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THE EFFECT OF IMMUNOBIOLOGICAL DRUGS IN CASE OF METSULFURON-METHYL INTOXICATION

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Abstract

Aims: To study the effect of immunomodulating agents (liquid bacterial interferon inducer and Antivirin - M) using the model of chronic human intoxication by the metsulfuron-methyl pesticide in experimental animals.

Main methods: The authors used immunological and statistical methods. The significance of differences was measured according to p- value. The differences were considered significant at $p \leq 0.05$. The paper presented results of studying the MSM impact on the cell culture in vitro and in vivo using a model of chronic intoxication caused by this pesticide.

Key findings:

The study showed that small doses of pesticide that did not cause cell degeneration, led to 25% decrease in cell production of α / β IFN.

Chronic intoxication modelling detected immunosuppression, which revealed itself in a 50% decrease in the infection resistance in the experimental animals. The use of immunomodulating agents - LBII and Antivirin – M stimulated the production of endogenous α / β IFN both in cell culture and in experimental animals. The experiment also showed normalization of immune functions and increase in the production of antiviral antibodies.

Significance:

The studied immunobiological drugs protect the human body from the immunosuppressive effect caused by this pesticide and enhance the ability of the animal organism to produce interferon. In this respect, the authors recommend using data related to immunological drugs with a view to enhance the body's resistance to the toxic effects of pesticides.

Key words: Pesticides, Metsulfuron-Methyl, Biological Pesticides, Environmental Pollutants, endogenous α / β-interferon, Interferon Inducers.

Introduction

Today, the arsenal of modern immunobiological drugs is huge. These are drugs for prophylactic vaccinations; immunoglobulins (mono- and polyglobulins) and immune (mainly antitoxic) sera; bacterial allergens used for allergodiagnostic treatment of certain patients with chronic localized forms (brucellosis, tuberculosis, toxoplasmosis), not amenable to other therapies; eubiotics; bacteriophages; immunomodulators of endogenous (immunocytokines, interferons) and exogenous (prodigiozan, etc.) origin.

In terms of their intended purpose and mechanism of action, all biopharmaceuticals used for immunization, are divided into three groups: 1) drugs providing active immunity (vaccines, toxoids); 2) drugs providing additional immunological protection of the human body due to the presence of antibodies (antitoxic, less often - antibacterial sera and immunoglobulins); 3) drugs that blockthe reproduction of pathogens in the infected organism (antirabic agents) and develop additional active immunity in the infected organism (tetanus and botulinum toxoids), used for emergency vaccination and preventive treatment.

Pesticides present most chemicals that get into the environment having an adverse effect on the human body; these chemicals are widely used to protect plants from pests, diseases and weeds. In this respect, herbicidesconstitute a significant part of pesticides.

Metsulfuron-methyl (MSM) presents a new generation of herbicides. Being a derivative of sulfuroncarbamide, used in agricultural production as a broad-spectrum herbicide to eliminate weeds and shrubs. This pesticide is considered to be low-toxic for the environment provided its low consumption rates. It is believed that this pesticide does not have an impact on the genetically modified crops.

The toxic effect of the new generation of pesticides is revealed in florid enzymopathy. It inhibits major enzyme activity of weed plant cells that provide their growth and development, thus resulting in their destruction [1]. It should be noted that the effect of pesticides on the immune system factors, in particular, on the interferon system is still little studied.

People, whose activities are connected with herbicides, face up to the risk of non-Hodgkin's lymphoma and soft tissue sarcoma; congenital heart disease along with their negative effect on the endocrine, immune and nervous systems. Research objective was to study the MSM effect on the functional ability of RD human cells to release α / β-interferon and on the development of specific immunity in animals. Practical significance of this paper is detrermined by the fact that pesticides, which are widely used in agriculturecause potential chemical pollution of the environment,

therefore, research findings may be useful for further studies aimed at increasing human resisting power to diseases caused by pesticides.

Although the global trends in the field of environmental protection are reduced to the cultivation of environmentally friendly arable crops, the use of pesticides (and herbicides) is still widespread, especially in the emerging markets. Consequently, modern science demands solution of issues related to immunity improvement and resisting power to diseases caused by the MSM herbicide.

