Neospora caninum-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival

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SUMMARY

The parasite, *Neospora caninum* is an important cause of abortion in cattle. It is transmitted vertically or horizontally and infection may result in abortion or the birth of a live, healthy but infected calf at full-term. Only a proportion of infected cattle abort and the pathogenesis of abortion is not understood. Groups of cattle were infected with 10^7 *N. caninum* tachyzoites intravenously at different times relative to gestation. Intravenous inoculation was chosen to reproduce the putative haematogenous spread of *N. caninum* following either recrudescence of endogenous infection or *de novo* infection. In all cattle, infection was accompanied by high γ-interferon and lymphoproliferative responses, and a biased IgG_2 response indicating that *N. caninum* infection is accompanied by a profound Th1 helper T cell-like response. Infection at 10 weeks gestation resulted in foetopathy and resorption of foetal tissues 3 weeks after infection in 5 out of 6 cows. Infection at 30 weeks gestation resulted in the birth of asymptomatic, congenitally-infected calves at full term in all 6 cows, whereas the 6 cows infected before artificial insemination gave birth to live, uninfected calves. These results suggest that the reason some cows abort is related to the time during gestation when they become infected or an existing infection recrudesces.

Key words: *Neospora caninum*, cattle, immune response, abortion, experimental infection.

INTRODUCTION

Neospora caninum is an intracellular protozoan parasite, closely related to *Toxoplasma gondii*. It was first described in 1984, in the brain of a dog which had died from a neuromuscular disease, and was named in 1988 (Dubey *et al*. 1988). *N. caninum* also occurs naturally in horses, goats, sheep and deer, but is of particular importance world-wide, as a cause of abortion in cattle (Dubey & Lindsay, 1996). *N. caninum* is the most commonly diagnosed cause of infectious abortion in cattle in the UK where it has been estimated that 12·5% of all abortions are attributable to *N. caninum* (Davison, Otter & Trees, 1999*a*) and in California, where *N. caninum* infection was identified in 42·5% of abortions in a prospective study of 19708 cattle (Anderson *et al*. 1995).

It is thought that the life-cycle of *N. caninum* is similar to that of other apicomplexan parasites. Three life-cycle stages of *N. caninum* have been described–the rapidly dividing tachyzoite stage, the bradyzoite stage in tissue cysts, found primarily in the central nervous system (Barr *et al*. 1991*b*), and an oocyst stage, identified in faeces of experimentally infected dogs (McAllister *et al*. 1998).

The immune response to *N. caninum* is thought to be similar to that stimulated by *T. gondii*, and dominated by the Type 1 cytokines, γ-interferon (γIFN) and interleukin (IL) 12. Mice are naturally resistant to infection with *N. caninum*, but this resistance can be ablated by *in vivo* depletion of γIFN or IL12 (Khan *et al*. 1997). Similarly, in cattle, experimental infection with *N. caninum* stimulates profound γIFN and *ex vivo* antigen-specific T cell responses (Lunden *et al*. 1998; Marks *et al*. 1998).

The major route of transmission of *N. caninum* is vertical, with transplacental transmission estimated to be about 95% efficient in the UK (Davison, Otter & Trees, 1999*b*). The majority of calves born from infected mothers are clinically normal, but are thought to remain infected for life with female progeny passing the parasite on to their offspring during gestation. However, a proportion of *N. caninum*-infected cattle abort. A seropositive cow is 3·5–7·4 times more likely to have an abortion than a seronegative cow and about 5% of infected cattle may abort in subsequent pregnancies (Anderson *et*

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al. 1995; Thurmond, Heitala & Blanchard, 1997; Moen *et al*. 1998; Davison *et al*. 1999*a*). The reasons why some cattle abort are not understood. There are 2 reports of the outcome of experimental *N. caninum* infection in pregnant cattle. In the first study, 2 cattle were infected on 81 and 126 days of gestation respectively and subsequently aborted *N. caninum*-infected foetuses and in the other study an abortion in a cow infected at 85 days of gestation was reported, whereas a cow infected at 120 days of gestation produced a *N. caninum*-infected, clinically-affected live calf at full-term (Dubey *et al*. 1992; Barr *et al*. 1994). These studies suggest that the time of infection of the foetus is important in determining the outcome of pregnancy, a hypothesis which is supported by studies in sheep infected with *N. caninum*, where the earlier in gestation infection is administered, the more pathogenic the outcome (Buxton *et al*. 1997, 1998). In this study we tested the hypothesis that the time of *N. caninum* parasitaemia, which may result either from the activation of an existing maternal infection or as a result of *de novo* infection, determines whether or not the foetus survives.

