VETERINARSKI ARHIV 82 (6), 617-627, 2012

Muramyl dipeptide, a stimulator of nonspecific immunity, induces the production of nitric oxide by Rainbow trout (*Oncorhynchus mykiss)* spleen and head kidney cells *in vitro*

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ŽUNIĆ ZVIZDIĆ, M., E. LICEK, C. LAM: Muramyl dipeptide, a stimulator of nonspecific immunity, induces the production of nitric oxide by Rainbow trout (*Oncorhynchus mykiss)* spleen and head kidney cells *in vitro*. Vet. arhiv 82, 617-627, 2012.

ABSTRACT

Immunoenhancing activity of synthetic muramyl dipeptide (MDP) was investigated in relation to the activation of rainbow trout spleen and head kidney T cells and macrophages. Cells cultured with classical T-cell mitogens exhibited significantly higher proliferation rates as compared to those of unstimulated cells. MDP, lipopolysaccharide (LPS) or MDP plus LPS did not stimulate cells to proliferate. In an attempt to show that MDP mediates function of macrophages, we measured the amounts of nitrites, an index of activation of macrophages and nitric oxide (NO) production, in culture supernatants. Incubation of the cells with either LPS, MDP, IFN-γ and their combinations, resulted in significant and time-dependent accumulation of nitrites in cultures. Taken together, our findings show that MDP is not mitogenic to the fish immune cells, but exerts its effect on macrophages through induction of nitric oxide synthesis. Monitoring of the production of NO can be a novel approach for the measurement of nonspecific immunity in fish.

Key words: macrophages, immunomodulators, muramyl dipeptide, lipopolysaccharide, nitric oxide, fish

Introduction

The contribution of aquaculture in rearing many marine and freshwater fish species is steadily increasing and constitutes an important food producing industry. But intensive rearing of fish in aquaculture is prone to high risks of infectious diseases caused by bacteria, virus, parasites or fungi. Such infectious diseases cause heavy losses in the

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ISSN 0372-5480
Printed in Croatia

production of fish worldwide (PRATHEEPA and SUKUMARAN, 2011). While antibiotics and other chemotherapeutic agents have been successfully used for the treatment or metaphylaxis in intensive aquaculture, there is overwhelming evidence that indiscriminate use of antibiotics has negative impacts including residual accumulation of the agents in fish tissue, development of drug resistance and suppression of the immune system (ANONYM., 2002). Understandably, many environmentally conscious consumers have low preferences for food prepared with fish reared in antibiotic-treated environment. The problematic relationship between aquaculture and use of antibiotics has led many experts to focus their attention on alternative approaches to prevent infectious diseases. The application of biological response modifiers represents an alternative that may be cost-effective in the rearing of delicate fish. Enhancing immunity is important to health as it provides a building block of defence mechanisms and protection against diseases. Several promising biological response modifiers have been tested in fish *in vivo* and *in vitro*. Extensive studies in a variety of fish species (ANDERSON, 1992) indicate that administration of agents such as chitosan, β-glucan, muramyl dipeptide, trace mineral and vitamin or their combinations as well as various products derived from plants and animals, are effective in stimulating or modulating both specific and non-specific defence mechanisms, and offer protection against viral and bacterial diseases in fish (LIU et al., 2011; BEHERA et al., 2011; CUESTA et al., 2002; KODAMA et al., 1993; MULERO et al., 1998; SIWICKI et al., 1994; SIWICKI et al., 2001). These agents can be added to the water in which the fish bathe (ZHANG et al., 2009). Bath administration is considered an ideal delivery route for mass manipulation since there is no need for individual handling. However, a controversy exists over the use of these substances, as in a few cases they have failed to render enhanced protection or to enhance immunity. In fish, immunoenhancing activity of the MDP was investigated in relation to the activation of rainbow trout phagocytes and to nonspecific protection against experimental infection with *Aeromonas salmonicida* through stimulating the production of phagocyte-activating factor (KODAMA et al., 1993). These findings have generated interest to learn the mechanisms by which MDP and its non-toxic derivatives exert their effects in enhancing specific and/or nonspecific immunity and for use in aquaculture. The present investigation was undertaken to investigate the immune enhancing effects of MDP in rainbow trout. For these purposes, the activation of lymphocytes and macrophages from rainbow trout (*Oncorhynchus mykiss*) by MDP were investigated using *in vitro* models.

Materials and methods

Animals. Rainbow trout (*Oncorhynchus mykiss*) were obtained from a hatchery in Upper Austria which is known to be free from infectious fish diseases. The mean weight and length of the trout was 25 g and 13 cm respectively. The fishes were held in water running

aquarium (±11 °C) and acclimatized to this environment for 3 weeks prior to use. The trout were fed with a daily ration of commercial pellets as recommended by supplier (Garant).

