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Effects of Some Organophosphate Pesticides on the Murine Immune System following Subchronic Exposure (I)

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Abstract □ Four technical grade organophosphate pesticides (Fenitrothion, Fenthion, Diazinon and EPN) were investigated for their effects on the murine immune function.

Among the immunotoxicological assay parameters of NIEHS, humoral immune parameter and pathotoxicological indicators were examined in this study.

Subchronic exposure of rodents to these pesticides resulted in marked suppression of humoral immune function and moderate histological changes of lymphoid organ without any significant alterations of clinical status.

Keywords □ Organophosphate, Subchronic exposure, Humoral immunity, Pathotoxicology

The world wide use of pesticides makes it urgent to know as much as possible about the effects of pesticides and their degradation products of human and animals.

Toxicologists have been 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. Exposure to pesticides, metals, and industrial chemicals has been shown to affect the immune response of laboratory animals (1-8).

Pesitcides known to affect immune response were well reviewed by Street, J.C. (9) and Moon, C.K. (10). Although their effects on the immune system were quite varied and could not be found identical action pattern even in the same lineage, most of them were shown to have negative effects on laboratory animals and human beings.

Fenitrothion, fenthion, diazinon and EPN, of which residues in foods are very high, are 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 have appeared far to our knowledge in relation to immunological effects and host resistance. So, in order to elucidate the immunotoxic potential of these pesticides, we began to investigate their effects on the immune parameters recommended by NIEHS, USA (1, 13).

In this study, we examined the humoral immune parameter and pathotoxicological indicators in animals treated with these pesticides subchronically.

EXPERIMENTAL METHODS

Materials

Agrochemicals used (Fenitrothion, Fenthion, Diazinon and EPN) were kindly supplied by Mr. H. Y. Kim, Korean Institute for Environmental Sciences.

PCB (Octa mixture of isomer) and Freund's Complete Adjuvant (FCA) were purchased from Wako Pure Chemical industry, and cyclophosphamide from Sigma Chemical Co.

All other reagents used were of reagent grade.

Animals and Treatment

Balb/c mice and Sprague Dawley rats were obtained from the Experimental Animal Breeding Center of Seoul National University. Animals were

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Table I. Body and Relative Organ Weights in Mice Administered Organophosphate Pesticides*.

Pesticide	Dosage (mg/kg)	Body wt. (g)	Liver/body (%)	Spleen/body (%)	Thymus/body (%)
Control	–	27.3±0.8	5.29±0.04	0.50±0.02	0.25±0.01
Fenitrothion	3.0	27.6±1.2	5.42±0.05[a]	0.47±0.01[b]	0.26±0.02
	30	27.4±1.0	5.64±0.05[b]	0.40±0.01[b]	0.18±0.01[c]
Fenthion	2.5	25.9±0.9	5.46±0.03[b]	0.53±0.03[a]	0.24±0.01
	25	26.1±0.9	5.71±0.07[c]	0.47±0.01[b]	0.19±0.01[c]
EPN	0.24	26.2±0.8	5.37±0.03	0.52±0.02	0.27±0.02
	2.4	26.3±0.7	5.48±0.05[b]	0.42±0.01[c]	0.18±0.01[c]
Diazinon	3.5	27.4±0.7	5.35±0.04	0.52±0.03	0.27±0.02
	35	27.3±0.6	5.42±0.06[a]	0.51±0.01	0.21±0.01[b]
PCB	10	26.7±0.7	6.06±0.03[c]	0.45±0.01[b]	0.20±0.01[c]
	100	26.9±0.8	8.37±0.07[c]	0.35±0.02[c]	0.12±0.02[c]
Clyclophosphamide	45	25.9±0.9	6.28±0.04[c]	0.23±0.03[c]	0.08±0.02[c]

* Body and relative orgen weights were determined 3 days after the last pesticide administration. Data are presented as mean ±SE. n=10 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 $p<0.01$

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 (7 AM to 7 PM) light-dark cycles. To minimize circadian effects, all animals were immunized, dosed and killed between 9 and 11 AM.

