Acta Veterinaria Hungarica 52 (1), pp. 61–69 (2004)

IMMUNE RESPONSE IN MICE INFECTED BY *ENCEPHALITOZOON CUNICULI* AND SUPPRESSED BY DEXAMETHASONE

Alexandra VALENČÁKOVÁ[1*], Monika HALÁNOVÁ[2], P. BÁLENT[1], Emília DVOROŽŇÁKOVÁ[3], Erika JAMBOROVÁ[2], F. LEŠNÍK[1], J. NEUSCHL[1], Ľ. PÁLENÍK[1] and Lýdia ČISLÁKOVÁ[2]

[1]University of Veterinary Medicine, Komenského 73, 041 81 Košice, Slovak Republic;
[2]Faculty of Medicine, P. J. Šafárik University, Košice, Slovak Republic;
[3]Slovak Academy of Science, Košice, Slovak Republic

(Received March 25, 2003; accepted June 19, 2003)

Several indicators of immune response were observed in immunocompetent mice of the ICR line and those suppressed by dexamethasone upon their experimental infection with the microsporidia of *Encephalitozoon cuniculi.* The mice were infected by one-shot intraperitoneal administration of 5×10^7 pathogenic spores. On Days 7, 14, 28 and 42 after infection, peripheral blood leukocyte phagocytic activity was determined and compared, including phagocytic index and the blastogenic response in spleen cells to mitogenic activation by concanavalin A and phytohaemagglutinin. The results point to the fact that *E. cuniculi* itself can cause a significant decrease in phagocytic activity of phagocytic leukocytes in the early stages of infection as well as a remarkable decrease in the proliferative response of spleen cells to T-cellular mitogens.

Key words: *Encephalitozoon cuniculi,* immunosuppression, mice, phagocytic activity, proliferative response

Recently there has been an increasing interest in the study of encephalitozoonosis in animals and humans, primarily due to the publication of evidence of its zoonotic character. One of several possible sources of infection is the consumption of water contaminated with microspores in locations with lower hygienic standards. Surface waters are generally known as the environmental source of microsporidia (Avery and Undeen, 1987). Large numbers of microsporidian spores can get into the environment from the body excretions of infected hosts. Spores can survive in the environment and remain infective for several weeks. Some spores can withstand extremes of temperature in distilled non-chlorinated

[*]Corresponding author; Alexandra Valenčáková, DVM, Department of Biology, University of Veterinary Medicine, Komenského 73, 041 81 Košice, Slovak Republic; E-mail: valencakova@uvm.sk; Fax: +421556325835

water, pH changes, as well as the process of multiple freezing and thawing, while survival may reach up to 10 years (Waller, 1979).

Most of the knowledge about encephalitozoonosis is based on the results obtained by the experimental infection of laboratory animals with *Encephalitozoon cuniculi*.

The information acquired by means of both natural and experimental infection of laboratory animals provides a basis for the understanding of the host-parasite relationship in human encephalitozoonosis.

The objective of this work was to find out whether the immunosuppression caused by dexamethasone can affect the activation of encephalitozoonosis in experimentally infected mice and, at the same time, whether *E. cuniculi* proper can have an immunosuppressive effect in the course of infection.

Materials and methods

Encephalitozoon cuniculi *spores*

The murine isolate of *E. cuniculi* was propagated on RK 13 cell line (Valenčáková et al., 2002). Fresh spores were used for intraperitoneal application in the dosage of 5×10^7/ml in 0.5 ml PBS 12 days after the beginning of dexamethasone application.

Dexamethasone

Dexamethasone in the form of sodium salt (Dexamed, Medochemie, Czech Republic) was diluted in water to be applied by injections so that the concentration of the applied volume of 0.025 ml per 10 g of murine weight was 0.4, 0.8, 1.2 and 1.6 mg/kg for i.m. application.

