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Borrelia afzelii and immune response of BALB/c mice

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Abstract

The aim of our study was to find a possible existence of various vaccination effects on the formation of antibodies against three individual *Borrelia afzelii* strains (dead cell suspension) isolated from a vector, potential vector and host of this spirochaete, which makes this experiment unique in the research of Lyme disease causative agents. Three strains of *Borrelia afzelii* were isolated from three different sources: *Ixodes ricinus* tick (BRZ 9), *Culex (Culex) pipiens molestus* mosquito (BRZ 14) and *Apodemus flavicollis* wild rodent (BRZ 21). Vaccination induced formation of IgM and IgG in the BALB/c mice (males) and these antibodies were detected in the serum by the Enzyme-Linked ImmunoSorbent Assay (ELISA). IgM antibodies were significantly increased in the mice immunized with BRZ 21 in the third sera testing ($P < 0.001$) and IgG antibodies in the mice immunized with the BRZ 9 and BRZ 21 strains ($P < 0.01$) during the whole three-sample collection. Significant differences were found in antibody concentration by comparison of groups immunized with BRZ 9/BRZ 21 and BRZ 14 ($P < 0.001$), probably caused by the amount of antigen of BRZ 14 strain. Such finding implies that the immune system of the host (rodent), attacked by the same genospecies of pathogenic borreliae (*B. afzelii*) coming from different sources reacts with the same intensity. This is the first study of reaction of the same borrelian antigen mixture coming from various sources.

Borrelia burgdorferi sensu lato, serum, immunization, ELISA

In Europe, at least three human pathogenic species of *Borrelia burgdorferi* sensu lato are responsible for Lyme disease (Baranton et al. 1992). Human pathogenic European strains of *Borrelia burgdorferi* sensu lato are much more heterogeneous than those of human pathogenic strains found in the United States. *Borrelia afzelii* belongs among the European strains (Wang et al. 1999). We successfully isolated three strains of *Borrelia afzelii* from three different sources: *Ixodes ricinus* ticks as a vector, *Culex* (*Culex*) *pipiens molestus* mosquitoes as a potential vector, and *Apodemus flavicollis* wild rodent as a reservoir of *Borrelia burgdorferi* life cycle. According to protein profiles characterization using the gradient Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), all proteins from 7 up to 200 kDa of all three isolated strains appeared to be the same. Characterization of these strains was also performed by protein profiles comparison with *B. burgdorferi* sensu stricto and *B. garinii*. The aforementioned fact about protein identity led us to investigate a response of the immune system to three strains of the same genospecies from three different sources in mammals. The aim of this study was to inoculate the whole homologous cell antigens and to find differences in the creation of IgM and IgG antibodies.

Materials and Methods

Animals

The 6-week-old male BALB/c mice were purchased from the Department of Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic. The animals were housed in a plastic cage, in an air conditioned room, at 20 - 24 $\pm$ 1 °C, with illumination from 07:00 h to 19:00 h. All animals were allowed to adapt to the environment for at least 2 weeks prior to immunization. The initial body weight of each animal was approximately 25 g. Duration of the experimental treatment was 9 weeks.

The male mice were divided into four groups (6 mice in each of them): first group was immunized with BRZ 9

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(BRZ refers to an internal mark of an individual isolated strain) *B. afzelii* whole cell antigen isolated from *Ixodes ricinus* (Pisárky, Brno) 16 × passaged, completed with 0.1% aluxid ($Al(OH)_3$); second group was injected with BRZ 21 *B. afzelii* whole cell antigen isolated from *Clethrionomys glareolus* (Studénka) 18 × passaged, completed with 0.1% aluxid; third group was injected with BRZ 14 *B. afzelii* whole cell antigen isolated from *Culex pipiens molestus* (Vysoké Mýto) 21 × passaged, completed with 0.1% aluxid; fourth group (control) was injected with saline completed with 0.1% aluxid.

Cultivation of isolated strains

Three individual species of *B. afzelii* (BRZ 9, BRZ 21, BRZ 14) were identified using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR, PCR - RFLP) and the gradient SDS-PAGE methods as reported by Janouškovcová et al. (2004). The isolated strains were cultivated in BSK-H medium (Sigma-Aldrich, USA) enriched with 6% rabbit serum, Riphampicin (50 μg/ml), Phosphomycin disodium salt (100 μg/ml) at 33 °C. The control sample of spirochaetes was observed by dark-field microscopy (DFM) at × 400 magnification. Living bacteria were cultivated until density of their suspension reached 10^6–10^7 cells/ml; afterwards, the bacteria were killed by freezing and used for immunization.

Immunization

The mice were injected on the first day of the immunization process and then after 14 and 35 days. Each mouse was injected with a mixture of 300 μl of the antigen solution completed with 0.1% aluxid. A concentration of 7.5 μg per mouse was applied for the BRZ 9 and BRZ 21 antigens and a concentration of 3.75 μg for BRZ 14. The dose of BRZ 14 antigen was used in a half amount due to the lack of bacterium suspense.

