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Original article

Influence of HMB (β-hydroxy-β-methylbutyrate) on antibody secreting cells (ASC) after in vitro and in vivo immunization with the anti-*Yersinia ruckeri* vaccine of rainbow trout (*Oncorhynchus mykiss*)

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Abstract – In practice, protection of fish against disease by immunization is of limited effectiveness. Therefore, research is concentrated on how to improve the potency and efficacy of vaccines and how to optimally activate the cell-mediated immunity and the specific antibody response. In the present study, the influence of HMB (β-Hydroxy-β-methylbutyrate) on the antibody secreting cells (ASC) after both in vitro and in vivo immunization of rainbow trout (*Oncorhynchus mykiss*) with the anti-Yersiniose vaccine was studied. For in vitro immunization, the spleens from 160 fish were sampled and placed each in 35 mm sterile wells with medium containing HMB at concentrations of 0, 0.1, 1, 5, 10, 25, 50 or 100 µg/mL of medium. The spleens from 80 fish were injected with the vaccine and incubated at 14 °C for 10 days. For the in vivo study, fish were fed pellets containing HMB at doses of 0, 10, 25 and 50 mg/kg bw per day. After 2 weeks of HMB supplementation, the fish were immunized by intraperitoneal injection of the vaccine. At 7, 14, 18, 21, 28 and 35 days after immunization, pronephros were taken from 10 fish in each group for testing. When analyzed by the ELISPOT assay, HMB increased the number of splenic ASC after in vitro immunization at concentrations between 10 and 100 µg/mL ($P < 0.05$). Dietary HMB also increased the number of total and specific ASC when the fish were vaccinated in vivo. In conclusion, the results of the present study showed that HMB increases the levels of specific ASC after both in vitro and in vivo immunization of rainbow trout with the anti-*Yersinia ruckeri* vaccine.

HMB / rainbow trout / anti-Yersiniose vaccine / antibody secreting cells

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Résumé – Influence du HMB sur les cellules productrices d'anticorps après vaccination in vitro et in vivo de la truite arc-en-ciel (*Oncorhynchus mykiss*) avec le vaccin Anti-Yersiniose. En pisciculture, la vaccination contre les maladies infectieuses ne présente qu'une efficacité limitée. En conséquence, la recherche est orientée vers les moyens d'augmenter l'efficacité des vaccins et de stimuler l'immunité cellulaire et la réponse en anticorps. La présente étude démontre l'influence du β-hydroxy-β-méthylbutyrate (HMB) sur les cellules productrices d'anticorps après immunisation in vitro et in vivo de la truite arc-en-ciel (*Oncorhynchus mykiss*) avec le vaccin « Anti-Yersiniose ». Pour l'étude in vitro, les rates de 160 poissons ont été prélevées et incubées dans du milieu contenant du HMB aux concentrations 0, 0,1, 1, 5, 10, 25, 50 ou 100 µg/mL de milieu. Les rates de 80 poissons ont été injectées avec le vaccin et incubées à 14 °C pendant 10 jours. Pour l'étude in vivo, des poissons (40-50 g) ont été nourris avec des granulés supplémentés en HMB à raison de 0, 10, 25 et 50 mg/kg de poisson par jour. Au bout de 2 semaines de supplémentation, les poissons ont été vaccinés par injection intra-péritonéale de vaccin. Aux jours 7, 14, 18, 21, 28 et 35 après vaccination, les pronephros ont été prélevés sur 10 poissons par groupe. In vitro, l'analyse ELISPOT a démontré que HMB augmentait le nombre de cellules produisant les anticorps spécifiques aux concentrations comprises entre 10 et 100 µg/µL ($P < 0,005$). In vivo, HMB distribué dans l'aliment a augmenté le nombre des cellules productrices d'anticorps spécifiques et non spécifiques après vaccination. En conclusion, in vitro et in vivo, HMB associé à la vaccination augmente, chez la truite arc-en-ciel, le nombre de cellules produisant les anticorps spécifiques.

HMB / truite arc-en-ciel / vaccin Anti-Yersiniose / cellules productrices d'anticorps

1. INTRODUCTION

The stimulation of specific protection of fish against infectious diseases by immunization has been developed with limited success, since some immunization techniques when actually applied to hatchery conditions are not as effective as they should be [1, 4]. One of the most frequent uncertainties regarding the use of vaccines is the effective protection over a long time. The immunization techniques by injection or immersion initiate a manipulation stress and consequently negative metabolic effects. In response to polyetiological stress, the adrenal gland is stimulated to release the hormone cortisol, which has a wide variety of effects, including a decrease in the nonspecific defense mechanisms and suppression of the specific immune response. The application of immunostimulants for the activation of the effectiveness of vaccines is a promising new development in aquaculture. Immunostimulants activate nonspecific defense mechanisms and specific immune responses if they are administered before, with, or after vaccines [1, 2, 7, 8, 17, 20, 21].