Laboratory animals

Forty-eight 4-month-old female mice of the ICR line (Faculty of Medicine, University of Pavol Jozef Šafarik, Slovak Republic) of 35–40 g average weight were included in the experiment. They were divided into four groups (n = 12) and placed in plastic trays with free access to water. They were fed standard mice-feeding granules – MP feeding mixtures (Dobrá Voda, Slovak Republic). The mice had been bred in compliance with the regulations. All mice were serologically examined for the presence of *E. cuniculi* antibodies before the experiment, with negative results.

Experimental scheme

Group 1	Group 2	Group 3	Group 4
K	**EC**	**DEXA + EC**	**DEXA**
Control	*E. cuniculi*	Dexamethasone + *E. cuniculi*	Dexamethasone

Physiological saline solution of 0.1 ml volume was applied i.m. in the first group of mice at the time intervals equivalent to those of dexamethasone application in the experimental groups. In the second group, a single dose of 5×10^7/ml of *E. cuniculi* spores was administered i.p. in a volume of 0.5 ml per mouse. In the third group, dexamethasone was applied every 48 h in the following doses: 1st dose of 0.4 mg/kg, 2nd and 3rd doses of 0.8 mg/kg, 4th and 5th doses of 1.2 mg/kg. Subsequently, starting from the 12th day up to the end of the experiment, 12 (6–17) consecutive doses of dexamethasone of 1.6 mg/kg were administered every 72 h. Additionally, on the 12th day of the experiment in this group, a single dose of 5×10^7/ml of *E. cuniculi* spores was administered i.p. in a volume of 0.5 ml per mouse. In the 4th group, dexamethasone was applied according to the same timetable as in Group 3.

Phagocytic activity and phagocytic index

At 7, 14, 28 and 42 days of the experiment, three mice selected from each group were treated with ether and killed by decapitation. Heparinised blood samples (5 units/ml) were used to test the phagocytic activity of all leukocytes mixed with hydroxymetacrylate particles (MSHP kit, ARTIM, Czech Republic). Phagocytic index. i.e. the ingestion capacity of leukocytes (the number of hydroxymetacrylate particles in one leukocyte) was determined according to Větvička et al. (1982).

Lymphocyte proliferation

Cells isolated under sterile conditions from spleen homogenate were washed twice with Dulbecco's PBS (pH 7.2) and once with RPMI 1640 (PAN, Biotech, Germany). Erythrocytes were lysed in 0.84% NH_4Cl. Lymphocytes were diluted to a concentration of 5×10^6 cells in 1 ml of medium. Both from SIGMA, phytohaemagglutinin (PHA) and concanavalin A (Con A) were used at 5 µg/ml concentration as T-cell polyclonal activators. The lymphocytes and mitogens were placed in a volume of 200 µl (RPMI 1940 with 15% fetal bovine serum and gentamicin diluted 1 ml/l) in closed Eppendorf tubes and kept under sterile conditions in a CO_2 incubator at 37 °C for 72 h. Then 3[4.5/dimethyl–thiazolyl]/2.5/diphenyltetrazolium bromide (MTT, SIGMA) of 2% concentration was added to the test tubes in a volume of 20 µl. After 4 h the tubes were centrifuged and 1 ml of dimethyl sulphoxide (SIGMA) was added to the pellet (Page et al., 1988). The level of proliferative activity was measured by colorimetry at 540 and 680 nm. The degree of proliferation was expressed as the stimulation index (SI) by the formula:

$$SI = \frac{540–680 \text{ (stimulated cells)}}{540–680 \text{ (unstimulated cells)}}$$

Indirect immunofluorescence antibody test (IFAT)

The indirect immunofluorescence method was used to test a positive serological titre (Chalupský et al., 1973). Antigens of *E. cuniculi* fixed on the slides were covered by the examined serum and swine anti-mouse immunoglobulin fluorescein isothiocyanate conjugate of 1:64 dilution (SwAM-FITC, SEVAC, Czech Republic).

Data evaluation

The results were statistically evaluated by one-way analysis of variance (ANOVA) with post hoc Bonferroni multiple-comparison test and expressed as mean ± SEM. Differences between groups at the level of $p < 0.05$ were considered significant.