The objective of this paper was to study the effect of immunomodulating agents (liquid bacterial interferon inducer (LBII) and Antivirin - M) using the model of chronic human intoxication by the metsulfuron-methyl (MSM) pesticide in experimental animals.

Materials and methods

Immunobiological drugs. The liquid bacterial interferon inducer (LBII) and Antivirin – M were used to stimulate interferon release.

LBII was developed in the Hamza Zhumatov Scientific Center for Hygiene and Epidemiology of the Consumer Protection Committee, Ministry of National Economy of the Republic of Kazakhstan; it was registered in the State Register of the Republic of Kazakhstan (IDA 42-33-68-11 (dated 19 January 2012).

*Antivirin - M*was obtained in the laboratory of the Hamza Zhumatov Scientific Center for Hygiene and Epidemiology (SCHE) (20% solution of this agent was prepared from beekeeping waste in a special extraction solution). The drug has passed all pre-clinical trials.

These drugs are effective immunocorrecting drugs. They have immunomodulatory effects, stimulate the phagocytic activity of tissue macrophages, enhance the phagocytic activity of immunocompetent cells and induce endogenous interferonogenesis [2,3].

*Pesticide.*MSM is a pesticide - herbicide (0,600 WG 2012-02-21 Polgar agrochem Ltd. Herbicide; Monitor WG). The authors used liquid solutions of this herbicide in required concentrations prepared ex tempore, by dissolving its dry granules in normal saline. The cytotoxic effect of different MSM concentrations on cell culture monolayer was calculated as the minimum concentration that caused 50% cytopathic effect (CPE_{50}) of pesticides on cell monolayer observed in an inverted microscope for 48 hours in comparison with the control cell monolayer.

Cells. The authors used monolayer cell cultures prepared from primary trypsinized mouse embryo fibroblasts (MEF),subinoculated mouse fibroblast cell line L_{929} and from the subinoculated human cell lines (RD).

Growth-supporting microenvironment. The MEM ("SIGMA") was used for cell culture. Cells were grown in plastic flasks of 25 cm^2 («Becman Dickinson»), in the growth medium supplemented with 10% of calf fetal serum –CFS ("LONZA") or bovine serum –BS ("PanEco") with 1 mM of glutamine and 100 ug / ml gentamicin («Gibco»). Cell removal was performed by using versene (0.5 liters) with trypsin (50 mg) ("Samson-Med").

Viruses and interferon inducers. Interferon titration was carried out by using test mouse encephalomyocarditis virus (EMC). EMC passages and titration were carried out in mouse fibroblast cells L_{929}. The authors used the EMC virus with infectious titer of 4,5- 5,5 lg $TCPD_{50}$ (tissue cytopathogenic doses). The α / β-interferon was induced by using vaccine strain of Newcastle disease, La Sota strain (NDV, Omsk), grown in chick embryos. In this regard, viruses with infectious titer of 5.5 to 6,0 lg embryo infectious doses (EID_{50}) were used.

Viruses and cells were stored in liquid nitrogen with addition of 7.5% DMSO as a cryoprotectant.

*Determination of α / β interferon*was carried out by micromethod. The authors used 96-well plates ("BD Falcon"). After virus induction, the culture medium was collected from the wells. Samples were treated with 0,1N HCL to pH 2.0 and placed at t + 4 °C for one day, whereupon the pH medium was restored by 0,1N NaOH to pH 7.2. Activity of the cellular α / β-interferonand the serum endogenous α / β- animal interferon was established by titration of interferon material in a monolayer of homologous cells. Interferon titer was calculated as the minimum dilution causing 50% inhibition of cytopathogenic effect of the test EMC virus in the homologous cell culture.

Animal experiments were carried out at the premises of the vivarium located at Hamza Zhumatov Scientific Center for Hygiene and Epidemiology in accordance with the regulations provided by the "Ethics of experiments on human beings and animals" and the sanitary-and-hygienic regulations of the vivarium, approved at the SCHE local committee meeting (minutes No.8, dated 21 December 2012).

