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APPLICATION OF IMMUNOSTIMULANTS AFTER SUPPRESSION INDUCED BY XENOBIOTICS: EFFECT OF LYSOZYME DIMER (KLP-602) AFTER IMMUNOSUPPRESSION INDUCED BY ATRAZINE IN RABBITS

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The interactions of xenobiotics with the immune system can finally induce immunosuppression or immunopotentiation. It is of great importance to study the early detection of immune deficiencies and modifications of the non-specific defence mechanisms. The immunostimulants are the agents that stimulate the response of effector cells such as macrophages, neutrophils and lymphocytes. They are not specific for a particular antigen, and are capable of enhancing non-antigen-specific resistance against infectious or neoplastic conditions. The purpose of this study was to determine the possibility of modulating the cellular immune response with the dimer of lysozyme (KLP–602) in rabbit after suppression induced by atrazine. Dimerized lysozyme is a natural immunomodulator obtained by monomer dimerization. It is an active substance of the preparation Lydium KLP, which is introduced to veterinary treatment in Poland. In the *in vivo* study rabbits were given an intraperitoneal injection of atrazine at the dose of $^1/_4LD_{50}$, then after 48 h they were administered the dimer of lysozyme (KLP–602) at the dose of 0.02 mg/kg b.w. After 2, 4, 7, 14 days post intoxication cells from blood of rabbits were isolated. In the study we determined the metabolic activity of phagocytes and the proliferative ability of T and B lymphocytes. The results indicate that this modulator is useful for stimulation of cellular and humoral immunity after experimentally induced suppression by the selected pesticide.

INTRODUCTION

Xenobiotics are factors of the external environment which cause significant change in the physiological functions in humans and animals. A diverse group of chemicals, including pesticides, heavy metals, dioxins, have been implicated as agents that may affect the immune system. The immune system is one of the most readily mobilisable defence barriers, and immunological responses have therefore been proposed as biomarkers of exposure to environmental pollutants [Fournier *et al.*, 2000].

Many common pesticides affect the immune system of laboratory animals and humans leading to its disregulation and to abnormal immune response. A disturbance of this steady state system might be harmful to the organisms by an inadequate and inefficient defense against infection or neoplasm (mainly lymphomas). Infections have been shown to be more frequent, often relapsing, more severe, and atypical, *e.g.* opportunistic infections. Alterations of the normal immune response can result in increased an autoimmune responses to the host antigens or allergic responses to common antigens present in the host environment. An increase in the prevalence of allergic diseases in the populations of industrialised countries may be in part related to the exposure of these populations to a growing number of xenobiotics [Banerjee, 1999; Barnett & Rogers, 1994; Burrell, 1995; Descotes & Vial, 1994; Stiller-Winkler *et al.*, 1999].

A large number of compounds with diverse chemical structure, *e.g.* synthetic products – levamisole, FK-565, muramylodipeptide – MDP, structural elements of bacteria – lipopolysaccharides, lipopeptides, capsular glycoproteins, animal or plant extracts – licorice, nutritional factors – vitamins B, C, hormones and cytokines – growth hormone, prolactin, interferon, lactoferrin are reported to be effective immunomodulators [Hadden, 1992].

The need to develop non-toxic and site-specific immunomodulators is greatly felt. One such compound, a Lydium KLP preparation, which is introduced to veterinary treatment in Poland by Nika Health Products, is known to be associated with antiviral, antibacterial and of various other biological activities. Unfortunately the mode of activity of the dimer of lysozyme has not been fully understood yet. Probably an increased activity can be generated by dimerization of lysozyme [Kiczka, 1994]. It may enhance the animals immune functions by the non-specific modulation of leukocyte activities.

The aim of this study was to determine the influence of the dimer of lysozyme (KLP-602) – active substance of the Lydium KLP preparation, on the selected parameters of immune cells isolated from rabbit after prior *in vivo* suppression of atrazine – a systemic herbicide.

MATERIAL AND METHODS

The study was carried out using 9 healthy rabbits (*Oryctolagus cuniculus*) weighing 2.5–3 kg. Animals were

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obtained from a commercial farm and remained under veterinary control.