Results

On Day 7 after infection, the phagocytic activity in the infected mice decreased rapidly in comparison with that in the controls, from 66.67% to 35.3% in Group EC to 30% in Group DEXA + EC, and finally to 16.3% ($p < 0.001$) in Group DEXA. This low phagocytic activity in Groups DEXA + EC and DEXA was maintained during the entire experiment. In Group EC, in contrast, the phagocytic activity on Day 14 after infection significantly increased in the infected animals as compared with that in the controls to 80.67% ($p < 0.05$), and on Day 28 to as much as 91.3% ($p < 0.001$). On Day 42, the phagocytic activity decreased again to 29% ($p < 0.001$, Fig. 1).

The phagocytic index dropped significantly ($p < 0.001$) in all samples in all three experimental groups compared to the controls (Fig. 2).

Proliferative response of T-lymphocytes to the Con A mitogen expressed by the stimulating index (SI) decreased on Day 7 after infection in Group EC with SI = 0.6 compared with that in the controls (SI = 1.9, $p < 0.01$), as well as in Groups EC + DEXA (SI = 0.7) and DEXA (SI = 0.7, $p < 0.05$). On Day 14 after infection, the SI in Group EC rose to 1.4 compared with the control group where it was 1.16. In the other experimental groups of EC + DEXA (SI = 0.6) and DEXA (SI = 0.8), on Day 28 the stimulating index obtained with Con A-activated lymphocytes decreased insignificantly as compared with the controls (IS = 1.6) (Fig. 3).

On Day 7 after infection, the proliferative activity of PHA-activated lymphocytes expressed by SI decreased in Group EC to 0.4 when compared with the controls (SI = 0.9, $p < 0.05$). On Day 14, a remarkable increase in SI was observed, similarly as with Con A, to SI = 0.9 as compared with the controls (SI = 0.8). In the other experimental groups the SI was remarkably lower than in the controls (SI = 0.9) during the whole experiment, with statistically relevant values in Group EC + DEXA (SI = 0.36) on Day 28 and in Group DEXA (SI = 0.33; SI = 0.36) on Days 28 and 42 ($p < 0.05$, Fig. 4).

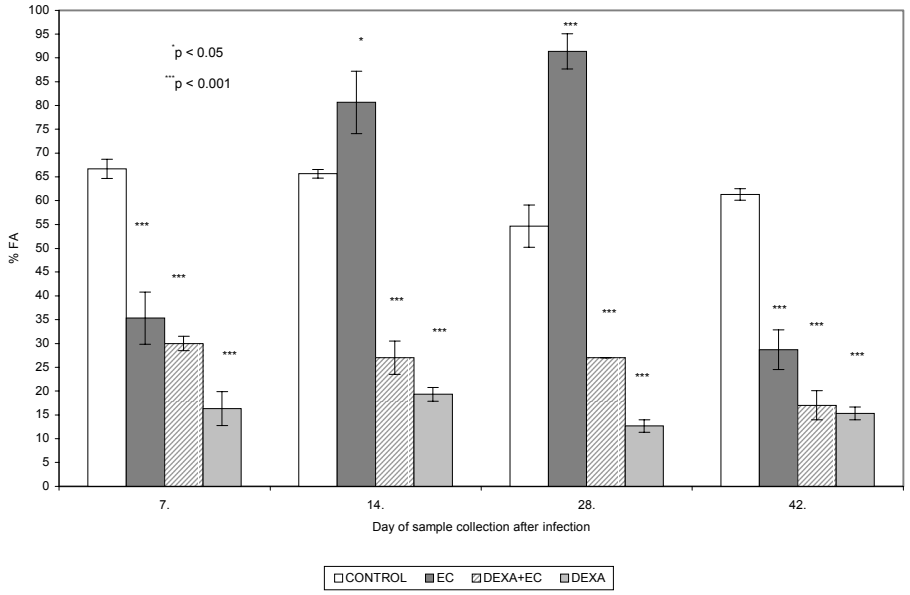

Fig. 1. Phagocytic activity of mice infected by *E. cuniculi* and suppressed by dexamethasone

