The effect of cyclophosphamide on the selected parameters of immunity in rats

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Abstract
The aim of the present study was to investigate the effect of cyclophosphamide on selected parameters of humoral and cellular immunity in rats. The experimental material comprised 48 adult Wistar rats aged 14 weeks, including 24 females and 24 males. The animals were initially divided into two groups: control (K) and experimental (C), each comprising 12 males and 12 females. Over a period of three consecutive days (days 1-3), 24 experimental group rats were administered cyclophosphamide intramuscularly at a rate of 50 mg/kg body weight/day. At the beginning of the experiment (day 0) and on the 8th, 15th and 22nd day of the study, six control group (K) rats (three males and three females) and six experimental group (C) rats (three males and three females) were sacrificed. Arterial blood samples were collected and diluted with heparin to determine and compare selected parameters of humoral and cellular immunity, including total protein levels, γ-globulin levels, lysozyme activity, ceruloplasmin activity, proliferative response of blood lymphocytes (MTT) after stimulation with LPS or ConA, the metabolic activity (RBA and Bursttest) and potential killing activity (PKA and Phatogest) of phagocytes. The results of the study indicate that cyclophosphamide had a significant immunosuppressive effect on all of the investigated parameters of humoral and cellular immunity in rats.

Key words: *cyclophosphamide, rats, immunosuppression, flow cytometry.*

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Introduction

Immunosuppression, i.e. the suppression of the immune response, may be considered undesirable, but it may also be induced deliberately under certain clinical circumstances, e.g. to inhibit or prevent an adverse reaction to treatment of allergic and autoimmune diseases, as well as to avoid transplant rejection. In some cases immunosuppressive therapy is continued until immune tolerance is achieved. Immunosuppression is a process that reduces the activation or efficacy of the immune system, including the mechanisms of both non-specific immunity, such as phagocytosis, cytokine production, interferon production, lysozyme activity, ceruloplasmin activity, and specific immunity, such as the levels of antibodies and lymphocyte proliferative activity [1-2].

Immunosuppression is induced by physical factors (stress, inadequate temperature, humidity and ventilation conditions, dust pollution, excessive stocking density), biological factors (bacteria, fungi, parasites and viruses) and chemical factors (microelement, macroelement and vitamin deficiencies, water and feed contamination, noxious gases, disinfectants, heavy metals, antibiotics, drugs) [3].

Due to the continuous progress in medical sciences, immunosuppressive drugs are more and more widely used in both human and veterinary medicine. Cyclophosphamide, administered in the present study, is a chemical immunosuppressive agent that belongs to the group of alkylating compounds.

The objective of this study was to make a preliminary evaluation of the effect of cyclophosphamide on selected parameters of humoral and cellular immunity in rats

Material and methods

Animals

Animal experiments were carried out in conformance with the Animal Protection Law (Journal of Laws of 24

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February 2005, no. 33, item 289) and the recommendations of the Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn. During the experiment, animals were kept in Faculty premises with the observance of adequate experimental conditions.

Experimental design

The experimental material comprised 48 adult Wistar rats aged 14 weeks, including 24 females with average body weight of 190 g and 24 males with average body weight of 320 g. The animals were initially divided into two groups: control (K) and experimental (C), each comprising 12 males and 12 females. The males and females of each group were kept in separate cages. All animals were fed Murigran pelleted feed for rodents (Akropol Motycz) and had ad libitum access to water. Over a period of 3 consecutive days (days 1-3), 24 experimental group rats were administered cyclophosphamide (*N*,*N*-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide CAS 50-18-0, SIGMA) intramuscularly at a rate of 50 mg/kg body weight/day in the form of 75 g/l PBS solution (phosphate buffered saline with the addition of calcium chloride and magnesium chloride, Biomed). At the beginning of the experiment (day 0) and on the 8th, 15th and 22th day of the study, 6 control group rats (K) (3 males and 3 females) and 6 experimental group rats (3 males and 3 females, group C) were sacrificed by an overdose of Narcotan (Halothanum, CAS 151-67-7, ZENTIVA, lot 3081006), arterial blood samples were collected and diluted with heparin (Heparinum natricum, CAS 9005-49-6 Warszawskie Zakłady Farmaceutyczne Polfa) to determine and compare selected parameters of biochemical, humoral and cellular immunity in rats (total protein levels, γ-globulin levels, lysozyme activity, ceruloplasmin activity, proliferative response of blood lymphocytes (MTT) after stimulation with LPS or ConA, the metabolic activity (RBA and Bursttest), potential killing activity (PKA and Phatogest) of phagocytes).

