

## THE IMMUNE RESPONSE IN A DOG TO *ENCEPHALITOOZON CUNICULI* INFECTION

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### ABSTRACT

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The immune response to *Encephalitozoon cuniculi* infection in a dog was investigated by means of the indirect fluorescent antibody test, the leucocyte migration inhibition test and the radial immunodiffusion test for serum IgG and IgM levels.

Specific antibodies were detected within 7 days of infection and they persisted for 370 days. A cell-mediated immune response was detected from Day 13 following infection until Day 97.

Histopathological examination showed plasma cell infiltration of the kidneys, meninges, lung, bladder, smooth muscle and spleen.

### INTRODUCTION

*Encephalitozoon cuniculi* is an obligate intracellular protozoan parasite that infects a wide variety of mammals (Canning, 1977). Transmission is either by the oral or transplacental route. In blue foxes, it has been shown that foetuses have to be infected *in utero* for clinical encephalitozoonosis to occur (Mohn & Nordstoga, 1982). In dogs, clinical signs of encephalitozoonosis occur mainly between 4-12 weeks of age (Stewart, Van Dellen & Botha, 1979). In an outbreak of the disease where both adults and young dogs had antibodies to *E. cuniculi*, clinical signs were entirely limited to younger dogs. Dosing of susceptible experimental dogs with *E. cuniculi* have failed to produce clinical signs; however, histopathological lesions as well as the presence of antibodies were demonstrated (Stewart, Botha & Van Dellen, 1979). In laboratory animals, *E. cuniculi*, also causes subclinical infection (Malherbe & Mundy, 1958).

These facts indicate that host-immune mechanisms may control the development of clinical signs and may therefore also have an influence on the multiplication of the parasite *in vivo*. In mice, this has been shown to involve T lymphocytes, since hypothyroid nude mice develop fulminating lethal infections after infection, whereas similarly infected euthymic mice develop only mild encephalitozoonosis (Nieder Korn, Shadduck & Schmidt, 1981). A transient enhancement of natural killer cell activity has also been demonstrated during infection in mice (Nieder Korn, Brieland & Mayhew, 1983). Depressed immune responsiveness, as represented by a lowered antibody response to sheep erythrocytes, has also been demonstrated (Nieder Korn *et al.*, 1981). In rabbits, infection leads to depressed humoral antibody response to the unrelated immunogens of *Brucella abortus* (Cox, 1977). Examination of serum proteins in naturally diseased blue fox pups revealed a marked hypergammaglobulinaemia (Mohn & Nordstoga, 1975).

In this study, the development of a cellular immune response was demonstrated by means of the leucocyte migration inhibition test (Schultz & Adams, 1978) and antibodies were demonstrated by the indirect fluorescent antibody (IFA) test (Stewart, Botha & Van Dellen, 1979).

### MATERIAL AND METHODS

**Infection of experimental animal:** A 3-year-old female beagle was obtained from the beagle colony of the Department of Genesiology and was housed in an isolated kennel.

Before the commencement of the trial the beagle was served by a male dog. As she did not become pregnant, she was again served by natural means when she came into oestrus 150 days later. Serum samples from the male dogs used on both occasions were negative to the IFA test for *E. cuniculi* antibodies. Five puppies were born to this mating. One pup was destroyed 2 hours after birth and another pup 4 days after birth, as they were in a weak condition. The remaining 3 puppies all died within 3 days after birth. The bitch was euthanized 370 days after commencement of the trial. *E. cuniculi* spores, grown in canine kidney cells (MDCK) (Stewart, Van Dellen & Botha, 1979), were harvested and washed, and  $5 \times 10^6$  spores were inoculated orally 10 days after the first service. At the same time and at regular intervals thereafter, 20 ml of blood was collected in heparin from the jugular vein for use in the leucocyte migration inhibition assay, and serum was collected in venoject tubes for serology and IgG and IgM determinations.

**Control animals:** Normal dog's blood was collected from 2 clinically normal dogs negative to the IFA test for *E. cuniculi* antibodies, and a leucocyte migration inhibition test was performed. Three normal beagle dogs were bled and their serum IgG and IgM levels were determined.

**Antigen:** *E. cuniculi* spores grown in tissue culture (Stewart, Van Dellen & Botha, 1979), were harvested and adjusted to  $5 \times 10^6$ /ml, sonicated 4 times in a Branson 52\* sonicator for 3 min with a 2 min interval between sonications and then stored at  $-18^\circ\text{C}$  until used. The interval between storage and use did not exceed 14 days.