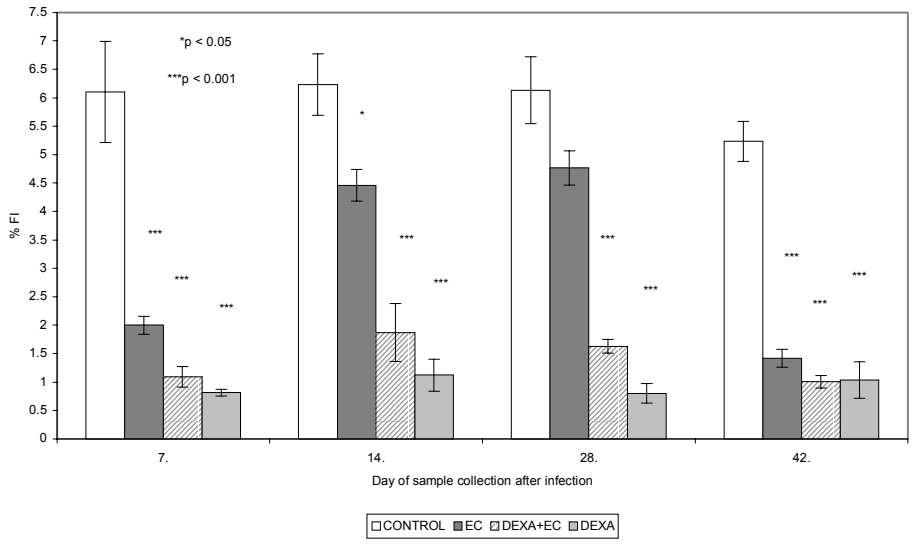

Fig. 2. Phagocytic index of mice infected by *E. cuniculi* and suppressed by dexamethasone

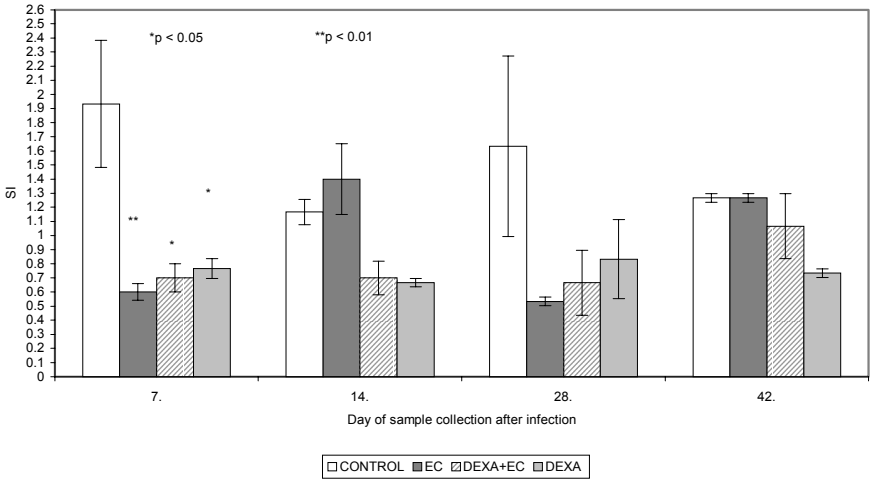

Fig. 3. Concanavalin A induced T-lymphocyte blastogenesis of mice infected by *E. cuniculi* and suppressed by dexamethasone

