



ENHANCEMENT OF ANTI-KLH IgG ANTIBODY PRODUCTION IN RABBITS AFTER TREATMENT WITH HABERLEA RHODOPENSIS EXTRACT

B. Popov¹, Zl. Dobрева^{1*}, Sv. Georgieva², S. Stanilova¹

¹Department of Molecular Biology, Immunology & Genetics, Faculty of Medicine

²Department of Genetics, Breeding and Reproduction, Faculty of Agriculture
Trakia University, Stara Zagora, Bulgaria

ABSTRACT

The immunomodulatory properties of *Haberlea rhodopensis* extract on primary and secondary specific antibody production were evaluated in New Zealand white rabbits. Rabbits were immunized with 50 µg of keyhole limpet hemocyanin (KLH). Simultaneously with antigen challenge groups of rabbits were injected with: 100 mg/kg *Haberlea rhodopensis* extract; 50 mg/kg cyclophosphamide or *Haberlea rhodopensis* extract + cyclophosphamide. The control group of rabbits was injected with KLH, only. Rabbits were reimmunized and treated with plant extract and cyclophosphamide on day 44. The specific anti-KLH total IgM,G, A and KLH-specific IgG antibody production were determined on day 0, 15, 22, 28 for primary and day 43, 51, 58, 66 for secondary antibody response by ELISA. Results showed that KLH-specific IgG antibody production had increased significantly after treatment with *H. rhodopensis* extract during the secondary humoral immune response. We concluded that, in tested concentration the extract from *Haberlea rhodopensis* showed a well expressed immunostimulatory activity on the secondary IgG and in the lower degree on the secondary total IgG,M,A antibody production.

Key words: immunostimulation, anti-KLH IgG, anti-KLH IgG,M,A, *Haberlea rhodopensis*

INTRODUCTION

Compounds that are capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response can be classified as immunomodulators. Those compounds which appear to stimulate the human immune response are being sought for the treatment of cancer, immunodeficiency diseases, or for generalized immunosuppression following drug treatment; for combinational therapy with antibiotics; and as adjuvants for vaccines (1). Plant extracts are among the attractive sources of new drugs and have been shown to induce promising immunostimulatory effects. Medicinal plants serve as therapeutic alternatives, safer choices, or in some cases, as

the only effective treatment. A large number of plant extracts and their isolated constituents have shown beneficial therapeutic effects, including anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial and immunomodulatory effects (2). Some of the plants with well established immunostimulatory activity are *Viscum album* (3), *Panax ginseng* (4), *Echinacea purpurea* (5) and *Cuscuta europea* (6).

The Balkan region endemite *Haberlea rhodopensis* is a world record-holder in desiccation tolerance, but its effect on the immune response is not investigated. Unfortunately, till now there are no strong historical and ethnobotanical data confirming the ancients' knowledge and use of *Haberlea* as medicinal attribute. However, one of the local plant names in the Rhodopi mountains is "shap" (food and mouth disease) which is considered as confirmation that the local people have used the plant against animal diseases (7). The aim of the present study is to

*Correspondence to: Zlatka Dobрева, Ph.D.
Department of Molecular Biology, Immunology & Genetics, Faculty of Medicine, Trakia University
Armeiska 11 St., 6000 Stara Zagora,
Bulgaria, E-mail address: zdobрева@mf.uni-sz.bg

investigate the influence of *Haberlea rhodopensis* extract on the specific primary and secondary antibody response.

MATERIALS AND METHODS

H. rhodopensis extract: Leaves of *H. rhodopensis* were collected from plants growing in their natural habitat (the vicinity of Bachkovo, Bulgaria) after licence of Ministry of environment and water of Bulgaria. Leaves were cut into small pieces and dried in room temperature for 1 month. After grinding the dry matter were macerated for 6 hours in 70% ethyl alcohol and were percolated for 48 h. Primary extract was concentrated by evaporation of ethanol in a vacuum environment to reach a ratio of 5% ethanol and 95% water. The obtained extract was filtered through filter paper to remove emulsified substances, chlorophyll and other particles. The extract was standardized in accordance with the method for determining the relative density (Bulgarian Pharmacopoeia Roll 2, p.19). The amount of extracted substances ranged between 0.098 and 0.113 g/cm³ (average 0.105 g/cm³).

