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Original article

Survival, immune responses and tissue cyst production in outbred (Swiss white) and inbred (CBA/Ca) strains of mice experimentally infected with *Neospora caninum* tachyzoites

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Abstract – The present work compared inbred (CBA/Ca) and outbred (Swiss white) strains of mice for their capacity to cope with a *Neospora caninum* infection and to consistently produce tissue cysts. In each experiment Swiss white and CBA/Ca mice were given three different doses of NC-1 tachyzoites. Lymphoproliferative and humoral responses as well as cytokine production were evaluated eight weeks after infection (PI) whereas tissue cyst production and histopathology were assessed 4, 6 and 10 weeks PI in immunosuppressed mice. Tissue cysts were observed 10 weeks after infection only in CBA/Ca mice receiving the two highest inoculum doses. Furthermore this strain showed the highest specific lymphoproliferative response. A mixed cytokine response with elevated IFN-γ and fairly low IL-4 and IL-10 secretion was recorded. In both strains, no lesions were observed in the tissues of infected mice. This study indicates that CBA/Ca female mice infected with 5×10^6 NC-1 tachyzoites represent a useful model for the study of specific maternal immune responses in pregnant animals.

Neospora caninum **/ immune response / resistance / cyst / mice**

1. INTRODUCTION

Neospora caninum is an apicomplexan protozoan, infecting a large range of mammals. In cattle, this parasite is now regarded as a major abortifacient [1, 5]. Several works have focused on the specific immune response to *N. caninum*. Resistance to *N. caninum* seems to rely on a type 1 cytokine response. IFN-γ was shown to inhibit in vitro the intracellular replication of *N. caninum* tachyzoites [8]. The important role of IFN-γ and IL-12 in the control of the infection was confirmed later [10]. Under experimental conditions, cattle infected with either tachyzoites or oocysts mounted a significant lymphoproliferative response to parasite antigens and secreted elevated amount of IFN-γ [3, 16, 22]. Specific antibody secretion was also observed and the IgG2 isotype was often noticed as preferentially produced [23]. The use of murine

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models together with the availability of genetically defective strains has allowed the study of numerous immune factors in the resistance to infection. For example, IFN-γ KO mice died during the acute phase of a *N. caninum* infection whereas wild type survived [19, 20]. B-cell deficient mice displayed higher susceptibility to *N. caninum* infection and lower IFN-γ production [6] and mice depleted of $CD4^+$ cells showed higher mortality than $CD8^+$ cell depleted or control mice which demonstrates the importance of effector lymphocytes in the resistance to infection [21]. Mouse models were used also to study vertical transfer of *N. caninum*. A differential rate of transmission depending on the period of gestation at the time of infection was reported [2]. Tissue cyst production in mice has also been assessed. Mc Guire et al. [18] tested six variables to improve tissue cyst production in mice. They showed that immunosuppressed male ICR mice produced more tissue cysts than females ICR, CBA/Ca and Balb/c mice. Nevertheless, not all the animals harboured cysts. A murine model would enable studies of the immune response accompanying the reactivation of a latent form of neosporosis such as is observed during pregnancy in cattle [7]. Although Kobayashi et al. [11] did not observe reactivation of *N. caninum* infection in mice receiving steroid hormones, pregnancy is a complex phenomenon and the use of hormones alone does not fully mimic the physiological environment existing during pregnancy. Resistance to *N. caninum* infection as well as tissue cyst production seems to depend on the strain of mouse used [14, 18]. In order to mimic cattle infection, a murine model should meet the following criteria: survival of the host after infection and establishment of a persistent infection i.e. the production of tissue cysts. In this study, we investigate the susceptibility of two strains of mice following inoculation with three different doses of NC-1 tachyzoites and production of tissue cysts in the brain.

