ISSN 1995-0780, Nanotechnologies in Russia, 2016, Vol. 11, Nos. 9–10, pp. 653–662. © Pleiades Publishing, Ltd., 2016.
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Influence of Iron-Molybdenum Nanocluster Polyoxometalates on the Apoptosis of Blood Leukocytes and the Level of Heat-Shock Proteins in the Cells of Thymus and Spleen in Rats

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Received January 28, 2016; in final form, June 14, 2016

Abstract—We have studied the influence of iron-molybdenum nanocluster polyoxometalates on the organism of animals (rats) in order to determine the correlation between the exposure and changes at the cellular level in the blood and immune system. Single and repeated administrations of polyoxometalates lead to an increase in the amount of cells containing heat-shock proteins (HSPs) HSP60 and HSP70 not only in the spleen but also in the thymus, despite the blood–thymus barrier. The enhancement of both early and late apoptosis in leukocytes after the administration of 30 doses of polyoxometalates may result from the disruption of homeostasis in immunopoietic organs. No changes in the blood parameters indicative of inflammatory process or anemia are observed at any exposure. The increased level of HSPs in the cells of thymus and spleen and the absence of changes indicative of inflammation in the blood leucocytes, apparently, support the viability of cells in studied organs, since no morphological disruptions are found in the thymus and the spleen.

DOI: 10.1134/S1995078016050049

INTRODUCTION

Nanocluster polyoxometalates (POMs) are in the group of keplerates, which are one of the most promising objects created recently. They were first synthesized by the research group of Professor A. Müller (University of Bielefeld, Germany). The process of synthesis is relatively simple. One of the typical representatives is keplerate $Mo_{72}Fe_{30}$, which contains oxygen polyhedra surrounding multicharged iron and molybdenum ions [1, 2]; the composition of this keplerate is as follows: $[Mo_{72}Fe_{30}O_{252}\ (CH_3COO)_{12}\ \{Mo_2O_7\ (H_2O)\}_2\ \{H_2Mo_2O_8\ (H_2O)\}\ (H_2O)_{91}] \cdot 150H_2O$.

It is water soluble and has a layered-caged structure; that is why this type of compound was called a keplerate, after Johannes Kepler, the creator of heliocentric planetary model. As can be seen from the formula, this POM contains stabilizing acetate ligands and water molecules. It is a spherical particle with a size of approximately 2.5 nm; it has internal cavity and pores (windows). Due to their structure and composition, POMs are of practical interest as catalytic and sensory material. Some of their properties (solubility and the ability to form multicharged ions in solution) indicate that POMs can be used for the targeted delivery of biologically active substances. Polyoxometalate ions can form associates with bioactive drugs and maintain their structure. Biocompatible polymers form a protective shell on the surface of POMs. Previously we found that POMs can be administered to animals percutaneously [3, 4]. Another advantage of $Mo_{72}Fe_{30}$ is its low toxicity (according to the data on acute and subacute toxicity after intramuscular injections in rats [5, 6] and toxicity after electrophoretic administration [3]). It is also important that POMs are decomposed gradually while delivering a drug. Polyoxometalates and the products of their biodegradation are not accumulated in tissues [6]. In our previous studies, we investigated the effect of single and multiple administrations of iron-molybdenum nanocluster POMs on a number of parameters [7], including the level of histones and heat-shock proteins (HSPs) in the blood and liver of rats [8]. Certainly, in order to estimate the possibility of using $Mo_{72}Fe_{30}$ for targeted drug delivery, further detailed studies on its effect on warm-blooded animals is needed. This article deals with this issue, in particular, with the possible distribution of POMs in the thymus and the spleen, as the blood-forming organs, and with the influence of iron-molybdenum POMs on the level of HSPs in these two organs and on the apoptosis in blood leukocytes.

Since recently, a number of studies have been published on the problem of inflammatory response in tissues after nanoparticle administration [9, 10]. One of

the causes of the damaging effect of nanoparticles can be their ability to act as haptens (by transforming the native proteins into foreign antigens) or as adjuvants (by absorbing antigens), which leads to immune, autoimmune, and inflammatory reactions [11]. An autoimmune response can result not only in the destruction of tissue, but also in the death of blood leukocytes through necrosis or apoptosis. A number of authors have found that the use of nanomaterials containing metal oxides can increase the rate of apoptosis in different tissues [10, 12–14]. Some authors even suppose that nanoparticles can be used for the promotion of apoptosis in cancer cells [15, 16]. The disruption of activation and regulation pathways of apoptosis by nanomaterials can cause various disorders. These disorders can result from either inhibition or activation of apoptosis. The structure of lymphoid organs is damaged by different stress factors. According to the results of experimental studies, apoptosis in the thymus and the spleen is induced by such stressors as immobilization, toxicants, sepsis, and hyperthermia [17, 18].

