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EFFECT OF CHLORPYRIFOS ON THE PROFILE OF SUBPOPULATIONS IMMUNOCOMPETENT CELLS B, T AND NK IN *IN VIVO* MODEL

Justyna Noworyta-Głowacka[1], Martyna Beresińska[1], Robert Bańkowski[1], Bożena Wiadrowska[1], Joanna Siennicka[2], Jan K. Ludwicki[1]*

[1] Department of Toxicology and Risk Assessment, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland

[2]Department of Virology, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland

ABSTRACT

Background. Current studies have indicated many environmental factors, such as pesticides, that cause immune system disorders through inducing changes in humoral and cellular responses thereby increasing the risk of contracting infectious diseases and cancer. The literature suggests that low exposures to certain organophosphorus pesticides stimulate the immune system, whilst high exposures result in decreased function. Precise mechanisms for the fall in immunocompetence are often unclear, however it can be predicted that the intimate interaction between the nervous and immune systems can potentially lead to toxicity.

Objectives. To determine the effects of organophosphorus pesticide, chlorpyrifos that is often used in Poland, on selected immunological responses, such as immune-competent cell proportions formed experimentally *in-vivo* by cells of *Wistar* rats during subchronic exposures after 45 and 90 days.

Materials and Methods. The test was carried out on ten male and ten female *Wistar* rats in each of three test groups, who received 3 chlorpyrifos doses for 90 days intragastrically, according to OECD guidelines (No. 401). Two control groups were given olive oil. After completion, the animals were deeply anaesthetised by a mixture of ketamine (Vetaketam) and xylazine (Vetaxym). Immuno-competent cells were profiled by a commercial monoclonal antibody method. In order to measure the dynamics of any changes, the aforementioned immunological responses were investigated after 45 days using the same procedures for obtaining the relevant biological test material.

Results. Test animals exposed to chlorpyrifos had altered number of white bood cells which were either increased or decreased relative to controls after 45 and 90 days for all exposure levels used.

Conclusions. The study demonstrated changes in white-blood cell (lymphocyte) response profiles, reflecting an immunomodulation although such changes were equivocal, where both suppression and stimulation were observed.

Key words*: immunomodulation, immune system, lymphocyte, organophosphorus pesticides, chlorpyrifos*

STRESZCZENIE

Wprowadzenie. Współczesne badania naukowe wskazują, iż wiele czynników środowiskowych, wliczając w to pestycydy, powoduje zaburzenia układu odpornościowego poprzez indukowanie zmian w odpowiedzi humoralnej i komórkowej, co w efekcie może prowadzić do zwiększonej zapadalności na choroby zakaźne i nowotworowe. Doniesienia literaturowe sugerują, że niskie poziomy narażenia na niektóre pestycydy fosforoorganiczne powodują stymulację układu odpornościowego, zaś wyższe, obniżenie funkcji immunologicznych. W wielu przypadkach nie jest poznany dokładny mechanizm spadku odporności, jednak poprzez związek funkcji neurologicznych i immunologicznych można przewidzieć potencjalne skutki działania toksycznego.

Cel. Celem badań było określenie wpływu chloropiryfosu na wybrane parametry odpowiedzi immunologicznej, takie jak skład odsetkowy komórek immunokompetentnych w modelu doświadczalnym *in vivo* szczurów laboratoryjnych szczepu *Wistar,* w trakcie ekspozycji subchronicznej 45 i 90 dniowej.

Materiał i metody. Badanie wykonano na samcach (10 szt.) i samicach (10 szt.) szczurów rasy *Wistar*. Zwierzętom z grupy badanej podawano dożołądkowo chloropiryfos przez 90 dni, zgodnie z zaleceniami OECD (Guideline No 401). Grupa kontrolna otrzymywała oliwę z oliwek. Po zakończeniu eksperymentu zwierzęta poddawano głębokiej narkozie przy użyciu mieszaniny: ketaminy (Vetaketam) i ksylazyny (Vetaxym). W celu określenia dynamiki ewentualnych zmian zbadano wyżej wymienione parametry immunologiczne po 45 dniach doświadczenia przy zachowaniu tych samych procedur uzyskania materiału badawczego.