The rabbits were divided into 3 groups (3 animals each): group I – control, group II – animals intoxicated by an intraperitoneal injection of atrazine at a dose of 125 mg/kg – $^1/_4LD_{50}$, and group III – animals intoxicated by an intraperitoneal injection of atrazine at a dose of 125 mg/kg – $^1/_4LD_{50}$. After 48 h the rabbits were injected intraperitoneally with the dimer of lysozyme (KLP-602) solution at a dose of 0.02 mg/kg of body weight.

The blood was obtained from rabbits of each group before and 2, 4, 7, 14 days after intoxication. In the studies the following parameters were assessed: metabolic activity of phagocytic cells, and proliferative response of lymphocytes to mitogens: cancanavalin (ConA), lipopolysaccharide (LPS).

Isolation of phagocytic cells and lymphocytes. Peripheral blood samples were collected from the ear vein and diluted 1:2 in RPMI 1640 without Ca^{2+}/Mg^{2+} (Biomed, Lublin, Poland). Cells were purified by centrifuging at 400×g for 40 min at an appropriate density gradient: Gradisol G (for neutrophil isolation) and Gradisol L (for lymphocyte isolation) (Aqua – Medica, Łódź, Poland). The interface cells were washed three times and suspended in the RPMI 1640 with 0.1% FCS (foetal calf serum, GIBCO, UK) for neutrophils and with 10% FCS for lymphocytes. Viable cells were determined by trypan blue exclusion and were evaluated to be greater than 98%. The viable phagocytes were adjusted to $3–5\times10^6$ cells mL^{-1} and lymphocytes to $1–3\times10^6$ cells mL^{-1}.

Evaluation of metabolic activity of neutrophils. Metabolic activity of phagocytes was measured by the respiratory burst activity (RBA test), using stimulation by PMA (phorbol myristate acetate, Sigma, Aldrich), as described by Rook *et al.* [1985]. Cells were dispensed into 96-well plates (100 μL) at a concentration of $3–5\times10^6$ cells mL^{-1} and left for incubation (2 h at 20°C, 5% CO_2). Then the non-adherent cells were washed and 100 μL of PMA (1 μg/mL) in 0.1% NBT (nitroblue tetrazolium, Sigma, Aldrich) solution in RPMI 1640 medium were added to each well. The mixture was incubated for 30 min at 20°C, 5% CO_2. After incubation, medium was removed and the cell pellet was washed with absolute ethanol. Then each well was filled with 120 μL of 2 mol KOH and 140 μL of DMSO (dimethylsulphoxide, POCh, Gliwice, Poland). The optical density was measured at 630 nm on a plate microreader Stat Fax 2600 (Awarness Technology, Finnland).

Mitogenic response of lymphocytes. The proliferative response of blood lymphocytes stimulated by mitogen conconavalin A - ConA (Sigma, Aldrich) or lipopolysaccharide – LPS (Sigma, Aldrich) was determined using the MTT colorimetric assay described by Mosmann [1983]. Lymphocytes suspension (100 μL/well) were dispensed into 96-well plates at a concentration of $1–3\times10^6$ cells mL^{-1}. Mitogens: 10 μL of conconavalin A (50 μg/mL RPMI) or 10 μL lipopolysaccharide (10 μg/mL RPMI) were added for stimulation of T or B lymphocyte proliferation, respectively. The microplate was incubated for 72 h at 20°C at 5% CO_2. After incubation, MTT (3-[4,5-dimethylthiazoly-2yl]-2,5-diphenyltetrazolium bromide, Sigma, Aldrich)) solution at a concentration of 7 mg/mL was added and the plate was incubated for the next 4 h. Then supernatant was removed and 100 μL DMSO was added to each well. The optical density was measured at 630 nm.

Statistical analysis. Statistical evaluation of results was performed using computer program Statistica 5.0. Data were analysed statistically by one-way analysis of variance (ANOVA). Duncan's post-hoc test was used to determine differences between groups. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

The presence of pesticides (chronic stressors, which affect the immune system) in environment is correlated with an increase in the incidence of infectious disease outbreaks and tumours. Atrazine and its metabolites are the most commonly detected contamination in surface and ground water in Europe. Some studies have shown that atrazine can disrupt normal immune system functions, enhancing the risk of infectious diseases or cancer [Böcher *et al.*, 1993; Hooghe *et al.*, 2000].

