FULL PAPER *Parasitology*

Steroid Hormones Do Not Reactivate *Neospora caninum* in Ovariectomized Mice

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ABSTRACT. The direct effects of three steroid hormones (progesterone, estradiol-17β and corticosterone) on the growth of *Neospora caninum* (*N. caninum*) tachyzoite were examined in Vero cells. Subsequently, ovariectomized BALB/c mice infected with *N. caninum* were treated with physiological concentrations of the steroid hormones for 1 or 2 weeks. These hormones had no direct effect on the parasite growth *in vitro*. In the infected mice, there was no significant difference in the parasite distribution and histopathological changes between the hormone-injected and control groups. No mice showed parasitemia at the time of autopsy. These results suggest that physiological levels of steroid hormones (progesterone, estradiol-17β and corticosterone) do not reactivate *N. caninum* in mice.

KEY WORDS: corticosterone, estradiol-17β, latent infection, *Neospora caninum*, progesterone.

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Neospora (N.) caninum is a recently described coccidial parasite that is closely related to *Toxoplasma gondii* (*T. gondii*) [4, 5]. *N. caninum* causes abortion and neonatal death in ruminants [19] and paralysis and death in young dogs [4]. Serological studies suggest that congenital transmission rather than post-natal infection play an important role in maintaining of *N. caninum* infection in a herd [7]. Infected cows repeat abortions or parturition of asymptomatic but congenitally infected calves [1, 3, 16], therefore it is suspected that *N. caninum* is sustained in a herd by vertical transmission for generations. In addition, serological evidence indicates that reactivation of the parasite occurs in infected cows at mid-pregnancy [17]. Reactivation of *N. caninum* and subsequent parasitemia were caused by pregnancy in latently infected mice [20]. From these findings, reactivation of the parasite followed by parasitemia is considered essential for vertical transmission. However, the mechanism of the reactivation remains unknown. Supraphysiological concentrations of estrogen and corticosterone decrease resistance to *T. gondi*i infection in mice [14]. Immunosuppression using prednisolone caused *N. caninum* reactivation and parasitemia in latently infected mice [20]. These findings indicate that steroid hormones have an effect on *N. caninum* reactivation.

In this study, the direct effects of three steroid hormones (progesterone, estradiol-17β and corticosterone) on the parasite growth were evaluated *in vitro*. Subsequently, we injected physiological concentrations of the three hormones into ovariectomized BALB/c mice infected with *N. caninum*. Parasite distribution and histopathological changes in these mice were examined to investigate the relationship between the steroid hormones and *N. caninum* reactivation.

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MATERIALS AND METHODS

Cell culture and mice: Vero cells were maintained in 25 cm^2 tissue culture flasks, using Eagle's Minimal Essential Medium (MEM) supplemented with 4 mM L-glutamine, MEM vitamin solution, MEM essential amino acids, MEM non-essential amino acids and 10% horse serum. Twenty-seven BALB/c A mice (4 weeks old, females) were purchased from CLEA JAPAN Inc. (Tokyo, Japan). They were housed in germ-free isolators with controlled air, light/dark cycle (12 hr), temperature (23 ± 1°C) and humidity (55 ± 7%) and fed sterile food (CLEA JAPAN Inc.) and water.

Parasite: *N. caninum* (BT-3 strain) was cultivated on Vero cells in 25 cm^2 tissue culture flasks, as previously described [22]. For purification of tachyzoites, a mixture of host cells and parasites harvested with a cell scraper was pelleted by centrifugation at 700 × g for 10 min and passed through a 27-gauge(G) needle to rupture host cells. Tachyzoites and host cell debris were layered on the top of a discontinuous three-layer percoll (Pharmacia, Uppsala, Sweden) gradient (30, 50 and 80% percoll diluted in phosphate-buffered saline (PBS) containing 0.15 M NaCl). Following centrifugation at 2,500 × g and 4°C for 30 min, the parasite fraction between 50 and 80% percoll was collected and washed with PBS two times. These tachyzoites were resuspended in PBS and counted by hemocytometer.

Hormone: Progesterone, estradiol-17β, and corticosterone (Sigma Chemical Co., St. Louis, MO) were dissolved in 99.5% ethanol for use *in vitro* and in sesami oil to inject mice.

Inoculation and experimental design: Monolayer cultures of Vero cells were infected with 1×10^6 tachyzoites. The medium was replaced with new medium containing appropriate concentrations of each steroid hormone at 8 hr post-inoculation. The concentration of each hormone was adjusted to physiological serum levels in pregnant mice (progesterone 100 *n*g/m*l*, estradiol-17β 2 *n*g/m*l*, and corticosterone 1,000 *n*g/m*l*) [2, 9, 11]. The effects of the hor-

mones at ten times higher or lower concentrations than the physiological level were also examined.

