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

Chang Kiu Moon, Yeo Pyo Yun*and Soo Hwan Lee

College of pharmacy, Seoul National University, Seoul 151, *Korea*

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Abstract □ Some of organophosphate pesticides which are the most heavily used in Korea, were examined for their effects on the murine immune system.

Immunotoxicological assay parameters adopted in this study were Arthus reaction for humoral immunity, delayed type hypersensitivity reaction for cell mediated immunity, carbon clearance for macrophage function and susceptibility to tumor challenge.

Subchronic exposure of rodents to the pesticides resulted in the marked suppression of immune functions and enhancement of susceptibility to tumor challenge.

Among the pesticides tested (fenitrothion, fenthion, diazinon and EPN), fenitrothion was the most suppressive in Arthus and delayed type hypersensitivity reaction.

Keywords □ Organophosphate pesticides, Arthus reaction, Delayed type hypersensitivity, Carbon clearance, Susceptibility to tumor challenge

Considering that excessively used pesticides could be exposed to human through several processes, it is important to know as much as possible about the effects of pesticides and their 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 in 1968 (7), 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.*

Fenitrothion, fenthion, diazinon and EPN, of which residues in food are very high, are the most heavily used in Korea (11). In a series of study to elucidate the immunotoxic potential of these pesticides, we began to examine their effects on the immune parameters recommended by NIEHS, USA (1, 12).

In the previous study, we investigated their effects on the humoral immunity and pathotoxicological indicators (13). In this study, we examined the Arthus reaction for humoral immunity, DTH reaction for cell mediated immunity, carbon clearance for macrophage function and susceptibility to tumor challenge following subchronic exposure of pesticides.

EXPERIMENTAL METHODS

Materials

Agrochemicals used (fenitrothion, fenthion, diazinon and EPN) were kindly supplied by Mr. H.Y. Kim, Korean Institute for Environmental Sciences. PCB and Freund's complete adjuvant were purchased from Wako Pure Chemical Ind. Co., cyclophosphamide 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 Experimental Animal Breeding Center of Seoul National University.

Animals were maintained as described in the previous report (13). Pesticide treatments were

* Present address; College of Pharmacy, Chung buk National University, Gyeshin-dong, Che ongju-city, Chungcheong-Buk-do 310, Korea

conducted at the dose of 1/10 LD_{50} and 1/100 LD_{50} for consecutive 10 days. Cyclophosphamide was dissolved in sterile saline immediately prior to use and injected into positive control group at the dose of 45mg/kg body weight for 4 days. Control group received corn oil alone.

Delayed and Antibody-mediated Hypersensitivity Assay

Cell mediated immunity was evaluated by delyed type hypersensitivity (DTH) reaction as described by Henningsen *et. al.* (14). Briefly, rats were sensitized on day 0 with 0.1mg bovine serum albumin (BSA) dissolved in 0.1ml of water-in oil emulsion composed of equal volumes of Freund's complete adjuvant (FCA) and sterile saline. Each rats was sensitized s.c. with 0.1ml of the control group was treated with 0.1ml saline. Seven days later, rats were challenged at the left rear footpad with 2% heat aggregated BSA (75ml). The right footpad was received an equal volume of sterile saline to serve as control site. Delayed type hypersensitivity reactions were determined 24hr after challenge by measuring footpad thickness with a micrometer caliper (Mitutoyo MFG Co., LTD., Japan).

Antibody mediated hypersensitivity reactions were determined 3hr after postchallenge.

Macrophage Function

The carbon clearance test for *in vivo* phagocytosis is based on the work of Biozzi *et. al.* (15)

Phagocytic activity was determined 2 days after the last pesticide adminstration. For the preparation of suspension of carbon, ink was diluted 1/6 with 1% gelatin and kept in a stoppered tube at 37℃ during the experiment. Injection was executed via the lateral tail vein at the dose of 0.01ml of colloidal carbon solution per gram of mouse. This corresponded to approximately 16mg carbon per 100g body weight of mouse. At the interval of 10 min, 20 min, 30 min and 40 min, 20 ml of blood sample was obtained from the retro-orbital plexus. The collected blood samples were expelled into each vial containing 1ml sodium carbonate and then measured absorbance against water blank at 600nm. 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 laboratory by serial i.p. passage in ICR mice.

Control and pesticide exposed ICR mice were inoculated s.c. in the right inguinal area with 4×10^4 cells 2 day 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.