Evaluation of humoral immunity parameters

γ-globulin levels in blood serum were determined by the precipitation method modified by Siwicki and Anderson [5], total protein levels in blood serum was determined by spectrophotometry as described by Lowry *et al.* [4] modified by Siwicki and Anderson [5], lysozyme activity in blood plasma was determined by the turbidimetric method [6] modified by Siwicki and Anderson [5] and ceruloplasmin activity in blood plasma – by the method developed by Siwicki and Studnicka [7].

γ-globulin levels

Whole blood samples were centrifuged for 5 min at 1000 g to separate blood cells from the serum. The optical density of total protein was determined in blood serum following the above procedure. 0.1 ml of serum was placed in the wells of microplates and 0.1 ml 12% polyethylene glycol (10 000 kDa) (Sigma Chemical Co.) suspended in deionized water was added. The microplates were incubated at room temperature for 2 hours, and well contents were stirred continuously. The microplates were centrifuged for 10 minutes at 5000 g to separate the γ-globulin fraction bound by polyethylene glycol (plate sediment) from the remaining total protein fraction which constituted the supernatant. The optical density of supernatant was measured in a microplate reader at 620 nm. The optical density of supernatant was subtracted from the optical density of total protein. γ-globulin level was determined using a standard curve (plotted earlier for total protein) as a reference, based on the ability of γ-globulins to bind with polyethylene glycol and precipitate.

Total protein level

Whole blood samples were centrifuged for 5 min at 1000 g to separate blood cells from the serum. Five µl of serum was placed in the wells of microplates and 25 µl of reagent A and 200 µl of reagent B was added (Rio-Rad, Hercules, CA). Well contents were gently stirred with a pipette. The microplates were incubated at room temperature for 15 min. Next optical density was measured in a microplate reader at 620 nm. Total protein level was determined using a standard curve as a reference. The standard curve was plotted based on optical density values for known protein dilutions.

Lysozyme activity

Whole blood samples were centrifuged for 5 min at 1000 g to separate blood cells from the serum. The serum was diluted 1 : 1 with phosphate buffer, and 0.1 ml of the solution was placed in the wells of microplates. 0.5 ml of *Micrococcus lysodeikticus* bacterial suspension (25 mg bacteria/100 ml phosphate buffer) (Sigma Chemical Co.) was added. Absorbance was measured directly after the addition of bacteria (E_0) and after 1, 2, 3 and 30 min (final E). The final absorbance was subtracted from the initial absorbance (E_0) to determine lysozyme activity with the use of a standard curve. The standard curve was plotted based on the optical density values for known lysozyme concentrations.

Ceruloplasmin activity

Whole blood samples were centrifuged for 5 minutes at 1000 g to separate blood cells from the serum. The following buffers were prepared: 1) acetate buffer (pH 5.2, containing crystalline acetic acid, sodium acetate trihydrate and 15 mg EDTA), 2) buffered substrate solution (0.2% p-phenyldiamine (PPD) in acetic buffer), 3) sodium azide solution (0.02% sodium azide solution in deionized water). 0.5 ml of buffered solution mix of the three buffers mentioned immersed in a water bath at a temperature of