Twenty-seven mice, treated subcutaneously (*s.c.*) with 4 mg of prednisolone at 5 and 6 weeks old, were inoculated *s.c.* with purified tachyzoites (1.5×10^6) suspended in PBS (0.2 m*l*) simultaneously with prednisolone at 6 weeks of age. Four weeks after the inoculation, three mice were sacrificed and it was confirmed that *N. caninum* DNA was not present in any organs other than the central nervous system (CNS). Thereafter, twenty-one mice were ovariectomized under pentobarbital anesthesia, and the remaining three mice served as a non-operated control. These ovariectomized mice were devided into seven groups (n = 3, respectively). From one week after the operation, the ovariectomized mice received a daily hormone injection *s.c.* for 7 or 14 days at concentrations described as sufficient to produce high serum levels similar to those in pregnant mice [10, 13, 23]. Briefly, progesterone at 100 μg/g/day, estradiol-17β at 4 μg/g/day or corticosterone at 18 μg/g $\times$ 2 times/day (intervals of 12 hr) was injected *s.c.* into mice of three groups for 7 days to examine the effect of each hormone. Furthermore, one group was treated with progesterone (100 μg/g/day) for 14 days to mimic the duration of high serum levels observed in pregnant mice. To examine the cooperative effect of progesterone and estradiol-17β, one group was treated with progesterone (100 μg/g/day) for 7 days and with a mixture of progesterone (100 μg/g/day) and estradiol-17β (4 μg/g/day) for a subsequent 7 days. Control groups of ovariectomized or non-operated mice received vehicle (sesami oil) at 5 μl/g/day for 7 or 14 days. All mice were sacrificed at the end of treatment, and tissue samples were collected. Half of each sample was stored at –20°C for polymerase chain reaction (PCR) analysis. The rest was fixed in 20% neutral buffered formalin for histological and immunohistochemical analysis. Serum samples collected from the mice were stored at –20°C until the examination.

Quantification of parasite growth: The number of tachyzoites was counted at the end of cultivation (36, 72 or 94 hr after hormone addition). Vero cells infected with tachyzoites were scraped and passed through a 27-G needle. Tachyzoites were enumerated by hemocytometer.

Histopathological and immunohistochemical examination: Tissue samples fixed in formalin were embedded in paraffin-wax and sectioned at 4 μm in thickness. These sections were stained with hematoxylin and eosin. Immunohistochemical staining was performed with anti *N. caninum* goat serum (1:6,400, VMRD Inc., Pullman, WA) and Histofine SAB kit (Nichirei Co., Tokyo, Japan).

Indirect fluorescent antibody test (IFAT): IFAT was performed with *N. caninum* (BT-3) tachyzoite antigen slides as previously described [22]. Test sera were titrated in a two-fold dilution from 1:50 to 1:3,200, and the serum from experimentally infected and normal mice was used at 1:200 as a positive and negative control. Fluorescein isothiocyanate (FITC)-labeled goat anti mouse IgG (Cappel, Durham, NC) was diluted at 1:400.

PCR: After thawing, tissue samples were homogenized, and DNA extraction and PCR were performed as previously described [21]. The final 50 μl mixture contained 200 μM dNTP, 1.25 units of Ampli Taq polymerasea (Applied Biosystems Inc., Foster city, CA), 1.5 mM $MgCl_2$ containing PCR buffer (Applied Biosystems Inc.), 25 pmol of the *N. caninum*-specific primers Np 21 and Np 6 [21] and 5 μl of template DNA. The PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide.

Statistical analysis: Tachyzoite growth rate, wet organ weight and serological data were represented as means ± S.E. Comparison analysis were performed using Student's *t*-test. All differences were considered significant at $P<0.05$.

RESULTS

Tachyzoite growth in Vero cells cultured with physiological concentrations of each hormone for 36 or 72 hr was not altered by any treatment (Fig. 1). Furthermore, there was no difference of parasite growth in Vero cells cultured with three graded concentrations of the hormones for 94 hr (Fig. 2).