The thymus and the spleen were chosen as the objects of this study, since the maturation of lymphocytes takes place in these organs; thus, they play an important role in the amount and proper functioning of blood lymphocytes. However, due to the blood–thymus barrier, the thymus is an immunologically privileged organ. The blood–thymus barrier prevents antigens of various origins from entering the thymus through the cortical capillaries, which provides positive selection, maturation, and differentiation of lymphocytes prior to the first contact with antigens. Thus, it is important to compare the effect of POMs on the morphological structure of the thymus and the spleen, which does not have such a barrier.

Over the recent years, it has been found that the protective effect of adaptation processes results from the production of HSPs, which belong to the group of stress-induced protective proteins [19, 20]. The expression of HSP is stimulated by any stress factors; therefore, the fractions of these proteins can serve as an indicator of the damaging effect of nanoparticles at the earliest stages, i.e., before the changes at the tissue level can be detected [21, 22].

HSPs can be found in any tissue of the organism. They have a highly conserved structure, which suggests that they play the key role in the regulation of cell cycle, apoptosis and necrosis. Any exogenous stress factors (including the administration of nanoparticles) can induce the expression of HSPs. The mode of protective action of HSPs is based on the disaggregation of abnormal protein complexes, utilization, and repair of damaged proteins and regulation of de novo protein synthesis [19, 20].

HSP70s take part in the regulation of apoptosis: they can act as antiapoptotic factors [23, 24]. These proteins are cytoprotectants that inhibit apoptosis at early stages (before irreversible processes begin) [25–27]; thus, they increase the survival rate of cell damaged by lethal toxicants. HSP70s form the stress-induced antiapoptotic signaling pathway that inhibits not only c-Jun N-terminal kinases, but also a number of caspases. In addition, HSP70s can recognize proteins denaturated by stress and proteins that entered the cell through the damaged membrane, which probably prevents autoimmune reactions. The regeneration of the native structure of proteins and folding is regulated by HSP70s, then by HSP60s [28]. HSP60 is the main mitochondrial chaperone, the expression of which is stimulated by the disruption of mitochondrial functions and oxidative stress. The changes in the level of this protein often correlates with those of TNFα. HSP60 is thought to be a trigger of innate and adaptive immune response at the early stages of inflammatory processes of various origins [29, 30].

In our previous study, we observed an increase in the level of HSPs in liver cells, which prevented the proinflammatory phenotype of blood lymphocytes, after the administration of iron-molybdenum polyoxometalates [8]. These findings correspond with the data of other authors, who proved that HSP70s have cytoprotective effect after the administration of selenium-containing nanoparticles [26].

Apoptosis was first detected in tissues by the morphological changes in cells (shrinkage of cells and nuclei, chromatin condensation, etc.) [31]. Then, in addition to morphological parameters, biochemical changes were observed, including the cleavage of DNA between nucleosomes and increased activity of caspases, which are involved in the signaling pathway of apoptosis [32]. However, later it was found that DNA fragmentation during apoptosis does not occur in some cells [33].

At the early stages of apoptosis, the cell membrane retains its integrity, but loses symmetry in the distribution of phospholipids [34]. Phosphatidylserine (PS) normally faces the inner side of the cell membrane; during the process of apoptosis, it is externalized, which is one specific signal for the recognition and destruction of apoptotic cells by macrophages [35]. Therefore, PS facing the outer side of the cell membrane is used as an indicator of early apoptosis. At the later stages, the membrane loses its integrity and DNA fragmentation begins. The exposition of PS during apoptosis is an evolutionary trait typical not only of mammals, but also of other species.

Although apoptosis is a universal biological process, when induced by the administration of nanoparticles it can lead to the excessive death of different cell populations, including leukocytes, which can cause systemic immune and inflammatory responses [36, 37]. Apoptosis is a separate type of cell death, but it can also be combined with other types (necrosis or autophagy) [36, 38]. Moreover, the enhancement of apoptosis in peripheral blood leukocytes can result from the damage of lymphopoietic organs that cannot be

detected at the tissue level. The parameters of apoptosis in leukocytes can also serve as a model of structural changes in organs at the cellular level.

The purpose of this work is to determine the level of HSPs in the cells of the immune system and the dynamics of the processes of apoptosis and necrosis in blood leukocytes after the administration of iron-molybdenum polyoxometalates of the keplerate type.