37°C. One test tube served as an experimental sample, and the other as control. Fifty µl of serum was added to the experimental sample which was incubated for 15 min at 37°C. Next 2 ml of a sodium azide solution was added to the experimental and control sample. Fifty µl of serum was added to the control sample, and both samples were mixed. The absorbance of the experimental sample was measured at a wavelength of 540 nm, using the control sample as a blind test. Ceruloplasmin activity was determined with the use of the standard curve. The standard curve was plotted based on the optical density values for known ceruloplasmin concentrations

Evaluation of non-specific cellular immunity parameters

The metabolic activity of blood phagocytic cells was determined based on the measurement of intracellular:

Respiratory burst activity (RBA) after stimulation with PMA (Phorobol Myristate Acetate, Sigma), as described by Chung and Secombes [8] and adapted for dogs by Siwicki *et al*. [9]. The isolated cells were resuspended in RPMI-1640 medium (Sigma) at 10^6 cells/ml. On 96-well U-shaped microplates, 100 µl of the isolated blood leukocytes was mixed with 100 µl of a 0.2% nitro blue tetrazolium (NBT, Sigma) solution in 0.2 M phosphate buffer at pH 7.2, and 1 µl of PMA at a concentration of 1 mg/ml in ethanol was added. After 30 min of incubation at 37°C, the supernatant was removed from each well. The cell pellet was washed with absolute ethanol and, three times, with 70% ethanol and it was dried at room temperature. The amount of extracted reduced NBT after incubation with 2 M KOH and DMSO (dimethylsulfoxide, Sigma) was measured colorimetrically at 620 nm in a microplate reader (Tecan Sunrise). All samples were tested in triplicate, and the results are presented as mean values.

Determination of oxidative metabolism of blood granulocytes and monocytes in rats – Bursttest (Phagoburst, Orpegen Pharma, Hiedelberg)

All test reagents were prepared in accordance with the manufacturer's recommendations, as indicated in the leaflet attached to the product. Each analyzed sample of whole heparinized blood was divided into four test tubes of 100 µl each and chilled to 0°C. Twenty µl chilled *Escherichia coli* bacteria was added to the first sample (experimental), 20 µl washing solution was added to the second sample (negative control), 20 µl fMLP was added to the third sample (low control), and 20 µl PMA was added to the fourth sample (high control). All test tubes were mixed and incubated for 10 min at 37°C (excluding the fMLP sample which was incubated for 7 min). After incubation, each test tube was supplemented with 20 µl substrate solution and was thoroughly shaken. All samples were incubated for 10 min at 37°C. After incubation, 2 ml lysing solution at room temperature was added. Test tubes were shaken and incubated at room temperature for 20 min. All samples were centrifuged for 5 min at 4°C (250 × g), and the supernatant was removed. All test tubes were rinsed once with 3 ml washing solution, centrifuged for 5 min at 4°C (250 × g), after which the supernatant was removed. Two hundred µl staining solution chilled to 0°C was added to each sample, test tubes were shaken and incubated for 10 min in an ice bath. Intracellular killing activity of phagocytes was determined in a cytometer (Beckmnan Coulter, Epics XL) in less than 30 min after the last reagent had been added. Three activators were used for cell stimulation: *E. coli* bacteria, PMA (4-phorbol-12-β-myristate-13-acetate) as the strong activator, and fMLP (*N*-formyl-met-leu-phe) as the weak activator. The added dihydrorodamine (123-DHR) was oxidized in mitochondria by H_2O_2 resulting from cell stimulation and was converted to cation rhodamine 123 (R123), the fluorescent emitter.

The potential killing activity (PKA) of mononuclear (MN) phagocytes and polymorphonuclear (PMN) phagocytes was determined in isolated blood leukocytes stimulated with killed microorganisms, according to the method presented by Rook *et al.* [10] and adapted for dogs by Siwicki *et al*. [9]. On 96-well U-shaped microplates, 100 µl of leucocytes was mixed with 100 µl of 0.2 % NBT in phosphate buffer at pH 7.2, and 10 µl of killed *Staphylococcus aureus* strain 209P (containing 10^6 bacteria) was added. The mixture was incubated for 1 h at 37°C and the supernatant was removed. The cell pellet was washed with absolute ethanol and, three times, with 70% ethanol and it was dried at room temperature. This was followed by the addition of 2 M KOH and DMSO to each well. The amount of extracted reduced NBT was measured at 620 nm in a microplate reader (Tecan Sunrise). All samples were tested in triplicate, and the results are presented as mean values.