Statistical Analysis

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

RESULTS

Pesticides tested significantly suppressed antibody mediated hypersensitivity (Arthus) reaction, and the results are shown in Table I.

Suppression of DTH reaction was observed in pesticide treated groups (Table II) and higher dosed group showed more obvious suppression. Relatively high sensitive responses of DTH to dose-variation was displayed in groups exposed to fenitrothion and fenthion. Fenitrothion showed the most suppression of DTH as in the case of humoral responses, but the suppression was not comparable to cyclophosphamide, which approximately two times potenter than fenitrothion.

Table I. Effect of Organophosphate Pesticides on Antibody-mediated Hypersensitivity (Arthus) reaction in Rats.

Pesticide	Dosage (mg/kg)	Degree of reaction footpad thickness (mm±SE)*	Index**
Control	-	5.27±0.19	1.18±0.05
Fenitrothion	3.0	3.95±0.31[b]	0.87±0.08[b]
	30	3.27±0.05[b]	0.71±0.01[b]
Fenthion	2.5	4.24±0.23[b]	0.92±0.04[b]
	25	3.40±0.03[b]	0.73±0.01[b]
EPN	0.24	4.28±0.07[b]	0.91±0.01[b]
	2.4	3.96±0.16[b]	0.87±0.03[b]
Diazinon	3.5	4.71±0.22[a]	1.02±0.05[a]
	35	3.80±0.33[b]	0.77±0.06[b]
PCB	10	3.97±0.21[b]	0.87±0.05[b]
	100	3.38±0.31[b]	0.74±0.07[b]
Cyclophosphamide	45	2.69±0.15[b]	0.58±0.03[b]

* Degree of reaction is measured as the difference in swelling between the antigen-challenged site and control site 3hr post-challenge on day 7.

** Index is the ratio of the difference in swelling between the antigen-challenged site and control site to the swelling of control site.

Data are presented as mean ±SE.

a) Significantly different from control group at $p<0.05$, b) Significantly different from control group at $p<0.01$

Corrected phagocytic indices were significantly decreased in the pesticide treated groups by the almost same levels at higher dosed groups (Table III). In comparison with the case of humoral and cellular immunity, suppression of phagocytic function was appeared regardless of the kinds of pesticides. Only PCB showed more suppressed phagocytic function than other tested pesticides.

Pesticide exposure resulted in a dose-dependent enhancement of susceptibility to sarcoma-180 tumor (Table IV). An increased frequency of tumor development occurred in pesticide treated groups compared to control group. The incidence of progressive tumor increased from 30% in control group to 90% in pesticide treated groups. Relatively higher susceptibility to tumor challenge was found in the groups exposed to EPN, fenitrothion and fenthion at higher doses. Especially higher tumor development was confirmed in the EPN high dosed group.

DISCUSSION

The effects of organophosphate pesticides on the immune functions and host susceptibility to tumor challenge were examined following subchronic exposure to nontoxic levels in rats and mice.

In the previous study, we could find a marked suppression of humoral immune response in the pesticide treated groups (13). Serum antibody concentrations were significantly reduced in all treated groups, especially in higher dosed groups.In accordance with these results, pesticides tested significantly suppressed antibody mediated hypersensitivity reaction (Arthus reaction). These results indicate that pesticide exposure may depress humoral imunity, not only through suppressing IgG production but also complement, neutrophil and mast cell.

DTH reactions were significantly depressed in pesticide treated aniamls as shown in Table II. Among the pesticides tested, fenitrothion was the most suppressive in DTH reaction as in the case of humoral immune responses. But, because of the complexity of DTH raction, there should be further studies for the elucidation of real elements or precise factors causing DTH-suppression.

Corrected phagocytic indices were significantly decreased in the pesticide treated groups by the almost same levels at higher dosed groups. These

Table II. Effect of Organophosphate Pesticides on Delayed Hypersensitivity Responses in Rats.