MATERIALS AND METHODS

The nanocluster POMs were obtained according to a tried and tested procedure developed by Professor Müller [1, 2]. Therefore, we will not describe the method in detail here. Starting reagents were chemically pure grade ammonium heptamolybdate $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, pure-for-analysis grade hydrazine sulfate $N_2H_4 \cdot H_2SO_4$, chemically pure ammonium acetate CH_3COONH_4, chemically pure glacial acetic acid CH_3COOH, high purity grade ammonium chloride NH_4Cl, iron chloride (III) hexahydrate $FeCl_3 \cdot 6H_2O$ Panreac (assay 97–102%), high purity grade hydrochloric acid HCl, and pure-for-analysis grade sodium chloride NaCl.

The next step of the preparation of $Mo_{72}Fe_{30}$ for further work was the certification of its composition and structure. The procedure used for certification was described in our previous paper [8]. All physicochemical parameters of obtained POMs were consistent (within the error tolerance) with literature data, including the parameters determined in our previous study [8].

The experiments were performed using healthy male rats of the Wistar line ($n = 55$) with weight ranging from 210 to 225 g maintained on the standard feed of vivarium. All animals were kept under the same conditions: 5 rats per a cage and free access to food and water at a temperature of 20 ± 2°C.

The conditions and treatment of animals corresponded with the recommendations of the international ethics committee (European Council Directive EC 2010/63/EU; Directive 2010/63/EU on the protection of animals used for scientific purposes, Official Journal of the European Union, September 22, 2010) and the ethics committee of the Institute of Immunology and Physiology, Russian Academy of Sciences (protocol No-D-PM-2015-27).

The animals were divided into four groups with ten rats in each group. Animals in the first group were injected with a single dose of POMs. Animals in the second group were injected daily for 7 days and in the third group for 30 days. The fourth group consisted of intact rats. The dose for each intramuscular injection was 0.15 mg/100 g, which is equal to a putative therapeutic dose. The concentration of molybdenum in one dose of POMs corresponded with the upper limit of the recommended daily intake; the concentration of iron was lower than the recommended daily intake by 21.5 times. The daily injections of POMs for 7 and 30 days can be considered a subacute effect; single dose can be considered an acute effect. For each of the experimental groups, we took control groups (five rats in each): 1c, 7c, and 30c. The animals from these control groups were administered distilled water instead of POMs in the same volume (0.3 mL) as in the experimental groups. All the animals were etherized 1 day after the last injection.

For the peripheral blood count, we used Celly 70 Biocode Hycel automatic analyzer designed for animal blood counts in studies and veterinary medicine. The blood leukocytes were isolated in ficoll-verografin mixture by the method based on differential centrifugation of blood components in the density gradient [39].

The number of apoptotic cells was counted by flow cytofluorometry [40]. The early stage of apoptosis was determined by the presence of PS on the surface of cell mebranes and its ability to bind to annexin V labeled with fluorescein isothiocyanate (annexin V-FITC). We used a commercial reagent kit ANNEXIN V-FITC/7-AAD KIT (Beckman Coulter, United States). The conjugation of annexin V with FITC enhances the fluorescence of PS-annexin V complex without affecting the ability of annexin V to bind to phospholipids. We used recombinant human annexin (purity over 99%), the maximum absorbance in the fluorescence of annexin V-FITC complex is 490 nm, and the maximum emission is 525 nm. The apoptotic cells are stained by annexin V before their morphological structure is damaged and the DNA hydrolysis begins.

The use of stains specific for DNA, such as propidium iodide or 7-aminoactinomycin (7-AAD), enables the detection of differences between the early and late apoptosis and necrosis. The latest, irreversible stage of apoptosis, when DNA is fragmented, are detected basing on the binding of 7-AAD with guanine-cytosine pair. The maximum absorbance in the fluorescence of DNA/7-AAD is 543 nm; the maximum emission is 655 nm. The suspension of leukocytes were mixed with annexin V-FITC and 7-AAD (1 μL and 2 μL, respectively, per 100 μL of suspension) and incubated at a temperature of 0°C in the dark for 15 min. Then the cells were analyzed by flow cytometry with the use of COULTER® EPICS® XL™ analyzer and CXP 2.2 software package.

The cells expressing HSP60 and HSP70 in the thymus and the spleen were detected by immunohistochemical testing by the indirect peroxidase method according to standard procedures [41] with the use of DAKO autostainer in formalin-fixed, paraffin-embedded samples with high temperature retrieval in Pascal DAKO autoclave. The formation of methylene bridges between amino groups of proteins during the fixation provides antigen masking in tissues. The

Table 1. Antibodies used for immunohistochemical testing

Antigen	Primary antibody: clone, brand, degree of dilution	Secondary antibody: clone, brand, degree of dilution
HSP60	Mouse antishock protein 60 (hsp60) clone LK1, Merck Millipore, United States, 1 : 50	Biotin Goat Anti-Mouse Ig (Multiple Adsorption), BD Biosciences, United States, 1 : 50
HSP70	HSP 70 (3A3): sc-32239, clone 3A3, Santa Cruz Biotechnology, Inc., United States, 1 : 50	

bonds between amino groups are cleaved by unmasking (enzymatic or high temperature retrieval). The method of retrieval is determined by the type of primary antibodies. For immunohistochemical staining, primary monoclonal antibodies were used (Table 1).