Determination of blood granulocyte and monocyte phagocytic activity in rats – Phagotest (Orpegen Pharma, Hiedelberg)

All test reagents were prepared in accordance with the manufacturer's recommendations, as indicated in the leaflet attached to the product. One hundred µl whole blood chilled to 0°C and 20 µl chilled bacteria were added to each of the two 5 ml test tubes (control and experimental) and shaken for around 3 s at low speed. The experimental sample was incubated for 10 min at 37°C, and the control sample – in an ice bath at 0°C. After incubation, 100 µl quenching solution was added to each sample and the samples were shaken. 3 ml of washing solution chilled to 0°C was added, samples were centrifuged for 5 min at 4°C (250 × g) and the supernatant was removed. The rinsing procedure was performed twice, and 2 ml lysing solution at room

temperature was added to each sample. The samples were shaken and incubated at room temperature for 20 min. Samples were centrifuged for 5 min at 4°C (250 × g), and the supernatant was removed. 3 ml washing solution chilled to 0°C was added to each sample, the samples were centrifuged for 5 min at 4°C (250 × g), and the supernatant was removed. Two hundred µl DNA staining solution chilled to 0°C was added, the samples were shaken and incubated for 10 min in an ice bath. Cellular phagocytic activity was determined in a cytometer (Beckmnan Coulter, Epics XL) in less than 60 min after the last reagent had been added. A Phagotest is performed with the involvement of fluorescein-stained *E. coli* bacteria which are phagocytized by macrophages. Cell nuclei are also stained. The test determines the number of phagocytizing cells, granulocytes and monocytes separately, and their phagocytic activity, i.e. the number of bacteria absorbed by a single cell in terms of fluorescence intensity.

Evaluation of specific cellular immunity parameters

Proliferative response of blood lymphocytes after stimulation with mitogens, concanavalin A (ConA) and lipopolysaccharide (LPS), were determined by MTT spectrophotometry (OD 570 nm) using (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide – 3-[4,5-dimethyl-2-thiazol]-2,5-diphenyl-2H-tetrazolium bromide), as described by Mosmann [11].

MTT (Sigma) was dissolved in PBS at a concentration of 5 mg/ml and filtered. On 96-well culture plates (Sarstetd, USA), 100 µl of blood lymphocytes in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% hepes buffer, penicillin/streptomycin (100 U/100 µg/ml) was mixed with 100 µl of RPMI 1640 containing mitogens ConA (5 µg/ml) or LPS (20 µg/ml). After 72 h incubation at 37°C in a 5% carbon dioxide atmosphere (Memmert Incubator), 50 µl of MTT solution was added into each well, and plates were incubated for 4 h at 37°C. After incubation the plates were centrifuged (1400 g, 15°C, 5 min). Supernatants were removed and 100 µl of DMSO (Sigma) were added into each well and incubated for 15 min at room temperature. After incubation the solubilized reduced MTT was measured colorimetrically at 570 nm in a microplate reader (Tecan Sunrise). All samples were tested in triplicate, and the results are presented as mean values. The final results are presented as the reactivity index (RI).

Statistical analysis

The obtained results were processed statistically in a one-factorial analysis of variance in an orthogonal design. The significance of differences between groups was verified by the Student's t-test with the use of GraphPad Prism 5 software.