β-Hydroxy-β-methylbutyrate (HMB) is a breakdown product of the amino acid leucine. Leucine is found in all dietary proteins and is an essential building block of protein in all tissues. Scientists have proposed that under stressful situations both animals and humans cannot eat or synthesize enough HMB in the body to meet the needs of the tissues [9–16]. The hypothesis that HMB could be an effective supplement in maximizing muscle growth and preventing muscle loss during stressful situations was first tested in animals. Several feeding studies with HMB have shown that this natural product may be able to help animals to survive environmental stresses and disease challenges [5, 6, 12, 14, 22].

In the present study, we determined the influence of HMB on the antibody secreting cells (ASC) after in vitro and in vivo immunization of rainbow trout (*Oncorhynchus mykiss*) with the anti-Yersiniose vaccine.

2. MATERIALS AND METHODS

2.1. Animals and immunomodulator

For the in vitro study, 160 healthy rainbow trout (*Oncorhynchus mykiss*) weighing 180-200 g were used, and for the in vivo experimental study, 600 healthy fish, weighing 40-50 g, were used. They were purchased from the Inland Fisheries Institute in Olsztyn (Poland).

β-Hydroxy-β-methylbutyrate came from Metabolic Technologies (Ames, IA, USA). The subjects received the supplement as the calcium salt (monohydrate) of HMB (Ca-HMB) feed grade (purity > 98%)

2.2. Experimental design

2.2.1. In vitro immunization

Spleens from 160 healthy rainbow trout were sampled and each one was placed in a 35 mm sterile well (six wells, Costar Cambridge, USA) containing 10 mL medium (Leibovitz-15, MBH Linz, Austria) with 2% foetal calf serum (FCS, Gibco Peisley, UK) and different concentrations of HMB: 0, 0.1, 1, 5, 10, 25, 50 and 100 μg/mL of medium, and injected with vaccine after 1 hour, according to the method previously described by Siwicki et al. [18]. For each concentration, spleens from 10 healthy fish were used. The spleens were each injected with 50 μL of anti-Yersiniose vaccine (Sanofi Libourne, France) at 1×10^6 bacteria mL^{-1} and incubated at 14 °C. Control spleens (10 organs for each concentration of HMB) were injected with phosphate buffered saline (PBS). The medium in each well was changed every day by removing 5 mL of old medium and replacing it with 5 mL of new medium with the appropriate concentrations of HMB. Spleens were sampled on day 10 after immunization and the cells were isolated by the technique presented by Siwicki and Dunier [19].

2.2.2. In vivo immunization

Healthy rainbow trout (*Oncorhynchus mykiss*), weighing 40-50 g were used in this experimental study. The fish were divided into four tanks of 150 fish each (500 L tanks with a recirculation system of water), at a temperature of 14 ± 1 °C. Fish were fed daily with pellets (45% protein, 1% body weight) containing HMB at doses of 0, 10, 25 and 50 mg/kg body weight/day, prepared by protocol used in Inland Fisheries Institute (Poland) for rainbow trout. After 2 weeks of HMB application, 75 fish from each group were immunized by intraperitoneal injection of 0.2 mL of the anti-Yersiniose vaccine (Sanofi Libourne, France) at 1×10^8 bacteria mL^{-1}. The control fish from each group (75 fish) were injected intraperitoneally with PBS and divided into four tanks (500 L with a similar recirculation system of water). The pronephros was collected from 10 fish of each group on days 7, 14, 18, 21, 28 and 35 after immunization.

2.3. Assay procedures

2.3.1. Isolation of leucocytes

A similar protocol for the purification of leucocytes after in vivo and in vitro immunization was used. Spleen (after in vitro immunization) or pronephros from vaccinated and non-vaccinated fish were removed and single cell suspensions were obtained by teasing the tissues in medium through a steel mesh. Cell suspensions were purified on a Gradisol L (density 1.077; Polfa Kutno, Poland) gradient. Counts of living cells from pronephros or spleen were made with trypan blue using a haemocytometer after three washings in the medium (Leibovitz-15).

2.3.2. Immunological assays

The ELISPOT assays for the quantification of total immunoglobulin secreting cells

(ISC) and specific antibody secreting cells (ASC) after in vivo and of ASC after in vitro immunization were used, according to the protocol presented by Siwicki and Dunier [18].

For the non-specific ELISPOT assay, multiscreen-HA 96 well filtration plates of cellulose esters (0.45 μm, Millipore, Bedford, MA, USA) were coated with 100 μL of the monoclonal anti-trout Ig (1-14) [3] diluted to 1:100 and incubated overnight at 4 °C. After three washings with PBS, the plates were incubated with 200 μL L15 + 5% FCS for 1 h at 37 °C to block the remaining binding sites.