***Corresponding author:** Robert Bańkowski, Department of Toxicology and Risk Assessment, National Institute of Public Health – National Institute of Hygiene, 24Chocimska Street, 00-791 Warsaw, Poland, phone: + 48 22 5421 332, e-mail: rbankowski@pzh.gov.pl

Wyniki. Uzyskane wyniki wskazują, że narażanie zwierząt doświadczalnych na chloropiryfos zmieniało skład odsetkowy komórek immunokompetentnych w modelu doświadczalnym *in vivo* zwiększając albo zmniejszając ich wartość w stosunku do poziomów oznaczonych u grupy zwierząt kontrolnych po 45 i 90 dniowej ekspozycji i na wszystkich poziomach narażania.
Wnioski. Odnotowane w wyniku przeprowadzonych badań zmiany w profilu białokrwinkowym świadczące o działaniu immunomodulującym były niejednoznaczne przyjmując formę zarówno immunosupresji jak i immunostymulacji.

Słowa kluczowe*: immunomodulacja, układ odpornościowy, limfocyty, pestycydy fosforoorganiczne, chloropiryfos*

INTRODUCTION

Undoubtedly, the widespread use of pesticides provides great benefits for their intended purposes, nevertheless their residual presence in foodstuffs results in agricultural workers becoming exposed to these substances, thereby constituting a health risk. In particular, those involved in the manufacture and distribution would be vulnerable, along with workers who come into direct contact with plant protection products or treated crops. Pesticide exposure can also occur domestically or in veterinary practices, including organophosphorus pesticides, where they are commonly used for pest control.

Organophosphorus insecticides are compounds that rapidly decompose and thus do not bio-accumulate, however trace amounts have been detected in the environment, even for several years after having been applied. For this reason they are considered as chemicals that are most widespread in the environment, where their presence constitutes an adverse health risk [21, 22]. A high risk of acute poisoning exists in only a small part of the general population who are exposed to high pesticide doses, where the effects are well known. Despite this, there are probably much greater numbers, for whom the exposure is small, arising from consuming pesticide residues in foodstuffs or being exposed through pest control measures applied either domestically, at the workplace or in general public areas. These exposures can be chronic and may lead to disorders of the immune system as well as endocrine gland function, further leading to disorders of development and reproduction [1, 6, 10, 15, 17, 19].

Direct effects of organophosphorus pesticides are to inhibit serine hydrolases (complement system) and esterases (cell membranes of lymphocytes and monocytes). They also cause oxidative damage to organs, signal transduction pathway changes and alterations in cell proliferation and differentiation. Indirect effects of these substances are immunotoxicity, where nervous system function is compromised and chronic effects are seen on metabolism in the immune system [9].

Numerous chemicals target the immune system, potentially causing many adverse outcomes. Indeed, many countries have demonstrated increased disorders with an underlying immunological basis, eg. hypersensitivity reactions, autoimmune disease and forms of cancer. Xenobiotics may facilitate and potentiate pathological immune mechanisms through inducing the mutation of those genes coding for immune-regulatory factors or modifying immunological tolerance and activation pathways. Amongst the currently used insecticides, organophosphorus compounds constitute the most dangerous risk of acute poisoning. The statistics indicate that pesticides have been responsible for around 4-5% of the total acute poisonings in Poland, of which 73% were due to organophosphorus insecticides [12]; the corresponding figure being 80% in the USA, where these insecticides are universally used [21].