Antigen concentration

The determination of antigen protein concentration was done according to Wannemacher et al. (1965). It is a biuret reaction, i.e. proteins and peptides react with biuret to form a violet complex. The absorbance of 570 nm was measured by Spekol 210 KIN-Programm (Carl Zeiss Jena) in 1 cm^3 cuvette.

Sample collection and analytical procedures

The mice were anaesthetized with diethyl ether and blood samples were collected from the tail vein (vena caudalis) of each of the mice after 14, 35 and 56 days from the beginning of immunization. At the end of experimental period, all animals were killed and blood samples were then collected from carotid artery (arteria carotis externa). Additional blood samples were taken 14 days after each injection in order to check production of specific antibodies. Sera were separated from other blood fractions by precipitation and centrifugation. Samples of serum were stored at -20ºC and after thawing examined using an Enzyme-Linked Immunosorbent Assay (ELISA).

ELISA

The sera were examined by the ELISA method modified according to our conditions (Vostal and Žákovská 2003). The ELISA method is available in commercial sets (TestLine, Brno, Czech Republic) and used in human medicine for diagnosis of Lyme borreliosis. A similar design of the ELISA method was reported by Štefančíková et al. (2000). Microplates in our examination were filled with antigen at a concentration of 2 μg/ml per each well, sera were diluted 1:400, anti-mouse IgG, IgM peroxidase conjugate (Sigma-Aldrich, USA) were diluted at 1:1000. Serum samples from the immunized mice were evaluated as positive. Serum samples from the control mice were evaluated as negative.

Statistical analysis

Statistical comparison of tested groups to control group was evaluated for each step of immunization by Kruskal-Wallis test (Statistica 9.0).

Results

We detected no significant differences between formation of IgM antibodies in the first, second and third sample of serum collection in the control group (Fig. 1), and a significant increase of IgG content between the first and third serum collection ($P < 0.05$) (Fig. 2). We found no significant difference ($P < 0.05$) in the formation of IgM antibodies in any of the experimentally immunized groups (1–3) in the first sample collection (after 14 days) (Fig. 1). Significant differences ($P < 0.01$) were detected in the formation of IgG antibodies in the groups BRZ 9 and BRZ 21 (Fig. 2). Results obtained in the second (after 35 days) and third serum (after 56 days) collections were almost identical for groups immunized with BRZ 9 and 21, except the third serum examination of BRZ 21 group, where significant increase ($P < 0.001$) of IgM antibodies was found. While the experimental groups immunized with BRZ 9 and 21 showed a significant increase ($P < 0.001$) in the formation of IgG antibodies, the group immunized with BRZ 14 did not.

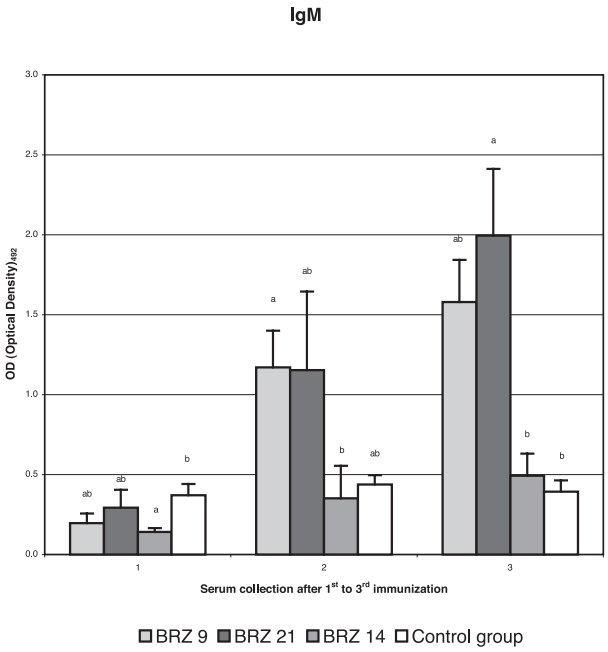

Fig. 1. Mice IgM responses to *Borrelia burgdorferi* strains (BRZ 9, BRZ 21, BRZ 14) antigens during the experimental period. Data points represent the mean absorbance (with SD) of serum samples from all 6 mice of each group in given collection after 1st to 3rd immunization. Significant differences between groups in each step of immunization are expressed by different letters.

The concentrations of IgG antibodies were high 14 days after the first immunization and slowly increased thereafter. In contrast, the concentrations of IgM antibodies were low initially and increased after each subsequent immunization. Both IgM and IgG antibodies in the third sample collection reached approximately double concentrationss compared to the concentrations in the first sample collection (Figs 1 and 2).