2. MATERIALS AND METHODS

2.1. Parasite strain and antigenic material

The tachyzoites of *N. caninum* (NC-1 strain) were maintained by continuous passages in Vero cells grown in RPMI-Stabilix (Biomedia, Boussens, France) supplemented with 100 UI/mL penicillin, 100 µg/mL streptomycin, non essential amino acids, sodium pyruvate and 2% horse serum at 37 °C and under an atmosphere containing 5% CO_2. In order to maintain virulence, tachyzoites used for experimental inoculations were passaged once in mice and thereafter collected from infected Vero cells (cell passage < 10) as described by Long and Bazler [15]. In order to separate the parasites from the cell debris, the suspension was extruded trough a 27-G needle, centrifuged at 165 *g*, washed once in PBS and the parasites were counted in a Neubaeur chamber. For the preparation of the antigenic extract, tachyzoites were treated by three freeze-thawing cycles followed by sonication [9]. Protein concentration was measured by the BCA method, and then the antigenic solution was divided into aliquots and stored at –20 °C until further use.

2.2. Experimental design

CBA/Ca and Swiss white (BK: W) mice aged 6 to 8 weeks were obtained from B and K (Hull, United Kingdom). Female mice were housed in conventional plastic cages and were provided with rodent chow and water ad libitum. Animal experiments were approved by the local ethics committee of the University of Liège.

2.2.1. N. caninum *tissue cyst production*

Three groups of seven CBA/Ca mice were each inoculated with 5×10^6, 1×10^6 or 2×10^5 NC-1 tachyzoites (groups 3, 4, 5) while group C1 (three mice) acted as control. Four groups of Swiss white mice were

Table I. Experimental design, clinical symptoms, mean brain lesion scores and mean cyst numbers (nb).

	Group	Inoculum dose	Number	Clinical outcome	Brain mean lesion score			Mean cyst nb
					4 weeks PI	6 weeks PI	10 weeks PI	10 weeks PI
SW	1	5×10^6	7	2 wk PI emaciation, 3 wk PI 1 dead + head tilting	2.25	1	1.5	0
	2	1×10^6	7	3 wk PI emaciation + hind paresis, head tilting	1.5	0.5	1.5	0
	3	2×10^5	7	3 wk PI emaciation, 1 dead	1.5	0	0*	0
	C1	Nil	3	No symptoms	0	0	0	0
CBA	4	5×10^6	7	2 wk PI 1 dead, hind limb paresis, skin necrosis	1.75	1	1.25	3.5
	5	1×10^6	7	3 wk PI skin necrosis	1.5	2.75§	1.75	2
	6	2×10^5	7	3 wk PI slight skin necrosis	2	1.25	0*	0
	C2	Nil	3	No symptom	0	0	0	0

* Values statistically different from those on week 6 PI.
§ Values statistically different from group 4 and 6 values.

treated similarly (groups 1, 2, 3 and C2) (see also Tab. I).

Mice received 2 mg of methylprednisolone acetate (MPA, Moderin, Pharmacia and Upjohn, Puurs, Belgium) 7 days before and on the day of infection. Surviving mice (two mice per group and one control) were sacrificed 4, 6 and 10 weeks after inoculation and their brains were removed. For each mouse, half the brain was fixed in 10% neutral buffered formalin, processed to paraffine wax and 4 µm-sections cut and stained with haematoxylin-eosin. Two sagital sections of each brain were examined. Lesions were graded using the following system: (0) no lesions; (1) slight (small inflammatory foci, perivascular cuffing, no necrosis); (2) moderate (larger inflammatory foci with necrosis of, up to 5% of the parenchyma); (3) severe (greater than 5% of the parenchyma). The other half of each brain was homogenated with PBS and three 10 µL-droplets were examined under the microscope at × 40 magnification to evaluate *N. caninum* tissue cyst burdens (cyst count).

2.2.2. Resistance to infection

Mice in three groups of three CBA/Ca mice were each inoculated with 5×10^6, 1×10^6 or 2×10^5 NC-1 tachyzoites (groups 3', 4', 5') while group C1' (three mice) acted as control. Four groups of Swiss white mice were treated similarly (groups 1', 2', 3' and C2'). Mice were sacrificed 8 weeks later and their brain, heart and liver were removed for histological examination. Their spleens were aseptically obtained to allow study of in vitro lymphoproliferation responses and cytokine production. Blood was obtained from the heart and the sera were collected for specific antibody detection (see Sect. 2.5).

