Acta Veterinaria (Beograd), Vol. 49. No. 2-3, 97-104, 1999.

UDK 619:612.017.12

HYPERIMMUNIZATION OF RABBITS WITH IN VITRO-GROWN ENCEPHALITOZOON CUNICULI ORGANISMS

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(Received, 20. January 1999)

Specific hyperimmune anti-Encephalitozzon cuniculi antisera were produced in immunocompetent rabbits. The animals were three times inoculated with cell culture-grown microsporidan spores. A-group rabbits were immunized intraperitoneally and B-group rabbits intravenously with gradually decreasing doses of the agent spores at 16-day intervals. All rabbits receiving E. cuniculi developed serum antibodies, within 4 to 12 days, with IFA IgG titres of at least 1:64. Positive titres continued to day 48. After the third inoculation, antibody levels rapidly increased and reached titres of 1: 1.024 up to 1:8.192. The dynamics of specific antibody production consisted of three stages in both infected rabbit groups. The titres progresively increased and first culminated on day 16 after the initial antigen doses. Descent of titres and dysbalance followed after the second inoculation, but the titres never dropped under values of 1:128. Post-third-injectional increase of IFA titres continued to day 44 when it culminated.

The presence of high IFA IgG titres in hyperimmunized rabbits indicates that both routes of inoculation as well as the doses used for in vitro-grown E. cuniculi spores as an antigen appear to be suitable for the preparation of specific hyperimmune antisera with sufficiently high antibody levels.

Key words: microsporidia, Encephalitozoon cuniculi, rabbit, hyperimmunization, indirect immunofluorescent antibody test.

INTRODUCTION

Encephalitozoon cuniculi (Levaditi, Nicolau and Schoen, 1923) is a relatively frequent mammalian opportustic pathogen belonging to the phylum

Microspora (Weber et al.,1994). The host range of this obligate intracellular protozoan parasite is rather wide. Its presence has been confirmed in many species of wild, farmed and laboratory animals such as lagomorphs and rodents, carnivores and primates. Moreover, microsporidia are increasingly recognized as important opportunistic pathogens in individuals suffering from human acquired immunodeficiency syndrome (AIDS) at the present time (Bryan et al., 1991).

As a typical microsporidial disease encephalitozoonosis is well-known with a chronic, usually latent, course in susceptible individuals (Stewart et al., 1986). Immunocompetent hosts infected naturally or experimentally with *E. cuniculi* developed only a few clinical signs of the disease. The microsporidia persist causing asymptomatic infection that lasts for the lifetime of the animal (Shadduck and Pakes, 1971). Thus, the clinical signs of encephalitis or nephritis and death are rarely observed in laboratory animals in spite of the high serological prevalence of the infection in rabbit colonies.

Histologically, the disease is characterized by both fibrosis and inflammatory microgranule formation without central necrosis in the central nervous and renal tissues (Pakes and Genity, 1994). As a rule, the microgranuloma cell infiltration consists of leukocytes with predominance of macrophages and lymphocytes, some plasma cells and eosinophils, usually with the presence of numerous microsporidian spores (Horvath et al., 1996).

Indirect and direct fluorescent antibody methods and avidin-biotin-peroxidase complex (ABC) were used to detect agent spore antigens in histological sections with commercial antibodies, (Park et al., 1993). Even one or two spores of *E. cuniculi* present in the affected tissue could be detected by immunohistochemical methods. These methods, however, require special equipment, complicated staining procedures and above all specific anti-E. cuniculi antisera of high quality. In laboratory practice, moreover, there is an increased necessity for antisera to detect microsporidial antigens in biological samples from suspected individuals of many other animal species.

Therefore, the authors of this work decided to prepare rabbit hyperimmune anti-*E. cuniculi* antisera of required quality and volume using in vitro-grown parasite spores as the antigen.

MATERIAL AND METHODS

Animals. Eighteen four-month-old New Zealand white rabbits of both sexes with an average weight of 2.7 $\pm$ 0.3 kg were included in the experiment. Rabbits of a conventional outbred strain came from a private E. cuniculi-free colony. The animals were divided into three groups of 6 (A, B, C) and were housed separately in standard rabbit cages (sized 60x40x36 cm) without bedding at room temperature (19 $\pm$ 2^{0}C), a relative humidity of 60 $\pm$ 5%, 5 changes of air per hour, and a light/dark ratio of 12/12 hours. Rabbits were fed with complete rabbit pellets and offered fresh water ad libitum. After the period of hyperimmunization, all rabbits were painlessly sacrificed on the day 50 of the experiment.