Experimental animals: 20 male New Zealand white rabbits (bred in the animal facilities of the Trakia University) were used in the study. They were in good health, 5-month-old and weighed between 3.5 and 4.0 kg at the beginning of the experiment. All animals received food and water *ad libitum*.

Immunization protocol: A subcutaneous (s.c.) injection of 50 µg KLH (keyhole limpet hemocyanin from *Megatura crenulata*; Sigma) in 0.5 ml saline was given to each rabbit and followed by the immunization protocol as it is described below:

- **Primary humoral response protocol:** The first injection with antigen was given on day 0 and booster doses of antigen in saline were administered on day 8.

- **Secondary humoral response protocol:** At the end of the primary response, reimmunization dose of 50 µg KLH in 0.5 ml saline was introduced in each rabbit on day 44.

Immunomodulation protocol: Experimental animals distributed in 4 groups were treated with immunomodulating substances, simultaneously with first and reimmunization antigen challenge as follow:

- 5 rabbits were treated with 100 mg/kg *Haberlea rhodopensis* extract (Hab) administered intramuscularly;

- 5 rabbits were treated with 50 mg/kg cyclophosphamide (CP) administered intramuscularly;

- 5 rabbits were treated with 100 mg/kg Hab + 50 mg/kg CP administered intramuscularly

- 5 rabbits were treated with KLH alone.

Blood samples: They were taken from each rabbit by venipuncture of ear veins on day 0, 15, 22, 28 for primary and on day 43, 51, 58, 66 for secondary antibody response. Sera were collected after centrifugation at 1800 rpm for 10 min and stored at -20°C

ELISA for serum anti-KLH antibodies: The specific KLH IgG and polyvalent (IgM, G, A) antibody production was determined in sera, by ELISA. The wells of Maxisorp flat-bottom immunomodules (Nunc, Roskilde, Denmark) were coated with 0.1 ml of 10 µg/ml solution of KLH in 0.05 M sodium carbonate buffer, pH 9.6 and incubated overnight at 4°C. Then, the immunomodules were washed and blocked for 1 h with PBS containing 1% BSA. Next, the samples (0.1 ml per well) diluted 1:50 in sample buffer (0.15 M PBS, pH 7.2 containing 1% BSA and 0.2% Tween) were added and immunomodules were incubate for 1 h at room temperature. After three washes, the detecting antibodies (0.1 ml per well) were added. For the detection of rabbit KLH-specific IgG, mouse monoclonal anti-rabbit IgG conjugated with peroxidase, γ chain specific (Sigma) was used. The total KLH-specific antibody production was revealed after the addition of mouse monoclonal anti-rabbit Ig peroxidase-conjugated, heavy chain specific to rabbit IgG, IgM and IgA. After incubation for 1 h at room temperature the immunomodules were washed and 0.1 ml of substrate solution (containing 5 µl 35% H₂O₂ and 2 mg chromogene - *o*-phenylenediamine, dissolved in 10 ml 0.1 M citric acid buffer (pH 5.0), was added to each well. The subsequent color reaction was stopped with 10% H₂SO₄ and the optical density was measured at 492 nm on an ELISA plate reader. All samples were duplicated. Results were expressed, as a percentage, in relation to control OD value of preimmune serum for each individual animal.

Statistical analysis: The data are expressed as a mean and standard error of the mean. Student's t-test was used to determine the statistical differences between groups. Differences were considered significant when the P-value was less than 0.05.

RESULTS AND DISCUSSION

Humoral immunity is mediated by the antibodies, which are produced by cells of the B lymphocyte lineage. The physiologic function of antibodies is to neutralize and eliminate the antigens that induced their formation. Primary humoral immune responses lead mainly to specific IgM antibody production followed by specific IgG antibodies and memory B cell formation. Secondary response is characterized by memory B cell activation, rapid production of IgG antibodies, followed by IgA and IgE antibodies. Primary and secondary antibody response to protein antigens differs qualitatively and quantitatively. Most pathogens elicit a humoral immune response that is characterized by an early rise of antigen-specific IgM, followed by affinity maturation, isotype switching, and the ensuing rise in antigen-specific IgG, IgA and IgE antibodies (8).