Pesticides and PCB were dissolved in corn oil and diluted so that rats and mice were given orally a volume of 0.2ml and 0.1ml respectively. Treated groups were exposed to following concentrations (mg/kg body wt. per day) for 10 days, which are

Table II. Effect of Organophosphate Pesticides on Humoral Immunity (Antibody Response to Bovine Serum Albumin) in Mice*.

Pesticide	Dosage (mg/kg)	Mean absorbance at 405 nm at serum dilution		
		1 : 8,000	1 : 16,000	1 : 32,000
Control	–	0.939±0.016	0.637±0.027	0.354±0.041
Fenitrothion	3.0	0.589±0.084[c]	0.441±0.002[c]	0.260±0.013[a]
	30	0.484±0.089[c]	0.301±0.048[c]	0.145±0.002[c]
Fenthion	2.5	0.812±0.056[a]	0.519±0.061	0.277±0.031
	25	0.534±0.038[c]	0.324±0.005[c]	0.177±0.040[b]
EPN	0.24	0.819±0.047[a]	0.531±0.058	0.282±0.022
	2.4	0.510±0.033[c]	0.321±0.027[c]	0.151±0.020[c]
Diazinon	3.5	0.694±0.120[a]	0.472±0.082	0.238±0.073
	35	0.465±0.005[c]	0.301±0.009[c]	0.124±0.025[c]
PCB	10	0.556±0.050[c]	0.432±0.015[c]	0.233±0.015[b]
	100	0.476±0.037[c]	0.378±0.011[c]	0.148±0.016[c]
Cyclophosphamide	45	0.275±0.010[c]	0.131±0.015[c]	0.038±0.008[c]

* A primary sc challenge of 2mg BSA in FCA (1 : 1) was given on day 0 ;
A second sc challenge of 1mg BSA in FCA (1 : 1) was given on day 20 ;
Blood was collected on day 26.
Data are presented as mean ±SE
a) $p<0.1$ b) $p<0.05$ c) $p<0.01$

Table III. Effect of Organophosphate Pesticides on the Numbers of Circulating Leukocytes in Mice*.

Pesticide	Dosage (mg/kg)	1 st day	2 nd day	4 th day	7 th day
Control	–	10900±200	10200±200	10100±300	12300±300
Fenitrothion	3.0	11200±200	9600±300	9500±400	6800±500
	30	11000±200	7600±400	8800±400	11400±500
Fenthion	2.5	10500±300	8100±400	8400±400	10800±400
	25	8700±400	6900±300	7300±300	9500±500
EPN	0.24	7600±200	8300±200	8900±400	10900±400
	2.4	6800±300	6800±300	7500±400	9400±500
Diazinon	3.5	9000±200	10600±300	10000±400	11900±400
	35	7700±200	9700±300	9300±500	10600±600
PCB	10	6600±400	10900±400	9900±300	8200±400
	100	6000±300	8200±300	9400±300	8200±400
Cyclophosphamide	45	3000±200	2800±300	4800±300	8700±300

* Data are presented as mean ±SE cells/mmi ; n=12 per group.
Mice were administered with pesticides for ten days.

1/10 LD_{50} and 1/100 LD_{50} respectively.

Cyclophosphamide as a positive control was dissolved in sterile saline immediately prior to use. Rats and mice were injected with 45mg/kg body wt. for 4 days. Control group received corn oil alone.

Humoral Immunity Assay

Each group consisted of 9 inbred Balb/c mice (male, 18-22g) and was administered pesticides orally for 10 days. Mice were immunized subcutaneously with 0.1ml sterile saline and Freund's Complete Adjuvant (FCA) (1 : 1) containing 2.0mg bovine serum albumin (BSA) at 28 day before assay. They were boosted with a subcutaneous injection of 1.0mg BSA in 0.1ml sterile saline and FCA (1 : 1) at 8 day before assay.

