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Seasonal fluctuations of the humoral immune response in the tortoise (*Testudo graeca* Pall.)

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The primary and the secondary immune response to a T-dependent (BSA) and a T-independent antigen (LPS) was studied on tortoises (*Testudo graeca* Pall.). Two separate experiments were conducted in summer and in winter. The titer and the class of antibodies produced was determined by ELISA. Our results show a considerable suppression in the anti-BSA and anti-LPS antibody production in winter, which is not only quantitative (titer), but also is qualitative (changes in IgM-IgG shift).

Key words: ELISA, humoral immunity, tortoise.

Introduction

A particularity of the humoral immunity in *Chelonia* is the characteristic sequence of "high molecular weight" (IgM), "low molecular weight" (LgG) and "extremely low molecular weight" (IgG lacking one or two domains of the F_c region) antibodies in the serum of repeatedly immunized *Testudo hermanni* Gmelin [1]. Other typical features are the increase in affinity of antigen-specific antibodies in *Testudo hermanni* and *Agrionemys horsefieldii* [7] and the longer intervals between the appearance of plaque-forming cells and haemolysins during the primary response of the latter species [10]. At the same time there is a lack of information dealing with the temperature and seasonal dependence on the humoral immune reactivity in a one and same Chelonian species and its response to T-independent antigens. Until now the tortoises have been immunized with various T-dependent antigens and in a restricted number of investigations hapten-carrier conjugates have been used [1, 8, 11, 12]. For this reason I decided to re-examine the possibility for seasonal fluctuations of the serum antibody production in the tortoise (*Testudo graeca* Pall.) when immunized with a T-dependent and a T-independent antigen.

Material and methods

Animals. Adult tortoises (*Testudo graeca* Pull.) of both sexes, weighting 1,0-3,0 kg, were collected in the Blagoevgrad south-west district of Bulgaria. In both seasons the animals were divided in two independent groups ("BSA" and "LPS"), 40 individuals each. The winter (December-February) and the summer (July-August) experiments were conducted on separate groups of tortoises, which were held troughout the year in a one and the same room at 15-20° C and were fed *ad libitum* with vegetables.

Antigens. The bovine serum albumin (BSA), fraction V-endotoxin free was purchased from SIGMA; the lipopolysaccharide (LPS) from *Escherichia coli*, strain 0111 : B4 : H2 – from IIPD, Sofia.

Antisera. Rabbit anti-tortoise IgM (whole molecule) and anti-tortoise LgM (whole molecule) were obtained as described previously [3, 4]. Goat anti-rabbit IgG (whole molecule), a peroxidase conjugate of IgG fraction of the antiserum was purchased from SIGMA.

Immunization schedule. The animals were immunized twice with BSA or LPS. Second injections were made at day 35 in summer and at day 50 in winter. The individual doses for BSA and LPS were 10 mg and 4 mg respectively. The antigens were administered subcutaneously on the thighs. The BSA was in an emulsion with incomplete Freund's adjuvant, the LPS – without adjuvant. Serum samples were collected three times after each injection, at various intervals as shown on the figures. The sera were prepared from blood, which was withdrawn by a cardiac puncture. The serum samples were kept frozen at – 20° C and were tested within a few days.

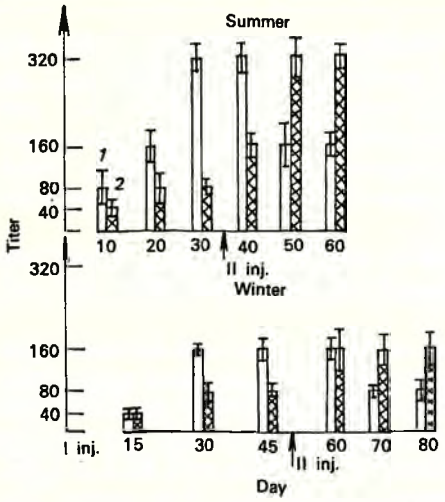
Enzyme-linked immunosorbent assay (*ELISA*). This was performed by the "amplified" method of Butler, McGivern and Swanson [6] with substantial modifications as described previously [5].

Statistics. The Student's *t*-test was used to compare the geometric means of antibody titers from every group of 40 tested sera. If $p < 0{,}05$, the difference was considered significant.

Results and discussion

In summer, the detection of anti-BSA antibodies revealed a primary response, which was predominantly of IgM class (titer 1 : 320 at day 30). The secondary response differed by a specific IgG increase (1 : 320 at days 50 and 60) and by a marked IgM decrease (until 1 : 160) for the same interval (Fig. 1). Similar to the summer one dynamics of the antibody production to BSA, but in significantly lower titers was observed in the winter experiment (Fig. 1). The development of the primary response was due mainly to IgM activity: highest titers 1 : 160 at days 30, 45 and 60. The secondary response was with "mixed" IgM-IgG production, the anti-BSA antibodies of IgG class being in higher titers at days 60, 70 and 80.

