In vitro assessment of the proliferative abilities of lymphocytes treated with Respisure vaccine as well as immunomodulators Lydium-KLP and Methisoprinol

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Abstract

There are no reports in the literature on the effect of vaccine antigen supplemented with immunomodulators on the activity of T and B lymphocytes. The aim of this study was to determine the proliferative activity of lymphocytes treated with a vaccine against mycoplasmal pneumonia of swine (MPS) and with selected immunomodulators in in vitro examinations. Five clinically healthy piglets at the age of 21 days were used in the experiment and blood for lymphocyte isolation was collected from them. Inactivated vaccine against MPS – Respisure (Pfizer) – at dilutions of 1 : 10, 1 : 100 and 1 : 1000 and immunomodulators – Methisoprinol (Polfa Grodzisk, Poland) and Lydium- KLP (Nika Health Products Ltd, USA), were also used. The test of mitogen-stimulated lymphocyte proliferation was performed according to the MTT method. T lymphocytes were stimulated with ConA, whereas B lymphocytes were stimulated with LPS. The experiment showed that combinations of immunomodulators: Lydium-KLP and Methisoprinol and the Respisure vaccine at dilutions of 1 : 100 and 1 : 1000 increase in vitro proliferative activity of ConA-stimulated T lymphocytes and LPS-stimulated B lymphocytes. A stronger proliferative response of both the lymphocyte types was observed for the combination of the Respisure vaccine and Methisoprinol.

Key words: *swine, lymphocyte proliferation, Respisure, Lydium-KLP, Methisoprinol.*

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Introduction

Mycoplasma hyopneumoniae (Mhp), which causes mycoplasmal pneumonia of swine (MPS) and is an important etiological factor of porcine respiratory disease complex (PRDC), is included in the group of some of the simplest microorganisms of the domain of bacteria. They possess a minimum set of organelles which are necessary for their growth and multiplication and are devoid of a cell membrane; this determines their polymorphism, low sensitivity to antibiotics and weak immunogenic properties [1].

Mycoplasma hyopneumoniae is transmitted primarily via the droplet route with respiratory system excretions, by direct contact of infected individuals and carriers with healthy animals [2, 3]. Infection results in immunosuppression related to decrease in lymphocyte activity and inhibition of macrophage phagocytic activity. *Mycoplasma hyopneumoniae* has been proven to cause immunosuppression of non-specific cellular response and increase the activity of suppressor T lymphocytes [4]. An infection results in excessive production and secretion of proinflammatory cytokines – tumour necrosis factor α (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6) and PGF_2

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prostaglandin to bronchio-alveolar fluid. It also activates complement system and increases the concentration of some acute phase proteins in serum, which triggers bronchopneumonia-type inflammatory reaction [5, 6]. One of the major methods of limiting losses caused by respiratory system diseases in pigs, apart from complying with production management principles and providing the animals with proper environment and feeding, is conducting the specific immunoprophylaxis and immunomodulation, which ensure maintaining high efficiency of the immune system, homeostasis and protection from invasion by pathogens. The beneficial effects of different types of biopreparations (vaccines, immunostimulants, immunomodulators) on the immune system have been thoroughly studied by numerous researchers and applied to support therapy, vaccinations and treating infections caused by various pathogens [7-18].

The aim of this study was to determine the proliferative abilities of T and B lymphocytes treated with a vaccine against MPS and selected immunomodulators in *in vitro* examinations.

Materials and methods

Five clinically healthy piglets at the age of 21 days were used in the experiment. Four ml of blood from *vena cava cranialis* was taken from each piglet to a test tube with heparin in order to isolate lymphocytes to assess their proliferative activity with an MTT test.

The following were used in the experiment: inactivated vaccine against MPS – Respisure (Pfizer Inc. Animal Health Group); the vaccine contains chemically inactivated entire Mhp cells and an oil adjuvant – Amphigen; Methisoprinol (Polfa Grodzisk, Poland) – a synthetic compound made up of one particle of inosine and 3 particles of 1-(dimethylamino)-2-propanol 4-(acetylamino)benzoate, at a concentration of 0.1 mg/ml and Lydium-KLP (Nika Health Products Ltd, USA) – a drug substance: lysozyme dimer, a protein of natural origin with enzymatic activity and N-acetylmuramide glycanohydrolase at a concentration of 0.2 mg/ml. Klein and Kiczka [10] report that a lysozyme dimer with the symbol KLP-602 was used in pre-clinical tests and is the drug substance of the preparation named Lydium-KLP.