In the mid-1970s, the first reports appeared on the immunotoxic action of organophosphorus insecticides [8, 10], however only a limited range of these compounds was tested [13]. Animal model experiments for elucidating the effects of organophosphorus compounds on the immune system, demonstrated initiation of a humoral immune response dependent on T-cell action in rodents, who had orally received cholinergic substances like parathion, malathion [3]. Studies on *Wistar* rats, that were given subcutaneous DDVP (an organophosphorus insecticide), clearly showed a decrease of Natural Killer (NK) cell activity, which play an important role in eliminating spontaneously occurring cancer cells and antibody dependent cytotoxic cells [27]. Short term experiments on male *Fisher* rats, however demonstrated the following effects of chlorpyrifos on the immune system [2];

- decreased T-cell blastogenesis, induced by concanalvin A
- decreased T-cell blastogenesis, induced by phytohaemagglutinin
- decreased humoral immunocompetence witnessed by decreased erythrocyte antigens
- relative increases in the proportion of CD5 and CD8 cell expression.

The presented study was thus aimed at determining the effect, *in vivo*, of chlorpyrifos (an organophosphorus insecticide), on the profile of immune-competent sub-populations of cells from experimental *Wistar* rats, after 45 and 90 days exposure to this substance.

Table 1. Influence of chlorpyrifos on the profile of subpopulations of immunoactive cells

Cell type	Experiment group in relations to the control group						Control group	
	1/20 LD_{50} (4.15mg/kg b.w.)		1/10 LD_{50} (8.3mg/kg b.w.)		1/5 LD_{50} (16.6mg/kg b.w.)			
	45 days exposure	90 days exposure	45 days exposure	90 days exposure	45 days exposure	90 days exposure	45 days exposure	90 days exposure
Lymphocyte-T	*72.3±5.7 ↓	68.3±5.1↑	*61.4±8.6 ↓	67.5±9.8 ↑	*58.3±7.4 ↓	69.5±7.7 ↑	83.5±5.7	62.7±7.7
Lymphocyte-B	*21.1±4.6↑	16.4±7.5 ↓	*28.5±8.7↑	22.8±7.2 ↓	*30.9±4.7↑	19.9±7.0 ↓	12.1±4.6	23.8±8.9
Lymphocyte-NK	6.6±2.6↑	15.3±7.2↑	*10.1±3.5↑	9.7±4.2 ↓	*10.8±5.6 ↑	10.5±5.4 ↓	4.4±2.4	13.5±2.9
Lymphocyte-$CD4^+$	75.4±2.5 ↓	*78.4±3.9 ↑	77.2±4.0 ↓	*77.4±3.5 ↑	*70.7±9.6↓	*78.0±3.0 ↑	78.9±1.9	73.7±3.4
Lymphocyte-$CD8^+$	*23.3±2.5↑	*20.8±3.9 ↓	21.9±4.0 ↑	21.9±3.8↓	*24.8±3.2 ↑	*20.7±2.8 ↓	19.9±1.8	25.5±3.4

*statistically significant result
↓ reduction in the percentage of cells in the test group relative to the control ± standard deviation
↑ increase in the percentage of cells in the test group relative to the control ± standard deviation

MATERIALS AND METHODS

An *in-vivo* animal model was chosen for the study using a rat species recommended by the OECD for sub--chronic toxicology experiments. Male and female *Wistar* rats were thus selected of mean body masses 160g and 230g respectively. During the 7 day acclimatisation period and ensuing duration of experiments, rats were fed a balanced diet of Labofeed B pellets with *ad libitum* watering. Rats were kept at 21-23°C throughout, with a humidity of 45-55%. The circadian rhythm of sleep and wakefulness was regulated by the rats receiving 12 hours of light per day.