The experiment was based on using white laboratory mice weighing 25 – 30 grams.

LBII was injected intraperitoneally by 1.0 ml.

Antivirin-Mwas injected per os by 0.5 ml 2 times a day, daily dose made 1.0 ml.

The EMC virus was administered to mice intraperitoneally by 1,0ml containing ten 50% lethal doses (LD_{50}).

Statistical analysis was performed through standard methods. Statistical significance was determined by the *p* value. Differences were considered significant in case of difference from the control values by $p \leq 0, 05$[4].

The potential danger of pesticides is caused by their toxicity for man and fauna, and, in some cases, for plants; as well as by their ability to cause side effects and long-term consequences. Pesticides contaminate soil with extrinsic

compounds, inhibit its biological activity, causing disorders in biocenosis composition and suppression of useful soil fauna, the occurrence of pest populations resistant to pesticides; provoke mutations that impair genetic purity of high-yielding varieties and the quality of agricultural products, etc. [5–7].

There are several classifications of pesticides, namely:

- by toxicity: highly toxic, moderately toxic, low-toxic;

- by volatility: safe, marginally hazardous, extremely hazardous;

- by their accumulation in the human body: overcumulative, with pronounced cumulation, moderately cumulation, low-cumulative;

- by penetration through noninjured skin: pesticides with pronounced properties, with moderate properties, with mild properties;

- by time of decay to non-toxic compounds: very resistant, resistant, with moderate resistance, with mild resistance.

Pesticides are mainly organic; some of them are organo-mineral or purely mineral compounds. There are organochlorine, organophosphate, organomercury pesticides, carbamic acid derivatives, carboxylic acid derivatives; nitrophenol compounds and derivatives, etc.

Small amounts of pesticides penetrating into the human body do not cause acute poisoning, but their systematic penetration may gradually lead to malfunctions and causechronic poisoning. This negative effect reveals itself in the occurrence of allergic reactions, immune reactivity reduction and other negative consequences].

High stability of many pesticides leads to profound changes in ecosystems. They can accumulate in living organisms in quantities that exceed manifold their natural content.

When in use, much of pesticides (70%) falls on the soil surface, which determines their migration. Pollution, caused by persistent organochlorines is quite substantial. In addition, these compounds are the most dangerous because they have different effects (toxic, mutagenic, carcinogenic). Their characteristic feature is the pronounced cumulative effect, which causes changes in the immune status of living organisms along with mutagenic and teratogenic effects [8–10]. It should be noted that the human immune system is working very hard. So often, even a small disruption can lead to immunodeficiency. The immune system works most intensively against bacteria and viruses. Normally, a person is born without any immunity to microorganisms and viruses. Through his life, every person constantly faces unknown microorganisms and viruses. The maximum number of germs exceeds tenfold the number of human cells (10^{13}) and exceeds the number of the immune system cells by100 times (10^{12}).

One needs to improve both nonspecific and specific immunity in the fight against pathogenic bacteria.

Nonspecific immunity is improved by increasing immunoglobulin A on the skin and mucosae, which requires sufficient content of vitamin A in the body. Against this background, the content of interferon alpha and betaincreases, inhibiting pathogens and viruses, which penetrated into the bloodstream.

The improvement of specific immunity implies antibody response to the new bacteria and viruses, which occurs in the first 5 - 6 days of disease. If it does not happen, one needs to improve immunity by means of immunomodulators, contributing to IFN production [11–13].

Propertiesof interferons originally discovered as natural antiviral antibiotics, are closely related to their anti-tumor - antiproliferative effect undertaken through inhibition of viruses by means of cell growth suppression. The process begins with the chain changes: interferon increases the activity of 2'5 'oligoadenylate synthetase enzymes, then - oligonucleotide synthesis catalyzation → activation of endonuclease enzymes → inhibition of cell proliferation with a view to degrade virus RNA [14].

