The influence of methisoprinol on the spleen phagocyte and blood lymphocyte activity in rats – *in vitro* study

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Abstract

The application of immunostimulators in human and veterinary medicine offers a wide of attractive method for inducing or modulating protection against infection diseases. In the present study we determined the in vitro influence of different concentrations of synthetic product methisoprinol on the proliferative response of blood lymphocytes stimulated by concanavaline A (ConA) and lipopolisaccharide (LPS) in rats. Also the metabolic ability and potential killing activity of spleen phagocytes were examined. For this study 10 adult male and 10 adult female rats were used. Peripheral blood was obtained by venous puncture (Vacutainer set). The proliferative ability of the blood lymphocytes stimulated by mitogens was determined by MTT assay. The respiratory burst activity (RBA) and potential killing activity (PKA) were determined by spectrophotometric assay. In each experiment, the concentrations of methisoprinol used in the RPMI 1640 medium were 0, 0.5, 1, 5, 10, 25 and 50 μg methisorinol/ml of medium. The present study shows that a concentrations between 5 to 50 μg/ml of methisoprinol statistically significantly (p < 0.05) increase the mitogens-induced proliferation rate of rats lymphocytes T and B and we have not observed statistically significant difference between male and female. The analysis of the results showed that the methisoprinol increased the metabolic ability (RBA) and potential killing activity (PKA) of spleen phagocytes at concentrations between 5 and 50 μg/ml, compared to the control. The highest RBA and PKA were observed at concentrations between 10 to 50 μg/ml and also we have not observed statistically significant difference between male and female animals.

Key words: methisoprinol, rats, lymphocyte proliferation, phagocyte activity.

Introduction

The immune system is a complex interaction of checks and balances that allows regulation by opposing stimulatory and inhibitory influences. The viral infections appear to be an important practical problem in human and animals, due to endemic spreading of the infections, high costs and generally low efficacy of the therapy. Therefore, a strategy of elimination of endemic infections should a battery prevention tasks aimed both to the elimination of the infective agents and the elevation of the non-specific anti-viral resistance of the organism. Immunomodulators comprise a group of biological and synthetic compounds and play an important role in modern alternative prevention and therapy of infectious diseases. The general opinion about the use of non-specific immunomodulators in prophylaxis of endemic infections is positive [1, 2]. A reasonable possibility for effective, non-toxic immunomodulation which should be considered for prevention of endemic infections in animals appears to be the application of potent activators of the macrophage-monocyte and lymphocyte systems [2-6]. Actually exist a large number of macrophage and lymphocyte activators of natural origin from bacteria, fungi, plants and other biological and non-biological materials [7-13]. These substances were tested in experimental and clinical investigations for the treatment of

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neoplastic diseases, as well as for the prophylaxis and treatment of infections in human and animals [1, 3, 8, 14, 15].

Methisoprinol (inosiplex) is a synthetic compound formed from the p-acetamidobenzoate salt of N-N dimethylamino-2-propanol and inosine in a 3 : 1 molar ratio. It exerts antitumour and antiviral activities *in vivo*, which are secondary to an immunomodulating influence on the non-specific and specific defence mechanisms in human and mouse [6, 18, 21, 22]. The immunomodulating effects of methisoprinol *in vitro* and *in vivo* has been assessed in many diseases including Herpes infections, the acquired immunodeficiency syndrome (AIDS) and autoimmune diseases in human [16, 21, 22].

The aim of the present investigation was to assess the *in vitro* influence of methisoprinol on the spleen macrophage activity and blood lymphocyte proliferation in rats.

Material and Methods

Animals and experimental design

For this study 10 adult male and 10 adult female Wistar rats, aged 12 weeks, were used. Animal experiments were carried out in conformance with the Animal Protection Law and the recommendations of the Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn. Peripheral blood was obtained by venous puncture (Vacutainer set – Vacuette Greiner Labortechnik) and spleens were separated after dissection with procedure used in laboratory animals.

For each experiment performed under identical conditions, collected peripheral blood were diluted with one part of RMPI 1640 culture medium (RPMI 1640, Sigma) supplemented with sodium pyruvate and 1% antibiotics (penicilin/streptomycin, Sigma) and lymphocytes were separated on Percoll gradient (Pharmacia, Sweden). The number of isolated cells was quantified microscopically after the cells were stained with trypan blue, using a modified Neubauer chamber. The spleen of each rat was removed aseptically and single-cell suspensions were obtained by teasing the tissues in medium through a steel mesh and isolating spleen phagocytes using either a Gradisol (Polfa, Poland)

The methisoprinol (Polfa Grodzisk, No: 330300, Poland) was used in the present studies. The isolated rats blood lymphocytes were stimulated by concanavaline A (ConA, Sigma Chemical) or lipopolysaccharide (LPS Sigma Chemical).