Considering the presence of synthetic compounds and environment pollutants in our life, studies on elaborating effective methods of enabling correction of the handicapped immunity are essential. The stimulation of the dimer of lysozyme (KLP-602) on metabolic ability of cells isolated from blood measured by RBA assay after prior *in vivo* suppression by atrazine is shown in Figure 1. The metabolic activity was statistically increased 2 days after injection of the modulator.

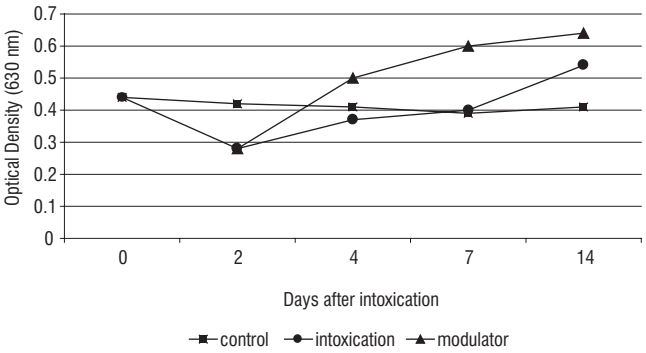

FIGURE 1. *In vivo* effects of the dimer of lysozyme (KLP-602) after prior *in vivo* intoxication with atrazine on metabolic activity of phagocytic cells (RBA). Data are shown as mean ±SD, n=10.

Immunomodulatory activity of the dimer of lysozyme on T lymphocyte proliferation is shown in Figure 2. The proliferative response of lymphocytes to concanavalin A significantly increased 2 days after administration of the modulator and the maximum response was seen 5 days after injection.

Proliferative activity of B lymphocytes was significantly reduced after intoxication by atrazine (Figure 3). The results of the present study demonstrate that the dimer of lysozyme is a very good stimulator of proliferative activity of B lymphocytes. Statistically significant increase was found 2 days after administration of the modulator and stimulatory response was still seen 12 days after administration of the KLP-602.

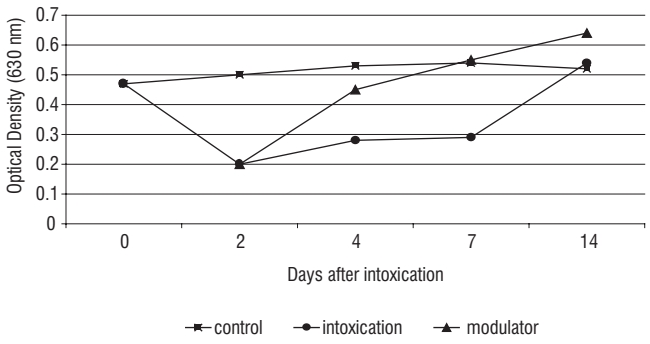

FIGURE 2. *In vivo* effects of the dimer of lysozyme (KLP-602) after prior *in vivo* intoxication with atrazine on proliferative ability of T lymphocytes stimulated by ConA. Data are shown as mean ±SD, n=10.

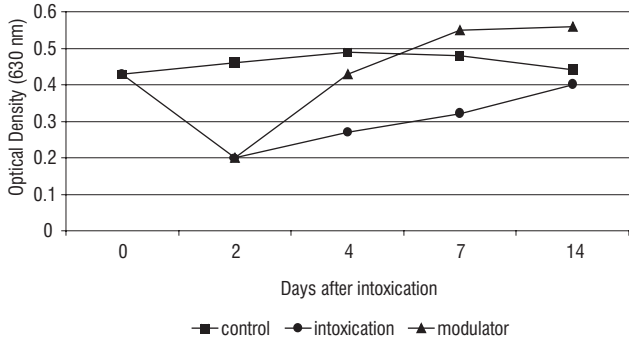

FIGURE 3. *In vivo* effects of the dimer of lysozyme (KLP-602) after prior *in vivo* intoxication with atrazine on proliferative ability of B lymphocytes stimulated by LPS. Data are shown as mean ±SD, n=10.

Our studies showed that this modulator was very useful for stimulation of cellular immunity after experimentally induced suppression with atrazine.