Currently, many countries produce available commercial drugs: human leucocyte interferon, lymphoblastic "Wellferon", fibroblastic ("Feron"); interferon and interferons produced by means of genetic engineering: recombinant alpha- ("Laferobion", "Roferon", "Realdiron", etc.), beta-and gamma-interferon ("Ingaron").

Some countries of the former Soviet Union produce interferon-containing OTC drugs in the form of droplets [15], ointments, suppositories, gel (e.g., Viferon, Grippferon, Genferon, etc.), defined by manufacturers as drugs aimed at treating various diseases, in particular, ARVI and influenza. According to WHO, there are no high-quality clinical efficacy studies or systematic observations of interferon-based treatment of influenza [16]. Recombinant interferons have a distinctive feature: they are obtained outside the human body (produced bythe bacterium E. coli, which DNA contains the integrated human interferon gene). This significantly reduces production costs and nullifies the likelihood of any infection transmission from the donor. Development of leukocyte and recombinant interferon techniques and highly effective methods of their purification gave the possibility to use these drugs in the treatment of viral hepatitis type B and C and certain types of neoplastic diseases (injectable preparation only). Interferon inducers present substances of natural or synthetic origin, which stimulate production or release of original interferons in the human body. Early preclinical studies of inducers in the 1970s did not reveal their apparent antiviral effect, but many of them appeared to be highly toxic. Therefore, development of this type of drugs depend on a thorough study in animals.

Inducers of naturalinterferons are divided into dsRNA, isolated from bacteriophages and yeasts and polyphenols isolated from plants. Synthetic inducers are presented by aromatic hydrocarbons and polynucleotides. Projected interferon inducers are presented by low-molecular CMA derivatives and various derivatives of fluorenones. Background paper testifies to the importance of studying the application of immunobiological drugs to enhance human immunity in cases of chronic exposure to pesticides (in particular, MSM as a new-generation herbicide).

Results

Researchoriginality lies in the fact that the authors first studied effects of immunomodulatory drugs in the presence of chronic intoxication caused by the MSM pesticide in experimental animals. To this end, various MSM properties were studied, namely: cytotoxicity, its impact on the production of interferons as well as its immunosuppressive and anti-toxic effects.

Determination of the MSM cytotoxic properties. Minimum MSM concentration, which did not cause 50% cell cytodestruction (CD_{50}) was determined during 48 hours, by micromethod in the RD cell monolayer. Daily monolayers were grown in 96-well flat-bottomed plates. Consistent MSM concentrations were introduced into the wells, ranging from 1: 500 - 0.5 mg (50 ug / ml) to 1: 32,000. The control wells were filled with the growth-supporting microenvironment without MSM. The state of monolayers were observed for 72 hours. After 12 hours of MSM contact within the concentration range 1: 500 and 1: 1000 degenerative changes were observed in the form of rounded cell form, the pH change to the alkaline values, in the absence of cytodestruction in these wells. During the same period, 50% of monolayer remained in a dilution of 1: 2000. Cell degeneration was not observed after 48-hour contact with MSM at dilutions of 1: 4000 and further to 1: 32000. Further studies of cell ability to produce interferon was based on average MSM concentrations - 0.05 mg / ml.

The impact of time-related MSM exposure on the production of α / β interferons in human RD cells.

The wells with a monolayer of RD cells were filled with 50 ug / ml of MCM, the control wells were filled with the growth-supporting microenvironment. The cells were placed in a thermostat at 37 ° C in a humidified chamber containing 5% CO_2 for various periods: 24, 48, 72, 96 and 120 hours; after that microenvironment was removed from all wells. Cells were washed with the medium 199; then 10^5 EID_{50} NDV was added to induce interferonogenesis. One day later, the microenvironment was collected from the wells and exposed to 0,1 N HCl to pH 2.0 at + 4 ° C for 24 hours, then the pH of the samples was reduced to 7,2 0,1 N NaOH. The obtained samples were stored at -20 ° C before use. Results of interferon activity studies in the obtained samples are shown in Figure 1.