Results

The intoxication of rats with cyclophosphamide (C) caused a statistically significant ($p \leq 0.001$) decrease in serum total protein, compared with the control group (K) (Table 1). As regards the investigated indicators of humoral immunity in rats, i.e. lysozyme activity, ceruloplasmin activity, and the serum concentrations of γ-globulins (Table 2), significantly ($p \leq 0.001$) lower values were noted in the group of cyclophosphamide-intoxicated animals. The administration of cyclophosphamide to rats (C) caused a decrease in lysozyme activity, ceruloplasmin activity and γ-globulin levels, in comparison with the control group. As concerns the indicators of non-specific cell-mediated immunity of rats, i.e. the respiratory burst activity (RBA) and the potential killing activity (PKA) of phagocytes, and the parameters of specific cell-mediated immunity of rats, i.e. the proliferation rates of lymphocytes (MTT assay) stimulated with LPS and ConA (Table 3), significantly ($p \leq 0.001$) lower values were observed in the group intoxicated with cyclophosphamide (C), compared with the control group (K).

The obtained results showed a significant drop in the phagocytic activity of granulocytes and monocytes in the group of rats intoxicated by cyclophosphamide (C), both in terms of the percentage of phagocytizing cells and average fluorescence intensity – the average number of phagocytized bacteria (Phagotest), in comparison with control (K) (Table 4).

In the group of animals intoxicated by cyclophosphamide (C), a drop in the number of granulocytes stimulated to kill bacteria and a decrease in the oxidative metabolism of granulocytes (in terms of average fluorescence intensity) stimulated with fMLP, PMA and

Table 1. The effect of cyclophosphamide on the total protein level in rats blood

Parameter	Group	Experimental day							
		0		8		15		22	
		$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD
Total protein level (g/l)	K	77.26	2.07	76.99	2.02	77.89	1,67	78.59	2.17
	C	76.95	3.90	68.05***	1.17	66.74***	3.21	68.11***	2.46

** p < 0.05; ** p < 0.01; *** p < 0.001*

Table 2. The effect of cyclophosphamide on the parameters of humoral immunity in rats

Parameter	Group	Experimental day							
		0		8		15		22	
		$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD
Lysozyme activity (mg/l)	K	8.79	0.18	9.07	0.19	9.03	0.08	9.10	0.22
	C	8.53	0.14	6.03***	0.21	6.11***	0.1	5.96***	0.19
Ceruloplasmin activity (mg/l)	K	98.15	5.01	100.34	3.31	102.11	2.33	99.75	3.06
	C	100.36	3.21	79.36***	4.67	81.02***	6.15	80.39***	2.81
γ-globulin level (g/l)	K	15.44	0.21	14.11	0.38	14.76	0.17	13.97	0.33
	C	14.97	0.34	9.98***	0.2	10.01***	0.51	9.78***	0.26

** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$*

Table 3. The effect of cyclophosphamide on the parameters of cellular immunity in rats

Parameter	Group	Experimental day							
		0		8		15		22	
		$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD
RBA (OD 620 nm)	K	0.41	0.05	0.42	0.06	0.42	0.03	0.40	0.04
	C	0.40	0.04	0.27***	0.02	0.22***	0.03	0.25***	0.05
PKA (OD 620 nm)	K	0.42	0.03	0.45	0.04	0.44	0.01	0.43	0.03
	C	0.42	0.04	0.24***	0.05	0.27***	0.03	0.27***	0.02
MTT-ConA (RI)	K	1.05	0.06	1.11	0.1	1.08	0.09	1.02	0.03
	C	1.07	0.02	0.55***	0.03	0.61***	0.03	0.62***	0.09
MTT-LPS (RI)	K	1.15	0.08	1.12	0.06	1.19	0.11	1.11	0.04
	C	1.12	0.09	0.48***	0.04	0.33***	0.21	0.47***	0.07

** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$*

Table 4. Percentage of phagocytic granulocytes and monocytes and average fluorescence intensity of granulocytes and monocytes in rat group determined in the Phagotest