MATERIALS AND METHODS

Cattle

Twenty-seven Holstein-Friesian maiden heifers were purchased from local farms that had no history of *Neospora*-associated abortion. Blood samples were taken from all 27 animals on arrival at the Liverpool University Animal Husbandry farm and sent to the Veterinary Investigation Centre, Preston and tested for evidence of exposure to Bovine Viral Diarrhoea Virus, Infectious Bovine Rhinotracheitis Virus and *Leptospira hardjo*. The cattle were negative for all 3 abortifacient agents. The cattle were also tested for evidence of exposure to *N. caninum* by antibody ELISA (MASTAZYME, Mast Diagnostics, UK; Williams *et al*. 1997, 1999) and cell proliferation assay and were negative in both tests.

The cattle were divided into 4 groups of 6 (Groups I–IV) and 1 group of 3 (Group V). Groups I–IV were infected with *N. caninum* at different times (see below) and Groups I–III and V were artificially inseminated. Group IV acted as non-pregnant infected controls and Group V were uninfected, pregnant controls.

Group I were infected with *N. caninum* 9 weeks before artificial insemination. Group II were infected at 10 weeks of gestation and Group III were infected at 30 weeks of gestation. Group IV consisted of 6 cows, 2 of which were infected at the same time as Groups I, II and III respectively.

Cattle were infected intravenously with 10^7 tachyzoites of *N. caninum* Nc Liverpool passage 9, in phosphate-buffered saline (PBS), pH 7·2 (Barber *et al*. 1997). Tachyzoites were grown in Vero cells and purified as described previously (Williams *et al*. 1997). Briefly, tachyzoites were isolated by mechanically disrupting the cell monolayer through 21-gauge needles followed by passage over Sephadex G10 (PD10 columns, Pharmacia, Sweden). The tachyzoites were then washed and counted. For each inoculum, 1 of the uninfected control cows in Group V was inoculated with uninfected Vero cells treated in exactly the same way as those which were infected with tachyzoites.

All 27 cattle were oestrus synchronized using PRIDS (Sanofi Animal Health, UK) and 21 inseminated artificially using standard techniques. Cattle ($n = 4$) which were found not to be pregnant after the first insemination were given a second insemination, then housed with a bull. Pregnancy was confirmed by transrectal ultrasonography at approximately day 30 of gestation. Animals were re-examined at regular intervals to determine foetal heart beat as an indication of foetal viability. Foetal growth was also assessed by comparing foetal trunk diameter and distance between the parietal bones of the head measured during ultrasonography.

Peripheral blood mononuclear cell proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated by isopycnic centrifugation over Lymphoprep (density 1·077 g/ml; Nycomed, Norway) followed by 4 washes in RPMI 1640 medium, to remove platelets. Viable PBMC were cultured for 5 days in RPMI 1640 medium, supplemented with 10% foetal calf serum, and penicillin/streptomycin (100 μg/ml; Bio-whittaker, USA) at a concentration of 2×10^6 cells/ml, with 1 μg/ml of *N. caninum* antigen, 1 μg/ml of Concanavalin A or medium alone. Each test was done in triplicate. Cells were incubated for 5 days at 37 °C in 5% CO_2 in air. After 5 days 1 μCi of [H^3]thymidine (Amersham International, Amersham, UK) was added to each well and the plates harvested 5 h later onto glass microfibre filter mats (Wallac, Finland) and then counted in a Wallac microbeta counter. The results are expressed as a ratio of the mean counts per min of test samples to the mean counts per min for the medium control (Stimulation Index).