This study was designed to establish the immunomodulating activity of the extract from *H. rhodopensis* on primary and secondary humoral immune response. The quantity of total anti-KLH specific antibodies, after primary and secondary immunizations in all groups of rabbits, was measured by ELISA using peroxidase monoclonal mouse anti-rabbit immunoglobulins which recognize an epitope located on the heavy chain of rabbit IgG, IgM

and IgA. After primary immunization, the total anti-KLH antibody production, peaked on Day 15. The level of increase was 1.3 times greater (130%) than the day 0 control value of nonimmune sera (**Fig. 1**). During primary antibody response increased antibody production remained at high levels on days 22 and 28. The presence of *H. rhodopensis* extract did not lead to a significant change of immune response in rabbits; there were no statistical differences between groups treated with immunistimulator, and with KLH alone. Rabbits treated with CP showed a well expressed reduction of specific total antibody synthesis, which is in accordance with the immunosuppressive activity of CP on the humoral immune response. The changes in the serum level of KLH-specific IgG primary antibody production are shown in **Fig. 2**. When a peroxidase-conjugated mouse anti-rabbit IgG was used, the level of anti- KLH antibodies from IgG isotype alone was determined. Results demonstrated that rabbits produced increased levels of anti-KLH IgG following the primary immunization. A maximum increase (130%) occurred on day 15 compared to day 0 and lasted until day 28. The treatment of rabbits with Hab or CP did not alter significantly the production of KLH-specific IgG antibodies during the primary immune response.

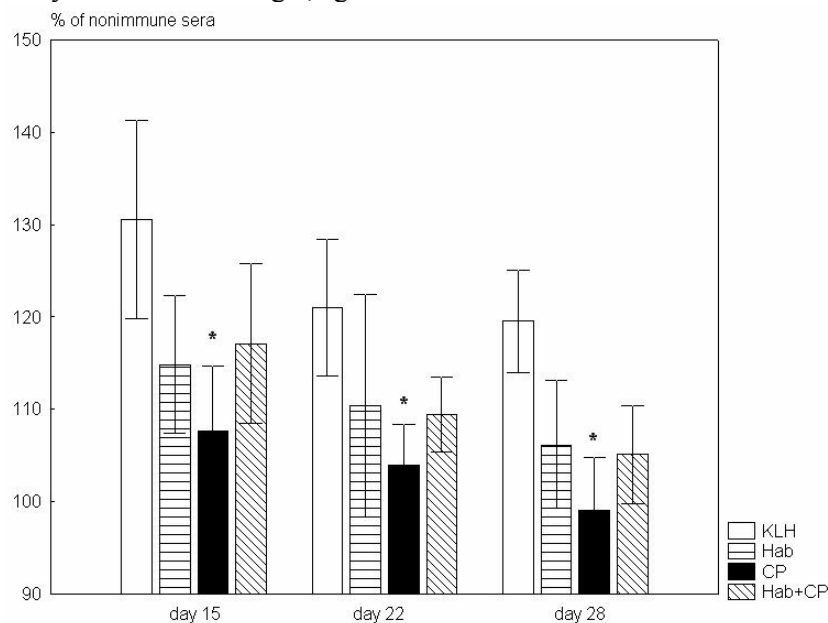


Fig. 1: Primary humoral immune response. Changes of total KLH-specific IgG, M, A antibody production in immunized rabbits. Rabbits were immunized with 50 µg KLH and treated with 100 mg/kg *Haberlea rhodopensis* extract (Hab), 50 mg/kg cyclophosphamide (CP) and 100 mg/kg Hab+50 mg/kg CP simultaneously with antigen challenge. The control groups of rabbits were immunized with 50 µg KLH. The obtained results in OD 492 are presented as % over OD value of nonimmune sera. *P<0.05 CP vs. KLH.