Cell culture reagents, medium and chemicals. N-acetymuramyl-L-alanyl-D-isoglutamine or MDP, penicillin, streptomycin, L-glutamine, 2-Mercaptoethanol, lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5, Phytohaemagglutinin (PHA), Concanavalin A (ConA), Pokeweed mitogen (PWM) were purchased from Sigma, St. Louis, MO, USA. MDP was dissolved in RPMI 1640 (Gibco; Life Technologies, Ltd., Paisley, Scotland, UK) supplemented with 5% or 10% heat-inactivated fetal calf serum (FCS, Gibco, Life Technologies, Paisley, UK). Recombinant mouse IFN-γ was obtained from Genzyme (Boston, MA, USA). RPMI 1640 was supplemented with 5 or 10% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), 2 mM L-glutamine and 5 $\times 10^{-5}$ 2-mercaptoethanol.

Cell cultures. Five rainbow trouts per group were used. Fish were anaesthetised with MS222, 132 mg/L for 3 min. (Thomson and Joseph Ltd, UK). Head kidneys and spleens were removed under sterile conditions, placed into ice-cold medium and teased into single cell suspensions. After allowing the big particles to sediment, the supernatants were collected. Mononuclear cells were purified from erythrocytes by using a high gradient Lymphoprep (Nycomed, Norway) and protocol described by the supplier.

Lymphocyte proliferation test (LPT). The cells (3×10^5 cells/well) were cultured with or without PHA (1.25 µg/mL), ConA (5 µg/mL), LPS (2.5-10 µg/mL) and MDP (10-100 µg/mL) in quadruplicate at 18 °C, 5% CO_2, 80% in air humidified atmosphere for 5 days. Proliferation was quantitated by pulsing the cell cultures for the final 18 hours of the incubation with (^{3}H)-Thymidine (1 µCi/well; Amersham, Bucks, UK). At the end of incubation period, the cells were harvested onto glass fibre filters with a cell harvester. Radioactivity of the cells was measured in a liquid scintillator ß-counter.

Nitrite determination. The cells were washed twice with RPMI 5% FCS and 2 $\times 10^6$ cells per 2 mL were cultured in 24-well plates (Nunc) with or without LPS (10 ng/mL), rmIFN-γ (10 U/mL) and MDP (1-100 µg/mL) at 18 °C, 5% CO_2, 80% in air humidified atmosphere for 24, 72 and 120 hrs. The accumulation of nitrite (NO_2^-), a stabile metabolite of NO, in cell culture supernatants was assayed by the standard Griess assay. Briefly, 50 µL aliquots of culture supernatants were reacted with 100 µl amounts each of 1% sulphanilamide and 0.1% of N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid in 96-well tissue culture plates and incubated for 10 min at room temperature. The absorbance at 540 nm was measured in an automated ELISA reader. Nitrite was quantitated by comparison with a $NaNO_2$ standard curve. Results are expressed as µM $NO_2^-/10^6$ cells/24 hrs or 120 hrs.

Statistical analysis. LPT results were compared by using multiple paired Student' *t*-test (after log. transformation of the values). P-values were adjusted according to Hochberg/Bonferroni.

Nitrite data were evaluated using ANOVA-Dunnet Post-test. P-values were adjusted according to Bonferroni. Results were expressed as mean ± standard deviation.

Results

Cells proliferated to T-cell mitogens, but not to MDP and LPS. To determine the effect of MDP on the lymphoproliferative activity of cells, spleen and head kidney were harvested from the rainbow trout and LPT of immune cells to MDP and various mitogens were assessed (Table 1). The proliferative responses of the spleen cells to PHA, ConA and PWM were significantly increased as compared to the control groups ($P<0.05$). Head kidney cells significantly proliferated after stimulation with PWM and ConA. In contrast, when stimulated with MDP or LPS, spleen and head kidney cells did not have higher proliferation in comparison to control cells.

Table 1. Proliferative response of the immune cells upon stimulation with mitogens and MDP

Antigen stimulation (μg/mL)	Organ, cpm †	
	Spleen	Head kidney
RPMI	2058 ± 241	1515 ± 340
PHA (2.5)	14296 ± 1200**	5550 ± 849
ConA (5)	7540 ± 1500**	8136 ± 568**
PWM (5)	14640 ± 1167**	6071 ± 1165**
LPS (2.5)	3147 ± 224	2033 ± 350
LPS (10)	3703 ± 458	2199 ± 250
MDP (10)	1912 ± 154	1928 ± 401
MDP (100)	1071 ± 345	1523 ± 386

† LPT of the rainbow trout spleen and head kidney cells incubated with or without PHA, ConA, PWM, LPS and MDP (10-100 μg/mL) for 5 days. Proliferation data (cpm) are representative of mean ± standard deviation, as determined by multiple paired Student'*t*-test for three independent experiments. P-values were adjusted according to Hochberg/Bonferroni. ** statistical significance P>0.001 - 0.01; cpm = counts per minute.