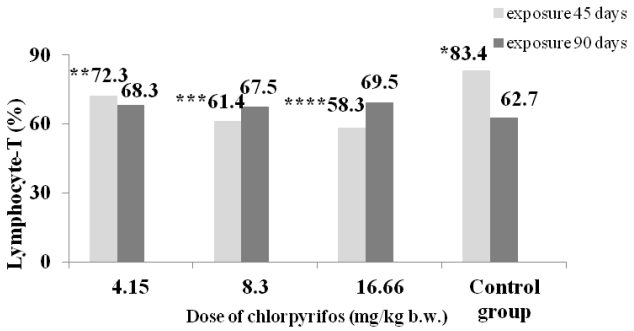
The rats were exposed to the test substance for 90 days. Three study groups were created, where rats received 1/5, 1/10 and 1/20 of the LD_{50} 97% chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate), together with 2 positive and two negative control groups who received just the test substance medium ie. olive oil. Each group consisted of 10 males and 10 females. The olive oil soluble form of chlorpyrifos was given once daily by gavage throughout the duration of the experiment. The control groups were given the following preparations according to the desired effect on the immune system;

- Dexasone – a glucocorticosteroid preparation with powerful immunosuppressive action; given once daily by intramuscular injection for a week prior to the 45 and 90 day experimental time points.
- Levamisole 7.5% solution for injection – evoking immune-stimulation; given once daily by intramuscular injection for two weeks prior to the 45 and 90 day experimental time points.

Such manoeuvres were intended to provide a view of the leukocyte system, when either immunologically stimulated or suppressed to which the test groups could be compared ie. those groups exposed to the three test doses of chlorpyrifos; 1/5, 1/10 i 1/20 LD_{50}. In order to follow the dynamics of immunological response, the sub-population profiles of immune-competent cells were analysed of B, T (CD4+,CD8+) and NK cells at the half way and end points of the experiment. For each time point, five animals from each of all the groups were taken for analysis, using the same procedures for obtaining the test material. The animals' welfare was assessed daily and weights were measured once weekly. On the appointed day of experimentation, the animals were deeply anaesthetised with a mixture of ketamine (Vetaketam) and xylazine (Vetaxym) and 4-6 mL of blood was sampled from the heart whilst the rats were still living and then appropriately stored into lithium-heparin blood tubes. The composition of B, T(CD4+,CD8+) and NK cells in the blood samples were determined with a Becton-Dickinson kit using monoclonal antibodies. Statistical analysis included qualitative tests - the non--parametric *Tukey's* multiple comparisons test.

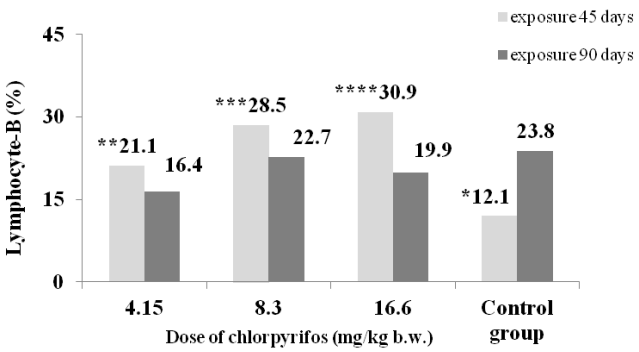
RESULTS

The subpopulation response profiles of immunocompetent B , T(CD4+,CD8+) and NK cells are presented jointly for male and female rats in effect disregarding any gender influence; thus reflecting normal conditions



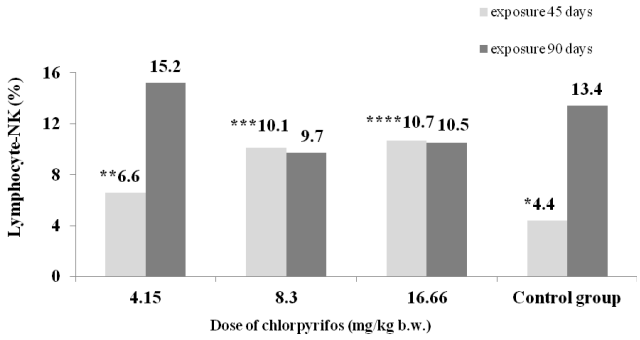
** vs.* - p<0.05 statistically significant difference
*** vs.* - p<0.05 statistically significant difference
**** vs.* - p<0.05 statistically significant difference
There were no significant differences between the experimental groups and the control group as a result of 90 days of exposure.