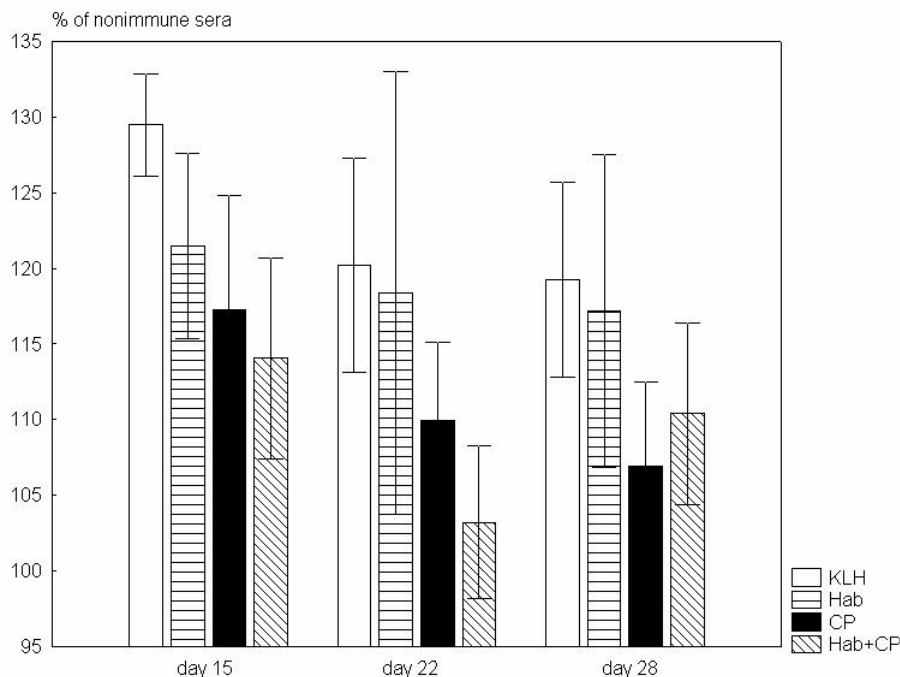


Fig. 2: Primary humoral immune response. Changes of KLH-specific IgG antibody production in immunized rabbits. Rabbits were immunized with 50 μ g KLH and treated with 100 mg/kg *Haberlea rhodopensis* extract (Hab), 50 mg/kg cyclophosphamide (CP) and 100 mg/kg Hab+50 mg/kg CP simultaneously with antigen challenge. The control groups of rabbits were immunized with 50 μ g KLH. The obtained results in OD 492 are presented as % over OD value of nonimmune sera. * $P < 0.05$ CP vs. KLH.

The quantity of specific-KLH antibodies reduced near to start level on day 44 and this day was chosen for the secondary immunization (reimmunization). A rapid and strong secondary humoral immune response was observed after reimmunization. The total specific antibody production (anti-KLH IgM, IgG, IgA) peaked on day 8 after reimmunization (day 51 in **Fig.3**) and decreased 15 days latter (day 66). In contrast to the primary antibody response rabbits treated with *H. rhodopensis* produced significantly more total specific antibodies from IgM, IgG, IgA isotypes compared to rabbits treated with KLH alone. However, the immunostimulating effect is transitory and does not occur at a later day. Results for anti-KLH IgG antibody production during the secondary immune response are presented in fig. 4. The presence of *H. rhodopensis* extract led to a marked augmentation of humoral immune response in rabbits. The observed differences between rabbits injected with immunostimulator and non-treated rabbits were statistically significant ($p < 0.05$) for day 51, 58 and 66. In addition, the increase of anti-KLH IgG, as a result of immunostimulation, was sustained for more than 23 days after reimmunization, as it is shown in **Fig 4**.

Humoral immune responses can be depressed by various external influences including emotional stress, physical stressors such as inadequate sleep or athletic overtraining, environmental and occupational chemical exposure, UV and other types of radiation, common viral or bacterial infections, certain drug therapies, blood transfusions and surgery (9-13). In all cases immunostimulation using medicinal plants can provide an alternative to conventional therapy when host defense mechanisms have to be activated under the conditions of impaired immune response (14). The basic sign of the immunostimulatory activity of the tested agent is the increased specific antibody production by activation of antigen-presenting cells and modulation of cytokine secretion.

Using an animal model, we were able to measure the immunomodulatory effects of herbal treatment on the production of specific immunoglobulins following primary and secondary exposure to an antigen KLH. Continuous collection of blood samples during the 11-week treatment period allowed us to define the time periods of maximal efficacy for *H. rhodopensis* extract. To our knowledge, this is the first study that has studied the effects of *H. rhodopensis* treatment on specific immunoglobulin production.

CONCLUSION

In tested concentration the total extract from *Haberlea rhodopensis* showed a well expressed

immunostimulatory activity on the secondary IgG and in the lower degree on the secondary IgM,G,A antibody production