In the corticosterone-treated mice, moderate atrophy of the spleen was observed. Mean wet weight of the spleen in these mice (53.27 ± 6.16 mg) was significantly decreased compared with that of the control group (112.87 ± 12.82 mg). Uterine weight in the mice treated with estradiol-17β (mean wet weight : 196.6 ± 16.91 mg), corticosterone (242.9 ± 36.63 mg) or both progesterone and estradiol-17β (150.9 ± 13.37 mg) were significantly increased as compared with that of the control group (67.93 ± 18.61 mg). The histopathological changes and immunohistochemical findings in hormone-treated mice were almost same as those

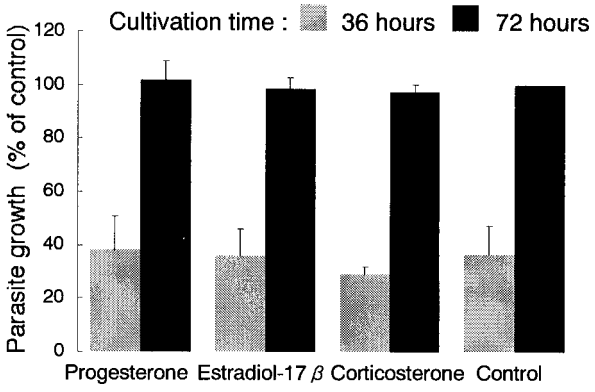

Fig. 1. Effect of steroid hormones on the growth of *N. caninum* tachyzoite with time. Vero cells infected with 1×10^6 tachyzoites were cultured with each hormone (progesterone 100 *n*g/m*l*, estradiol-17β 2 *n*g/m*l* or corticosterone 1,000 *n*g/m*l*) for 36 or 72 hr. Control group was treated with 50 μl of vehicle (99.5% ethanol). The tachyzoites were enumerated at the end of culture. Results are represented as the mean ± S.E. There is no significant difference between hormone-treated and control groups.

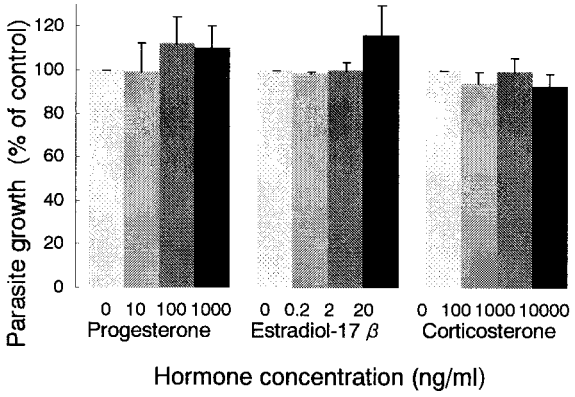

Fig. 2. Effect of steroid hormones on the growth of *N. caninum* tachyzoite at three graded concentrations. Vero cells infected with 1×10^6 tachyzoites were cultured with graded concentrations of each hormone for 94 hr. Control group was treated with 50 μl of vehicle (99.5% ethanol). The tachyzoites were enumerated at the end of culture. Results are represented as the mean ± S.E. No steroid hormone affected tachyzoite growth at any concentration.

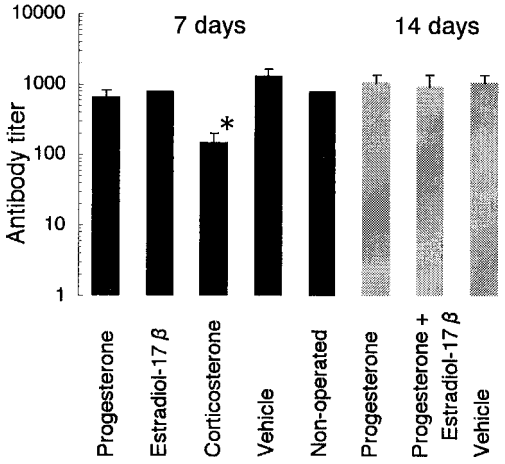

Fig. 3. Anti *N. caninum* antibody titers of infected mice treated with steroid hormones for 7 or 14 days after overiectomy. The antibody titer was determined by IFAT with *N. caninum* tachyzoite antigen slides. The data are expressed as the mean ± S.E. of three mice. *: According to Student's *t* test, the differences between the vehicle and corticosterone-treated group are significant (P=0.012).

of the control mice (Table 1). In the brain, multifocal mild perivascular cuffings composed of lymphocytes, plasma cells and macrophages were observed. There were small necrotic foci with vacuolation of white matter, calcification and moderate infiltrations of macrophages and lymphocytes. Free and clusters of tachyzoites reacting with anti *N. caninum* serum were observed adjacent to the inflammatory lesions and the necrotic foci. Multifocal, mild infiltration of lymphochytes and macrophages were found in the liver. The corticosterone-treated group tended to have mild lesions compared with the other groups. In all mice, *N. caninum* antigens were detected only in the brain. Anti *N. caninum* antibody titers rose to the same levels in all but corticosterone treated mice (Fig. 3). These mice showed significantly lower antibody titers than the vehicle-treated mice (P=0.012). The presence of *N. caninum* DNA was confirmed by PCR in the brains and/or spinal cords of all mice. But other tissues and blood samples were all negative by PCR (Table 1). There was no appreciable difference in the concentration of the PCR products between hormone-treated and control mice (data not shown).