The test of mitogen-stimulated lymphocyte proliferation was performed by the colorimetric method (MTT) as described by Mosmann [19] and modified by Siwicki *et al.* [17]. Samples of peripheral blood were diluted at the ratio of 1 : 1 with the RPMI 1640 medium (Sigma). Lymphocytes were isolated in Histopaque 1077 gradient (Sigma). The isolated lymphocytes were suspended in the RPMI 1640 medium at concentration 5×10^6/ml of medium with an addition of 10% foetal calf serum (FCS, Sigma) and distributed to 96-well plates (NUNC) at 100 µl/well. 20 µl of Methisoprinol at the concentration of 0.1 mg/ml or Lydium-KLP at 0.2 mg/ml or RPMI 1640 was added to each well and subsequently, after 24 hours of incubation, 50 µl of Respisure vaccine was added at dilutions of 1 : 10, 1 : 100 or 1 : 1000 and the whole sample was filled up to 220 µl, adding 50 µl of mitogen: concanavalin A (Con A, Sigma) at the concentration of 6 µg/ml or lipopolysaccharide (LPS, Sigma) obtained from *Escherichia coli*, serotype 0111:B4, at the concentration of 20 µg/ml. Plates with a suspension of lymphocytes and immunomodulators were incubated for a further 48 hours at a temperature of 37°C (5% CO_2). In order to determine the proliferative activity of T and B cells in the presence of the Respisure vaccine alone, 50 µl of the preparation was added to each well at dilutions of 1 : 10, 1 : 100 or 1 : 1000 and, subsequently, 50 µl of mitogen Con A was added at a concentration of 6 µg/ml or LPS at concentration 20 µg/ml, and the whole sample was filled up to 220 µl with the RPMI medium and incubated for 48 hours at a temperature of 37°C (5% CO_2). RPMI 1640 in the amount of 100 µl/well was used as a control. Following the incubation, 10 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-5,2diphenyl tetrazolium bromide, Sigma) solution at the concentration of 5 mg/ml PBS was distributed to each well and incubated for 4 hours at a temperature of 37°C (5% CO_2). The plates were subsequently centrifuged for 5 minutes at 800 rpm, then the supernatant was removed and 100 µl of dimethylsulfoxide (DMSO, Polskie Odczynniki Chemiczne S.A.) was added to each well. After 10 minutes, the absorbance readout was performed in a MRX 1.1 microreader (Dynex) at a wavelength of 620 nm.

The results were analyzed statistically with the analysis of variance test for comparing multiple means (the NIR test) at $p < 0.05$ and the standard deviations were determined.

Results

The effect of combined application of immunomodulators Lydium-KLP or Methisoprinol, as well as various dilutions of the Respisure vaccine on the proliferative response of ConA-stimulated T lymphocytes and LPS-stimulated B lymphocytes in the blood of healthy piglets is shown in Table 1 and Figure 1.

In terms of proliferative response of T lymphocytes, a significantly lower ($p < 0.05$) response was observed when the Respisure vaccine was applied at the 1 : 10 dilution or when it was applied in combination with Lydium-KLP or Methisoprinol as compared to such combinations at the 1 : 100 and 1 : 1000 dilutions. The proliferative activity of T lymphocytes when the vaccine at the dilution of 1 : 1000 was administered in combination with Methisoprinol was also statistically higher ($p < 0.05$) as compared to the combination of Respisure at the dilution of 1 : 100 with Methisoprinol or Lydium-KLP.

The assessment of the proliferative response of B lymphocytes when Respisure was administered at the

Table 1. The influence of combined application of Lydium-KLP or Methisoprinol and Respisure vaccine at various dilutions on the levels of T-lymphocytes proliferative response stimulated by Con A and B-lymphocytes stimulated by LPS

		EXTINCTION (620 nm)									
GROUPS		**R** 1 : 10	**KLP+R** 1 : 10	**MET+R** 1 : 10	**R** 1 : 100	**KLP+R** 1 : 100	**MET+R** 1 : 100	**R** 1 : 1000	**KLP+R** 1 : 1000	**MET+R** 1 : 1000	**RPMI**
Con A	X	0.12^{Ab}	0.08^{AFa}	0.09^{AFa}	0.29^{E}	0.31^{BCb}	0.37^{Bbc}	0.25^{Ea}	0.45^{BFb}	0.58^{BDFbd}	0.15
	SD	0.04	0.01	0.01	0.05	0.11	0.11	0.04	0.15	0.14	0.01
LPS	X	0.14^{A}	0.09^{Aa}	0.09^{Aa}	0.21^{A}	0.41^{BCbc}	0.44^{BCbc}	0.20^{A}	0.83^{BDbd}	0.88^{BDbd}	0.14
	SD	0.06	0.01	0.01	0.04	0.07	0.09	0.02	0.24	0.07	0.03

R – Respisure, KLP – Lydium-KLP, MET – Methisoprinol, AB, CD, EF – differences between groups KLP+R, MET+R i R with $p < 0.05$
ab, cd – differences between dilutions within groups with $p < 0.05$