**The leucocyte migration inhibition (LMI) test:** Blood was centrifuged at  $500 \times g$  for 4 min and the buffy coat was removed, then mixed with 10 ml of normal saline and layered over a Ficoll/Hypaque density gradient (SG=1,077) and centrifuged at  $900 \times g$  for 45 minutes. The band of cells at the interface was removed, washed and adjusted to  $5 \times 10^7$  cell/ml in Hank's/Eagle's medium with 10% foetal calf serum (FCS). Twenty  $\mu\text{l}$  of antigen suspension was added to 0,5 ml cells and 20  $\mu\text{l}$  of normal saline was added to other 0,5 ml cells to act as control cells. Micro capillary tubes were filled with

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equal volumes of cells, sealed with "cetoseal" and centrifuged at 500 × g for 4 min. Four or more replicates of cells with antigen and control cells were used each time. Each tube was then cut as closely as possible to the cell pellet-fluid interface and positioned horizontally in a leucocyte migration chamber\*. This was then filled with Hank's/Eagle's medium plus 10 % FCS and sealed with a coverslip. After incubation for 24 h at 37 °C, the migration area was projected on paper via an epidiascope. The outlines of the migration area and tube were drawn, and the area was measured with a planimeter.

The results were expressed as follows:

$$\text{Migration Index (MI)} = \frac{\text{Mean of area of migration of cells with antigen}}{\text{Mean of area of migration of control sample}}$$

A chi-squared test was performed to determine the significance levels.

**Immunoglobulin determinations:** IgG and IgM determinations were carried out, using the radial immunodiffusion kits for the quantitative determination of canine immunoglobulin G and M†. The test was carried out according to the manufacturer's instructions, except that each serum sample was measured accurately with an Hamilton syringe and then added to the relevant well.

**Indirect fluorescent antibody test:** This was carried out as previously described (Stewart, Botha & Van Dellen, 1979).

**Necropsy:** A complete post-mortem examination was carried out following euthanasia of the bitch and 2 of her puppies.

One kidney from each animal was removed in an aseptic manner and primary kidney cultures were prepared as previously described (Steward, Van Dellen & Botha, 1979). Tissues from the major organs were fixed in 10 % neutral formalin. Sections for histopathological examination were cut and stained with haematoxylin and eosin (HE) and Gram stain.

RESULTS

**Leucocyte migration inhibition test:** (Table 1). The MI was 0,76 on Day 13 post-infection, decreased to 0,23 by Day 36 and then slowly increased until Day 97 post-infection and thereafter no significant inhibition could be detected. Two negative dogs used as controls showed no inhibition.

**Serology:** The IFA titre was negative on Day 0, rose to 160 by Day 7 post infection, reached a peak of 320 by Day 15 post-infection and remained positive for the remainder of the trial.

**IgG and IgM determinations:** (Table 2). By Day 7 post-infection the IgG had risen to above the pre-infection levels and, apart from Days 35 and 47 post-infection, it remained above this level for the remainder of the trial. The IgM level rose to above the preinfection levels on Day 10 post-infection followed by another slight rise on days 20 and 28 post-infection.

**Necropsy and histopathology:** At the time of euthanasia the bitch appeared to be in good health and showed no clinical signs of encephalitozoonosis. Macroscopically, hepatomegaly and splenomegaly were present and the

TABLE 1 Leucocyte migration inhibition test in a bitch experimentally infected with *E. cuniculi*

Days post-infection	Area of migration ± SD		Migration index	Significance level
	With antigen	Control cells		
0	121 ± 13	128 ± 23	0,945	NS *
13	306 ± 14	405 ± 40	0,76	0,01
20	60 ± 18	131 ± 34	0,46	0,05
36	124 ± 29	533 ± 53	0,23	0,01
50	225 ± 28	347 ± 60	0,65	NS
63	95 ± 29	154 ± 23	0,61	0,05
70	141 ± 23	262 ± 19	0,54	0,05
97	337 ± 40	465 ± 00	0,73	0,05
111	631 ± 141	639 ± 120	0,99	NS
118	295 ± 17	315 ± 17	0,94	NS
<i>Control dogs</i>				
1	532 ± 17	531 ± 12	1,00	NS
2	505 ± 13	524 ± 13	0,96	NS

\* NS = Not significant

TABLE 2 Serological titre and IgG and IgM levels in a bitch experimentally infected with *E. cuniculi*

Days post-infection	IFA titre	IgG (mg/l)	IgM (mg/l)
0	Neg	11 000	1 800
7	160	21 000	1 750
10	80	21 000	4 000
13	320	22 000	1 250
20	80	23 000	2 200
28	40	24 000	2 100
35	160	19 000	400
47	80	18 000	400
49	80	24 000	1 800
62	80	21 000	1 750
69	40	21 000	1 750
76	40	22 000	1 800
110	40	24 000	1 500
159	80	24 000	1 800
181	40	21 000	900
285	160	24 000	1 800
295	160	24 000	1 500
370	80	25 000	1 800
3 control dogs (x̄)		13 820	1 560

capsule of the right kidney stripped with difficulty. Histopathologically, the kidneys showed multifocal interstitial nephritis characterized by a plasma cell infiltration and membranoproliferative glomerulonephritis. Plasma cell infiltration was also observed in the smooth muscle of the bladder, meninges, lung and, severely, in the spleen. Megakaryocytes were present in the spleen. There was present a mild interstitial pneumonia, a severe purulent endometritis and vaginitis.