Group	Granulocytes		Monocytes	
	% phagocytic cells (± SD)	average fluorescence intensity (± SD)	% phagocytic cells (± SD)	average fluorescence intensity (± SD)
Experimental day	**Control (K)**			
0	84.11 ±3.24	38.24 ±4.24	60.35 ±3.11	19.01 ±2.02
8	85.32 ±2.33	36.17 ±2.66	61.05 ±2.47	18.03 ±1.1
15	83.98 ±3.13	35.82 ±2.81	61.64 ±3.24	17.97 ±1.09
22	84.67 ±2.88	36.49 ±1.19	60.89 ±1.91	18.88 ±1.23
Experimental day	**Cyclophosphamide (C)**			
0	85.03 ±1.79	37.65 ±3.05	61.47 ±3.21	18.95 ±1.87
8	66.12*** ±2.14	16.23*** ±1.98	37.41*** ±2.23	6.32*** ±0.68
15	62.64*** ±1.36	15.98*** ±2.65	39.11*** ±3.33	5.44*** ±1.31
22	63.23*** ±2.43	16.09*** ±2.22	39.38*** ±1.69	5.21*** ±0.98

** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$*

Table 5. Average intracellular killing activity of granulocytes and average fluorescence intensity in rat group after stimulation with fMLP, PMA and *E. coli* as determined in the Bursttest

		Group	Experimental day	K	C
Granulocytes	**fMLP**	% stimulated cells (± SD)	0	19.21 ±1.05	19.26 ±1.39
			8	19.03 ±1.12	7.64*** ±0.64
			15	18.95 ±0.92	7.55*** ±1.01
			22	19.15 ±1.26	7.88*** ±0.97
		average fluorescence intensity (± SD)	0	4.2 ±0.67	3.97 ±0.96
			8	3.94 ±0.32	1.73*** ±0.77
			15	3.84 ±0.76	1.86*** ±0.72
			22	3.92 ±0.71	1.75*** ±0.86
	PMA	% stimulated cells (± SD)	0	86.32 ±1.75	86.23 ±3.01
			8	85.76 ±1.67	68.38*** ±1.97
			15	85.89 ±2.13	66.54*** ±2.11
			22	86.79 ±1.74	66.94*** ±1.86
		average fluorescence intensity (± SD)	0	24.99 ±3.05	25.59 ±2.61
			8	25.55 ±2.37	10.23*** ±1.11
			15	25.86 ±1.46	10.51*** ±1.08
			22	24.76 ±2.25	9.98*** ±1.96
	***E. coli* bacteria**	% stimulated cells (± SD)	0	81.13 ±2.35	82.57 ±1.86
			8	82.18 ±3.11	60.08*** ±2.04
			15	81.87 ±1.99	59.11*** ±1.01
			22	80.99 ± 2.02	58.31*** ±1.67
		average fluorescence intensity (± SD)	0	25.19 ±0.96	25.64 ±0.74
			8	24.82 ±1.16	11.66*** ±0.99
			15	24.64 ±0.91	12.05*** ±1.14
			22	24.24 ±1.17	12.06*** ±1.07

** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$*

E. coli was noted in comparison with control (K) (Table 5). Although the values of the investigated parameters were generally lower, an analysis of the intracellular killing activity of monocytes in the group of control (K) and intoxicated (C) rats (Table 6) did not reveal significant differences only in respect of the oxidative metabolism of cells stimulated with fMLP in the group of animals intoxicated by cyclophosphamide (C).

Discussion

Cyclophosphamide belongs to the group of DNA-alkylating agents. It is one of the most popular drugs used to treat different forms of cancer, and autoimmune diseases. In transplantology, it is applied as a preparative regimen for allogeneic bone marrow transplant recipients [1]. Cyclophosphamide targets fast-dividing cells which replicate rapidly, like cancer cells, as they are most sensitive to alkylating agents. However, the action of cyclophosphamide is not selective, and therefore the drug can also damage the fast-proliferating cells of healthy tissue, including bone marrow cells (in particular the developing blood cells), activated lymphocytes (that proliferate and produce antibodies), fetal cells, immune cells, hair follicle cells, and intestinal epithelial cells [12]. Cyclophosphamide weakens both the cell-mediated and humoral immune response. Its effect is dose-dependent, but even a single administration may temporarily impair the immune system.