2.3. Lymphocyte proliferation assay

Spleens were forced aseptically through a wire mesh. The cells were washed twice in DMEM and diluted in RPMI culture medium containing 50 µM 2-mercaptoethanol. The cell concentration was adjusted to 1.25×10^6 cells/mL and 2.5×10^5 cells in 200 µL were plated in triplicates into 96-well microplates. The wells contained either 20 µL of NC-1 Ag (100 µg/mL), PBS or Concanavalin A (ConA, 50 µg/mL). The plates were incubated for 3 days; then the cells were pulsed for 24 h with 0.5 µCi/well of [methyl-3H] thymidine, harvested onto nitrocellulose filters and the incorporated radioactivity was measured with a β counter (Beckman Instruments, Fullerton, USA) for 3 min. The results were expressed as mean count per minute (CPM) of stimulated wells minus mean CPM of control wells (Δ CPM).

2.4. Cytokine measurements

The in vitro production of different cytokines was evaluated lymphoproliferative supernatants harvested at 72 h, using commercial IFN-γ, IL-4 and IL-10 sandwich enzyme-linked immunosorbent assay kits (mIFN-γ, mIL-4 and mIL-10 Cytoscreen TM ELISA Kit, Biosource International, Camarillo, USA). These assays were performed in duplicate and supernatants were diluted to 1:10 for IFN-γ and IL-10, whereas undiluted supernatants were used for IL-4 testing. According to the manufacturer's specifications, the detection limits were 0.625–80 pg for IL-10, 15.6–1 000 pg for IL-4 and 7.8–500 pg for IFN-γ.

2.5. Quantification of *Neospora*-specific serum IgM, IgG1 and IgG2a

Maxisorp (Nunc, Rocksilde, Denmark) microplate-wells were coated with 100 µL of NC-1 and Vero cell Ag solutions of 15 µg for IgM and 10 µg/mL for IgG measurements. Plates were incubated for one and seven hours at 37 °C and at room temperature (RT) respectively. To block non-specific antibody binding, the plates were coated with casein hydrolysate solution overnight at RT. Sera were run in duplicate at two fold-dilutions (1:100 to 1:12 800) and incubated at 37 °C for 1 h. After washing, 100 µL of biotinylated rat antibody (0.5 µg/mL) to mouse IgM, IgG1 and IgG2a (Serotec, Oxford, United Kingdom) were added for 60 min at 37 °C. After washing, streptavidin-bound peroxidase (Amersham, Piscataway, USA) was added at a dilution of 1:1 000 and left to incubate for one hour. The reaction was then visualised using TMB (3,3', 5,5'-tetramethyl-benzidine) as substrate. The reaction was allowed to develop for 10 (IgG1 and IgG2a) or 15 min (IgM). The 450 nm OD of each well was determined using an electronic plate reader. For each assay, a positive control standard was run. The OD values obtained with Vero cell wells were subtracted from the values recorded for NC-1 coated wells. Each OD value was converted to a titre. A titre was considered as positive when above the mean of negative control + 3 standard deviations. Results from each plate were related to the positive standard.

2.6. Immunohistochemistry

Two paraffin wax sections were cut from the brains of the experimental mice (tissue cyst production) and mounted on Poly-Lysin-coated glass slides. Each individual section was separated from the next one by 500 µm. The sections were processed with an automated capillary immunostainer (Ventana ES Instrument, Tucson, USA). Briefly, sections were incubated with an anti-*N. caninum* rabbit antiserum (1:800). Then, an anti-rabbit biotin-conjugated antibody was added followed by an avidin-peroxidase complex solution. Reactions were visualised by application of 3-amino-9 ethylcarbazole (AEC). Sections were counter-stained with haematoxylin-eosin.