MDP, LPS and IFN-γ stimulated macrophages to produce nitrite, a stable metabolite of NO, accumulation in culture supernatants. Expression and activity of inducible nitric oxide (iNOS) were monitored by estimating nitrite, the stable end-product of NO production. The presence of nitrite was detected in the supernatants of the unstimulated spleen and head kidney cells already at 24 hours of culture.

Incubation of the spleen cells with MDP (1-100 μg/mL), LPS (10 ng/mL) or IFN-γ (10 U/mL) induced high levels of nitrite in culture supernatants after 72-120 hrs when

compared to 24 hrs MDP (1-100 μg/mL) (Table 2). Co-culture of the spleen cells with both LPS and MDP resulted in synergism of nitrite generation after 72 and 120 hrs. Synthesis of NO was significantly higher at the concentration of 100 μg/mL MDP. In contrast, MDP did not synergize with IFN-γ in the production of nitrites.

Table 2. MDP, LPS and IFN-γ induced nitrite accumulation in spleen cells

A

	Nitrite, μM at †		
Modulating agent (μg/mL)	24 hrs	72 hrs	120 hrs
Control, vehicle	1.5 ± 0.7	2.5 ± 0.7	3 ± 0
MDP (1)	1.5 ± 0.7	1 ± 0	5 ± 0**
MDP (10)	2 ± 0	1.5 ± 0.7	1 ± 0
MDP (100)	3.5 ± 0.7	4 ± 0	8.5 ± 0.7***

B

	Nitrite, μM at †		
Modulating agent (μg/mL)	24 hrs	72 hrs	120 hrs
LPS	2 ± 0	2 ± 0	5.5 ± 0.7
LPS + MDP (1)	0.5 ± 0.7	4 ± 1	4 ± 0
LPS + MDP (10)	2 ± 1.4	4 ± 1.4	5.5 ± 0.7
LPS + MDP (100)	1.5 ± 0.7	9.5 ± 2.1***	15 ± 2.8***

C

	Nitrite, μM at †		
Modulating agent (μg/mL)	24 hrs	72 hrs	120 hrs
IFN-γ	1.5 ± 0.7	3.5 ± 0.7	5.5 ± 0.7
IFN-γ + MDP (1)	0.5 ± 0.7	2 ± 0	3 ± 0
IFN-γ + MDP (10)	1.5 ± 0.7	4.5 ± 2.1	6.5 ± 0.7
IFN-γ + MDP (100)	1.5 ± 0.7	4.5 ± 0.7	7.5 ± 0.7

† Rainbow trout spleen cells were incubated with or without LPS, IFN-γ and MDP (1-100 μg/mL). After 24, 72 and 120 hrs of incubation, supernatants were used to estimate nitrite accumulation by the Griess method. Nitrite data are representative of mean ± standard deviation, as determined by ANOVA-Dunnet Post-test for two independent experiments. P-values were adjusted according to Bonferroni. ** statistical significance P>0.01 - 0.001; *** statistical significance P<0.001.

Incubation of the head kidney cells with LPS or IFN-γ resulted in accumulation of high levels of nitrites in cultures measured first at 72 and 120 hrs (Tables 3 B, C). In addition, co-culture of the cells with MDP (10 μg/mL) resulted in the significant generation of nitrite in supernatants seen already at 24 hrs of incubation (Table 3 A). MDP at 100 μg/mL synergize with IFN-γ in the production of nitrites at 120 hrs (Table 3 C). A similar synergistic effect of MDP and LPS on the release of nitrites from head kidney cells was also observed at the concentration of 1-100 μg/mL, but only after 120 hrs of co-culture.