In the present study, cyclophosphamide had an inhibitory effect on protein production, causing a decrease in total protein and protein fractions. The administration of this drug was followed by a decrease in γ-globulin levels and ceruloplasmin activity. The above most probably resulted from the adverse side effects of cyclophosphamide on hepatocytes which produce protein. Cyclophosphamide inhibits the activity of hepatic enzymes responsible for protein synthesis. The analyzed drug indirectly affects the levels of ceruloplasmin (and other acute-phase proteins) through the impairment of the synthesis of cytokines which stimulate the production of proteins in the liver, i.e. IL-1,

Table 6. Average intracellular killing activity of monocytes and average fluorescence intensity in rat group after stimulation with fMLP, PMA and *E. coli* as determined in the Bursttest

		Group	Experimental day	K	C
Monocytes	**fMLP**	% stimulated cells (± SD)	0	9.77 ±0.23	9.82 ±0.31
			8	9.92 ±0.54	5.89*** ±0.57
			15	9.65 ±0.44	5.81*** ±0.61
			22	10.01 ±0.51	6.03*** ±0.49
		average fluorescence intensity (± SD)	0	7.21 ±0.89	7.14 ±0.64
			8	6.88 ±0.63	5.91** ±0.88
			15	6.73 ±0.51	5.46*** ±0.72
			22	7.09 ±0.64	6.01*** ±0.24
	PMA	% stimulated cells (± SD)	0	41.44 ±1.87	42.03 ±1.33
			8	42.31 ±0.94	30.01*** ±1.62
			15	41.75 ±1.64	29.14*** ±1.21
			22	40.09 ±1.88	28.61*** ±1.16
		average fluorescence intensity (± SD)	0	12.35 ±0.74	11.96 ±1.03
			8	11.89 ±1.07	3.54*** ±0.83
			15	12.54 ±0.86	4.06*** ±1.01
			22	12.05 ±0.93	3.96*** ±0.93
	***E. coli* bacteria**	% stimulated cells (± SD)	0	45.07 ±3.01	43.67 ±2.63
			8	44.22 ±2.12	20.14*** ±1.76
			15	43.73 ±1.9	22.1*** ±3.84
			22	43.92 ±2.24	21.43*** ±2.34
		average fluorescence intensity (± SD)	0	12.25 ±0.76	12.58 ±0.63
			8	13.04 ±1.03	7.02*** ±0.37
			15	11.95 ±0.74	6.26*** ±0.71
			22	12.69 ±0.81	5.65*** ±0.45

** p < 0.05; ** p < 0.01; *** p < 0.001*

IL-6 and TNF-α secreted mostly by monocytes and macrophages. A decrease in the levels of immunoglobulin, the main fraction of γ-globulins, may be indirectly caused by the inhibition of B cell proliferation by cyclophosphamide. A lower number of daughter cells is tantamount to a lower number of antibodies synthesized by these cells. Cyclophosphamide exerts a direct impact on plasma cells, and it inhibits protein synthesis. King and Perry [13] described the toxic effects of various chemotherapeutics on the liver, including cyclophosphamide which was found to inhibit selected hepatic enzymes. According to these authors, the drug exerts a specific, and not directly toxic, effect on the cytochrome P450 system which converts cyclophosphamide to active metabolites. As the process intensifies (following the administration of higher doses of the drug), hepatocyte dysfunction is observed, including disturbances in the synthesis of selected proteins.

In the present study, cyclophosphamide contributed to a decrease in the activity of lysozyme (which is also a γ-globulin), produced by phagocytes. Zhao *et al.* [14] also administered this drug to rats and reported a decrease in serum lysozyme levels.