2.7. Statistical analysis

The non-parametric Kruskal-Wallis and Mann-Whitney *U*-tests were used to compare the lymphoproliferative response, cytokine production, brain lesion score and cyst production. All statistical analyses were considered significant at the $p < 0.05$ level.

3. RESULTS

3.1. Tissue cyst production in the brain

First clinical signs appeared two weeks after parasite inoculation. Two Swiss white and one CBA/Ca mouse died. Nervous symptoms consisting in hind limb paresis and/or head tilting were observed (Tab. I). At post mortem examination these mice exhibited a generalised organ congestion. In animals sacrificed four and six weeks after infection macroscopic white spots were seen in the brain. Four weeks after infection, histological examination revealed some perivascular cuffing and a multifocal subacute encephalitis in all mice. In mice inoculated with 5×10^6 NC-1 tachyzoites numerous foci of necrosis, sometimes quite large were present. On week 6, P.I. CBA/Ca mice presented the same pattern but Swiss white mice exhibited some subacute encephalitis without necrosis. Ten weeks after inoculation mean lesion scores in mice receiving 2×10^5 NC-1 tachyzoites were significantly lower than in the other groups ($p < 0.005$). In all mice, lesions were characterised by fibrosis. No cysts were observed in the haematoxylin-eosin stained sections. Microscopic observation of brain homogenate from mice euthanised four and six weeks after inoculation was also negative for cysts. Ten weeks after infection, cysts surrounded by a thick wall were observed in all group 4 mice and in two out of three mice of group 5. Cysts were not observed in any of the other groups.

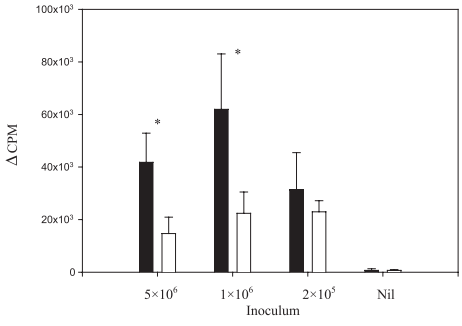

Figure 1. Specific proliferative response of spleen cells from CBA/Ca (■) and Swiss white (□) mice inoculated with 5×10^6, 1×10^6 or 2×10^5 NC-1 tachyzoites. Control mice (C) were given 2×10^5 Vero cells SC. Δ CPM refers to the difference between stimulated and unstimulated cells and is expressed as the means of triplicate values ± SD. * Values significantly different from those of corresponding SW group.

3.2. Resistance to infection

All mice remained clinically normal throughout the experiment. Brain, heart and liver sections were free of lesions. *Neospora caninum* specific IgM, IgG1 and IgG2a antibody levels were recorded in all groups. Levels of IgG1 and IgG2a isotypes were very high, as the signal was still positive at a 1:12 800 dilution in all infected groups; there was no preferential secretion of any isotype. Cell-mediated immunity as assessed by in vitro lymphocyte proliferation assay in the presence of NC-1 Ag, ConA or PBS indicated that eight weeks PI a significantly higher response was present in CBA/Ca mice when compared to Swiss white animals ($p < 0.05$) (Fig. 1). The mean value of Swiss white mice was about half the response of CBA/Ca mice whereas negligible specific lymphoproliferative responses were recorded in control groups. Interleukin-4, 10 and IFN-γ production was measured in the supernatants of stimulated or unstimulated spleen cells maintained in vitro (see above). IL-4, IL-10 or IFN-γ were undetectable in supernatants