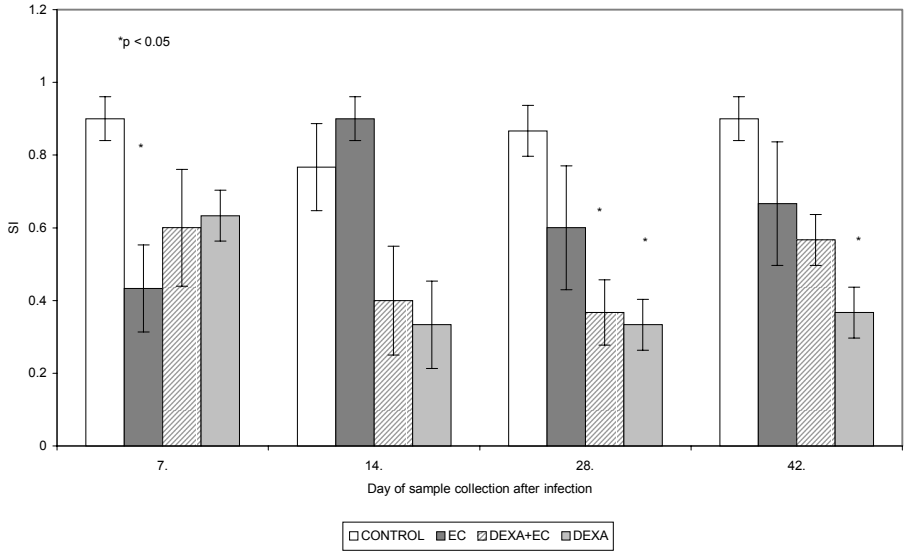

Fig. 4. Phytohaemagglutinin-induced T-lymphocyte blastogenesis of mice infected by *E. cuniculi* and suppressed by dexamethasone

Discussion

It is generally known that encephalitozoonosis causes latent infections in immunocompetent laboratory animals, whereas in immunodeficient or immunosuppressed hosts it leads to the development of clinically apparent infections that

may be lethal (Didier and Shadduck, 1997). Macrophages play an important role in the defence against the disease caused in a host organism by *E. cuniculi.* Infected macrophages assist in spreading the infection; however, they also form an integral part of the immune response to these microsporidia (Didier, 1995). The main effector functions of macrophages include cytokine production (IL-1, IL-3, IL-6, INF-gamma, TNF-alpha), phagocytosis, intracellular killing of pathogens, production of toxic oxygen metabolites, nitrogen oxide and its reactive intermediate products that may serve as indicators of the intensity of phagocytosis. They are able to present an antigen to T-lymphocytes, which makes them engaged indirectly in the T- and B-cellular immune response (Štercl, 1993).

Our results indicate a significant suppression of phagocytic activity and of the ingestion capacity of phagocytes on Day 7 after the infection of immunocompetent mice of line ICR with the spores of *E. cuniculi*. The values are comparable with those obtained in the group of mice suppressed with dexamethasone. In the group of mice with an immune system suppressed with dexamethasone and concurrently infected with *E. cuniculi*, the decrease in the phagocytic activity and in the ingestion capacity of phagocytes was even more remarkable.

Although on Days 14 and 28 the phagocytic activity greatly increased in the mice infected with *E. cuniculi*, giving evidence of the intensive defence of the host organism against infection, the ingestion capacity of phagocytes in these mice was lower. This may result in the survival of *E. cuniculi* and its consequent multiplication and proliferation. Similarly, also in the experiment of Halánová et al. (1998), 15 (93%) and 30 (81%) days after infection the phagocytic activity was increased in mice experimentally infected with *E. cuniculi*, as compared with the controls. This confirms that after infection of the host organism with a foreign pathogen, activation of macrophages and their increased phagocytic activity occur.

Similarly, the spleen cells of mice infected with *E. cuniculi* manifested a significantly lower blastogenic response to the T-cell mitogens concanavalin A and phytohaemagglutinin on Day 7 after infection than those of the uninfected mice. Moreover, the values obtained were lower than those in the group of mice suppressed with dexamethasone, which is consistent with the results reported by Didier and Shadduck (1988). After two weeks the blastogenic response to T-cellular mitogens in the mice infected with *E. cuniculi* returned to normal, but it slightly exceeded the values obtained in the controls even on Day 14 after infection.