The results of our experiment indicate that cyclophosphamide had a negative effect on the respiratory burst activity (RBA test and Bursttest) and the potential killing activity (PKA test and Phagotest) of phagocytes. The values of the investigated parameters dropped in intoxicated rats. Cyclophosphamide affects cells by alkylating DNA, RNA and enzymes with a protein structure. Therefore, it not only influences proliferating cells, but it may also retard their intermitotic functions. The above may inhibit phagocytic activity at various stages of phagocytosis. Phagocyte suppression may result from inhibited synthesis of cytokines, enzymes which regulate the functions of phagocytes, participate in the synthesis of substances found in phagocyte grains as well as receptors that recognize microorganisms and complement receptors. By attaching alkyl groups to enzymes, cyclophosphamide may retard the respiratory burst activity by disrupting the synthesis of reactive oxygen species at one or several stages,

disrupting lysozyme activity and/or inhibiting the activity of enzymes participating in its synthesis. Cyclophosphamide may also indirectly influence phagocytes by affecting lymphokine production by lymphocytes. In two studies, Winkelstein [15, 16] demonstrated cyclophosphamide's inhibiting effect on macrophage populations at inflammation sites. According to the author, the above is due to disturbances in the monocyte production process which creates macrophages. Winkelstein also argued that this process could lower cellular reactivity. In a study of cyclophosphamide, Cairo *et al.* [17] noted a drop in the production of reactive oxygen species by phagocytes. Zhao *et al.* [14] also administered this drug to rats and reported a decrease of the phagocytic percentage and the phagocytic index of macrophages. A study of mice [18] revealed lower levels of nitrogen oxide (NO) following the administration of cyclophosphamide.

As shown by the present experiment, cyclophosphamide reduced the proliferative capacity of T cells and B cells in rats after mitogenic stimulation. This suggests that both lymphocyte populations are sensitive to cyclophosphamide. The decrease in proliferation rates results from the action of this drug on cell DNA. Cyclophosphamide prevents cell division by cross-linking DNA strands, thus inhibiting the production of daughter lymphocytes. It also adds alkyl groups onto molecules where they do not belong, giving rise to abnormal, dysfunctional daughter cells with reduced activity. Cyclophosphamide probably inhibits also the expression of genes for IL-2. In addition, proliferation impairment may be a consequence of cyclophosphamide's adverse effect on monocytes/macrophages which participate in regulating the functions of lymphocytes. As regards in vivo proliferation, an even greater decline in the proliferative activity of B cells can be expected, due to the fact that the majority of antigens are T-dependent. This means that cyclophosphamide affects B cells directly, but also indirectly through the inhibition of T-cells. A decrease in the proliferation rates of T-cells is followed by lower stimulation of B-cells, while the reduced proliferative capacity of B-cells leads to lower antibody production by these cells. Angulo *et al.* [19] also reported a negative impact of cyclophosphamide on the proliferative capacity of lymphocytes. In their study mice were treated with 3 intraperitoneal (i.p.) injections of cyclophosphamide at a dose of 100 mg/kg body weight at 72-hour intervals. 98% suppression of T-cell proliferation in response to cyclophosphamide was observed already on day 6 after the administration of the last dose of the drug. According to the above authors, such a significant decrease in the proliferative activity of T-cells resulted from both the direct effect of cyclophosphamide and the presence of a heterogeneous immature myeloid cell population in the spleen, which suppressed the activation of T-cells. Another experiment [20] investigated the immunosuppressive effect of low-dose cyclophosphamide, administered i.p. for 5 consecutive days, on the proliferation rates of lymphocytes from the peripheral blood, spleen and lymph nodes. A decline in the proliferative capacity of circulating lymphocytes was reported already at the lowest doses (5 and 10 mg/kg) of cyclophosphamide and stimulation with both mitogens (ConA and PHA), but a statistically significant result was noted at 20 mg/kg and PHA stimulation. The 20 mg/kg dose of cyclophosphamide was also sufficient to significantly reduce the proliferative activity of lymphocytes from the peripheral blood, spleen and lymph nodes after antigen (PPD – purified protein derivative of tuberculin) stimulation.

The obtained results clearly indicate that cyclophosphamide has an immunosuppressive effect, and that it can be effectively used to chemically induce immune system impairment.

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