Duplicate cell cultures were set up and after 3 days, supernatants were harvested by centrifugation at 10000 ***g*** for 5 min, and stored at −20 °C for use for γIFN and TNFα analysis.

Antigen preparation

N. caninum antigen used in cell cultures was a whole homogenate of *N. caninum* Nc Liverpool tachyzoites derived from Vero cell cultures as described above. Purified tachyzoites were snap frozen in liquid nitrogen twice and then sonicated in a sonicating

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water bath for 3 min. The sonicate was centrifuged at 10000 ***g*** for 30 min at 4 °C, the supernatant collected and the protein concentration estimated using Coomassie Plus (Pierce Ltd, USA).

Determination of γIFN in culture supernatants

γIFN levels were measured in culture supernatants using the BOVIGAM γIFN kit (CSL, Victoria, Australia). A standard curve, derived from a series of dilutions of a recombinant bovine γIFN standard (0·95 ng/ml; Ciba-Geigy, Switzerland) was used to quantify levels in the test samples.

Measurement of tumour necrosis factor α

TNFα activity in either tissue culture supernatants or serum was detected using a WEHI-164 cytotoxicity assay using the method of Adams & Czuprynski (1994). Actinomycin D (Sigma), treated WEHI-164 cells (ECACC, Porton Down, Wiltshire, UK) were incubated overnight with supernatants from PBMC cultures, serum or a range of dilutions of recombinant human TNFα as the standard (R&D Systems) and cytotoxicity measured using a Non-radioactive Cell Proliferation kit (Promega) which measures live cells.

*Anti-*N. caninum *specific antibody ELISA*

N. caninum-specific antibodies were measured using the MASTAZYME ELISA kit (Mast Diagnostics). Optical density readings were expressed as a percentage of a high positive control (percentage positivity (PP) 33). IgG_1 and IgG_2 responses were measured using the same ELISA kit but using mouse anti-bovine IgG_1 or IgG_2 at a dilution of 1:500 as the secondary antibodies (Serotec, Oxford, UK), followed by a sheep anti-mouse Ig conjugated to horse-radish peroxidase at a dilution of 1:2000 (Serotec). Results are expressed as optical density (OD) readings with the mean OD of 12 replicates of 3 negative control sera deducted. IgG_1 and IgG_2 titres were calculated as the reciprocal $\log_{10}$ dilution that gave an OD greater than the negative control. The negative control was calculated as the mean of 12 replicate wells each of the 3 negative control sera (Group V) plus 2 standard deviations ($n = 36$).

Necropsy of adult cows and neonatal calves

Calves were euthanized within 7 days of birth by intravenous injection of concentrated pentobarbitone Na (Euthatal, Merial Animal Health Ltd, Harlow, UK). The adult cattle were killed 8 weeks after calving, at an Over Thirty Months (OTM)-registered establishment (under BSE regulations). Samples of tissue were collected for PCR, parasite isolation *in vitro*, mouse inoculation and histological examination. Tissues for histological examination were fixed in 10% formalin, embedded in paraffin wax and stained with haematoxylin and eosin using standard techniques. For isolation of parasites by *in vitro* culture the method of Barber *et al*. (1997) was used. Cultures were checked for evidence of tachyzoites at 4 and 8 weeks after inoculation. For mouse inoculation, samples of brain tissue were homogenized and injected intraperitoneally into BALB/c mice which had been treated with 0·05 ml Depomedrone (methylprednisolone acetate, 40 mg/ml: Pharmacia & UpJohn Ltd, Corby, UK), 24 h previously. Mice were bled at 3 and 6 weeks after inoculation and tested for *N. caninum*-specific antibodies by IFAT (Trees *et al*. 1993).