After removal of the blocking medium, 100 μL of the cell suspension was added to each well at 1×10^6 cells per well. The cells were incubated for 6 h at room temperature (22 °C). A control was carried out for each cell type (from spleen or pronephros) in which the first antibody (monoclonal 1-14) was omitted. After incubation, the plates were washed three times in PBS and eight times in PBS + 0.05 Tween 20, then 100 μL peroxidase labeled goat anti-trout Ig (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), diluted to 1:2000 in PBS-Tween + 1% FCS, was added to each well and incubated overnight at 4 °C. After three washings in PBS, 100 μL TMB Membrane Enhancer diluted to 1:10 in the peroxidase substrate and the B peroxidase solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added to each well. The plates were incubated for 15 min before washing in tap water and drying. The blue spots were counted using a microscope at low magnification and the results were expressed as spot-forming cells per 10^6 leucocytes from pronephros. The assays were done in quadruplicate for each fish.

For the specific ELISPOT assay, the multiscreen-HA plates were coated with 100 μL of 1×10^8 *Yersinia ruckeri* bacteria in PBS and incubated overnight at 4 °C. After three washings in PBS the previously described methodology for the non-specific ELISPOT assay was applied, using 1×10^6 cells per well for pronephros of vaccinated or non-vaccinated fish.

2.4. Statistical analysis

For statistical analysis, the means and standard deviations (SD) for all test values were calculated, and the Student's t-test was used to determine differences between two groups (control and experimental group with HMB). The significance level used was $P < 0.05$.

3. RESULTS

The in vitro study of the influence of different concentrations of HMB on the immune response analyzed by enumeration of specific antibody secreting cells (ASC) of immunized and non-immunized spleens are presented in Figure 1. HMB concentrations between 10 and 100 μg/mL of medium significantly ($P < 0.05$) increased the number of splenic ASC compared to the control without HMB. The highest number of ASC was observed with a concentration of 50 μg/mL HMB.

The in vivo effects of HMB applicated per os, at doses of 10, 25 and 50 mg/kg body weight, on the kinetics of total ISC are presented in Figure 2, and those on the kinetics of specific ASC are presented in Figure 3. The results showed that HMB applicated to food two weeks before vaccination, at doses between 10 and 50 mg/kg b.w., statistically increased ($P < 0.05$) the total Ig and specific antibody secreting cells after in vivo immunization, compared to the HMB-free group of fish. The numbers of total Ig and specific antibody secreting cells increased rapidly and the highest levels were observed in each experimental group of fish between 18 and 21 days after vaccination.

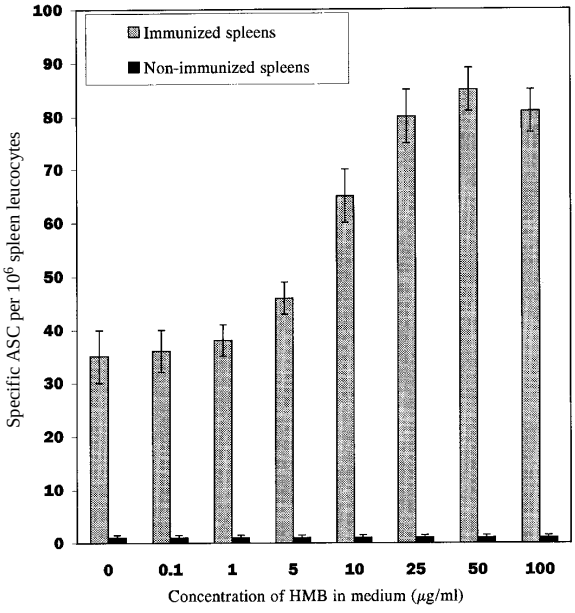

Figure 1. The in vitro influence of different concentrations of HMB on the number of specific antibody secreting cells (ASC) from spleens immunized with the anti-Yersiniose vaccine and from non-immunized spleens (10 days after injection); (mean ± SD; n = 10).

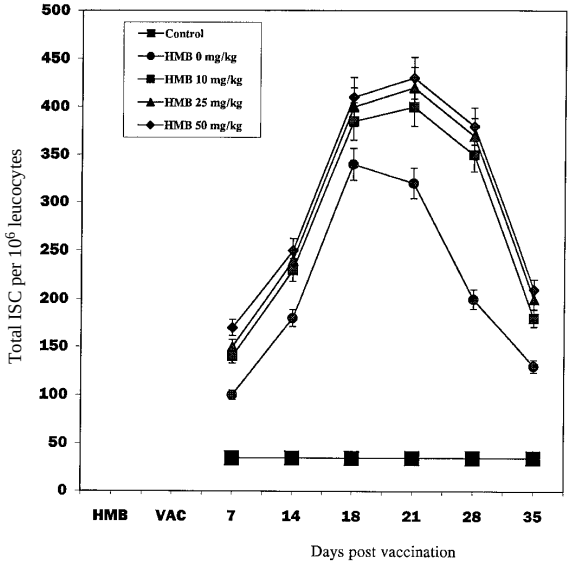

Figure 2. The influence of different doses of HMB on the kinetics of the numbers of total Ig secreting cells (ISC) per 10^6 pronephros leucocytes from rainbow trout immunized with anti-Yersiniose vaccine and in non-vaccinated fish (Control); (mean ± SD; n = 10).

