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EXPERIMENTAL ENCEPHALITOZOONOSIS IN THE BLUE FOX

TRANSPLACENTAL TRANSMISSION OF THE PARASITE

By

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MOHN, S. F., K. NORDSTOGA and O. M. MØLLER: *Experimental encephalitozoonosis in the blue fox — Transplacental transmission of the parasite.* Acta vet. scand. 1982, *23*, 211—220. — Spores of Encephalitozoon cuniculi were recovered from foetal and placental tissues from blue fox females orally inoculated with the parasite. The results provided evidence for transplacental transmission of the causative agent of fox encephalitozoonosis.

blue fox; encephalitozoonosis; transplacental transmission.

Encephalitozoon cuniculi (E. cuniculi) is an intracellular microsporidian parasite infecting homeothermic animals, including man (*Wilson* 1979). Encephalitozoonosis is commonly seen in conventional rabbit colonies, usually with a subclinical course of disease. In rabbits the infection is generally accepted to be transmitted horizontally almost invariably via the oral route by ingestion of infected or contaminated food or by infected urine. Evidence for vertical transmission via the rabbit placenta has, however, been reported in a few cases (*Hunt et al.* 1972, *Owen & Gannon* 1980). The natural mode of transmission of the parasite in other mammalian species is largely unknown, although vertical transmission has been suggested and transplacental transmission has been reported in cross-fostering studies with mice (*Perrin* 1943) and in a few cases of spontaneous encephalitozoonosis in the squirrel monkey (Saimiri sciureus) (*Anver et al.* 1972, *Brown et al.* 1973).

During recent years great losses associated with E. cuniculi have been reported among young blue foxes (Alopex lagopus) in Norway (*Nordstoga et al.* 1974, *Nordstoga & Westbye* 1976). Vertical transmission of the parasite has been suggested to be the most likely mode of infection as the disease has only appeared spontaneously in pups and young foxes. A similar pattern has been observed in experimental cases after oral and intrauterine inoculation of vixens just before and during the gestation period (*Mohn et al.* 1974, *Nordstoga et al.* 1978). Transplacental transmission of the parasite seems to be the likely mode of infection although other vertical routes cannot be excluded; evidence for horizontal transmission, however, has never been provided. The aim of the present study was to confirm the theory of transplacental transmission of the parasite in the blue fox.

MATERIALS AND METHODS

Organism

A strain of E. cuniculi previously isolated from a blue fox which died from spontaneous encephalitozoonosis was propagated in monolayer cell cultures as decribed elsewhere (*Mohn et al.* 1981). Spores harvested from the cell culture medium were inoculated intraperitoneally into 30 mice of the outbred stock Bom: NMRI f (SPF). Ten days post inoculation the mice were killed, and their bodies cut into pieces and mixed with conventional food.

Vixens

Equal portions of the food were then immediately fed to 10 healthy E. cuniculi sero-negative vixens about 10 months of age which were within the period of 5 to 34 days before mating. The vixens were kept in wire mesh cages in a separate shed. After inoculation they were fed normal food and treated as the other vixens in the farm, including 2 matings with an interval of 2 days.

Offspring

Litters were delivered by caesarian section on the 50th to the 53rd day after the second mating. Some of the pups were euthanized immediately after delivery and their organs as well as the placentae removed for mouse inoculation and histological examination. The other pups were, immediately after delivery,

marked and transferred to non-inoculated presumably E. cuniculi-free vixens which had given birth normally on the previous or the same day. These pups as well as the foster-mothers' own pups were all followed clinically and by serological examination.

Serological examinations

Venous blood samples were collected from pups after 3 weeks of age. The sera were tested for E. cuniculi antibodies by the modified india-ink immunoreaction (IIR) (*Kellett & Bywater* 1978). The antigen was made as described by *Waller* (1977) using an E. cuniculi strain isolated from a spontaneous case of fox encephalitozoonosis.

Pathological examinations

Post mortem examinations were carried out on pups either found dead or killed. Histological sections of various organs and of the placentae were stained with haematoxylin and eosin and with a modified Gram method (*Petri* 1969). Photographs were recorded on Kodak 2415 film.

Mouse inoculations

Homogenized placentae and brain, heart and kidney tissues from the pups were suspended in sterile 0.15 mol/l saline and volumes of 0.5 ml of the suspension were injected intraperitoneally into groups of 4 Swiss albino mice, stock Bom:NMRI f(SPF). Ten days after injection smears of the peritoneal exudate of the mice were examined for E. cuniculi spores using the modified Gram method and the indirect fluorescent antibody test (IFAT). If no parasites were detected after the primary mouse passage, peritoneal saline washings mixed with homogenized liver, spleen and kidney tissues from the mice were passaged at least 3 times into new mice before the samples were concluded to be negative.