Pesticide	Sosage (mg/kg)	Degree of reaction footpad thickness (mm ± SE)*	Index**
Control	–	2.73 ± 0.06	0.61 ± 0.01
Fenitrothion	3.0	2.05 ± 0.12[b]	0.45 ± 0.02[b]
	30	1.43 ± 0.02[b]	0.31 ± 0.01
Fenthion	2.5	2.21 ± 0.15[a]	0.48 ± 0.04[a]
	25	1.66 ± 0.05[b]	0.36 ± 0.01[b]
EPN	0.24	2.02 ± 0.15[b]	0.43 ± 0.03[b]
	2.4	1.97 ± 0.07[b]	0.43 ± 0.01[b]
Diazinon	3.5	2.19 ± 0.17[a]	0.47 ± 0.10[a]
	35	1.83 ± 0.02[b]	0.37 ± 0.01[b]
PCB	10	1.92 ± 0.06[b]	0.42 ± 0.02[b]
	100	1.37 ± 0.07[b]	0.29 ± 0.01[b]
Cyclophospha mide	45	1.27 ± 0.05[b]	0.27 ± 0.01[b]

* Degree of reaction is measured as the different in swelling between the antigen-challenged site and control site 24hr post-challenge on day 8.
** Index is the ratio of the difference in swelling between the antigen-challenged site and control site to the swelling of control site.
Data are presented as mean ± SE.
a) Significantly different from control group at $p < 0.05$, b) Significantly different from control group at $p < 0.01$

Table III. Effect of Organophosphate Pesticides on the Carbon Clearance Activity (phagocyte activity) in Mice.

Pesticide	Dosage (mg/kg)	Phagocytic Index* (K)	Corrected** Phagocytic Index
Control	–	0.017 ± 0.003	3.73 ± 0.08
Fenitrothion	3.0	0.018 ± 0.002	3.71 ± 0.15
	30	0.014 ± 0.003	3.33 ± 0.09[b]
Fenthion	2.5	0.014 ± 0.002	3.45 ± 0.14
	25	0.015 ± 0.002	3.39 ± 0.16[a]
EPN	0.24	0.015 ± 0.002	3.37 ± 0.14
	2.4	0.012 ± 0.004	3.30 ± 0.18[b]
Diazinon	3.5	0.015 ± 0.001	3.65 ± 0.14
	35	0.016 ± 0.002	3.48 ± 0.09[a]
PCB	10	0.014 ± 0.002	2.88 ± 0.29[b]
	100	0.014 ± 0.006	1.96 ± 0.29[c]
Cyclophosphamide	45	0.017 ± 0.002	3.23 ± 0.27[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 the weight of the liver and spleen.
a) $p < 0.1$ b) $p < 0.05$ c) $p < 0.01$

Table IV. Tumor Development following Injection of Sarcoma-180 in Pesticide -treated Mice*.

Pesticide	Dosage (mg/kg)	No.of mice tested	No.of tumor developed	Percentage (%)
Control	-	10	3	30
Fenitrothion	3.0	11	6	54.5
	30	10	7	70
Fenthion	2.5	11	7	63.6
	25	10	7	70
EPN	0.24	10	3	30
	2.4	10	9	90
Diazinon	3.5	8	4	50
	35	10	6	60
PCB	10	11	6	54.5
	100	7	4	57.1
Cyclophosphamide	45	8	7	87.5

* Mice were challenged with 4×10^4 sarcoma-180 cells sc in the right inguinal area

** Percentage is Tumors per Number of tested animal ratio

results indicate that pesticides suppress the reticuloendothelial system. But incompatible with above results, there was a recent report that fenitrothion ehhanced phagocytosis in sheep at the dose of 1/10 LD_{50} (16). Although not fully explanable, this discrepancy can be accounted in part by differences in treat schedule and experimental animals.

Pesticide exposure resulted in a dose-dependent enhancement of susceptibility to sarcoma-180 tumor. It is well known that non-specific immunity plays an important role in host resistance againt 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 pesticide 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 organophosphates 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 organophosphate pesticides. Others demonstrated that organophosphate induced immunosuppression might result from direct action of acetylcholine upon the immune system (19). In support of this observation, cholinergic receptors have been identfied on lymphocytes and macrophages (20).

An alternative hypothesis to be considered is that the observed immunosuppression might be mediated by glucocorticoids released in response to the toxic chemical stresses associated with the cholinergic crisis. In support of this hypothesis, Szot and Murphy (21) have demonstrated the elevation of plasma corticosteroid concentrations in rats given sublethal doses of parathion. The immunosuppressive action of corticosteroids are well known (22, 23). 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 notoxic levels of the pesticides suppresses various immune functions (humoral immunity, cell mediated immunity and phagocytic function) and enhances host susceptibility to tumor challenge. Because of the wide spread use of these pesticides 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 these pesticides. And also, the effects of contaminants presented in these technical pesticides should be clarified.

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