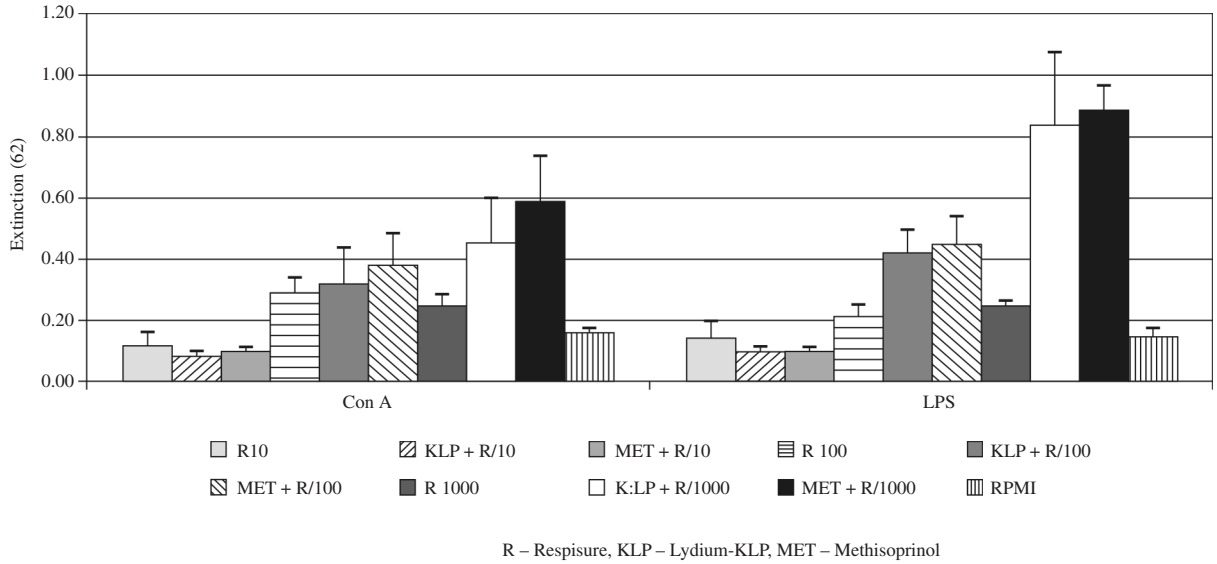

Fig. 1. Comparison of the proliferative response of T and B lymphocytes subjected to Respisure vaccine and Lydium-KLP or Methisoprinol activity

dilution of 1 : 10 or when it was administered in combination with Lydium-KLP or Methisoprinol showed their statistically lower ($p < 0.05$) activity as compared to such combinations of the vaccine at the dilutions of 1 : 100 and 1 : 1000. Significantly higher ($p < 0.05$) proliferative response of B lymphocytes was also observed after the vaccine at the dilution of 1 : 1000 was administered in combination with Lydium-KLP or Methisoprinol as compared to the combination of the Respisure vaccine at the dilution of 1 : 100 with Lydium-KLP or with Methisoprinol.

The study has shown a significantly higher ($p < 0.05$) proliferative response of B lymphocytes stimulated with LPS as compared to the proliferative response of T lymphocytes stimulated with ConA when the Respisure vaccine was used in combination with immunomodulators Lydium-KLP and Methisoprinol, both at the dilution of 1 : 100, and 1 : 1000. In addition, a stronger proliferative response of T and B lymphocytes was observed when the combination of Respisure and Methisoprinol was applied.

Discussion

An important role in the response of an organism to an infection is played by cellular immune mechanisms [20, 21]. A lesser role seems to be played by humoral response, both colostral [22, 23], and active. Markowska-Daniel and Glapiak [12] observed seroconversion in pigs not before 7 weeks after immunization with the Respisure vaccine, whereas Smith *et al*. [24] detected it in only 14% of the pigs immunized with the Respisure One vaccine, implying that the serological status has no particular influence on vaccine-