Parasites. *Encephalitozoon cuniculi* organisms of murine origin were grown within, "E6" cells of VERO Green Monkey Kidney Cultures. The infected cell culture was grown in modified RPMI 1640 media supplemented with 5% foetal calf serum and antibiotics (Penicilline, Streptomycin and Amphotericine B). Spores freshly collected from the culture supernatants according to Koudela et al. (1993) were used as an antigen for both animal inoculation and IFAT serological examinations.

Way of hyperimmunization. To produce specific anti-*E. cuniculi* hyperimmune antisera, experimental animals were three times inoculated with in vitro-grown microsporidian spores on day 0,16 and 32 of the experiment. Six rabbits in the A-group were immunized i.p. with gradually decreasing doses of E. cuniculi spores ($1x10^8$, $1x10^6$ and $1x10^4$ respectively) in a single 1.0 ml volumes of phosphate buffered saline (PBS) at 16-day intervals. The 6 rabbits in group B were inoculated i. v. into the marginal ear vein (vena auricularis lateralls) with gradually decreasing doses of 1×10^4, $1x10^3$ and $1x10^2$ agent spores in a single 0.5 ml volumes of PBS at the same intervals. The other six animals (group C) were not inoculated and served as the negative controls.

Serum sample collection. Samples of blood sera from rabbit groups A and B were collected regularly every 4 days during the hyperimmunization. Serum samples from rabbits in group C were obtained three-times namely, on day 0, 20 and 40 of the experiment. Blood was withdrawn from the marginal ear vein in the standard way. On the spore inoculation days (0, 16, 32) the blood samples were taken just prior to injection. Sera were stored frozen at -20 ^{0}C until used.

The IFA test. The indirect immunofluorescence of anti-*E. cuniculi* antibodies in the rabbit serum was assessed according to the method described in detail by Chalupsky et al. (1973). Sera that reacted at a dilution of 1:64 or higher were considered to be positive. The humoral responses of hyperimmunized animals were compared with those of uninoculated control animals as well as with positive rabbit serum. The commercial swine-anti-rabbit conjugate (SwARb/FITC) used in the test was obtained from SEVAC a. s., Prague, Czech Republic.

Statistical evaluation. The correlation coefficients of different variables were calculated using the F-test. Differences among groups at the level of $p < 0.05$ were considered significant.

RESULTS

Clinical and pathological findings. No evidence of either encephalitozoonosis-releated clinical sings or hypersensitivity reactions was observed in any rabbits hyperimmunized during the period of the experiment. None of the animals infected died before the end of the hyperimunization. Moreover, no pathological changes were found by necropsy of the experimental and control rabbits on day 50.

IFA titers. The regular examinations of rabbit serum samples by IFAT monitored the humoral immune response to the *Encephalitozoon cuniculi*-antigen

given to each experimental animal. In comparison to control animals the immune systems of the infected rabbits reacted by developing specific antibodies ($p<0.05$).

Before hyperimmunization, the experimental animals did not produce IFA IgG to the microsporidium (day 0). All immunized rabbits started developing anti-E. cuniculi antibodies within 4 days after the first inoculation. The first positive titres (1:64) occurred in three i. v. inoculated rabbits (B-7, B-9 and B-11) and in rabbit A-5 on the day 4 (Tab.1.) All i. v. infected rabbits (group B) produced specific antibody levels corresponding to positive IgG titres from the day 8 until the last serum sample collection. In the i. p. inoculated group (A), all six rabbits were found to be positive later on the day 12.

In the A-group, the levels of specific IFA IgG several times reached titres of 1:4.096 on days 40 to 44 of the experiment. The IFA IgG titre 1:8.192 occured once in rabbit B-11 as the absolutely highest value. In both groups of inoculated rabbits, levels of blood serum anti-*E. cuniculi* antibodies had titers of only 1:512 during the period between days 24 and 32 of the experiment in comparison to higher titres (at least 1:1024) observed before day 24 and after day 32. The control rabbits did not have IFA titers (table 1).

Table 1: IFA titres of rabbit serumm anti-*Encephalitozoon cuniculi* antibodies (1:n) obtained during the hyperimmunization.