For the visualization of immune complexes, we used a NovolinkTM Polymer Detection System (Leica Biosystems, Germany) commercial test system. Then the nuclei were stained with hematoxylin. The cells expressing HSP60 and HSP70 were detected by the brown color of the cytoplasm, provided by tetrahydrochloride 3,3-diaminobenzidine (DAB) that binds to immune complexes.

Since the outer and inner layers of immune organs have morphological and functional differences, the concentrations of HSPs were determined in the cortical and medullary portions of the thymus and in the red and white pulp of the spleen. The cells containing HSP60 and HSP70 were detected using a Leica DM 2500 microscope with a Leica DFC 420 digital camera. The cells were counted at a magnification of 40× in 20 fields of vision (area of F.v. = 0.0827 cm2) with the use of standard procedures of VideoTest Morphology 5.0 (VieoTest, St. Petersburg). Then the mean number of HSP60- and HSP70-positive cells per unit area was counted.

The statistical analysis of obtained data was performed using Statistica 6.0 (Stat. Soft. Inc.) software package and Microsoft Exel 2003. Statistical hypothesis were tested with a confidence level $p < 5\%$ ($P < 0.05$).

RESULTS AND DISCUSSION

No changes in the appearance and behavior of rats were observed after either single or multiple (for 7 or 30 days) administration of polyoxometalates. The histological analysis of lymphopoietic organs (the thymus and the spleen) 30 days after the administration of POMs showed that there were no structural changes (Figs. 1, 2). The boundaries of the red and white pulp in the spleen were well-defined; the lymphoid follicles of the white pulp were divided into well-defined zones (Fig. 2). The cortical and medullary portions of the thymus had well-defined boundaries as well. No dystrophic changes were observed in thymocytes (Fig. 1).

In the intact animals, no cells with a color specific for HSP70s were found in either cortical or medullary portions of the thymus. The number of HSP60-containing cells (per field of vision) in the cortical or medullary portions was 1156 ± 189 and 90 ± 27, respectively. The number of stained cells containing HSP70 and HSP60 (per field of vision) in the spleen of intact

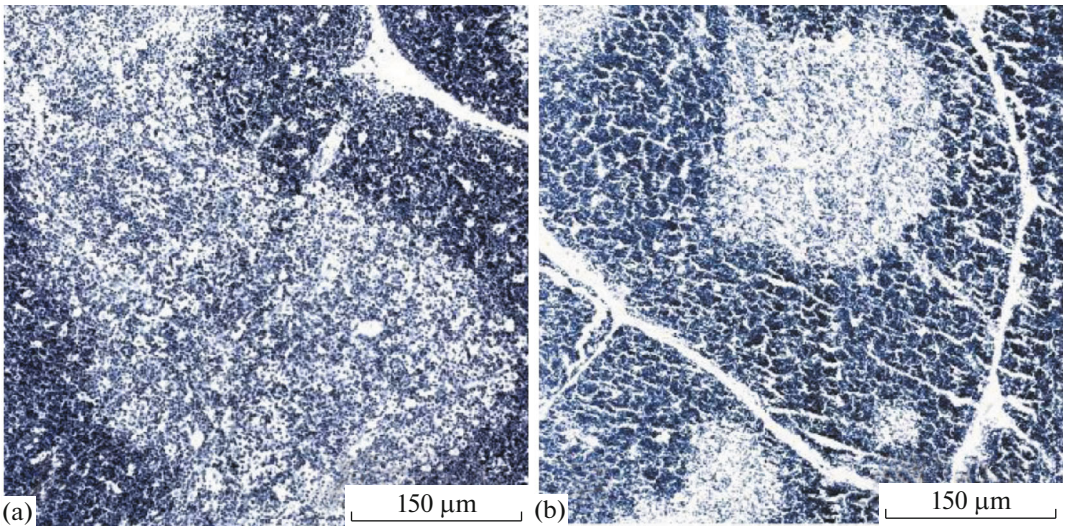

Fig. 1. (Color online) Morphological structure of the intact thymus (a) and the thymus of rats injected with POMs for 30 days (b). Bar 150 μm.