Detection of N. caninum *DNA in brain tissue by polymerase chain reaction* (*PCR*)

Two PCR methods were used. First a 2-tube nested PCR, based on the method described by McMillan *et al*. (1997), was used. Six 50 mg samples from different regions of each brain were homogenized and a 50 mg aliquot taken for DNA extraction. DNA was extracted using a QIAamp tissue kit (Qiagen Ltd, Crawley, Sussex, UK). The external primers, NF1 [forward (GCGTGATATACTACTCCCTG-TG) and SR1 [reverse (AAACTCCTGGAAGTT-AAAG)] were used in the second reaction at a concentration of 0·8 μM. These primers are complementary for the internal transcribed spacer – 1 (ITS1) region of the ribosomal DNA. For each reaction 50 μl of mixture was used containing 1·75 mM $MgCl_2$, 0·2 mM of each deoxynucleotide, 0·8 Units of Red Hot DNA polymerase (Advanced Biotechnologies, Surrey, UK). Five μl of DNA was used in the first reaction and 1 μl transferred to the second reaction. The first PCR consisted of 95 °C for 5 min followed by 15 cycles of 94 °C for 30 sec, 56 °C for 90 sec and 72 °C for 30 sec followed by a final extension at 72 °C for 10 min. The second PCR was conducted using the same conditions but the amplification comprised 30 cycles. Positive controls were uninfected bovine brain preparation spiked with tachyzoite-derived DNA and spiked reaction mix alone. Brain tissue from a *N. caninum*-negative cow and water were used as negative controls. Twenty μl aliquots of the reaction mixtures were analysed on 2% agarose gels, incorporating ethidium bromide and the PCR products visualized under UV light.

PCRs were repeated twice for each animal using DNA extracted from different sample of tissue on each occasion. As all the tissues tested using these primers were negative by PCR, a second PCR was employed using the primers and technique described by Uggla *et al*. (1998). For this PCR the external primers and internal primers used were F6 and 5·8B and PN3 and PN4 respectively. This PCR produced a product of 249 basepairs compared to the former

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Table 1. Summary of clinical outcome of the experiment in which cattle were infected intravenously with *N. caninum* tachyzoites at different times relative to artificial insemination

Group	Number of cattle	Time of infection relative to AI (weeks)	Estimated foetal age at 16 weeks	Number of live calves born at full term (40 weeks)
I	6	−9	16 weeks	6/6
II	6	+10	13 weeks* 16 weeks†	1/6
III	6	+30	16 weeks	6/6
IV	6	Non-pregnant, infection controls	N.A.‡	N.A.‡
V	3	Non-infected pregnant controls	16 weeks	3/3

* In 5 dead foetuses.
† In 1 live foetus.
‡ N.A., Not applicable.

Table 2. Summary of serological status and detection of parasites in calves born from dams experimentally infected with *Neospora caninum* tachyzoites before or during pregnancy

(Serum antibody levels were measured by MASTAZYME ELISA and the results are expressed as a percentage of the high positive control (percent positivity).)

Group	Antibody status of calves at birth	Mean percent positivity (±1 S.D.)	Detection of *N. caninum* DNA in brain tissue by PCR
I	6/6 negative	2 (1·3)	None detected
II	1/1 negative	0	None detected
III	6/6 positive	92 (12)	1/6 positive
V	3/3 negative	5 (1)	None detected

PCR which produced a product of 146 basepairs. Samples were heated for 2·5 min at 94 °C; thereafter each cycle consisted of a 30 sec denaturation at 94 °C, 40 sec at the annealing temperature of 50 °C followed by a 1 min extension at 72 °C. Amplification was performed with F6 and 5·8B over 25 cycles, followed by a final extension at 72 °C for 3 min. In the second PCR the annealing temperature was increased to 54 °C, extension decreased to 30 sec and the amplification was performed with PN3 and PN4 over 30 cycles.

Statistical methods

The data were analysed using the Minitab statistical program (Minitab for Windows, Minitab Inc., State College, PA, USA). Differences in PBMC proliferation were examined using the Kruskal–Wallis test. Differences between IgG_1 and IgG_2 titres were investigated using the 2-Sample *t*-test. The Critical Probability was taken to be $P = 0{\cdot}05$.