Blood samples were collected via a cardiac puncture and the obtained serum was stored at -20℃

Table IV. Effect of Organophosphate Pesticides on the Serum Proteins of the Rats*.

Pesticide	Dosage (mg/kg)	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio
Control	–	6.78±0.12	3.52±0.12	3.27±0.04	1.08±0.04
Fenitrothion	3.0	7.33±0.29[a]	3.70±0.06	3.63±0.27[a]	1.03±0.08
	30	6.93±0.13	3.50±0.26	3.43±0.14	1.03±0.12
Fenthion	2.5	7.23±0.39	3.30±0.15	3.93±0.26[c]	0.84±0.04[c]
	25	7.20±0.40	3.43±0.18	3.76±0.32[a]	0.92±0.08
EPN	0.24	7.17±0.33	3.53±0.14	3.63±0.43	1.00±0.14
	2.4	7.53±0.53[a]	3.13±0.49	4.40±0.25[c]	0.72±0.11[c]
Diazinon	3.5	7.80±0.47[b]	3.53±0.23	4.27±0.24[c]	0.83±0.01[c]
	35	7.60±0.10[c]	3.10±0.15[a]	4.40±0.25[c]	0.69±0.07[c]
PCB	10	7.67±0.66	3.07±0.60	4.60±0.17[c]	0.67±0.13[c]
	100	8.57±0.47[c]	3.50±0.10	5.07±0.53[c]	0.71±0.09[c]
Cyclophosphamide	45	6.63±0.39	2.47±0.28[c]	4.17±0.26[c]	0.59±0.08[c]

Rats were administered with pesticides for ten days.
Data are presented as mean ±SE.
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 V. Enzyme Activities in Serum of the Rats Achministered Organophosphate Pesticides .

Pesticide	Dosage (mg/kg)	ALT (SGPT) (U/L)	AST (SGOT) (U/L)	Alkaline Phosphatase (U/L)
Control	–	22.4±1.08	60.1± 3.9	85.2± 6.4
Fenitrothion	3.0	20.7±0.44	56.7± 5.9	96.7±19.4
	30	20.2±2.4	53.1± 2.7	83.2± 8.2
Fenthion	2.5	19.6±2.0	53.2± 5.8	98.6± 8.8
	25	23.3±1.1	70.3± 2.6	105.1±21.2
EPN	0.24	18.6±1.2	74.8±10.7	84.8± 7.6
	2.4	27.4±4.2	80.7±14.6	73.5± 5.1
Diazinon	3.5	19.7±1.8	59.3± 8.4	88.3±15.3
	35	19.7±2.2	59.8± 5.7	98.3± 7.4
PCB	10	20.0±2.3	62.1± 0.9	75.1±19.2
	100	20.8±1.6	99.8±10.9[a]	68.9±16.5
Cyclophosphamide	45	15.4±2.5[b]	37.5± 1.9[c]	46.2± 1.6[c]

* Rats were administered with pesticides for ten days.
Data are presented as mean ±SE.
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 $p<0.01$

until analyzed.

A Titertek Multiskan automatic analyzer was used to analyze anti-BSA antibody titer in the mouse serum.

Analyses were performed by a modification (14, 15) of the method of Engvall and Perlmann (16).

The Number of Circulating Leukocyte

Blood was collected from the retro orbital plexus, on 1st, 2nd, 4th and 7th day after the last pesticide treatment. Collected blood was about $80\mu l$ and was mixed with $320\mu l$ 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.

Clinco-chemical Values

Blood samples were cellected via a cardiac puncture and allowd 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 SBA 300 Automated Selective Clinical Chemistry Analyzer (Gilford).

Histopathology

Aniamls 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 (4 micron) were cut by microtome and stained with hematoxylin and eosin (H & E), and examined histologically.