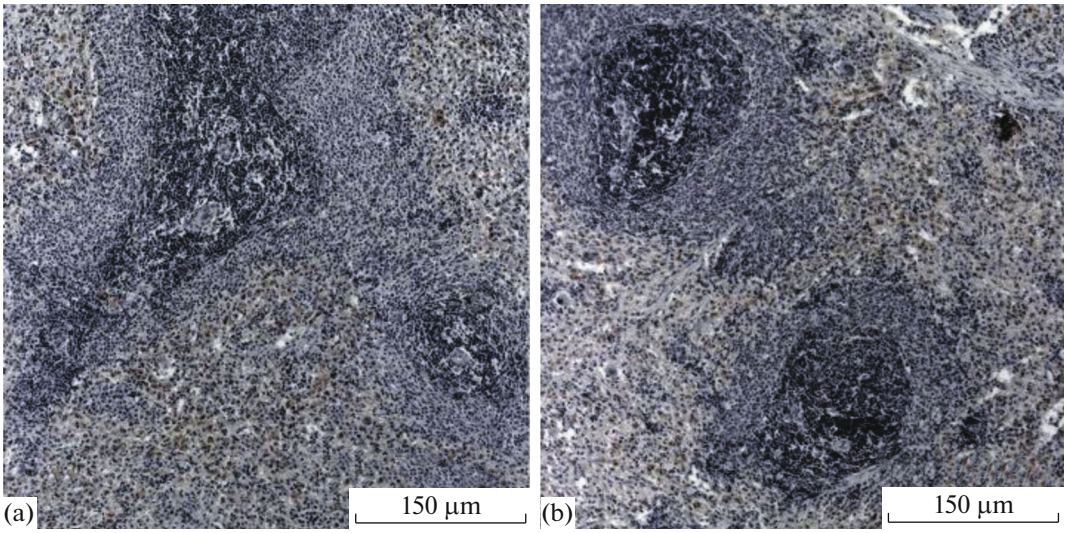

Fig. 2. (Color online) Morphological structure of the intact spleen (a) and the spleen of rats injected with POMs for 30 days (b). Bar 150 μm.

animals was 1510 ± 52 and 1459 ± 83 in the red pulp and 96 ± 25 and 111 ± 27 in the white pulp of the spleen, respectively.

In the control groups of rats (1c, 7c, and 30c), no cells containing HSP70 were found in the thymus; however, cells with HSP60 were detected; this may result from the sensitivity and limit of detection of the method of detection (Table 2). In the experimental groups, the cells containing HSP70 were also found in the thymus, and the amount of cells containing HSP60 was increased, which indicates the disruption of homeostasis probably caused by the permeation of POMs or their metabolites through the blood–thymus barrier. The apoptosis of lymphocytes occurs constantly during the process of negative selection, which is needed for the elimination of autoreactive thymocytes. Stress factors, such as xenobiotics and hyperthermia, can stimulate the apoptosis of lymphocytes in the cortical portion of the thymus and lead to dystrophic changes [17, 18, 42]. Since the number of maturing lymphocytes is higher in the cortex of the thymus, here the amount of cells producing HSP60 and HSP70 was higher than in the medullary portion in all groups. Although the number of cells containing HSPs in the medullary portion was lower, the tendency of elevation in this value in response to the administration of POMs was the same. These findings suggest that an increase in the number of cells expressing HSPs is a typical reaction to the administration of POMs. In the cortical portion of the thymus, a more significant increase in this value is needed to prevent the disruption of maturation and differentiation of thymocytes. Similar changes in the amount of cells producing HSPs were observed in the spleen (Table 2). In the red pulp, where the old red blood cells are phagocytosed, the number of cells containing HSP60 and HSP70 is increased by almost two times. In the white pulp, only the number of cells expressing HSP60 is increased by two times. These findings may result from the differences in the functional activity of the red and white pulp, since the level of HSPs in the red pulp of both intact and control rats is almost 15 times higher than that in the white pulp. Several recent studies have shown that no increase in the level of HSPs in response to stress factors indicates the disruption of adaptation processes [8, 43, 44]. Since the HSPs are known to have antiapoptotic effect [23–27], we suggest that an increased amount of cells expressing HSP60 and HSP70 can be a compensatory reaction needed for maintaining the structure of the thymus and spleen. Thus, the elevated amount of cells containing HSPs in the experimental group compared with the control animals suggests that the administration of nanoparticles acts as a stress factor for the organism and triggers the compensatory reaction.