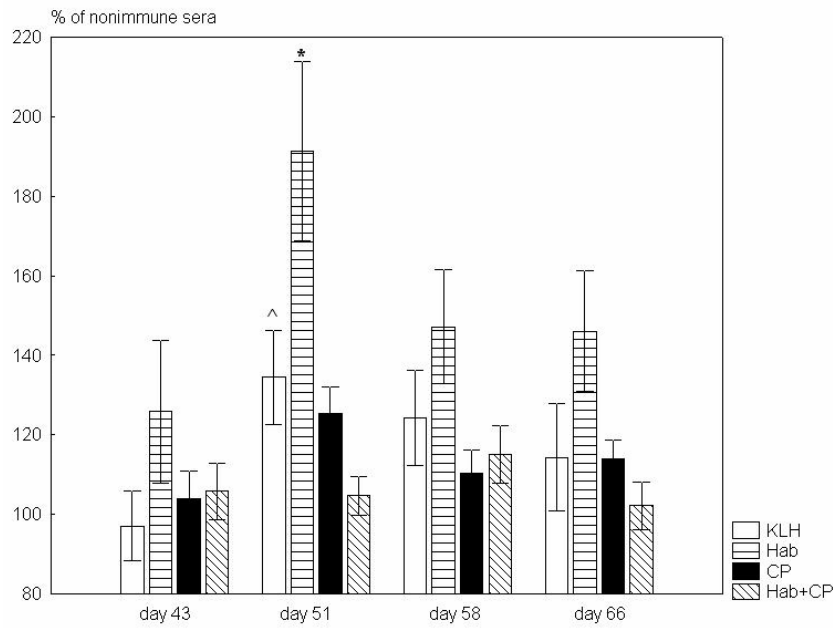


Fig. 3: Secondary humoral immune response. Changes of total KLH-specific IgG, M, A antibody production in immunized rabbits. Rabbits were reimmunized with 50 µg KLH and treated with 100 mg/kg *Haberlea rhodopensis* extract (Hab), 50 mg/kg cyclophosphamide (CP) and 100 mg/kg Hab+50 mg/kg CP simultaneously with antigen challenge. The control groups of rabbits were reimmunized with 50 µg KLH. The obtained results in OD 492 are presented as % over OD value of nonimmune sera. *P<0.05 CP vs. KLH. *P<0.05 Hab vs. KLH; ^P<0.05 KLH₄₃ vs. KLH₅₁

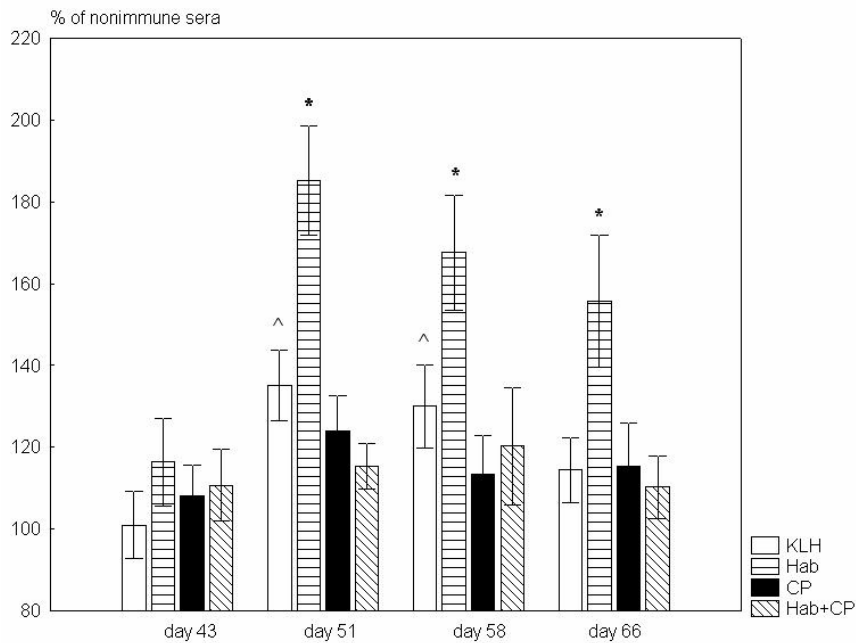


Fig. 4: Secondary humoral immune response. Changes of KLH-specific IgG antibody production in immunized rabbits. Rabbits were reimmunized with 50 µg KLH and treated with 100 mg/kg *Haberlea rhodopensis* extract (Hab), 50 mg/kg cyclophosphamide (CP) and 100 mg/kg Hab+50 mg/kg CP simultaneously with antigen challenge. The control groups of rabbits were reimmunized with 50 µg KLH. The obtained results in OD 492 are presented as % over OD value of nonimmune sera. *P<0.05 CP vs. KLH. *P<0.05 Hab vs. KLH; ^P<0.05 KLH₄₃ vs. KLH₅₁ or KLH₅₈

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