4. DISCUSSION

Successful vaccination strategies may depend upon memory formation by B cells and may activate antibody-secreting cells. Humoral immune responses in which B lymphocytes and plasma cells are the effector cells, have been studied by the detection of specific antibody secreting cells or by the study of their products, i.e. antibodies in serum [1, 4]. In our study, we determined the influence of the natural product HMB on the number of antibody secreting cells after in vitro and in vivo immunization with the anti-*Y. ruckeri* vaccine. HMB was administered orally two weeks before the stressful time of fish immunization by intraperitoneal injection of the vaccine. This observation was the first analysis of the influence of orally administered HMB on ASC cells after in vitro and in vivo immunization of animals.

Leucine is transaminated to 2-oxoicocaproate (KIC) and then partially oxidized to 3-hydroxy-3-methylbutyrate (HMB). These two catabolites have been shown to have a positive effect on human and animal health when used as a dietary supplement [5, 6, 9, 11, 12, 14]. HMB has been shown to reduce animal mortality and this decrease may possibly be attributed to nonspecific stimulation of the immune system [10, 13, 22]. Oral administration of KIC and HMB to steers increased DNA synthesis by mitogen-stimulated lymphocytes in animals [13, 15]. The in vitro study showed that HMB has a stimulating effect on the macrophage activity of chickens; it induces proliferation of macrophages in culture as well as enhances

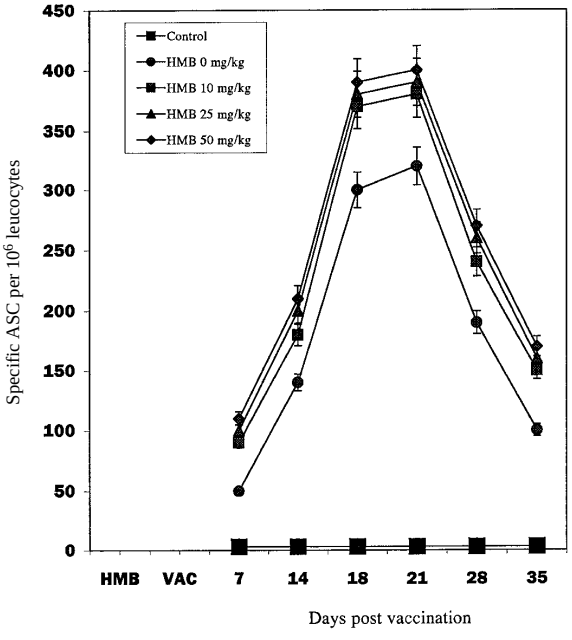

Figure 3. The influence of different doses of HMB on the kinetics of the numbers of specific antibody secreting cells (ASC) per 10^6 pronephros leucocytes from rainbow trout immunized with anti-Yersiniose vaccine and in non-vaccinated fish (Control); (mean ± SD; n = 10).

macrophage effector functions such as nitrite production and phagocytosis [16].

In our study, the immunostimulating effect of HMB on the humoral immune response was observed. The in vitro study showed that HMB had no toxic effect on the immunocompetent cell activity and at concentrations between 10 and 100 μg/mL it increased the level of specific antibody secreting cells. The in vivo study indicated a similar pattern. Oral administration of HMB to fish before immunization, at doses between 10 and 50 mg/kg b.w. enhanced the effectiveness of the vaccine, analyzed by the levels of total Ig and specific antibody secreting cells. The observed differences in the number of ASC measured in spleen and pronephros are the effect of organ specificity and experimental condition. Similarly to the effect of dimerized lysozyme (KLP-602) applied before the anti-*Y. ruckeri* vaccine [21], HMB increased the levels of the effector cells, a very important part of the specific immune response, and had a positive influence on the humoral immune response in fish. The in vitro and in vivo studies showed that HMB had a positive effect on the humoral immunity and may be used orally before immunization to increase the effectiveness of vaccines and protection against bacterial diseases. Future studies will include determining optimal doses, influence on the nonspecific defence mechanisms and protocol for feeding this substance to maximize protection, given the constraints of fish culture and economics.

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