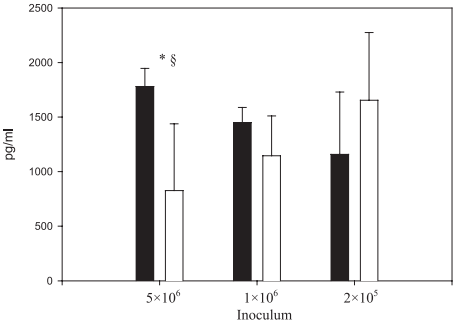

Figure 2. Production of IFN-γ by spleen cells from CBA/Ca (■) and Swiss white (□) mice receiving 5×10^6, 1×10^6 and 2×10^5 NC-1 tachyzoites. Cells were stimulated for 72 h by NC-1 antigen. IFN-γ was measured by ELISA and expressed as the mean result of three mice ± SD. The presented data report the value of stimulated cells. * Values significantly different from those of CBA receiving 1×10^6 NC1. § Values significantly different from those of corresponding SW group.

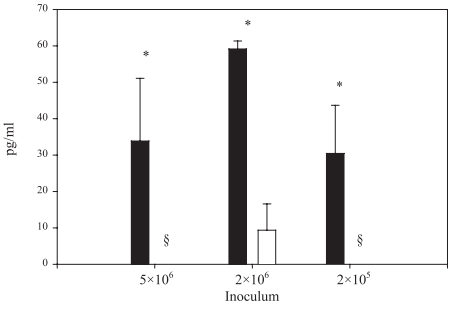

Figure 3. Production of IL-4 by spleen cells from CBA/Ca (■) and Swiss white (□) mice receiving 5×10^6, 1×10^6 and 2×10^5 NC-1 tachyzoites. Cells were stimulated for 72 h by NC-1 antigen. IL-4 was measured by ELISA and expressed as the mean of three mice ± SD. The presented data report the value of stimulated cells. * Values significantly different from those of corresponding SW group. § Undetectable levels.

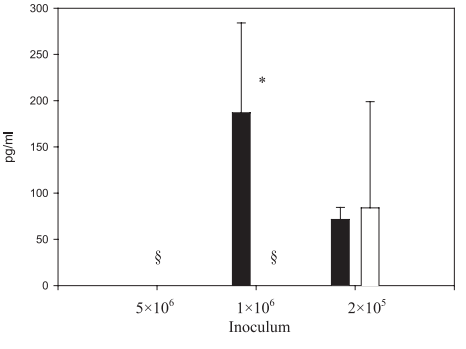

Figure 4. Production of IL-10 by spleen cells from CBA/Ca (■) and Swiss white (□) mice receiving 5×10^6, 1×10^6 and 2×10^5 NC-1 tachyzoites. Cells were stimulated for 72 h by NC-1 antigen. IL-10 was measured by ELISA and expressed as the mean of three mice ± SD. The presented data report the value of stimulated cells. * Values significantly different from those of corresponding SW group. § Undetectable levels.

from unstimulated cultures whereas splenocytes from infected mice in the presence of NC-1 Ag produced elevated levels of IFN-γ. The amount of IFN-γ produced was significantly higher in mice of group 4 than in the corresponding groups of Swiss white mice (Fig. 2). Th2 cytokines such as IL-4 and IL-10 gave highly variable results in the different groups. IL-4 was detected in all groups of CBA/Ca mice (Fig. 3) whereas IL-10 secretion was undetectable in the groups given the highest inoculum dose (groups 1' and 4') (Fig. 4). IL-4 and IL-10 level were detected in groups 2' and 3' respectively but IL-4 level was lower than in group 5' mice ($p < 0.05$).

4. DISCUSSION

In the present study, the immune response to experimental infection with *N. caninum* of two strains of mice was evaluated as well as the production of tissue cysts in the brain. Tissue cysts were observed in immunosuppressed mice 10 weeks after infection but cyst production could have occurred earlier as the preceding examination was performed four weeks before. The two groups of CBA/Ca mice receiving the highest doses of inoculum harboured tissue