The figure shows that test samples were subject to the suppressive impact of MSM oninterferonogenesis, which increased, in direct proportion to an increase in the duration of the MSM impact on the RD cells. Thus, during 24 hours the MSM concentration in the target cellscaused dramatic suppression of interferon activity from 920 ± 64,6 U/ml in the control wells to 56 ± 12,6 U / ml. Further incubation of cells with MSM lead to a gradual decrease in interferon titres produced by the RD cells.

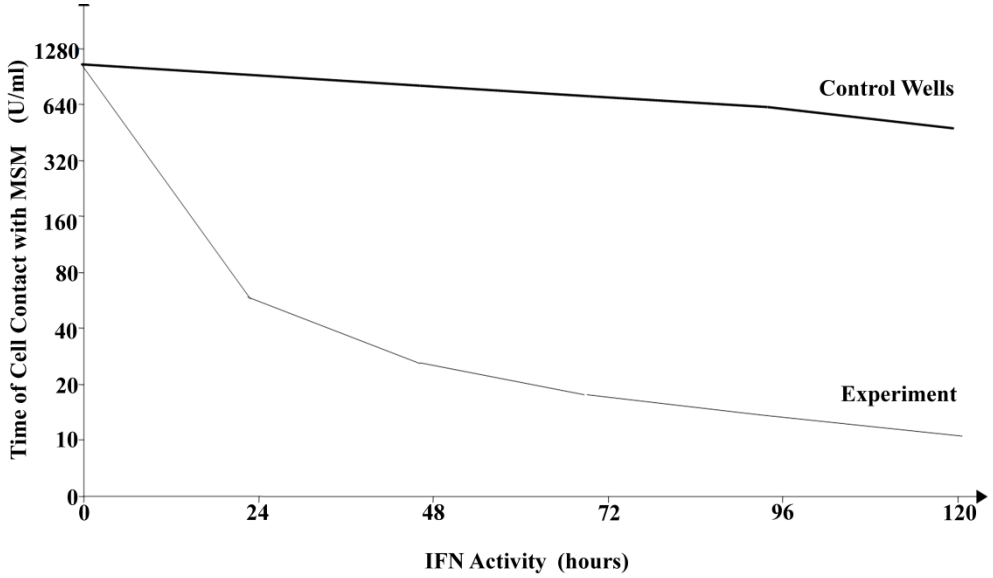

Figure 1. IFN production after the contact of RD cells and MSM.

Study of the comparative MSM effect on various cells - human RD and mouse L_{929}in concentrations ranging from 0.005 to 0.05 mg / ml was also showed a decrease in directly-proportional production of α / β- interferon on average from 64.0 ± 12.1 U / ml to 12 ± 1,4 U/ mL (in control wells - 198.2 ± 1.3 U/ml). However, at the same time, the study detectedhigher sensitivity of human RD cells to the pesticidea as compared with the mouse cells - L_{929} and EMC, determined in tests related to cytotoxicity and interferon production.

Interferon activity in the samples, where cells were exposed to MSM during 120 hours, made only 12 ± 1,8 U/ml. Control wells with a monolayer of RD cells that were not exposed to the pesticide, produced interferon during 120 hours, at almost the same rate. The impact of LBII and Antivirin-M on the immunosuppressive MSM effect and on the specific immune development (production of antibodies to influenza A / Aichi (H3N2).

The group of mice (32 individuals, 8 mice in each subgroup) was exposed to MSM according to the above scheme. In 28 and 35 days after the introduction of MSM along with LBII and Antivirin-M in the same way, all the animals were immunized with influenza A / Aichi (H3N2), 10^5 EID_{50}, intraperitoneally, by 1.0 ml. 7 days after the last immunization blood serum was obtained from all animals, separated and heated at 60º for 30 minutes with a view to

inactivate serum heat-labile inhibitors, then the serum was treated with potassium periodate to inactivate thermostable serum inhibitors; after that the activity of specific influenza antibodies was determined in samples by micromethod in the hemagglutination-inhibition reaction (HAI) using a 1% suspension of chicken erythrocytes. Cumulative findings are presented in Table 1. These data show that long-term administration of MSM provided immunosuppressive effect on the formation of specific immunity to influenza virus in animals from the third subgroup, whereas administration of LBII and Antivirin-M in animals from the first and second subgroups fostered reduced immunosuppression, as detected in comparison with the control (fourth) subgroup. Introduction of immunobiological drugs stimulated production of antibodies to the influenza virus A / Aichi (H3N2), respectively, in the second and in the third animal subgroups.