Figure 1. Effect of chlorpyrifos on percentage of T cells



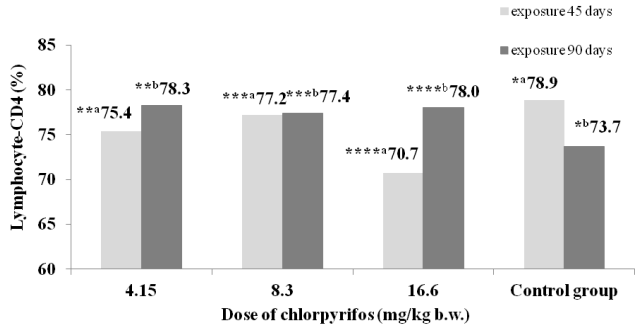
** vs.* - p<0.05 statistically significant difference
*** vs.* - p<0.05 statistically significant difference
**** vs.* - p<0.05 statistically significant difference
There were no significant differences between the experimental groups and the control group as a result of 90 days of exposure.

Figure 2. Effect of chlorpyrifos in the percentage of lymphocytes B



*** vs.* - p<0.05 statistically significant difference
**** vs.* - p<0.05 statistically significant difference
There were no significant differences between the experimental groups and the control group as a result of 90 days of exposure.

Figure 3. Effect of chlorpyrifos in the percentage of lymphocytes NK



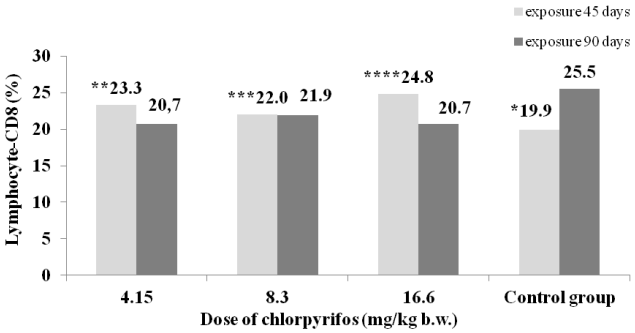
****a vs *a - p<0.05 statistically significant difference
** b vs *b - p<0.05 statistically significant difference
***b vs *b - p>0.05 statistically significant difference
****b vs *b - p>0.05 statistically significant difference

Figure 4. Effect of chlorpyrifos on percentage of CD4+ lymphocytes

found in nature. A significant modulating effect on leucocytes was observed in the experimental animals after 45 days of exposure. Such effects were equivocal with both immunosuppression and immunostimulation demonstrated; results are presented in Table 1.

Proportions of T-cells were found to be lowered, irrespective of the doses used, compared to controls after 45 days of exposure however, after 90 days exposure the situation was reversed for all doses, where T-cells were raised compared to controls; Figure 1. Furthermore, increased proportions of B-cells, irrespective of dose, were seen compared to controls after 45 days exposure, but these were all decreased after 90 days exposure under the same conditions; Figure 2. An increase in NK cells was also seen only after 45 days exposure compared to controls, irrespective of dose; Figure 3.

Regardless of the chlorpyrifos dose, the CD4 cell proportions were lowered compared to controls after 45 days of exposure but were all found to increase at 90 days exposure; Figure 4. In the case of CD8 cells, exposure after 45 days showed them to increase, irrespective of dose, compared to controls but a decrease in all analogous tests was observed after 90 days exposure; Figure 5.



**** vs * - p<0.05 statistically significant difference
There were no significant differences between the experimental groups and the control group as a result of 90 days of exposure.