cysts whereas none were observed in any of the Swiss white mice. Swiss white mice are naturally resistant to *N. caninum* infection and cysts have only been occasionally observed from 21 days PI in immunosuppressed mice [12]. Tissue cyst production has been demonstrated in immunosuppressed CBA/Ca mice inoculated with NC-2 or NC-Liverpool tachyzoites but not all of the mice investigated harboured tissue cysts and the rate of mortality was high (31%) [18]. The NC-1 strain was used in the present study as it is known that different strains exhibit differing levels of pathogenicity and abilities to produce tissue cysts. In this respect, Lindsay and Dubey [13] observed higher mortality rates in mice inoculated with the NC-1 versus NC-2 strains but they did not compare the number of tissue cysts produced, whereas Mc Guire et al. [18] observed greater numbers of tissue cysts when using the NC-Liverpool rather than the NC-2 strain. As *N. caninum* is an intracellular pathogen, resistance to infection relies mainly on the development of a cellular immune response. Some data suggest that the resistance to *N. caninum* infection depends on the Th1:Th2 cytokine ratio rather than on a strict Th1 response. In this respect, Long et al. [14] reported that the IFN-γ:IL-4 ratio was higher in B10D2 resistant mice than in susceptible strains as demonstrated by reduced cerebral lesions and parasite load in B10D2 mice. In the present study, a highly specific lymphoproliferative response was observed in immunocompetent mice eight weeks after a primary infection; this lymphoproliferative response was higher in CBA/Ca mice and was accompanied by a mixed cytokine secretion pattern, characterised by high levels of IFN-γ and lower levels of IL-4 and IL-10, these latter cytokines being especially high in CBA/Ca mice. Despite this Th2 cytokine secretion, the infection was well tolerated as demonstrated by the absence of clinical symptoms and histological lesions. Furthermore no difference in susceptibility was observed between the outbred and inbred strains of mice although Th2 cytokine levels were higher in inbred mice. Lunden et al. [17] showed that resistance against neosporosis in immunised BALB/c is related to high IFN-γ secretion and that protection is observed even in the presence of high IL-4 and IL-5 levels. Th2 cytokine production is also observed after a *T. gondii* infection, in which it is considered to allow the control of an excessive Th1 cytokine secretion and subsequent immunopathology [4]. In the present study, no preferential isotype secretion was observed corresponding to the mixed pattern of the cytokine response. As demonstrated in the present study, the mouse strain and inoculum doses used are important variables that will influence the results obtained when using a mouse model. These two strains of mice were equally resistant to *N. caninum* infection despite a quite different immune response but only one was able after immunosuppression to produce consistently detectable levels of tissue cysts. In conclusion, the present study indicated that immunosuppressed female CBA/Ca mice given 5×10^6 NC-1 tachyzoites are able to survive and to consistently develop tissue cysts. This mouse model fulfils the criteria mentioned above and could be useful for further investigations on the host-parasite relationships.

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REFERENCES

[1] Buxton D., Protozoan infections (*Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis*

spp.) in sheep and goats: recent advances, Vet. Res. 29 (1998) 289–310.

[2] Cole R.A., Lindsay D.S., Blagburn B.L., Dubey J.P., Vertical transmission of *Neospora caninum* in mice, J. Parasitol. 81 (1995) 730–732.

[3] De Marez T., Lidell S., Dubey J.P., Jenkins M.C., Gasbarre L., Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular immune responses, Int. J. Parasitol. 29 (1999) 1647–1657.

[4] Denkers E.Y., Gazzinelli R.T., Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection, Clin. Microbiol. Rev. 11 (1998) 569–588.

[5] Dubey J.P., Neosporosis in cattle, J. Parasitol. 89 Suppl. (2003) S42–S56.

[6] Eperon S., Bronnimann K., Hemphill A., Gottstein B., Susceptibility of B-cell deficient C57BL/6 (μMT) mice to *Neospora caninum* infection, Parasite Immunol. 21 (1999) 225–236.

[7] Guy C.S., Williams D.J.L., Kelly D.F., McGarry J.W., Guy F., Bjorkman Smith R.F., Trees A.J., *Neospora caninum* in persistently infected, pregnant cows: spontaneous transplacental infection is associated with an acute increase in maternal antibody, Vet. Rec. 149 (2001) 443–449.