All changes in the organs of immunopoiesis are accompanied by changes in the composition and parameters of the peripheral blood. The parameters of the peripheral blood of intact rats were the following: hematocrit = 36.8 ± 0.6%, hemoglobin = 129.8 ± 3.3 g/L, red blood cells = 6.85 ± 0.16 mln/μL, and platelets = 329.0 ± 55.9 ths/μL. The total number of leukocytes was 13.0 ± 0.6 ths/μL; the concentrations of the fractions of leukocytes were the following: lymphocytes 8.95 ± 0.97 ths/μL, monocytes 2.10 ± 0.50 ths/μL, and granulocytes 2.00 ± 0.30 ths/μL; the percent proportion of these types of leukocytes were 68.4 ± 6.5%, 16.0 ± 4.1% and 15.6 ± 2.5%, respectively. In the control groups of rats, which were injected with distilled water for 1, 7, or 30 days (1c, 7c, and 30c), the peripheral blood parameters did not differ significantly from those in intact rats (Table 3).

The flow cytofluorometry of blood leukocytes in intact rats showed that 91.80 ± 1.01% of the cells are

Table 2. HSPs in the cells of the immune system of rats in experimental and control groups (the number of stained cells per field of vision

Parameters	Organs		Groups					
			1c	7c	30c	Day 1	Day 7	Day 30
HSP70	Thymus	Cortical portion	0	0	0	478 ± 58*	1728 ± 369*[1]	311 ± 41*[7]
		Medullary portion	0	0	0	2.4 ± 0.4*	193 ± 66*[1]	372 ± 86*[1]
	Spleen	Red pulp	1560 ± 154	1470 ± 50	1520 ±49	2670 ± 88*	1519 ± 88[1]	2324 ±86*[1,7]
		White pulp	108 ± 19	81 ± 20	90 ± 24	120 ± 34	61 ± 11	82 ± 13
HSP60	Thymus	Cortical portion	1110 ± 140	1186 ± 222	1192 ± 109	2226 ± 148*	3723 ± 427*[1]	2801 ± 612*
		Medullary portion	96 ± 22	108 ± 26	87 ± 21	471 ± 88*	212 ± 15*[1]	333 ± 56*
	Spleen	Red pulp	1538 ± 104	1388 ± 68	1450 ± 101	2217 ± 138*	2188 ± 80*	2704 ± 107*[1,7]
		White pulp	119 ± 26	100 ± 17	103 ± 22	322 ± 42*	223 ± 26*	324 ± 27*[7]

*Statistically significant difference from the parallel control group, $p < 0.05$;
[1,7]Statistically significant difference from the experimental groups of rats injected with a single dose (1) of 7 doses (7) of POMs.

Table 3. Parameters of the peripheral blood in control and experimental groups

Parameters		1c	7c	30c	Day 1	Day 7	Day 30
Hematocrit, %		37.1 ± 0.9	36.9 ± 0.6	35.9 ± 0.9	43.6 ± 0.4*	41.1 ± 1.0*	43.3 ± 0.8*
Hemoglobin, g/L		127.2 ± 2.9	132.8 ± 3.3	129.0 ± 3.1	151.6 ± 1.4*	155.0 ± 3.6*	152.0 ± 4.1*
Red blood cells, mln/μL		7.00 ± 0.26	6.75 ± 0.19	7.02 ± 0.18	8.54 ± 0.07*	8.06 ± 0.19*	8.25 ± 0.10*
Plateles, ths/μL		345.6 ± 43.5	345.6 ± 42.9	330.2 ± 45.5	597.8 ± 25.7*	654.2 ± 22.6*	627.0 ± 114.0
Total leukocyte count, ths/μL		13.0 ± 0.5	12.8 ± 0.4	12.9 ± 0.6	6.2 ± 0.5*	6.2 ± 0.8*	9.3 ± 1.7
Lymphocytes	ths/μL	8.92 ± 0.66	8.64 ± 0.84	8.94 ± 0.81	4.54 ± 0.41*	4.20 ± 0.48*	6.20 ± 1.10
	%	68.5 ± 4.1	66.9 ± 4.5	69.0 ± 4.0	73.4 ± 0.7	68.6 ± 4.2	66.5 ± 2.1[1]
Monocytes	ths/μL	2.20 ± 0.40	1.96 ± 0.39	1.88 ± 0.41	0.58 ± 0.07*	0.56 ± 0.11*	1.10 ± 0.30
	%	16.7 ± 3.4	15.6 ± 3.2	14.9 ± 3.4	9.2 ± 0.7	8.6 ± 0.8	11.8 ± 1.0[7]
Granulocytes	ths/μL	1.94 ± 0.24	2.24 ± 0.19	2.08 ± 0.26	1.08 ± 0.08*	1.48 ± 0.40*	2.00 ± 0.40
	%	14.8 ± 1.5	17.5 ± 1.6	16.1 ± 1.8	17.4 ± 0.8	22.8 ± 3.6	21.8 ± 2.3