RESULTS

Clinical outcome of infection

Group I cattle were infected 9 weeks prior to insemination. The viability of the foetuses was monitored throughout gestation and all 6 survived to full term (Table 1). Six live calves were born, no *N. caninum*-specific antibodies were detected in the calves prior to ingestion of colostrum and no *N. caninum* DNA was detected by PCR when the calves were necropsied at 7 days of age (Table 2).

Group II cattle were infected at 10 weeks of gestation. Foetal viability was determined at 11 and 16 weeks gestation. At 11 weeks gestation, all 6 foetuses had detectable heart beats but at 16 weeks no heart beats were detected in 5 of the 6 foetuses. The 5 dead foetuses had an estimated foetal age of 13 weeks, indicating that, assuming their growth was normal up to the point of death, the foetuses had died 3 weeks after infection. The remaining foetus had an estimated age of 16 weeks (Table 1). This foetus survived to term and was born normally. No serum antibody was detected in this calf at birth and no *N. caninum* DNA was detected in its brain, when necropsied at 7 days of age (Table 2). The dam of this calf developed a serum antibody response (Fig. 3), suggesting she was infected as a result of the inoculation.

Group III cattle were infected at 30 weeks gestation. All 6 animals gave birth to live calves at full term (Table 1), and all the calves were *N. caninum*-antibody positive at birth, prior to ingestion

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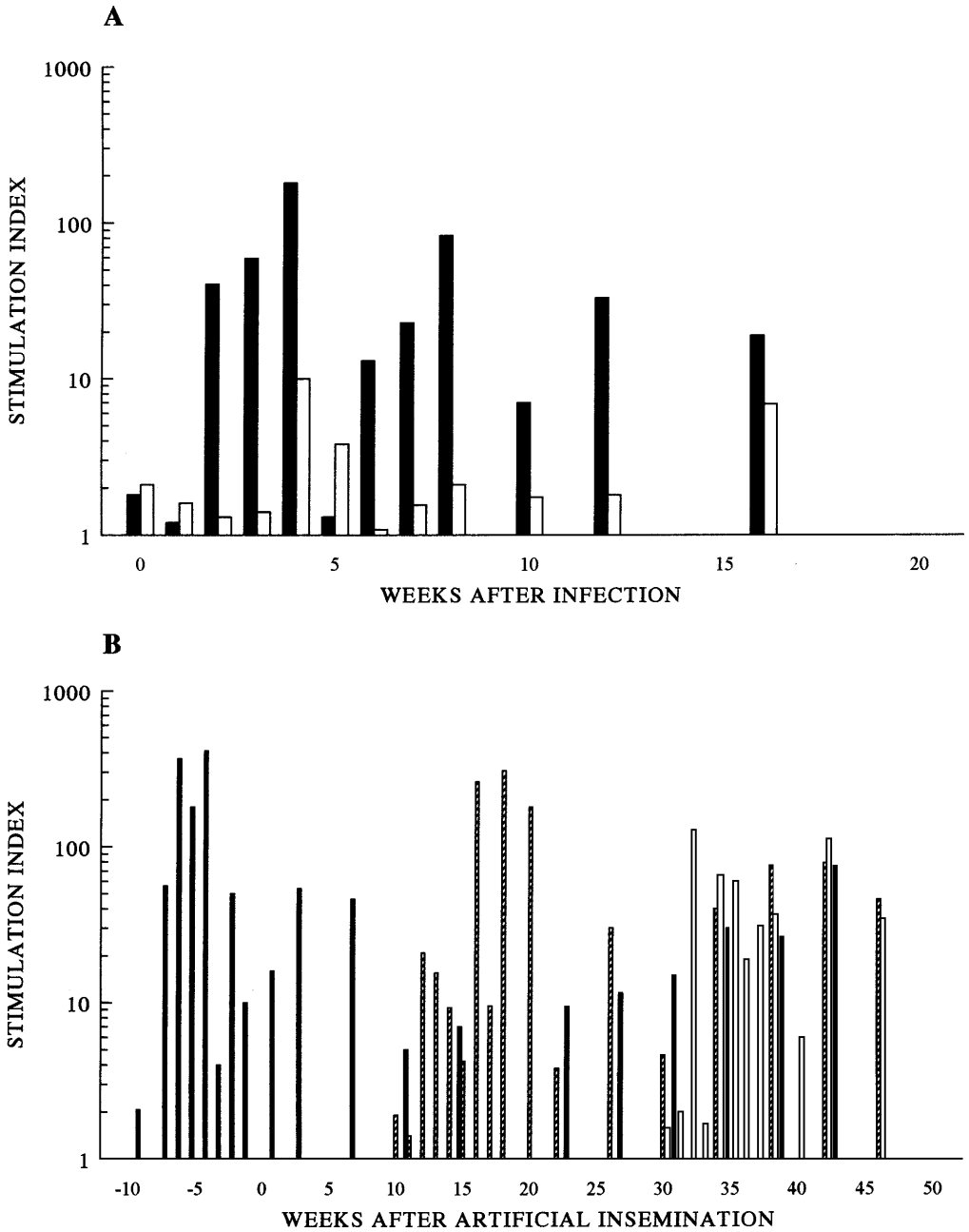