Statistical Analysis

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

RESULTS

While none of the pesticide-treated animals died or revealed overt toxicity during experimental period, the pesticide exposure resulted in marked suppression in humoral immunity.

There was no significant alteration in body and relative organ weights in mice administered or-

Table VI. Clinico-chemical Values in Rats Administered Organophosphate Pesticides*.

Pesticide	Dosage (mg/kg)	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Control	–	47.9± 1.8	84.8± 8.2
Fenitrothion	3.0	54.7± 3.6[a]	67.5± 5.2
	30	50.1± 5.3	72.5±11.9
Fenthion	2.5	50.4± 4.4	44.6±2.7[b]
	25	56.1±3.8[b]	54.9±4.9[b]
EPN	0.24	49.3± 1.4	54.6±7.4[b]
	2.4	79.5±5.2[c]	45.6±5.2[b]
Diazinon	3.5	52.7± 2.8	57.4±4.2[a]
	35	54.5±1.4	40.4±3.1[c]
PCB	10	84.0±3.6[c]	69.9±21.7
	100	112.3±12.4[c]	104.6±44.2
Cyclophosphamide	45	65.7±10.2[b]	51.9±2.1[b]

* Rats were administered with pesticides for ten days.
Data are presented as mean ±SE.
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 $p<0.01$

ganophosphate pesticides although positive controls showed decreased immunoorgan/body weight ratio (Table I).

The effects of pesticides tested on humoral immunity in male Balb/c mice were measured and the results were shown in Table II. Pesticides tested produced a marked suppression of antibody titers against bovine serum albumin. Serum antibody concentrations were significantly decreased in all tested groups exposed to pesticides especially in higher dosed groups.

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 groups were lower than those of control group as shown in Table III.

In the pesticide treated group serum globulin levels were elevated in spite of the suppression of humoral response and serum albumin is slightly decreased (Table IV).

Table V shows the enzyme activities in serum of the rats administered organophosphate pesticides. Examination of serum enzyme acitivities indicated that s-ALT (s-GPT), s-AST (s-GOT) and alkaline phosphatase were not affeted by pesticide exposure in this experimental condition.

Table VII. Clinico-chemical Values in Rats Administered Organophosphate Pesticides*.

Pesticide	Dosage (mg/kg)	BUN (mg/dl)	Glucose (mg/dl)
Control	–	24.6±1.8	72.5± 2.7
Fenitrothion	3.0	19.1±0.9	64.7±12.9
	30	24.4±0.5	71.6±11.3
Fenthion	2.5	17.1±1.2	74.8± 5.9
	25	24.0±1.9	66.1±12.8
EPN	0.24	25.3±1.4	64.2± 8.5
	2.4	30.1±3.0	63.5±12.0
Diazinon	3.5	20.1±1.6	67.5±12.7
	35	21.9±0.8	74.8± 4.6
PCB	10	24.8±2.7	63.1± 5.9
	100	39.5±3.0[c]	62.5± 8.7
Cyclophosphamide	45	22.5±1.2	71.4± 3.4

* Rats were administered with pesticides for ten days.
Data are presented as mean ±SE.
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 VIII. Histological Changes in Rats Administered Organophosphate Pesticides.

Pesticide	Dosages (mg/kg)	Thymus atrophy	Thymus congestion	No. of lymphocyte in spleen white pulp
Control	–	–	–	–
Fenitrothion	3.0	–	–	D
	30	+	+	D
Fenthion	2.5	–	+	–
	25	+	+	D
EPN	0.24	–	+	–
	2.4	+	+	D
Diazinon	3.5	–	+	–
	35	+	+	D
PCB	10	+	+	D
	100	+	+	D
Cyclophosphamide	45	++	+	D

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

Table VI shows the effects of pesticides on serum lipids. The results indicated that serum cholesterol levels were increased in the groups exposed to fenitrothion, fenthion and diazinon. But obviously strong hyperglycemic effect was found in the groups exposed to EPN and PCB. But serum triglyceride levels were decreased in all tested groups except higher dose PCB treated group.