Perhaps, spleen cells recovered at 1 week postinfection were not capable of responding to additional proliferation signals from the mitogens *in vitro* due to already having reached their maximum responsiveness. Their ability to recognise mitogenic signals may be saturated or exhausted, thus accounting for the lower than normal responses to mitogens expressed by spleen cells from 1-week infected mice. How such decreased responses to mitogens may aid parasite persistence is difficult to define, especially in the light of the findings that the spleen

cells of *E. cuniculi* infected mice recognise *E. cuniculi* and proliferate in response to it *in vitro* more than spleen cells from uninfected mice. Deficient immune responses lead to clinical signs of disease or to death, as it has been reported to occur in *E. cuniculi* infected hypothymic nude mice (Didier and Shadduck, 1988).

Similar conclusions can be drawn from the work of Niederkorn et al. (1983) who emphasised the relationship between peritoneal infection with *E. cuniculi* with a slightly increased proliferative response of spleen cells to mitogens in resistant BALB/c mice compared with the control group of uninfected mice and a significantly suppressed response in sensitive C57BL/6 mice.

In summary, it should be stated that encephalitozoonosis is a present-day and serious infection chiefly from the point of view of laboratory animals that are employed as animal models for the study of immune mechanisms participating in its suppression. Mice can be infected spontaneously and the absence of manifestations of a disease may lead to unnoticed use of infected animals in research; therefore, medical examination of laboratory animals is inevitable before their use in an experiment.

Acknowledgement

The present work was performed within the framework of grant projects VEGA No. 1/9269/02 and No. 1/0580/03 of the Slovak Ministry of Education.

References

Avery, S. W. and Undeen, A. H. (1987): The isolation of microsporidia and other pathogens from concentrated ditch water. J. Am. Mosq. Control. Assoc. **3,** 54–58.

Chalupský, J., Vávra, J. and Bedrník, P. (1973): Detection of antibodies to *Encephalitozoon cuniculi* in rabbits by the indirect immunofluorescent antibody test. Folia Parasitol. **20,** 281–284.

Didier, E. S. (1995): Reactive nitrogen intermediates implicated in the inhibition of *Encephalitozoon cuniculi* (phylum Microspora) replication in murine peritoneal macrophages. Parasit. Immun. **17,** 405–412.

Didier, E. S. and Shadduck, J. A. (1988): Modulated immune responsiveness associated with experimental *Encephalitozoon cuniculi* infection in BALB/c mice. Lab. Anim. Sci. **38,** 680–684.

Didier, E. S. and Shadduck, J. A. (1997): Immunobiology of microsporidiosis in mammals. In: Abstracts, 2nd Workshop on Microsporidiosis and Cryptosporidiosis in Immunodeficient Patients, České Budějovice, 15 p.

Halánová, M., Bálent, P., Štefkovič, M., El Naas, A., Czizsmárová, G., Hipíková, V., Levkutová, M., Jurčina, A. and Maslej, P. (1998): Observations of the effect of *Encephalitozoon cuniculi* infection on the immune system in mice. Helminthologia **35,** 223–226.

Niederkorn, J. Y., Brieland, J. K. and Mayhew, E. (1983): Enhanced natural killer activity in experimental murine encephalitozoonosis. Infect. Immun. **41,** 302–307.

Page, M., Bejaoui, N., Cinq-Mars, B. and Lemieux, P. (1988): Optimization of the tetrazolium based colorimetric assay for the measurement of cell number and cytotoxicity. Int. J. Immunopharmacol. **10,** 785–793.

Štercl, J. (1993): The Immune System and Its Physiological Functions (in Slovak). Immunol. Society, Prague, 480 pp.

Valenčáková, A., Bálent, P., Malčeková, B., Lešník, F., Halánová, M. and Hipíková, V. (2002): A comparison of mammalian microsporidia species replication in various cell lines. Biologia **57,** 773–776.

Větvička, V., Fornúsek, I., Kopeček, J., Kamínková, J., Kašpárek, I. and Vránová, M. (1982): Phagocytosis of human leukocytes: A simple micromethod. Immunol. Lett. **5,** 97–100.

Waller, T. (1979): Sensitivity of *Encephalitozoon cuniculi* to various temperatures, disinfectants and drugs. Lab. Anim. **13,** 227–230.