	Titer on the indicated day of hyperimmunization												
Rabbits	0	4	8	12	16	20	24	28	32	36	40	44	48
Group A (i.p.)													
A-1	0	16	256	512	1.024	1.024	256	512	1.024	512	2.048	1.024	1.024
A-2	0	32	256	256	1.024	512	128	512	2.048	1.024	4.096	4.096	2.048
A-3	0	16	16	128	256	256	128	128	1.024	512	2.048	1.024	1.024
A-4	0	16	32	256	1.024	1.024	256	128	512	1.024	2.048	4.096	2.048
A-5	0	64	512	1.024	1.024	512	512	256	512	1.024	4.096	4.096	2.048
A-6	0	32	128	256	512	512	256	512	1.024	1.024	2.048	4.096	1.024
Group B (i.p.)													
B-7	0	64	512	1.024	1.024	1.024	256	1.024	512	512	1.024	2.048	2.048
B-8	0	16	128	512	1.024	256	128	1.024	512	1.024	4.096	2.048	1.024
B-9	0	64	256	512	1.024	512	256	512	512	512	2.048	4.096	1.-24
B-10	0	16	128	512	512	256	128	128	256	512	1.024	1.024	4.096
B-11	0	64	512	1.024	1.024	512	256	512	1.024	2.048	4.096	8.192	2.048
B-12	0	32	256	1.024	1.024	512	128	256	256	1.024	2.048	4.096	2.048
Group C													
C-13 - C-18	0	NT	NT	NT	NT	0	NT	NT	NT	NT	0	NT	NT

NT – not tested

Initial inoculation Second inoculation Final inoculation

The dynamics of the antibody production was characterized by three different stages in both infected rabbit groups (Fig1.):

1. *Progressive ascent of the IFA IgG titres after the initial antigen doses* (1 x 10^8 and 1x10^4 respectively) from day 4 to day 16.

2. *The descent and dysbalance of the antibody levels where the titers never dropped under the value of 1:128 between days 20 and 28.*

3. The expressed increase and day 44 culmination of antibody production followed by a slight titre descent on day 48.

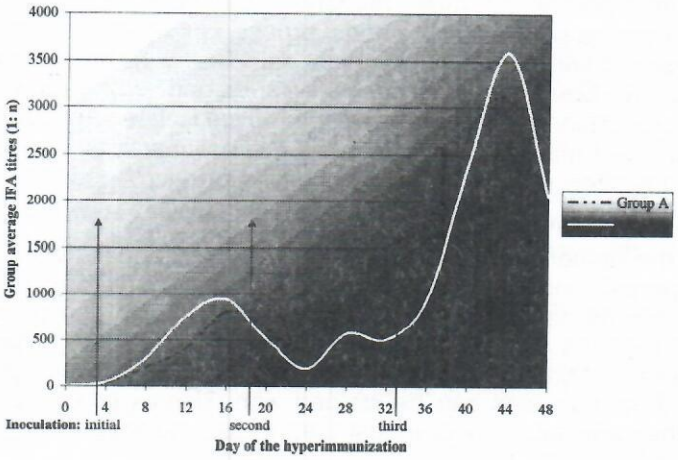

Figure 1. Dynamics of anti *E. cuniculi* antibody production in hyperimmunized rabbit groups as recognized by the IFA test.

DISCUSSION

Previous results have generally shown that laboratory rabbits are really suitable animals for producing hyperimmune antisera after multiple inoculation of microsporidian *Encephalitozoon cuniculi.* Equally, cell culture processed agent spores of different species origin (murine) are utilizable in developing specific antibodies to *E. cuniculi* However, in spite of relatively high infective doses, in vitro grown spores do not appear to be sufficiently pathogenic because clinical signs and pathological changes were missing in infected animals.

In our experiment, each of the immunized rabbits developed anti-*E cuniculi* antibodies after first inoculation. Ascent of the humoral immune response was more marked in intravenously inoculated rabbits (group B), where positive titres of at least 1:64 occurred in 3/6 already on day 4 and in 6/6 on day 8. The highest production of the antibodies in both immunized groups was observed after the third infection on days 40 and 44 respectively, when the levels

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HIPERIMUNIZACIJA KUNIĆA SA IN VITRO KULTIVISANIM ENCEPHALITOZOON CUNICULI

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SADRŽAJ

Specifični hiperimuni antiserum na *Encephalitozoon cuniculi* proizveden je na imunikompetentnim kunićima. Imunizacija je sprovedena trokratno sa sporama mikrospoidija koje su odgajene na kulturi ćelija. Kunići A grupe su imunizovani intraperitonealnom, a B grupe intravenskom aplikacijom agensa, dozama sa opadajućim brojem spora u 16-todnevnim intervalima. Antitela su se pojavila kod svih kunića u roku od 4–12 dana, a titar se održavao do 48 dana, kada je, nakon treće inokulacije naglo porastao nivo antitela. Produkcija specifičnih antitela je pokazivala određenu dinamiku i uočene su tri faze i titar antitela je prvo kulminirao 16 dana, da bi se uočilo blago opadanje nakon druge inokulacije, a nakon treće doze titar je naglo rastao i kulminirao 44 dana. Ovo ukazuje da je primenjeni režim imunizacije pogodan za pripremu specifičnog hiperimunog seruma.