The anti-LPS antibodies in summer tortoises were mainly of IgM class and they reached the highest titer 1 : 320 at day 30 after the initial antigenic stimulation (Fig. 2). Their level was without alteration even after the second injection and it persisted until day 50. Ten days later (day 60) they were lowered to a titer 1 : 160. Along with the anti-LPS response of IgM origin, a presence of IgG antibodies was found. It was in too low titers – 1 : 40 at day 30 and 1 : 80 at days



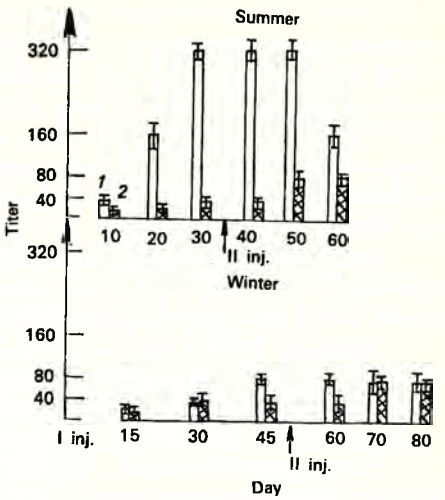


Fig. 1. ELISA for measuring antibodies to bovine serum albumin in the tortoise (*Testudo graeca* Pall.). Each column represents the geometric mean of antibody titer ± standard deviation ($n=40$)
1 – IgM; *2* – IgG

Fig. 2. ELISA for measuring antibodies to lipopolysaccharide in the tortoise (*Testudo graeca* Pall.). Each column represents the geometric mean of antibody titer ± standard deviation ($n=40$)
1 – IgM; *2* – IgG

50 and 60 (Fig. 2). The winter response of *Testudo graeca* Pall. was characterized by longer intervals for the appearance in the serum of low-titer antibodies (until 1 : 80) of both immunoglobulin classes. It is notewothy that in the secondary response the IgG titer became equal to that of the IgM.

In earlier studies on *Testudo hermanni* Gmelin [1] it has been shown that by a combination of suitable conditions (choice of antigen, adjuvant and multiple immunizations) a production of high-titer haemagglutinating antibodies could be achieved. Probably, the lower ELISA titers that were found in *Testudo graeca* Pall. could be explained by several ways: weak immunogenicity of the BSA, which was administered only twice at greater intervals between injections and in lower individual doses. A similarity was established with respect to the class of anti-BSA antibodies in *Testudo graeca* and to that of anti-pig serum protein antibodies in *Testudo hermanni* [1]. It concerns the early (at day 10) appearance of IgM antibodies, which persisted in the serum of *Testudo graeca*. A discrepancy was found in the production of IgG antibodies. While in *Testudo hermanni* the "low-molecular weight" antibodies (IgG or IgY) appear after three months (confirmed by passive haemagglutination test of fractions from gel-filtration of the immune sera), low-titer antibodies were detected in *Testudo graeca* ten days after the first injection. However, it would be inappropriate to compare directly results from ELISA and passive haemagglutination test, since they are quite different assay systems. The latter is also valid for the interpretation of our results from experiments with the T-independent antigen LPS and the data about hapten-carrier conjugates [2, 7]. The observed, in some cases, anti-dinitrophenol (or anti-trinitrophenol) haemagglutinins in *Testudo hermanni*, which were in higher titers than that of the anti-LPS antibodies in *Testudo graeca*, could be due

to a greater epitope density of the haptens on the carrier surface, stimulation of a T-helper subpopulation against the carrier, pre-existence of natural antibodies [2] and/or to a weaker immunogenicity of the lipopolysaccharide. These results rather differ from similar experimental data on mammalian species where the same antigen (LPS) has been used [9].

The results of our winter experiment could be compared, with some limitations, with the data of similar studies on *Testudo ibera* [8] and *Testudo hermanni* [1]. The latter investigation has been performed on animals that were immunized 12 times in the course of several years. Hence, such data could be used with respect to their final conclusion only: for a delay in the antibody production of haemagglutinins to pig serum proteins [1] and of precipitins against *Brucella* antigens [8].

The most common conclusive remark, which could be made is that the immune system of the tortoise (*Testudo graeca* Pall.) shows clear seasonal quantitative and qualitative fluctuations in its function to produce serum antibodies to BSA and to LPS.

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