No *E. cuniculi* were observed in any of the organs, despite an extensive search of the HE and gram-stained sections.

No abnormalities were detected in the 2 puppies either on necropsy or on histopathological examination, and there was no evidence of encephalitozoonosis.

**Primary kidney cultures:** Kidney cultures were established from both puppies and the mother. Unfortunately, both cultures from the puppies were lost through bacterial contamination.

The culture from the mother was subpassaged once, and as cells were starting to degenerate, MDCK cells were added to the culture. The culture was maintained for a further 4 subpassages (28 d), after which it was discarded as negative.

DISCUSSION

These results show that both humoral- and cell-mediated immune responses developed following infection with *E. cuniculi* as was demonstrated by the

\* Sterilin

† Miles Laboratories

development of antibodies as well as a positive LMI reaction.

Antibodies were detected 7 days post-infection and remained relatively low, thus confirming previous results (Stewart, Botha & Van Dellen, 1979), except that they were detected earlier in the present case. The variation in titre probably represents an inability to determine precisely the end-point of the IFA test. Similar results have been observed in blue foxes (Mohn, Nordstoga & Dishington, 1982), where antibodies were detected for 200 days following experimental infection. However, significant titres had not been detected by the following breeding season. It would appear that antibodies may persist longer in dogs than in foxes. This may be correlated with the fact that the only histopathological lesion observed in adult foxes is endometritis (Mohn *et al.*, 1982), whereas in the present study a multifocal plasmacytic interstitial nephritis, together with a plasma cell infiltration of the meninges, smooth muscle of the bladder, lungs and spleen were seen. The purulent endometritis seen in this bitch was not thought to be due to *E. cuniculi* infection, as the lesion was characterized by infiltration of neutrophils and not mononuclear cells. It probably represents an early pyometra. In experimentally infected foxes, endometritis is a common finding with infiltration of predominantly mononuclear inflammatory cells, including some plasma cells.

The relatively long interval between infection and successful service may account for the failure to demonstrate transplacental transmission. Cases have been recorded where an infected vixen has given birth to more than 1 infected litter. However, this is rare (Mohn *et al.*, 1982).

The positive LMI reaction which occurred in this bitch and the fact that it persisted for 97 days indicates that a cell mediated immune response plays a role in immunity to canine encephalitozoonosis. What role these mechanisms play in protective immunity is not known; but the fact that this animal did not show any clinical signs of encephalitozoonosis during this trial suggests that adult dogs are able to develop a strong immunity to infection. A similar situation has also been observed in blue foxes where clinical encephalitozoonosis has not been observed in adult foxes (Mohn, Nordstoga, Krogsrud & Helgebostad, 1974). The presence of histopathological lesions and the development of specific antibodies in this trial is confirmation that the organism did become established. The failure to obtain a statistically significant reduction of migration on Day 50 post-infection was probably due to technical error, as there were insufficient cells to set up more than 3 replicates of the test.

The interstitial nephritis and glomerulonephritis could be due either to persisting antigen or antigen/antibody complexes or possibly to the development of autoimmunity (McCluskey & Colvin, 1978). The failure in this trial to reisolate *E. cuniculi* would favour the latter hypothesis. No attempt was made in this study to demonstrate autoantibodies.

Tizard (1982) gives the normal serum immunoglobulin levels of dogs as 10 000–20 000 mg/l for IgG and 700–2 700 mg/l for IgM. The IgG level was above normal by Day 7 post-infection and apart from Day 37 and 47 post-infection, it remained above normal for the remainder of the trial. The IgM level rose to above the

normal range only on Day 10 post-infection. These results confirm the results of others who have shown a hypergammoglobinaemia in encephalitozoonosis of foxes (Mohn, 1982). However, because this bitch was shown to have an endometritis, which was thought to be unrelated to the encephalitozoonosis, this may have interfered with the finding of hypergammoglobinaemia making interpretation of these findings difficult.

#### CONCLUSIONS

These results show that, in canine encephalitozoonosis, lesions develop even in mildly infected animals, thus confirming the previous results of Botha, Van Dellen & Stewart, (1979). These are accompanied by an immunological response which initially is shown by positive IFA titres which persisted for up to 370 days following infection and a positive LMI test which persisted for 97 days.

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