[8] Innes E.A., Panton W.R.M., Marks J., Trees A.J., Holmdahl J., Buxton D., Interferon gamma inhibits the intracellular multiplication of *Neospora caninum*, as shown by incorporation of 3H Uracil, J. Comp. Pathol. 113 (1995) 95–100.

[9] Innes E.A., Panton W.R.M., Sanderson A., Thomson K.T., Wastling J.M., Maley S., Buxton D., Induction of $CD4^+$ and $CD8^+$ T cell response in efferent lymph responding to *Toxoplasma gondii* infection: analysis of phenotype and function, Parasite Immunol. 17 (1995) 151–160.

[10] Khan I.M., Schwartzman J.D., Fonseka S., Kasper L.H., *Neospora caninum*: role for immune cytokines in host immunity, Exp. Parasitol. 85 (1997) 24–34.

[11] Kobayashi A., Katagiri S., Kimura T., Ochiai K., Umemura T., Steroid hormones do not reactivate *Neospora caninum* in ovariectomized mice, J. Vet. Med. Sci. 64 (2002) 773–777.

[12] Lindsay D.S., Dubey J.P., *Neospora caninum* (Protozoa: Apicomplexa) infections in mice, J. Parasitol. 75 (1989) 772–779.

[13] Lindsay D.S., Dubey J.P., Infections in mice with tachyzoites and bradyzoites of *Neospora caninum* (Protozoa: Apicomplexa), J. Parasitol. 76 (1990) 410–413.

[14] Long M.T., Baszler T.V., Mathison B.A., Comparison of intracerebral parasite load, lesion development, and systemic cytokines in mouse strains infected with *Neospora caninum*, J. Parasitol. 84 (1998) 316–320.

[15] Long M.T., Baszler T.V., Neutralisation of maternal IL-4 modulates congenital protozoal transmission: comparison of innate versus acquired immune responses, J. Immunol. 164 (2000) 4768–4774.

[16] Lunden A., Marks J., Maley W.M., Innes E.A., Cellular immune response in cattle experimentally infected with *Neospora caninum*, Parasite Immunol. 20 (1998) 519–526.

[17] Lunden A., Wright S., Allen J.E., Buxton D., Immunisation of mice against neosporosis, Int. J. Parasitol. 32 (2002) 867–876.

[18] McGuire A.M., McAllister M.M., Jolley W.R., Anderson-Sprecher R.C., A protocol for the production of *Neospora caninum* tissue cysts in mice, J. Parasitol. 83 (1997) 647–651.

[19] Nishikawa Y., Tragoolpua K., Inoue N., Makala L., Nagasawa H., Otsuka H., Mikami T., In the absence of endogenous gamma interferon, mice acutely infected with *Neospora caninum* succumb to a lethal immune response characterized by inactivation of peritoneal macrophages, Clin. Diagn. Lab. Immunol. 8 (2001) 811–817.

[20] Ritter D.M., Kerlin R., Sibert G., Brake D., Immune factors influencing the course of infection with *Neospora caninum* in the murine host, J. Parasitol. 88 (2002) 271–280.

[21] Tanaka T., Hamada T., Inoue N., Nagasawa H., Fujisaki K., Suzuki N., Mikami T., The role of $CD4^+$ or $CD8^+$ T cells in the protective immune response of BALB/c mice to *Neospora caninum* infection, Vet. Parasitol. 90 (2000) 183–191.

[22] Trees A.J., McAllister M.M., Guy C.S., McGarry J.W., Smith R.F., Williams D.J.L., *Neospora caninum*: oocyst challenge of pregnant cows, Vet. Parasitol. 109 (2002) 147–154.

[23] Williams D.J.L., Guy C.S., McGarry J.W., Guy F., Tasker L., Smith R.F., McEachern K., Cripps P.J., Kelly D.F., Trees A.J., *Neospora caninum*-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival, Parasitology 121 (2000) 347–358.