Assay procedure

The proliferative ability of the blood lymphocytes stimulated by mitogens were determined by MTT assay, first described by Mosmann [23] and used in animals by Siwicki et al. [9] and Wagner et al. [24]. MTT [3-(4,5-Dimethyl thiazol-2-yl0-2,5-diphenyl) tetrazolium bromide] (Sigma) was dissolved in PBS at concentration of 5 mg MTT/ml and

sterilized by filtration. This stock solution was used for the MTT assay. One hundred µl of isolated lymphocytes were distributed in 96-well culture plates (Costar, USA) at concentration 1×10^6 cells/ml in RPMI 1640 medium containing 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% Hepes buffer, penicillin/streptomycin (100 U/100 µg/ml) and 10% fetal calf serum (FCS) with or without methisoprinol at different concentrations. In each experiment, the concentrations of methisoprinol used in the RPMI 1640 medium (Sigma) were 0, 0.5, 1, 5, 10, 25 and 50 µg methisorinol/ml of medium. The mitogens: ConA at concentration 5 µg/ml or LPS at concentration 20 µg/ml were then added (100 µl/well) to each well. All samples were tested in triplicate. The mixture containing cells in medium with different concentration of methisoprinol and mitogen were incubated for 72 h at 37°C, with 5% carbon dioxide atmosphere (Asab incubator, Sweden). After incubation, 50 µl of MTT (5 mg/ml PBS) solution were added to each well and incubated 4 h in 37°C. The microtiter plates were centrifuged (1400 × g, 15°C, 5 min) and the supernatant was removed carefully by an Eppendorf pipette. To each well 100 µl of a DMSO (Sigma) were added and after 10-15 min. incubation, the solubilized reduced MTT was evaluated in an ELISA microreader (Alab Plate Reader, USA) at 620 nm wavelength.

The activity of spleen phagocytes was analysed using respiratory burst activity (RBA) assay and potential killing activity (PKA) assay.

Respiratory burst activity was determined in spleen phagocytes after stimulation with PMA (Phormol Myristate Acetate, Sigma), as described by Chung and Secombes [25] and adapted for animals by Siwicki et al. [9]. One hundred ?l of isolated phagocytes were distributed in 96-well culture plates (Costar, USA) at concentration 1×10^6 cells/ml in RPMI 1640 medium containing 10% fetal calf serum (FCS) with or without methisoprinol at different concentrations. In each experiment, the concentrations of methisoprinol used in the RPMI 1640 medium (Sigma) were 0, 0.5, 1, 5, 10, 25 and 50 µg methisorinol/ml of medium. After 2 of incubation, cells was mixed with 100 µl 0.2% nitro blue tetrazolium (NBT, Sigma) and 1 µl of PMA at concentration of 1 mg/ml in ethanol. After 30 min. of incubation at 37°C, the supernatant was removed from each well. The cell pellet was washed with absolute ethanol and also three times with 70% ethanol. The amount of extracted reduced NBT after incubation with 2M KOH and DMSO (dimethylsulfoxide, Sigma) was measured colorimetrically at optical density (OD) 620 nm in microplate reader (Tecan, Sunrise).

The potential killing activity (PKA) of spleen phagocytes was determined after stimulation cells with killed *Staphylococcus aureus* strain 209P, according to the method presented by Rook et al. [26] adapted for animals by Siwicki et al. [9]. One hundred µl of isolated phagocytes were distributed in 96-well culture plates (Costar, USA) at concentration 1×10^6 cells/ml in RPMI 1640 medium

Table 1. *In vitro* influence of different concentrations of methisoprinol on the proliferative response of blood lymphocytes stimulated by Con A and LPS in male (M) and female (F) rats (mean ±SD, n = 10, *significant difference p < 0.05) compared to the control (only mitogens)

| Concentration of methisoprinol in medium (µg/ml): | Mitogens | | | |
| | Con A | | LPS | |
	M	F	M	F
Control (0) only mitogen	0.48 ±0.04	0.45 ±0.05	0.43 ±0.03	0.40 ±0.05
0.5	0.50 ±0.04	0.47 ±0.03	0.45 ±0.04	0.42 ±0.05
1.0	0.53 ±0.05	0.50 ±0.05	0.48 ±0.05	0.46 ±0.06
5.0	0.59 ±0.03*	0.56 ±0.04*	0.52 ±0.04*	0.49 ±0.03*
10.0	0.81 ±0.05*	0.78 ±0.03*	0.68 ±0.05*	0.65 ±0.04*
25.0	0.85 ±0.05*	0.81 ±0.06*	0.75 ±0.05*	0.72 ±0.04*
50.0	0.86 ±0.07*	0.83 ±0.06*	0.77 ±0.04*	0.74 ±0.05*