Indirect fluorescent antibody test (IFAT)

Smears of peritoneal exudate were air-dried and fixed in acetone for 35 min. They were then covered by a 1:80 dilution of a fox serum with E. cuniculi antibody titers of 3,200 and 12,800 measured by IIR and IFAT, respectively. After incubation for 1 h at 37°C the samples were rinsed 3 times in phosphate buffered saline (PBS) and covered by a 1:10 dilution of the rabbit-anti-

fox-gammaglobulin fluorescin isothiocyanate (FITC) labelled conjugate previously described (*Mohn & Ødegaard* 1977). After incubation for 1 h at 37°C the preparations were rinsed 3 times in PBS and finally counterstained with a 1:10,000 dilution of Evans blue for 10 min. Controls for the various reagents were included in the test. The stained samples were examined in a Zeiss reflected-light fluorescence microscope equipped with an epi-condenser IV F, Osram HBO 50 W super pressure mercury lamp, blue excitation filter KP 490 and barrier filter orange LP 528. Photographs were recorded on Ilford HP5 film.

Urine

Urine sampled from the bladder was centrifuged and heat-fixed smears of the sediment were stained with the modified Gram method and examined microscopically for E. cuniculi spores.

RESULTS

One of the vixens was not mated, and another mated normally but pregnancy was never confirmed. Eight vixens were found to be pregnant. Five of these whelped normally, while the litters of the other 3 were delivered by caesarian section. Table 1 summarises the results of the material from the latter 3 vixens which are labelled Nos. 1, 2 and 3. The results of the other 5 vixens will be published in detail elsewhere.

Table 1. Recovery of E. cuniculi from placentae and organs of caesarian derived pups after oral inoculation of their mothers.

Vixen No.	Number of pups delivered	Number of pups examined	Number of placentae examined	Recovery of E. cuniculi from placentae[a]			Recovery of E. cuniculi from organs[a]		
				Histology	Mouse inoculation		Histology	Mouse inoculation	
					Gram-stained smears	IFAT		Gram-stained smears	IFAT
1	8	7	None	n.p.	n.p.	n.p.	+ (1)	+ (1)	n.p.
2	2	2	None	n.p.	n.p.	n.p.	+ (1)	+ (1)	n.p.
3	13	8	13	+ (4)	+ (13)[b]	+ (13)[b]	—	+ (8)[c]	+ (8)[c]

n.p.: Not performed
\+ : E. cuniculi recovered
— : E. cuniculi not recovered
a : Number of positives in brackets
b : One batch of 13 placentae
c : Organs of 2 pups and one batch of organs of 6 pups

Vixen No. 1. Eight pups were delivered by caesarian section. Four pups each were transferred to 2 foster-mothers which on the same day had given birth to 7 and 8 pups, respectively. On the following day only 2 of the transferred pups were still alive, one with each foster-mother. Neither pathological evidence nor spores of E. cuniculi could be detected histologically in the organs of 5 of the dead pups. Mouse inoculations did not reveal any parasites. Serological examinations of the 2 living pups at 40 days of age revealed a titre of 10 in one and was negative in the other. The sero-positive pup gradually became restless and developed signs of ataxia and posterior weakness. At the age of 82 days it had a serum titre of 1600 to E. cuniculi and the pup was euthanized. Lesions indicative of fox encephalitizoonosis, i.e. hydrocephalus, meningo-encephalitis, interstitial nephritis and polyarteritis nodosa (*Nordstoga & Westbye* 1976), were observed. Gram-positive oval bodies about 1.5 µm×2.5 µm resembling spores of E. cuniculi were detected microscopically in histological sections of its organs and in urine smears. Similar spores were also recovered in macrophages in the peritoneal exudate of inoculated mice.

At the age of 47 days, 2 of the foster-mothers' own pups appeared sick, exhibiting weakness and neurological signs, their sera revealing E. cuniculi-titres of 100 and 800 respectively, while serum sampled from the foster-mother on the same day showed a titre of 25. The latter pup was euthanized and lesions similar to those described in the caesarian derived pup were found by necropsy together with numerous parasites resembling E. cuniculi in its organs and urine. During the course of the study encephalitozoonosis was diagnosed in 4 of the 8 pups of this litter.

The pup transferred to the other foster-mother was killed at the age of 178 days. During the study evidence of encephalitozoonosis could not be found either in this pup or in the 7 pups borne by the foster-mother.