Table 1.Effect of interferon inducers on the IFN-antibody response in mice exposed to the MSM impact.

Subgroups of animals, exposed to	IFN activity, I.U./ml* (M± m)	Influenzaantibodytiters **
MSM+LBII (subgroup 1)	246,0±13,2	61,8±12,2
MSM+Antivirin-M (subgroup 2)	78,2±2,4	14,2±2,4
MSM (subgroup 3)	54±12,1	23±4,6
Controlgroup (subgroup 4)	62,8±4,6	48±6,8

*- inverse averages of interferon titers expressed in International Units I.U./ml.

**-inverse geometric mean values of antibody titers.

Antitoxic action of liquidbacterial interferon inducer and Antivirin M in case of chronic intoxication caused by MSM pesticide.

Using the model of chronic intoxication, according to the above scheme, the authors determined the MSM impact on cumulative animal mortality indicators in the context of stimulating the endogenous α / β-interferon by administering LBII and Antivirin-M. To this end, MSM was administered during 25 days in concentration of 0.025 μg/ml to mice, divided into three groups (20 animals in each group). At the same time, the animals from the experimental groups were administered LBII and Antivirin-M according to the above scheme. The control group of animals received only MSM, without LBII and Antivirin-M.

Upon expiration of the intoxication simulation (25days) animals from the experimental and control groups were exposed to a single 50% lethal dose of EMC(1 LD_{50}); after that, cumulative mortality of animals was recorded within 10 days (observation period).

Figure 2 shows that the virus-synthesizing ability of cells in the test animals exposed to MSM hardly decreases, while the interferon-producing capacity is suppressed.

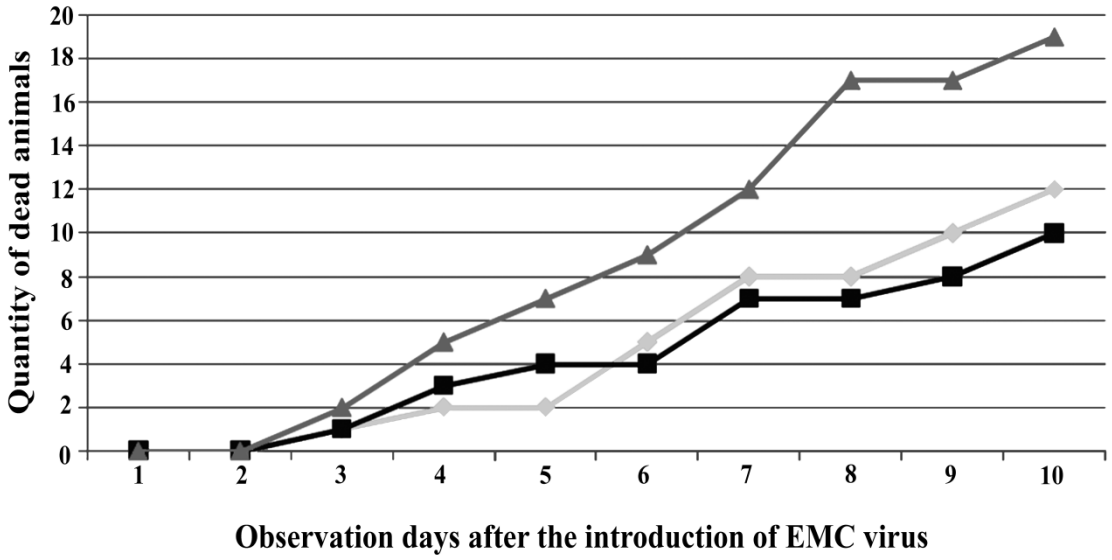

Figure 2. The decrease in cumulative mortality of animals caused by the MSM pesticide intoxication given the effect of immunobiological drugs.