induced protection against Mhp infection. Hence, a study was initiated to perform *in vitro* assessment of the immunomodulating effect of selected biopreparations on isolated lymphocytes. Lysozyme dimer, successfully applied in animal therapy, was the first to be registered and its use has been authorized in Poland as a preparation named Lydium-KLP [9]. It has been shown not to have any toxic, cytotoxic or mutagenic effect, even after multiple therapeutic doses have been applied. In the body (in the oral cavity, in the eye, in neonates' alimentary tract, in chicken embryos) it is a factor of local anti-infection resistance which consists in enzymatic lysis of bacterial cells through cleavage of β-1,4 glycoside bonds between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan. It has been shown in pre-clinical *in vitro* studies of cells stimulated by Con A that, depending on its concentration, lysozyme dimer supports the production of IFN-α and modulates the synthesis and secretion of IL-2, IL-6 and TNF-α [10]. The mechanism of immunostimulating effect consists in stimulation of phagocytosis, activation of T and B lymphocytes, synthesis of immunoglobulins: IgG and IgM and secretion of cytokines [15, 18, 25, 26]. It was found that a single application of lysozyme dimer at 20 μg/kg of body weight in weaned piglets results in increased synthesis and secretion of IL-1, IL-2, TNF-α and IFN-α by mononuclear cells, isolated from the piglets' blood [26]. Isoprinosine (methisoprinol) is applied in therapy as an immunostimulator and a medicine to eliminate some viral infections. It has been shown in *in vitro* experiments to amplify the effect of mitogens (PHA, Con A) on T lymphocytes proliferation, as well as to stimulate humoral and cellular immunity, and non-specific defense processes. Isoprinosine activates macrophages, stimulates phagocytosis, enhances T lymphocytes maturation, stimulates the activity of CD4+ and CD8+ lymphocytes and NK cells towards infected cells [27-29]. Because of the regulating effect on the activity of helper and suppressor T lymphocytes, it indirectly affects the humoral response of the organism. It can also stimulate maturation of T lymphocytes and synthesis of specific antiviral antibodies [27].

Research conducted by the authors has shown the poor proliferative activity of T and B lymphocytes treated with the Respisure vaccine alone or treated simultaneously with the Respisure vaccine at the dilution of 1 : 10 and isoprinosine or lysozyme dimer. A considerable increase in the proliferative response was observed at a higher dilution of the vaccine (1 : 100), administered separately or in the presence of both immunomodulators. However, the strongest stimulating effect on T and B lymphocytes was observed in exposure to the vaccine at the dilution of 1 : 1000 together with isoprinosine, and slightly weaker following the application of lysozyme dimer. No differences were observed for the effect of the vaccine alone at the dilutions of 1 : 100 and 1 : 1000 on the proliferative activity of T and B lymphocytes. The results of *in vitro* experiments have clearly shown that the proliferative response of T and B lymphocytes stimulated with selected mitogens is closely dependent on the vaccine antigen. A significantly higher proliferative response was observed at the highest antigen dilution of 1 : 1000 than at the dilution of 1 : 10. The aim of the *in vitro* experiments was primarily to determine the effect of the vaccine antigen on the activity of T and B lymphocytes and to show whether various concentrations of the vaccine antigen may adversely affect the activity of T and B lymphocytes. The preliminary results of *in vitro* experiments indicate that excessively high doses of the vaccine antigen may adversely affect *in vitro* proliferative response of T and B lymphocytes. In view of the emphasized greater role of cellular as compared to humoral response, one may be puzzled by the results of the authors' experiment in which the proliferative response of LPS-stimulated B lymphocytes was significantly higher ($p < 0.05$) than the proliferative response of ConA-stimulated T lymphocytes when the Respisure vaccine at the dilutions of 1 : 100 and 1 : 1000 was administered in combination with immunomodulators Lydium-KLP and Methisoprinol. There are no reports in the available literature on the effect of the vaccine antigen applied together with immunomodulators on the activity of T and B lymphocytes. However, the positive effect of selected immunomodulators on the proliferation of T and B lymphocytes has been shown by other authors [10, 16, 17, 27, 28]. A study conducted by Siwicki *et al*. [17] showed that injection of lysozyme dimer (KLP-602) stimulated cellular and humoral mechanisms of immunity and provided protection against furunculosis in salmonids. Isoprinosine is known to amplify the effect of mitogens on T and B lymphocytes [8]. Siwicki and Mizak [16] carried out *in vitro* examination of the effect of methisoprinol at various concentrations on the proliferative response of ConA- and LPS-stimulated lymphocytes in dogs. They showed that methisoprinol at concentrations ranging from 1 to 50 μg/ml increased the proliferation of canine T and B lymphocytes, its effect being more positive in older dogs. Isoprinosine also shows affinity to macrophages, enhancing their proliferation and phagocytic properties [30]. It also stimulates the synthesis of immunoglobulins and the activity of interferon [11]. It should be stressed for lysozyme dimer that the experiments which have revealed its positive effect have been mainly carried out in *in vivo* conditions, as it is very difficult to mimic *in vitro* conditions of its activity in a live organism [9].

To conclude the research, it may be claimed that combinations of immunomodulators Lydium-KLP or Methisoprinol, and the Respisure vaccine at dilutions of 1 : 100 and 1 : 1000 enhance *in vitro* proliferative activity of ConA-stimulated T lymphocytes and LPS-stimulated B lymphocytes, with the proliferative activity of both types of lymphocytes being stronger for the combination of Respisure and Methisoprinol.

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