Vixen No. 2. Two pups were delivered by caesarian section and transferred to a foster-mother which the previous night had given birth to 14 pups. One of the 2 pups died the following day due to a bacterial pneumonia. Spores of E. cuniculi could not be detected on histological examination or after mice inoculation of its organs. The other pup gradually became weak and signs of ataxia and lameness were observed. The pup died at the age of 42 days. Necropsy revealed hydrocephalus, enlarged and pale

kidneys and thickened coronary arteries with prominent nodular lesions. Polyarteritis nodosa was seen on histology of brain, kidney and myocardium. Numerous Gram-positive oval spores with an average size of 1.5 μm×2.5 μm were found in urine smears and in histological sections of the organs. Gram-positive oval spores, mainly as intracytoplasmic colonies, were also detected in macrophages from the peritoneal exudate of inoculated mice.

Four of the foster-mother's own pups were transferred to another vixen. Two of the remaining pups died at the age of 21 days due to Escherichia coli septicemia. The other pups of the litter remained healthy and no signs of encephalitozoonosis were observed throughout the study.

Vixen No. 3. A total of 13 pups were delivered by caesarian section. Immediately after birth 2 pups were euthanized and 11 pups were transferred to a foster-mother which the previous day had given birth to 13 pups. Histological examination of sections of organs from the culled pups did not reveal pathological evidence or spores of E. cuniculi. However, Gram-positive oval bodies resembling E. cuniculi spores were detected in macrophages in the peritoneal exudate of mice injected with a suspension of their organs. In FITC-stained smears of the exudate examined by IFAT, numerous intracellular colonies of oval bodies with an average size of 1.5 μm×2.5 μm showed a bright yellow-green fluorescence uniformly distributed on the periphery of the colonies and on each single spore, whereas the central area of the spores had a purple-red colour (Fig. 1). Control preparations did not exhibit specific fluorescence. Histological examination was carried out on Gram-stained sections of the 13 placentae. A few intracellular colonies of Gram-positive oval bodies measuring approximately 1.5 μm×2.5 μm (Figs. 2 and 3) and some scattered spores of similar shape and size lying free in the blood vessels were seen in 4 of the placentae. Similar spores, located mainly as intracytoplasmic colonies in macrophages, were observed in Gram-stained smears of the peritoneal exudate from mice inoculated with a suspension of the placentae. Smears of this exudate examined by IFAT revealed clumps of intracellular oval spores with a bright yellow-green fluorescence as described above (Fig. 1). No evidence of pathological change was found in the placentae on histological examination.

Two of the foster-mother's own pups died during the first days of life. Nine of the remaining pups were transferred to 2

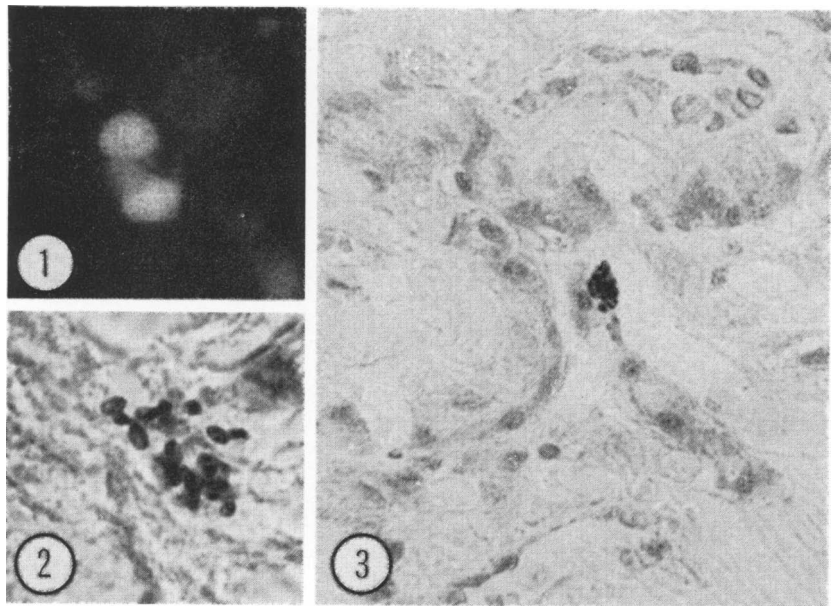

Figure 1. Fluoresceing clumps of E. cuniculi spores in macrophages in peritoneal exudate of mouse after intraperitoneal inoculation with a suspension of organs from caesarian derived pups. Fluorescin isothiocyanate labelled rabbit-anti-fox-gammaglobulin conjugate in the indirect fluorescence antibody test. × 100.

Figure 2. Collection of vacuolated E. cuniculi spores in the tissue of a ceasarian derived blue fox placenta. Modified Gram staining. × 400.