Table 3. MDP, LPS and IFN-γ induced nitrite accumulation in head kidney cells

A

	Nitrite, μM at †		
Modulating agent (μg/mL)	24 hrs	72 hrs	120 hrs
Control, vehicle	2 ± 0	2 ± 0.5	2 ± 0.8
MDP (1)	3 ± 0.5	3 ± 0.5	4 ± 1.5
MDP (10)	4 ± 0.8*	3 ± 0	3 ± 0.4
MDP (100)	1 ± 0	3 ± 1	7 ± 2.4*

B

	Nitrite, μM at †		
Modulating agent (μg/mL)	24 hrs	72 hrs	120 hrs
LPS	2 ± 0.7	4 ± 2.2	5 ± 2.5
LPS + MDP (1)	2 ± 0	4 ± 1.8	7 ± 2
LPS + MDP (10)	2 ± 0.5	3 ± 1.5	6 ± 1.8
LPS + MDP (100)	3 ± 0	3 ± 0.5	8 ± 2.2

C

	Nitrite, μM at †		
Modulating agent (μg/mL)	24 hrs	72 hrs	120 hrs
IFN-γ	2 ± 0	5 ± 2.4	7 ± 2
IFN-γ + MDP (1)	3 ± 0.5	3 ± 1.2	6 ± 2.2
IFN-γ + MDP (10)	3 ± 0.8	6 ± 1	5 ± 0.5
IFN-γ + MDP (100)	3 ± 1	4 ± 0.5	9 ± 2.5

† Rainbow trout head kidney cells were incubated with or without LPS, IFN-γ and MDP (1-100 μg/mL). After 24, 72 and 120 hrs of incubation, supernatants were used to estimate nitrite accumulation by the Griess method. Nitrite data are representative of mean ± standard deviation, as determined by ANOVA-Dunnet Post-test for two independent experiments. P-values were adjusted according to Bonferroni. *statistical significance P>0.01 - 0.05.

Discussion

Aquaculture represents one of the fast growing food producing sectors of the world and aims to increase productivity per unit space. The economic loss due to the diseases outbreak in the aquaculture sector can be considerable. Among various kinds of cultivated organisms, many marine and freshwater finfish and shellfish species constitute an important industry with their production increasing every year. Because of intensive farming practices, infectious diseases pose a major problem in aquaculture industry, causing heavy losses to farmers.

In order to address this problem, fish culture is under pressure to decrease the use of synthetic antibiotics and chemotherapeutics because of the risk caused to humans by chemical residues in food and by antibiotic resistance being passed on to human pathogens. Consequently, efforts are being made to exploit plants, plant extracts or natural plant compounds as potential alternatives to synthetic chemicals for the stimulation of immune responses and disease resistance in fish. As the results of this investigation show here that co-culture of fish immune cells with MDP and LPS did not stimulate spleen and head kidney lymphocytes measured by proliferation compared to the unstimulated or mitogen-stimulated control cells.

In contrast, MDP specifically activated macrophages to induce iNOS and therefore to generate nitrite in culture supernatants. Although the produced nitrites were lower than in the mouse model (ZUNIC et al., 1998), the produced NO activity was readily detectable by the Griess assay. Unlike in mouse model, unstimulated fish macrophages produced nitrite at detectable amounts too. Interestingly, the water soluble MDP derivative was by itself capable of activating the macrophages. The proof for its activating capability was provided by the synergy observed between MDP and IFN-γ or LPS. The observed effects were greater than additive effects of the two immunostimulants (see Table 2) The mechanisms for the synergy are unclear but will be addressed in an-ongoing study.

Earlier work from DRAPIER et al. (1988) showed that the MDP molecule synergized with IFN-γ in the activation of macrophages to produce NO in a manner that was partially inhibitable by anti-TNF-α antibody. It was proposed that the TNF-α produced acted in a feedback loop to activate IFN-γ-primed cells so as to increase the amounts of NO. It is therefore possible that also in a teleost MDP has the ability to synergize with IFN-γ in the induction of TNF-α and other factors required for the synergy in the macrophages from head kidney. On the other hand, it could be that upon stimulation with exogenous IFN-γ and MDP, the high amount of produced NO inhibited the NO synthase activity as reported with other NO-donors (MORIN et al., 1994). Identification of the factors responsible for the synergy must await further study. It is, however, clear that LPS and IFN-γ act through totally separate receptors and therefore induce the transcription of different genes (WRIGHT et al., 1990; WRIGHT et al., 1994; WANG et al., 2001). Our findings that MDP synergized LPS

induced effects in fish is consistent with a number of reports in the literature showing that MDP molecule can synergize with LPS in the activation of mammalian macrophages (PALACIOS at al., 1992; SHI et al., 1995; VERMEULEN et al., 1987). For example, PALACIOS et al. (1992) showed that MDP activated murine lung and thioglycollate-elicited peritoneal macrophages to produce mediators of nonspecific immunity.