DISCUSSION

In the present study, we investigated the effects of three steroid hormones (progesterone, estradiol-17β, and corticosterone) on *N. caninum* reactivation. These hormones had no direct effect on tachyzoite growth at physiological, and

Table 1. Distribution of inflammatory lesions and *Neospora caninum* DNA in latently infected mice treated with steroid hormones after ovariectomy

Treatment	Number of mice	Lesion/DNA[a)]				
		Brain	Liver	Lung	Pancreas	Blood
7 days treatment						
Progesterone	3	3/3	3/0	0/0	0/0	–/0
Estradiol-17β	3	3/3	3/0	0/0	0/0	–/0
Corticosterone	3	3/3	1/0	0/0	0/0	–/0
Vehicle	3	3/3	3/0	0/0	1/0	–/0
Non-operated	3	3/3	3/0	0/0	1/0	–/0
14 days treatment						
Progesteron	3	3/3	2/0	0/0	0/0	–/0
Progesteron + Estradiol-17β	3	3/3	3/0	0/0	0/0	–/0
Vehicle	3	3/3	3/0	0/0	0/0	–/0

a) Number of mice with inflammatory lesions/number of mice in which *N. caninum* DNA was detected.

even at ten times higher, concentrations *in vitro.* Ovariectomized mice infected with *N. caninum* were treated with physiological concentrations of each hormone. Parasite distribution and histopathological lesions were not changed, and parasitemia was not observed in any mice. These results suggest that physiological levels of these steroid hormones do not activate *N. caninum in vitro* and *in vivo.*

In this study, mice were treated with each hormone at concentrations described as sufficient to produce high serum levels similar to those in pregnant mice [10, 13, 23]. To mimic the condition in pregnant mice, each hormone was administered for 7 days and progesterone was injected for 14 days [2, 9, 11]. Another group was treated with progesterone for 7 days and with a mixture of progesterone and estradiol-17β for a subsequent 7 days to examine the cooperative effect of these hormones. The uterines of mice treated with estradiol-17β, corticosterone or both progesterone and estradiol-17β were increased in size and two to three times heavier than those of vehicle-treated mice. In addition, corticosterone-treated mice showed moderate atrophy of the spleen, suppression of inflammatory cell infiltration and lowered anti *N. caninum* antibody titers. These findings suggest that high serum levels of these hormones were sufficiently maintained in the hormone-treated mice.

Progesterone did not cause *N. caninum* reactivation in the ovariectomized mice in this study. Progesterone, which is present at higher levels in placenta than in plasma, has a suppressive effect on the cell-mediated immune response at the placental concentrations *in vitro* [12, 18], and progesterone have a regional immunomodulatory effect rather than a systemic effect [18]. Even supraphysiological concentrations of progesterone have no effect on mouse resistance to *T. gondii* infection [14]. These studies support that progesterone does not have an indirect effect on *N. caninum* reactivation via suppression of host immunity. Similarly, estradiol-17β and corticosterone did not cause reactivation directly or indirectly in the present study, while these hormones have variable immunomodulatory effects at physiological concentrations. A smaller amount of estradiol-17β than that used in this study decreased the production of TNFα and IFNγ and inhibited recruitment and activation of inflammatory cells in C3H/Ne mice [15]. Restraint stress induces an elevation in serum corticosterone levels accompanying a marked decrease of NK activity and IFNγ production in spleen cells [8]. It has also been reported that supraphysiological levels of estradiol-17β and corticosterone decrease resistance to *T. gondii* infection in mice [14]. Our results suggest that physiological levels of estradiol-17β and corticosterone may not play a crucial role in *N. caninum* reactivation in spite of their suppresive effect on cell-mediated immunity.

In conclusion, physiological levels of steroid hormones, including progesterone, estradiol-17β and corticosterone, may not reactivate *N. caninum* in mice. Other hormones and immunomodulatory factors are active during pregnancy, *e.g.* chorionic gonadotrophin, α-phetoprotein and α_2-macrogroblin [6], and these factors should also be examined as possible causes of *N. caninum* reactivation.

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