The results showed that animal deaths were observed in the control group of mice (triangles) from the first day. 19 animals died on the 10th day, while in the group of mice, which received Antivirin - M (diamonds), only 12 out of 20 animals died during the same period, and in the group receiving LBII (squares) only 10 out of 20 animals died. The control group, which did not receive LBII and Antivirin – M, demonstrated twofold increase in mortality resulting from administration of a lethal EMC virus.

These results show the effectiveness of the studied drugs as regards the immune system of the experimental animals.

Discussion

Being widely used in agriculture, pesticides remain a potential factor of chemical pollution of the environment. In this regard, up to 95% of chemicals penetrate into the body with food, and can pose a potential threat to public health. As regards study of their impact mechanism on theanimal cells, their action is also directed at enzymatic activity inhibition of cell systems– acetylcholinesterase, 2'5 'oligoadenylate synthetases, etc. [17]. The MSM mechanism is based on the plant growth inhibition at the cellularlevel. In this regard, studying the impact of pesticides and their transformation products on human tissues and organs is very relevant[18]. Previous studies showed that glyphosate

(organophosphorus herbicide) inconcentrations, which did not cause cytotoxic effects on cells, provided 96-120 hour duration of its effect on the human RD cells, could cause a reduction in α / β interferon by 20% [18].

This study of MSM effect on the functional capacity of α / βinterferon production in the human RD cells also showed its inhibitive effect on interferon production. The importance of interferon, produced by low molecular weight proteins to maintain homeostasis, is confirmed by the fact that its function is extensively encoded in genetic terms: in 20 genes - inα-interferon, in 5 genes - in β-interferon and in one gene - in γ-interferon. Leukocytes produce mainly α-interferon, whereas β-interferon is produced in fibroblasts, γ-interferon is released by T-helpers and macrophages. At the same time, probably, these molecules can be synthesized by all core cells [3]. Activation of the interferon genes occurs under the influence of adverse environmental factors, the introduction of viruses and/or bacteria into the human body, as well as by any agents containing foreign DNA.

Thus, interferon contained in the studied immunobiological drugs is the most important cytokine; its action is directed at providing basic functions of a non-specific innate immunity: antiviral, anticancer, anti-inflammatory, immunomodulatory and radioprotective [3]. Immunosuppression that occurs in the body under the influence of adverse factors of exogenous and endogenous origin in the body reduces interferon productionin cells. One should note that immunosuppression is the main laboratory and clinical sign suggesting a decrease in the body's resistance to many diseases - from cancer to various kinds of infections, and, perhaps, a sign of the toxic impact caused by pesticides [19–21]. Studies show the effectiveapplication of the studied immunobiological drugs aimed at improving human immunity provided negative effects of herbicides.

Given relevance of this research, there is a need to continue comprehensive development of similar immunobiological drugs, as well as to carry out system monitoring of pesticide residues in food products and in the environment [22–25].

Conclusion

1. Long-term (25 days or more) exposure of experimental animals (mice) to low doses of MSM leads to reduction in cell ability to produce interferon, caused by the cumulative effect of this pesticide.
2. The virus-synthesizing ability of cells in the test animals exposed to MSM hardly decreases, while the interferon-producing capacity is suppressed. This indicates that immune factors are more sensitive to the damaging effect caused by the pesticide.

3. The MSM effect on the experimental animals revealed their reduced resistance to lethal viral infections, which was expressed in the accelerated cumulative mortality of animals by 20-25% in terms of modeling chronic intoxication with low doses of MSM.

4. The studied immunobiological drugs increase the ability of the animal organism to produce interferon, which is shown by the reduction in cumulative mortality of the experimental animals by 20 - 25%.

In the future, the authors plan to explore new immunological drugs that increase immunity in the presence of chronic toxicity caused by the new generation of herbicides.

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Daniyar Aspetov, Mariya Omarova, Lyazat Orakbay and Bayan Zhumatova declare that they have no confl ict of interest. All the authors confirm that the study was conducted at the expense of personal funds and deny any external interference, which could affect research results.

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