Figure 5. Effect of chlorpyrifos for the percentage of CD8+ lymphocytes

DISCUSSION

The results reported, form a continuation on from previous studies [16], where the exposure time to chlorpyrifos was limited to 28 days, with doses used of 25, 17, 12.5 and 8 mg/kg body mass of experimental rats. Likewise, samples of peripheral blood had been collected to determine immune-responses by the proportions of B, T (CD4+, CD8+) and NK cells. Resulting analyses had then indicated a modest negative correlation between exposure levels and the number of NK cells. There was however no immunosuppression, compared to the immunosuppressive controls using dexamethasone. In addition, phagocytic granulocyte activity in all the test groups was not significantly different from that in controls.

Literature reports suggest that low orgnophosphorus pesticide exposures provoke immune system stimulation, but higher doses decrease immunological function.

In many instances however, the precise mechanisms of decreased immune function are unknown but through the intimate association of neurological and immune system functions it is possible to predict any potentially toxic effects. T-cells and to a lesser extent B-cells contain all components of the cholinergic system present in neurones; ie. acetylcholine (Ach), choline acetyltransferase (ChAT), high-affinity choline transporter (CHT), muscarinic acetylcholine receptors (mAChR), nicotinic acetylcholine receptors (nAChR), and acetylcholinesterase (AChE). When subjected to organophosphorus pesticides, T and B-cells react in similar ways to neurones. During the interaction of T cell receptor-mediated TCR / CD3 antigen presenting lymphocytes, an increased synthesis and secretion of ACh is observed. As a result, stimulation of mAChR occurs along with synthesis of IL-2 and its receptor. Contemporaneously, T-cell proliferation becomes intensified. Under normal conditions, secreted ACh concentrations from lymphocytes are low due to high cholinesterase activity. Under conditions of acute exposure and high concentrations of organophosphorus pesticides, intense production and accumulation of ACh occurs in the immunological synapses (interface between T-cells and antigen presenting cells) together with blocking of the mAChR and reduced rate of IL-2 synthesis and that of its receptor. Proliferation of T--cells becomes thus inhibited. When exposed to low organophosphorus compound exposure, cholinesterase becomes only partially inhibited, but the ACh generated from T-cell secretions accumulates in immunological synapses and may as a result stimulate ACh dependent IL-2 synthesis. Taking this evidence into account, it may be expected that the effect of organophosphorus compounds on T-cells inhibits their proliferation under high concentrations of pesticides, but are stimulated under low ones [14].

Both *in vitro* and *in vivo* studies on organophosphorus compounds indicate histological changes in tissues of the immune system, along with disorders in the maturation and function of immunocompetent cells including, a participating proportion of B and T-cells [25, 26].

In human studies, organophosphorus compounds have shown effects on the production of antibodies [3], interleukin IL-2 [18] and T-cell proliferation induced by IL-2 [5]. Such relationships may also cause a decrease in non-traditional B lymphocytes containing the CD5 cell surface marker (B1a), but an increase in CD26 cells and auto-antibodies associated with allergy and hypersensitivity towards antibiotics [23, 24]. These compounds may inhibit complement, alter the Th1/Th2 cytokine profile [4, 7] along with decreasing the activities of NK cells, lymphokine-activated killer (LAK) cells and cytotoxic T lymphocytes (CTL). During infection, when neutrophil activation increases, an exposure to organophosphorus compounds may lead to the immunological function of neutrophils being lowered [11]. Human *in vitro* studies [17] on T-cells and bronchial epithelial cells exposed to chlorpyrifos, in the presence or absence of an oxidative stress agent, demonstrated the cytotoxicity of this pesticide already at concentrations ≥ 250 μM. The immunomodulatory action of this compound may therefore be similar to most of the studied cytokine promoters, especially since the presence of a oxidative stress agent, potentiates the outcomes of chlorpyrifos action.

CONCLUSIONS

1. Following 45 day chlorpyrifos exposure, the lymphocyte system cells became significantly modulated in the test animals. Only CD4 lymphocytes, irrespective of dose, became significantly altered after both 45 and 90 days exposure.
2. The observed changes to the lymphocyte system profile, attesting to immunomodulatory action, were equivocal where both suppression and stimulation were observed.

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Conflict of interest

The authors declare no conflict of interest.

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