As shown in Table VII BUN and blood glucose levels were found quite normal in all tested groups.

On gross examination, thymus in particular showed a mild atrophy at higher dosage levels in pesticide treated groups (Table VIII). Thymus congestion was also observed in the pesticide treated groups. Any 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 pesticide treated groups. Especially cyclophosphamide treated group presented necrosis and depletion of the lymphocytes in spleen white pulp. Microscopic examination of thymus revealed no significant lesions in pesticide treated groups. However, cyclophosphamide presented a severe atrophy of thymus cortex and depletion of thymocytes. Histological examination revealed no significant lesions in the liver in pesticide treated groups.

DISCUSSION

In this study, we examined the effects of organophosphate pesticides on the humoral immunity and pathotoxicological parameters following subchronic exposure of rats and mice to nontoxic levels of the pesticides. While none of animals exposed to pesticides died or revealed overt toxicity during experiment, the treated groups showed marked suppression in humoral immunity.

There have been many reports on the immunosuppressive effects of the pesticides (8, 17). For example, some organophosphate pesticides -malathion, methyl parathion and dichlorovos *etc.*, have been shown to suppress humoral immune responses in laboratory animals (18,19). The pesticides tested in this study produced marked suppression of antibody titers against BSA. Serum antibody concentrations were significantly decreased in all treated groups, especially higher dosed groups. The recent reports on fenitrothion and diazinon were somewhat different results from those found in this study. That is, fenitrothion increased the serum IgG level in sheep and worker (20), and diazinon increased serum $IgG_{1,2}$ level in mice (21). But, we found in several articles that organophosphate pesticides showed somewhat varied immunosuppressive patterns, according to dose, administration route, test subjects and environmental factors, *etc* (9, 10). So, above discrepancies could be accounted, at least in part, by these factors.

While the cyclophosphamide treated group induced severe leukopenia, the pesticide treated groups showed more varied and less marked alterations. Generally, the number of circulating leukocytes in the pesticide treated groups were lower than those of control group.

At first, decrement in the relative spleen and thymus wights 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 support the immunosuppressive potential of organophosphate pesticides tested and were compatible with their effects on the humoral immunity.

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 suggests that the pesticide exposure might alter the liver function. This condition might be compared to the early phase of acute inflammation or necrotic process, in which slight elevation of α_2-globulin and slight decrease in albumin concentration.

Examination of serum enzyme activities indicated that s-ALT (s-GPT), s-AST (s-GOT) and alkaline phosphatase were not affected by pesticide exposure in this experimental conditions. Even though the activities of s-ALT, s-AST and alkaline phosphatase were found in normal range, it 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 in the groups exposed to fenitrothion, fenthion and diazinon. But obviously strong hyperglycemic effect was found in the groups exposed to EPN and PCB. However, serum triglyceride levels were decreased in all test -groups except higher dosed PCB treated group. But fenitrothion and fenthion showed increasing tendency by the elevation of doses. These results suggest that lipid metabolism or lipid releasing mechanism of the liver is negatively influenced by these pesticides.

Blood glucose levels and BUN values of all tested groups were found in normal range. But increasing tendency of BUN was observed in all tested groups by the increasing dose variation. It indicates a possibility that these kinds of pesticides might produce lipid metabolic disturbance by higher dose treatment or long term exposure.

There have been many publications that corticosteroids have the immunosuppressive actions (22-24), and Szot and Murphy (25) demonstrated sublethal doses of parathion elevated plasma corticosteroid concentration in rats. And so, it cannot be excluded that above immunosuppression might be mediated by glucocorticoids released in response to the toxic chemical stresses associated with the cholinergic crisis and clinical status.

In summary, pesticide exposure did not induce any signs of overt toxicity but, suppressed humoral immune function. Beacause of the wide spread use of these pesticides and potential for human exposure, further studies on their effects on other immune parameters are in progress.

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