Only a few studies have reported the relationship between pollutants and immunomodulators. It has been described that the application of natural biological products in fish culture for the modulation of depressed immunity is very important practically. The study conducted by Studnicka *et al.* [2000] found that the dimer of lysozyme (KLP-602) could be used for the restoration of cellular and humoral defence mechanisms and elimination of the immunosuppressive effects of selected xenobiotics (oxytetracycline, oxolinic acid, lindane) in carp. The results are consistent with those obtained by our *in vitro* studies. The application of different concentrations of this modulator (50, 20, 10 μg/mL of medium) increased the metabolic activity of macrophages and proliferative ability of lymphocytes after *in vivo* suppression induced by selected pesticides [Rymuszka *et al.*, 2000].

These results were similar to those obtained with other immunostimulants. Dunier *et al.* [1995] analysed the immunotoxic influence of lindane. The suppression of immune system in rainbow trout was reversed by administration of vitamin C. In the other study Siwicki and Dunier [1994] found the efficacy of Nitrogranulogen NGG, (Polfa, Poland) after *in vivo* suppression induced by lindan.

Many papers have reported that the preparation Lydium KLP, whose active substance is dimer of lysozyme (KLP-602) and which is introduced to veterinary treatment in Poland by Nika Health Products, can modify the activity of phagocytes and other components of the immune system or increase resistance against diseases in higher vertebrates. Preclinical tests showed that Lydium KLP – is a non-toxic, well-tolerated and safe medication [Garbuliński, 1994; Klein & Kiczka, 1994]. Clinical tests with diseased animals of various species showed fast and effective action against diseases of viral and bacterial etiology [Pejsak *et al.*, 1994; Wiśniewski *et al.*, 1994].

The study by Kołodziejczyk *et al.* [2002] indicated the positive effect of Lydium KLP in treating coliform mastitis in sows and mixed respiratory infections in swine. Mukezamfury *et al.* [1996] in the study of influence of Lydium-KLP showed, that this preparation significantly increased the humoral immune response of rabbits. Antibody titre level after application of Lydium-KLP was twice higher than that in the control group. Moreover, Siwicki *et al.* [1997] in the study, which was performed on pigs, showed that the dimer of lysozyme (KLP-602) stimulated the proliferative activity of T and B lymphocytes and the metabolic activity of phagocytes. The authors observed significantly increased secretion of IL-1, IL-2, IFN-γ and the increased level of lysozyme. Kiczka and Dudko [1994] investigated the effect of intravenous administration of Lydium-KLP on selected clinical and laboratory parameters of blood in healthy calves. The authors found increased percentage of granulocytes, mainly neutrophiles. Injection of this preparation increased the percentage of phagocytic cells, phagocytic index and percentage of NBT-positive neutrophils in healthy and diseased calves [Kiczka *et al.*, 1994a]. The study by Pomorski *et al.* [1994] confirmed the efficacy of Lydium KLP therapy in some diseases in dogs. The best results were obtained in the acute cases, the worse in the chronic ones. It was found that the preparation intensified efficiency of antibiotics therapy. These results were in agreement with those obtained by Kiczka *et al.* [1994b], which was performed on foals. The tested preparation neither affected the foals clinical status. Instead, it caused mobilisation of the immune system by increasing the percentage of segmented neutrophils.

Immunostimulants are used as an alternative approach to antibiotic treatment (because of their negative effects on the environment) and vaccination (because of their limited effects) in the prevention of diseases.

The study conducted by Samorek-Salamonowicz *et al.* [2001] showed that application of Lydium-KLP and Levamisol in reproductive flocks during their vaccination against Derzsy's disease gave favourable results. It was also found, that injection of Lydium KLP improved results of intramammary treatment with antibiotics in cows [Malinowski *et al.*, 1994]. Moreover, the immunostimulatory effects of the immunomodulator were more significant in the fish before immunization than after immunization. The study performed by Morand *et al.* [1999] in sheatfish showed that dimerized lysozyme stimulated the non-specific cellular and humoral mechanisms and protection against MAS – *motile aeromonas septicaemia*.

CONCLUSION

In conclusion, the present results showed that administration of the dimer of lysozyme (KLP-602) stimulated and corrected the suppressed immunity and can be applicated as a prophylactic agent.

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