Discussion

Borrelia burgdorferi sensu lato has so far been isolated from various vertebrates, including humans, and also from ticks or even insects (Hubálek et al. 1998; Halouzka et al. 1999). Isolated strains of *B. burgdorferi* sensu lato showed considerable phenotypic diversity. Several studies found that mosquitoes harbour spirochaetes. In those studies, blood-sucking arthropods were examined by darkfield microscopy and PCR for the presence of borreliae (Sanogo et al. 2000; Žákovská et al. 2004; Kosik-Bogacka et al. 2006). Material used in our study additionally confirms the presence of *Borrelia afzelii* in females of *Culex* (*Culex*) *pipiens* biotype molestus. According to the protein profiles characterization using gradient SDS-PAGE method as described by Janouškovcová et al. 2004, all visualised proteins of all three isolated strains appeared to be the same. This fact led us to investigate a response of three identical antigens from three different sources in mammals.

Live *Borreliae* are risky to manipulate. We, therefore, investigated the possibility of using a dead *Borrelia* antigen as an alternative means of inducing protective immunity. Generally, the best suitable and most often used animal models are mice. Studies with

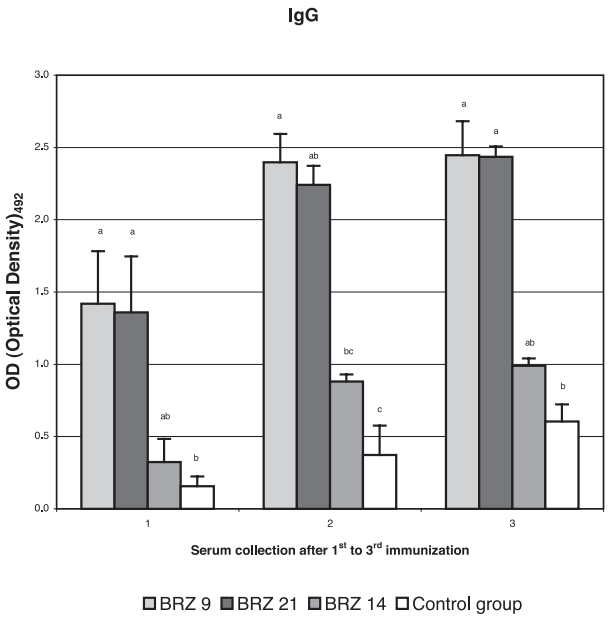

Fig. 2. Mice IgG responses to *Borrelia burgdorferi* strains (BRZ 9, BRZ 21, BRZ 14) antigens during the experimental period. Data points represent the mean absorbance (with SD) of serum samples from all 6 mice of each group in given collection after 1st to 3rd immunization. Significant differences between groups in each step of immunization are expressed by different letters.

laboratory mice provide different results of immunization against *Borrelia burgdorferi* s. l. in the production of interleukins (Giambartolomei et al. 1998), in differences among *Borrelia* genospecies (Tuomi et al. 2002), in clinical signs (Wright et al. 1990) and in the production of antibodies against two of the most focused borrelian proteins OspA (Zhong et al. 1997a,b) and OspC (Scheiblhofer et al. 2003). The immunization resulted in the development of an immune response to each of the three strains. Concentrations of IgM and IgG antibodies were monitored in individual mice 14, 35 and 56 days after the first immunization. The results of this experiment showed that the concentrations of IgM antibodies were low initially and increased after each subsequent immunization. In contrast, the concentrations of IgG antibodies were high 14 day after the first immunization and slowly increased thereafter. The concentration of IgG antibodies was significantly increased between the first and the third step in control group. These trends of rising concentrations of all immunoglobulins were probably caused by the effect of aluxid, although we used the lowest concentration sufficient for binding the antigen (Harlow and Lane 1988). Both IgM and IgG antibodies in the third sample collection doubled their contents compared to the contents in the first sample collection. IgM antibodies were significantly increased in BRZ 21 group in the third sample collection. Two groups of mice injected with BRZ 9 and BRZ 21 showed significant values in the increasing amounts of specific IgG antibodies during the three-time vaccination and three-sample collection. No significant increase of antibodies was observed in the group immunized with BRZ 14 probably due to the use of a half antigen dose. It means that the amount of antigen (3.75 mg) was not in the range of 5–50 mg, optimal for experimental immunization (Harlow and Lane 1988). The data on kinetics

of antibody responses in BALB/c mice confirms and extends a similar study performed on inoculated white-footed mice (*Peromyscus leucopus*) (Schaible et al. 1991).

In conclusion, our results show that the amounts of antibodies depend on the amount of the served antigen and that the immunized mice were more susceptible to a low dose of the antigen as recommended by immunization manual (Harlow and Lane 1988). The data presented here show that BALB/c mice immunization with *Borrelia afzelii* antigens isolated from various sources induced similar production of specific antibodies that could possibly increase protective immunity against infection.

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