Figure 3. Clump of E. cuniculi spores in the cytoplasm of an endothelial cell, unassociated with inflammatory response, in the tissue of a caesarian derived blue fox placenta. Modified Gram staining. × 160.

other vixens outside this study, while the other 2 pups remained with their mother. Two days after addition of the caesarian derived pups, the vixen killed all the 13 pups in its cage. Gram-positive oval bodies resembling E. cuniculi spores were detected in the peritoneal exudate of mice injected with an organ suspension from 6 of the dead caesarian derived pups. In smears of exudate examined by IFAT, intracellular colonies of bodies with the shape and size of E. cuniculi spores showed bright yellow-green fluorescence as described above (Fig. 1). Histological examination of organs from the pups was not performed due to advanced decomposition of the carcases. Parasites could not be detected in the peritoneal exudate of mice inoculated with a suspension of organs from the foster-mother's own pups. None of the pups borne by this foster-mother and transferred to other vixens exhibited signs of encephalitozoonosis.

DISCUSSION

The parasite recovered from the placentae, organs and urine of pups delivered by caesarian section appears to have the morphology and staining properties characteristic of spores of E. cuniculi. Pups transferred to foster-mothers immediately after delivery developed typical signs of encephalitozoonosis including a significant rise in humoral E. cuniculi antibody levels. The appearance of clumps of intracytoplasmic spores in macrophages of the peritoneal exudate from inoculated, presumably E. cuniculi-free mice, also supports the presence of E. cuniculi. The specific fluorescence observed with positive E. cuniculi antiserum in the IFA'T indicates that the parasite is antigenically identical with E. cuniculi. Likewise the isolates appear to be indistinguishable from the strain with which the vixens were originally inoculated.

The recovery of E. cuniculi from the placentae and organs of the pups borne to vixen No. 3 provides strong evidence for transplacental transmission of the parasite. Two pups transferred to foster-mothers developed typical encephalitozoonosis and parasites resembling E. cuniculi were recovered. The pups borne by one of the foster-mothers remained healthy throughout the experiment and no signs of encephalitozoonosis were observed. However, some of the pups borne by the other foster-mother developed natural encephalitozoonosis. E. cuniculi antibodies were

detected in the serum of this foster-mother and it seems likely that E. cuniculi had, in addition, been spreading among the foxes in the farm during the study. Neonatal infection of the 2 pups can, therefore, not be excluded, although cross-fostering experiments and neonatal inoculations of pups have failed to establish clinical encephalitozoonosis (*Mohn*, unpublished); transplacental transmission of the parasite seems to be the most likely mode of infection in these pups also.

A few spores of E. cuniculi, scattered within the cells and blood vessels, were seen in sections of the placentae. The number of parasites present in the organs of infected pups killed at birth was probably also small. This presumably explains the failure to demonstrate spores microscopically in the organs of these pups. The recovery of the parasite by means of intraperitoneal inoculations into mice allows detection of small numbers of the parasite after multiplication in the peritoneal cavity of the host. One mouse passage was sufficient, in all cases, for recovery of the parasite, which indicates that there were at least 10^4 spores per mouse dose injected (*Petri* 1969). This concentration of spores may, however, be difficult to detect microscopically in histological sections, especially since they occur singly or in groups of a few spores without infiltration of inflammatory cells or other tissue lesions.

Parasites could not be detected in the organs of 6 pups from 2 litters examined within 24 h of birth. This could be due either to a failure to detect low numbers of parasites in the organs, or to a low incidence of infection despite transplacental spread, or to a combination of these possibilities. The latter theory is in accordance with observations in natural and experimental cases of encephalitozoonosis where only a proportion of pups in an affected litter may develop the disease (*Mohn*, upnublished). One of the pups of vixen No. 1 was probably not infected in utero, since it showed no signs of encephalitozoonosis and was seronegative throughout its life, although the possibility cannot be excluded, that it overcame the infection during the first weeks of life.

After oral inoculation of vixens, the parasite is likely to spread by the haematogenous route to the uterus and the maternal part of the placenta. In the endotheliochorial type of placenta, characteristic of the Carnivora (*Amoroso* 1952), a cell membrane barrier separates the maternal and foetal blood. The

parasite, therefore, probably has to multiply intracellularly in the placental tissues before it can cross this barrier and enter the foetal blood stream. The detection of intracytoplasmic parasite colonies in the placental tissues, which were probably parasitophorous vacuoles of E. cuniculi, and of free spores in the placental blood vessels, seems to confirm this theory.

Transplacental transmission of the parasite is likely to be a common mode of infection in fox encephalitozoonosis, although infection via other vertical routes cannot be excluded. The mechanism of transmission and the stage of gestation at which the placentae and foetuses are infected are, however, still unknown.

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SAMMENDRAG

Eksperimentell encephalitozoonose hos blårev — Transplacental overføring av parasitten.

Sporer av Encephalitozoon cuniculi ble påvist i fosterhinner og i organer av fostre etter oralt podete blårevtisper. Resultatene viser at parasitten kan overføres fra mor til avkom via placenta.

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