*Statistically significant difference from the parallel control group, $p < 0.05$;
[1,7]Statistically significant difference from the experimental groups of rats injected with a single dose (1) of 7 doses (7) of POMs.

alive, 7.51 ± 0.96% are at the early stage of apoptosis, 0.55 ± 0.09% are at the later stage of apoptosis, and 0.16 ± 0.02% are necrotic. In the control groups of rats injected with distilled water for 1, 7, or 30 days (1c, 7c, and 30c); these parameters and the mean values of the parameters of apoptosis did not differ significantly from those in intact rats (Table 4).

The analysis of the peripheral blood in experimental groups (Table 3) showed that no animals had anemia, which is a sign of chronic intoxication: the level of hemoglobin and erythrocytes did not decrease during the study. However, the level of hemoglobin and erythrocytes in all experimental groups was higher than that in parallel control groups, which corresponds with the tendency of an increase in hematocrit in these groups. The elevation of hemoglobin concentrations after the administration of iron-molybdenum POMs is easy to explain. Iron takes part in the synthesis of hemoglobin by binding to the iron response element (IRE) on aminolevulinate synthase mRNA and initiating the translation of this enzyme, which catalyzes the synthesis of heme [45]. The level of platelets, which can also increase hematocrit, was elevated after 1 and 7 days of administration and then remained almost constant to day 30.

Neither the single nor repeated administration of polyoxometalates did caused the elevation of the total concentration of leukocytes and the levels of their fractions (Table 3), which indicates that no systemic inflammatory process was induced. However, the total concentration of leukocytes after 1 and 7 days of administration of POMs was lower than that in paral-

Table 4. Leukocytes at early and late stages of apoptosis and necrosis in the blood of rats in control and experimental groups (% of the total number of cells)

Parameters	1c	7c	30c	Day 1	Day 7	Day 30
Alive cells	90.68 ± 0.71	91.42 ± 1.00	91.32 ± 1.02	92.12 ± 1.25	92.17 ± 1.05	66.53 ± 6.14*[1,7]
Early apoptosis	8.26 ± 0.47	7.40 ± 0.84	7.46 ± 0.97	7.16 ± 1.17	6.65 ± 0.95	23.77 ± 3.78*[1,7]
Late apoptosis	0.86 ± 0.32	1.02 ± 0.32	1.02 ± 0.17	0.52 ± 0.10	0.91 ± 0.13[1]	9.10 ± 3.19*[1,7]
Necrosis	0.20 ± 0.03	0.16 ± 0.02	0.20 ± 0.03	0.20 ± 0.06	0.27 ± 0.03*	0.60 ± 0.30

*Statistically significant difference from the parallel control group, $p < 0.05$;
[1,7]Statistically significant difference from the experimental groups of rats injected with a single dose (1) of 7 doses (7) of POMs.

lel control groups (1c and 7c); the absolute values (ths/μL) were lower for all the fractions: lymphocytes, granulocytes and monocytes. At the same time, the percentage of fractions in these groups did not change significantly compared with the control groups. Leukopenia caused by the reduction of all fractions after 1 and 7 days of administration of POMs can result from the enhanced production of erythrocytes and platelets in the bone marrow and, consequently, a shortage of substrates and energy for leukopoiesis. By day 30, the total concentration of leukocytes and all fractions became normal.

Since the total and differential leukocyte counts in the group of rats injected with POMs for 30 days were normal and previous studies have shown that iron and molybdenum are not accumulated in tissues [6], we suggest that the organism adapts to the subacute administration of POMs after 30 days and leukopenia is a transient effect.

The rate of apoptosis in blood leukocytes was analyzed by flow cytofluorometry. We obtained a two-parameter histogram of log fluorescence determined at 525 nm and log fluorescence determined at 625 nm; the total amount of analyzed cells can be divided into four separate subpopulations (Figs. 3, 4). Alive cells have low FITC signal and low 7-AAD signal (quadrant 3); cells at the early, reversible stage of apoptosis have high FITC and low 7-AAD signal (quadrant 4); and cells at the later stage of apoptosis have high FITC and high 7-AAD signal (quadrant 2). Necrotic cells have low FITC and high 7-AAD signal (quadrant 1).

These four subpopulations were counted in each of blood samples. All the mean values and standard errors in each group are presented in Table 4. The number of leukocytes at the early stage of apoptosis was significantly higher than that at the later stage and necrosis (Table 4).