In the presence of LPS, however, the production of mediators was significantly upregulated, suggesting that there was synergism between LPS and the MDP molecule in the activation of the cells. Similarly, stimulation of canine monocytes with MDP and LPS resulted in synergistic activation of cells as measured by IL-6 production (SHI et al., 1995). A similar picture was seen in human monocytes where MDP was found to synergize with LPS in the induction of mRNA accumulation of IL-1 (VERMEULEN et al., 1987). Thus the mechanism of the interactions between MDP and LPS could be mediated at least in part, at the level of cytokine gene transcription. Moreover, expression of cytokines is regulated by common transcription factor NF- κB which is regulated by TNF-α and IL-1 (KRASNOW et al., 1991).

In our study, MDP up-regulated LPS-induced NO suggesting that MDP exerts its activity through up-regulation of common transcription factor. Thus, it is possible to attribute the biological activities of muramyl peptides in teleosts to differential effects on cytokine gene transcription and activation of cellular transcription factors as observed in mammals. In this report, MDP exerted *in vitro* the enhancement of a NO-dependent pathway. This pathway has been shown to be an important event in the release of the cytokines by mammalian cells *in vitro* and *in vivo*. Indeed, NO was shown to directly induce the *in vitro* release of cytokines, such as TNF-α, IL-6, IL-8 (EIGLER et al., 1993; BECHEREL et al., 1994; ANDREW et al., 1995; ZINETTI et al., 1995) and to synergize with the released cytokines in the induction of other mediators of nonspecific immunity. Cytokines such as fish T-cell-derived "gamma interferon" and human TNF-α, synergize in elevating the NO synthase pathways of trout macrophages, which leads to production of bactericidal NO. In addition, numerous authors reported inhibition of macrophages proliferation by MDP (KUTSUKAKE et al., 1990; NAGAO and TANAKA, 1983) and NO (CHENAIS and TENU, 1994; HUOT et al., 1993). It does not therefore come as a surprise that in our experiments enhancement of NO production by MDP was associated with a marked anti-proliferative effect. But, the potency of different bacteria-derived constituents in their capacity to induce nitrite production by fish macrophages has not been investigated. NEUMANN et al. (1995) showed that LPS-stimulated goldfish kidney macrophage cell-line induced low levels of NO, but at the very high dose of 30 μg/mL. Therefore, the key finding of this report that head kidney and spleen macrophages are able to produce NO upon stimulation with immunomodulator is of importance not

only for the study of the evolution of nonspecific immune system, but also for targeting the important immune mediators in developing the fish vaccines. It is known that NO acts as a cytotoxic compound in bacterial and viral infections. In addition, KODAMA et al. (1993) showed that MDP-activated rainbow trout cells produced phagocyte-activating factor and injection of MDP to fish provided protection against challenge with virulent *A. salmonicida*. Therefore, through its enhancement of non-specific immunity, there is a potential therapeutical application of non-pyrogenic and non-toxic analogs of MDP in the prophylaxis or treatment of fish diseases.

Acknowledgements

We would like to gratefully acknowledge Marijke Nefzger for her helpful advice and discussions.

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Received: 6 February 2012
Accepted: 27 June 2012

ŽUNIĆ ZVIZDIĆ, M., E. LICEK, C. LAM: Muramil dipeptid, stimulator nespecifične imunosti, potiče tvorbu dušikova oksida u stanicama jetre i nadbubrežne žlijezde pastrve (*Oncorhynchus mykiss*) *in vitro*. Vet. arhiv 82, 617-627, 2012.

SAŽETAK

Povećanje imunosne aktivnosti sintetičkim muramil dipeptidom istraženo je s obzirom na aktivaciju T limfocita i makrofaga jetre i prednjeg bubrega kalifornijske pastrve. Stanice uzgajane s klasičnim T-staničnim mitogenima pokazuju značajno veću proliferaciju u odnosu na nestimulirane stanice. Muramil dipeptid (MDP), lipopolisaharid (LPS) ili MDP u kombinaciji s LPS nisu potaknuli proliferaciju stanica. Utjecaj MDP na funkciju makrofaga dokazan je kroz mjerenja proizvedenog dušika u nadtalogu staničnih kultura kao pokazatelja aktivacije makrofaga i proizvodnje dušikova oksida. Inkubacija stanica s LPS, MDP, IFN-γ ili njihovih kombinacija rezultirala je u značajnom i vremenski ovisnom nakupljanju dušika u staničnim kulturama. Rezultati pokazuju da MDP nije mitogen za riblje imunološke stanice, ali djeluje na makrofage putem aktivacije sinteze dušikova oksida. Praćenje proizvodnje dušikova oksida može biti novi pristup u mjerenju nespecifične imunosti riba.

Ključne riječi: makrofagi, imunomodulatori, muramil dipeptid, lipopolisaharid, dušikov oksid, ribe