of a_2-globulin and slight decrease in albumin concentraion.

Examination of serum enzyme activities indicated that ALT(sGPT), AST(sGOT) and alkaline phosphatase were not affect by pesticide exposure in this experimental conditions. Even though the activities of ALT, AST and alkaline phosphatase were found in normal range, they might be so interpreted that hepatobiliary damages were happened so limited as not to allow the detection with the applied assay methods.

Serum lipid examination indicated that serum cholesterol levels were increased, but serum trigl-

yceride levels were decreased in the group exposed to Diazinon. These results suggest that lipid metabolism or lipid releasing mechanism of the liver was negatively influenced by this pesticide.

Corrected phagocytic indices were significantly decreased in the pesticide treated group at higher dose group. This result indicates that Diazinon suppresses the reticuloendothelial system.

Diazinon exposure resulted in enhancement of susceptibility to sacorma-180 tumor in a dose-depent fashion. It is well known that non-specific immunity plays an important role in host resistance against tumor, and so, it could not be excluded that not only specific immunity but also non specific immunity especially, NK-cell activity might be suppressed by Diazinon exposure, but conclusions should be reserved until more precise studies are completed.

Taking into consideration of immunotoxic potential of organophosphate pesticides, at first, we suppose that organophosphate pesticides or their metabolites might directly act on the immune system. Musson and Becker[17] and Taurog *et al.*[18] have demonstrated *in vitro* immunosuppression by cholinesterase inhibitors (phosphonates) structurally similar to the esterase inhibiting metabolites of some organosphate pesticides. Others demonstated that organophosphate induced immunosuppression might result from direct action of acetylcholine upon the immune system.[16] In support of this observation, cholinergic receptors have been identified on lymphocytes and macrophages.[19]

An alternative hypothesis to be considered is that the observed immunosuppression might be madiated by glucocorticoids released in response to the toxic chemical stresses associated with the cholinergic crisis. In support of this hypothesis, Szot and Murphy[20] have demonstrated the elevation of plasma corticosteroid concentrations in rats given sublethal doses of parathion. The immunosuppressive action of corticosteroids are also well known.[21,22] Both *in vitro* and *in vivo* experiments, which exclude involvement of the hypothalamus-pituitary-adrenal system, are required to determine the relative importance of cholinergic stimulation, stress and esterase inhibition, as well as other possible nonstress effects in organophosphate induced immunosuppression.

In conclusion, results described in this report demonstrate that subchronic exposure of the experimental animals to nontoxic levels of Diazinon suppresses immune functions and enhances host susceptibility to tumor challenge. Because of the wide spread use of this pesticide and the potential for human exposure, it is important that further studies should be conducted to define the cell types and functions of the immune system altered by this pesticide.

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