Fig. 1. *Neospora caninum* antigen-stimulated proliferation by PBMC from cattle following intravenous inoculation with 10^7 tachyzoites. PBMC were collected at weekly intervals, cultured in the presence of 1 μg/ml of *N. caninum* antigen for 5 days and proliferation measured by incorporation of [H^3]thymidine. (A) Longitudinal response in infected Group IV (■) and uninfected, pregnant Group V (□). (B) Responses in Group I (■), infected 9 weeks before artificial insemination, Group II (▬) infected 10 weeks after artificial insemination, and Group III (□) infected 30 weeks after artificial insemination. Results are presented as the means of triplicate cultures for all the animals in the group.

of colostrum. Parasite DNA was detected by PCR using the primers described by Uggla *et al.* (1998), in the brain tissues examined from 1 of the 6 calves tested (Table 2).

Brain, myocardium and spinal cord samples were collected from all calves and adult cattle at necropsy. Samples from each tissue were homogenized and inoculated onto Vero cells in tissue culture, into mice and were examined histologically and by immunocytochemistry. None of the mice seroconverted and no *N. caninum* tachyzoites were isolated in culture. No histological evidence of *N. caninum* infection was detected in any of the calves or adult cattle.

Proliferation responses

The proliferation response of peripheral blood mononuclear cells to *N. caninum* antigens was followed in Groups IV and V from 2 weeks before infection to 16 weeks after infection and in the 3 inseminated groups from 2 weeks prior to infection through to parturition. The results are shown in Fig.