Table 2. *In vitro* influence of different concentrations of methisoprinol on the respiratory burst activity (RBA) and potential killing activity PKA) of spleen phagocytes in male (M) and female (F) rats (mean ±SD, n = 10, *significant difference p < 0.05) compared to the control

| Concentration of methisoprinol in medium (µg/ml): | Activity of spleen phagocytes: | | | |
| | RBA | | PKA | |
	M	F	M	F
Control (0)	0.40 ±0.05	0.38 ±0.04	0.37 ±0.04	0.35 ±0.05
0.5	0.42 ±0.05	0.43 ±0.05	0.38 ±0.05	0.37 ±0.05
1.0	0.43 ±0.06	0.45 ±0.07	0.39 ±0.04	0.40 ±0.06
5.0	0.51 ±0.04*	0.50 ±0.05*	0.45 ±0.05*	0.46 ±0.05*
10.0	0.63 ±0.04*	0.64 ±0.05*	0.58 ±0.04*	0.55 ±0.06*
25.0	0.72 ±0.04*	0.70 ±0.05*	0.65 ±0.04*	0.63 ±0.05*
50.0	0.75 ±0.05*	0.72 ±0.04*	0.68 ±0.05*	0.62 ±0.06*

containing 10% foetal calf serum (FCS) with or without methisoprinol at different concentrations. In each experiment, the concentrations of methisoprinol used in the RPMI 1640 medium (Sigma) were 0, 0.5, 1, 5, 10, 25 and 50 µg methisoprinol/ml of medium. After 2 h incubation cells were mixed with 100 µl of 0.2 % NBT and 10 µl killed bacteria (containing 106 bacteria). The mixture was incubated for 1 h at 37°C and the supernatant was removed. The cell pellet was washed with ethanol and it was dried at room temperature. This was followed by the addition of 2M KOH and DMSO to each well. The amount of extracted reduced NBT was measured colorimetrically at optical density (OD) 620 nm in microplate reader (Tecan, Sunrise).

Statistical analysis

The results were analyzed using the Student's *t*-test. Means and standard deviations for all values were calculated and presented in the figures. Differences between the treatment means were considered statistically significant if p < 0.05 with use of GraphPad Prism 5 software.

Results

In presented study we examined the *in vitro* influence of different concentrations of synthetic immunomodulator methisoprinol on the proliferative response of blood lymphocytes T and B as measured by the MTT assay. The

influences of different concentrations of methisoprinol on the proliferative response of blood lymphocytes T stimulated by ConA are presented in the Table 1. The results showed that the methisoprinol at concentration between 5 and 50 µg/ml significantly increased the proliferative response of T lymphocytes, compared to the control presented by cells incubated without methisoprinol (only ConA). The highest proliferative responses of lymphocytes were observed at concentrations between 10 to 50 µg/ml, but we have not observed statistically significant difference between male and female animals..

The influences of different concentrations of methisoprinol on the proliferative response of blood lymphocytes B stimulated by LPS are presented in the Table 1. The analysis of the results showed that the methisoprinol also increased the proliferative response of B-cells at concentrations between 5 and 50 µg/ml, compared to the control presented by cells incubated without methisoprinol (only LPS). The highest proliferative responses of B-cells were observed at concentrations between 10 to 50 µg/ml. The present study shows that the concentrations between 5 to 50 µg/ml of methisoprinol increase the mitogens-induced proliferation rate of rats blood lymphocytes T and B, without difference between male and female rats.

The statistically significantly ($p < 0.05$) higher values of the phagocytic ability and potential killing activity of spleen phagocytes were observed at concentrations of methisoprinol between 5 to 50 µg/ml, compared to the control presented by cells incubated without methisoprinol. The influence of different concentrations of methisoprinol on the respiratory burst activity and potential killing activities are presented in the Table 2. The analysis of the results showed that the methisoprinol increased the metabolic ability (RBA) and potential killing activity (PKA) of spleen phagocytes at concentrations between 5 and 50 µg/ml, compared to the control. The highest RBA and PKA values were observed at concentrations between 10 to 50 µg/ml and statistically significant difference between male and female animals was not observed.

Discussion

Several publications have compiled a large amount of data on the immunomodulating activity of methisoprinol [17, 21]. This product potentiates or augments immunological events by triggering agents such as mitogens, antigens or lymphokines. Mitogen or antigen-stimulated T-lymphocyte differentiation and proliferation are accelerated by concurrent exposure to methisoprinol [15]. The methisoprinol strongly stimulated differentiation of cells of the T-lymphocyte lineage [20]. The methisoprinol-induced increase in the concanavalin A-stimulated blastogenic response was more apparent in cell cultures showing an initially low blastogenic response [19]. Similarly, *in vitro* methisoprinol markedly enhanced concanavalin A-stimulated T-lymphocyte cell proliferation in cells from patients with severely impaired cell-mediated responsiveness, including patients with AIDS or persistent

generalised lymphadenopathy [5]. The pokeweed mitogen-stimulated T-cell-dependent B-cell proliferative responses in cells from patients with AIDS or AIDS-related complex have also been reported to be enhanced by methisoprinol [14]. By direct interaction *in vitro*, methisoprinol has been shown to stimulate various parameters of metabolic activity of macrophages and monocytes [18, 21]. Incubation with methisoprinol *in vitro* stimulated the production of interleukin-1 in human monocytes and macrophages, and increased the levels of lisosomal enzymes in the latter [21].

In our preliminary study the strong stimulating influence of different concentrations of methisoprinol on the blood proliferative response of T and B-lymphocytes were observed. Also methisoprinol at different concentration activated metabolic ability and potential killing activity of spleen PMN and MN phagocytes. This is very important for restoration or modulation of immunity after suppression induced by different viruses or xenobiotics.

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