According to the data presented in Table 4, single and repeated administration of iron-molybdenum POMs does not decrease the survival rate of cells, since no significant differences in the mean values of alive and apoptotic cells were observed between experimental and control groups. In the group of rats injected with nanoparticles for 7 days, the level of leukocytes at the later stage of apoptosis was higher than that in rats injected with a single dose; the level of necrotic leukocytes was higher than that in the parallel

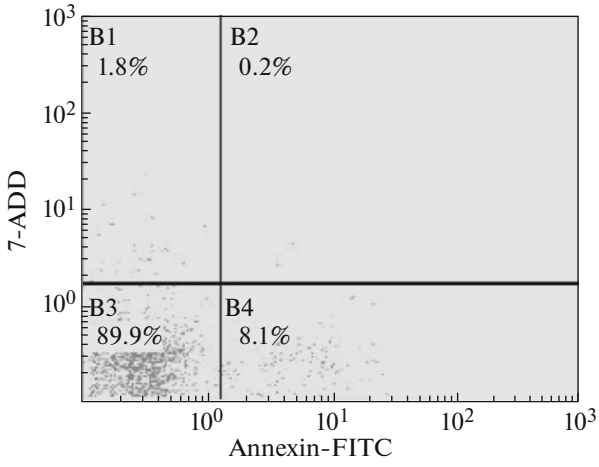

Fig. 3. Two-parameter histogram of FITC signals (annexin V-FITC binded to phosphatidylserine on the surface of the cell membranes) and 7-AAD signals (7-AAD binded to fragmented DNA) in intact rats.

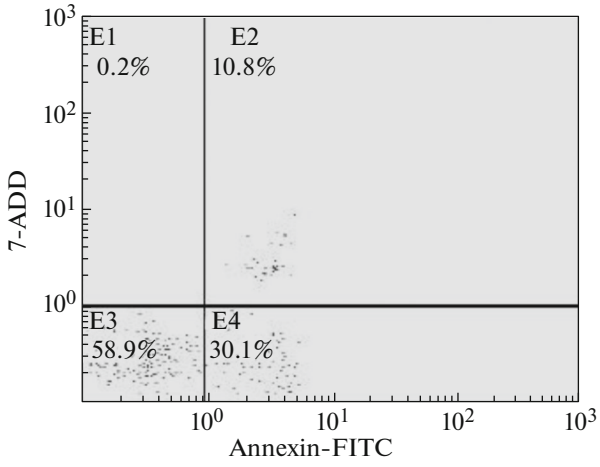

Fig. 4. Two-parameter histogram of FITC signals (annexin V-FITC binded to phosphatidylserine on the surface of the cell membranes) and 7-AAD signals (7-AAD binded to fragmented DNA) in rats injected with POMs for 30 days.

control group. After 30 days of administration of POMs, the changes in the survival rate of leukocytes were more significant (Table 2). The number of alive cells decreased to 66.53%. The frequency of irreversible changes (late apoptosis and necrosis) reached almost 10%; this value mainly accounts for apoptosis, since an increase in the frequency of necrosis was not statistically significant. The level of leukocytes at the early stage of apoptosis after 30 days was almost 3 times higher than that in the other two experimental groups and the group 30c. These data help explain the findings of our previous study [8] that the level of histones increases after 30 days of administration of POMs: according to the literature data, the accumulation of histones is needed for the following DNA compacting and chromatin condensation before the fragmentation during apoptosis [46].

Thus, the level of cells containing HSPs in the thymus and the spleen and the apoptosis rate of blood leukocytes showed a well-defined tendency to increase. The enhancement of both early and late apoptosis in leukocytes indicates the disruption of homeostasis in immunopoietic organs. The rate of apoptosis in blood leukocytes can serve as an indicator of changes at the cellular level before the damaging effect can be detected at the organ level.

CONCLUSIONS

The administration of POMs leads to an increase in the level of cells containing HSP60 and HSP70 not only in the spleen but also in the thymus, despite the blood–thymus barrier. We suggest that POMs can have similar effect on all immunologically privileged organs.

An increase in the level of HSP in the thymus and the spleen indicates that the administration of POMs is a stress factor for these organs.

The level of cells expressing HSP60 and HSP70 in the thymus and the spleen after both single and multiple doses of POMs leads to no morphological disruptions being found in these organs after 30 days of administration.

The administration of POMs leads to the elevation of apoptosis rate only by day 30, which means that the time of exposition of these nanoparticles should be changed in case of their therapeutic use.

The analysis of the peripheral blood of injected rats showed no signs of anemia of inflammation after either single or multiple doses of iron-molybdenum POMs.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 15-03-03603) and the Ural Federal University competitive growth program.

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Translated by Ya. Lavrenchuk