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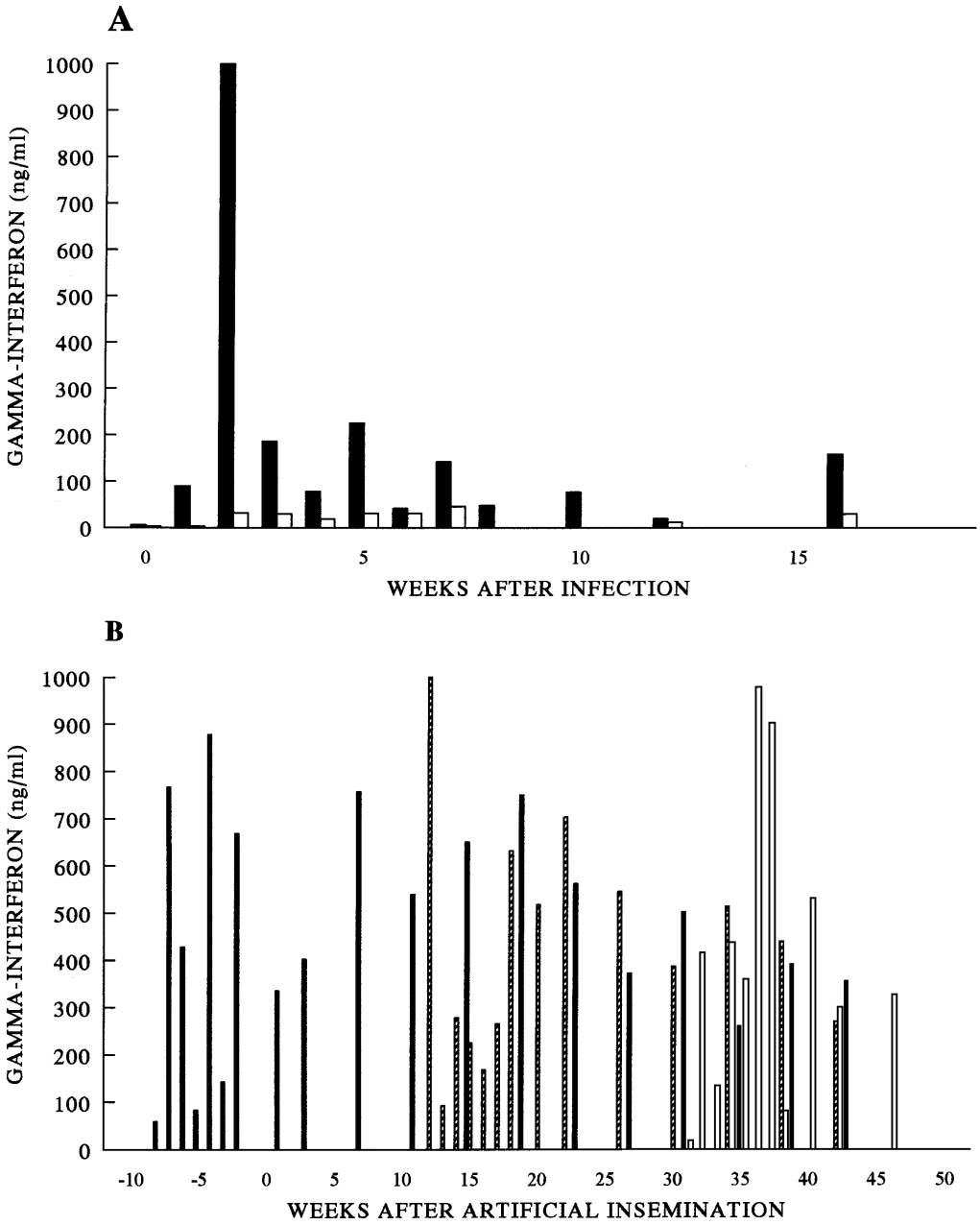

Fig. 2. Gamma-interferon production in culture supernatants from PBMC taken from cattle following intravenous inoculation with 10^7 tachyzoites. PBMC were collected at weekly intervals and cultured in the presence of 1 μg/ml of *Neospora caninum* antigen for 3 days. (A) Longitudinal response in infected Group IV (■) and uninfected, pregnant Group V (□). (B) Responses in Group I (■), infected 9 weeks before artificial insemination, Group II (▬) infected 10 weeks after artificial insemination, and Group III (□) infected 30 weeks after artificial insemination. Results are presented as the means for all the cows in each group.

1. In Groups I, III and IV the proliferative response was first detected 2 weeks following infection, and reached a peak between 4 and 10 weeks after infection. There was a delay in the proliferation response in Group II – proliferation was significantly lower in Group II compared with Groups I, III and IV on weeks 4 and 5 after infection ($P = 0{\cdot}006$ and $P = 0{\cdot}002$ respectively). Whereas there was considerable animal to animal variation in the proliferation response in Groups I, III and IV, the 6 animals in Groups II all had similar low responses. High levels of proliferation were detected 6 weeks after infection in Group II. Thereafter proliferation was detected in all 4 infected groups throughout the period of observation.

γIFN and TNFα responses

γIFN was measured in supernatants from PBMC cultured with *N. caninum* antigen for 3 days. The results are shown in Fig. 2. High levels of γIFN were detected in all infected animals from 2 weeks after infection until parturition, when the experiment was terminated.

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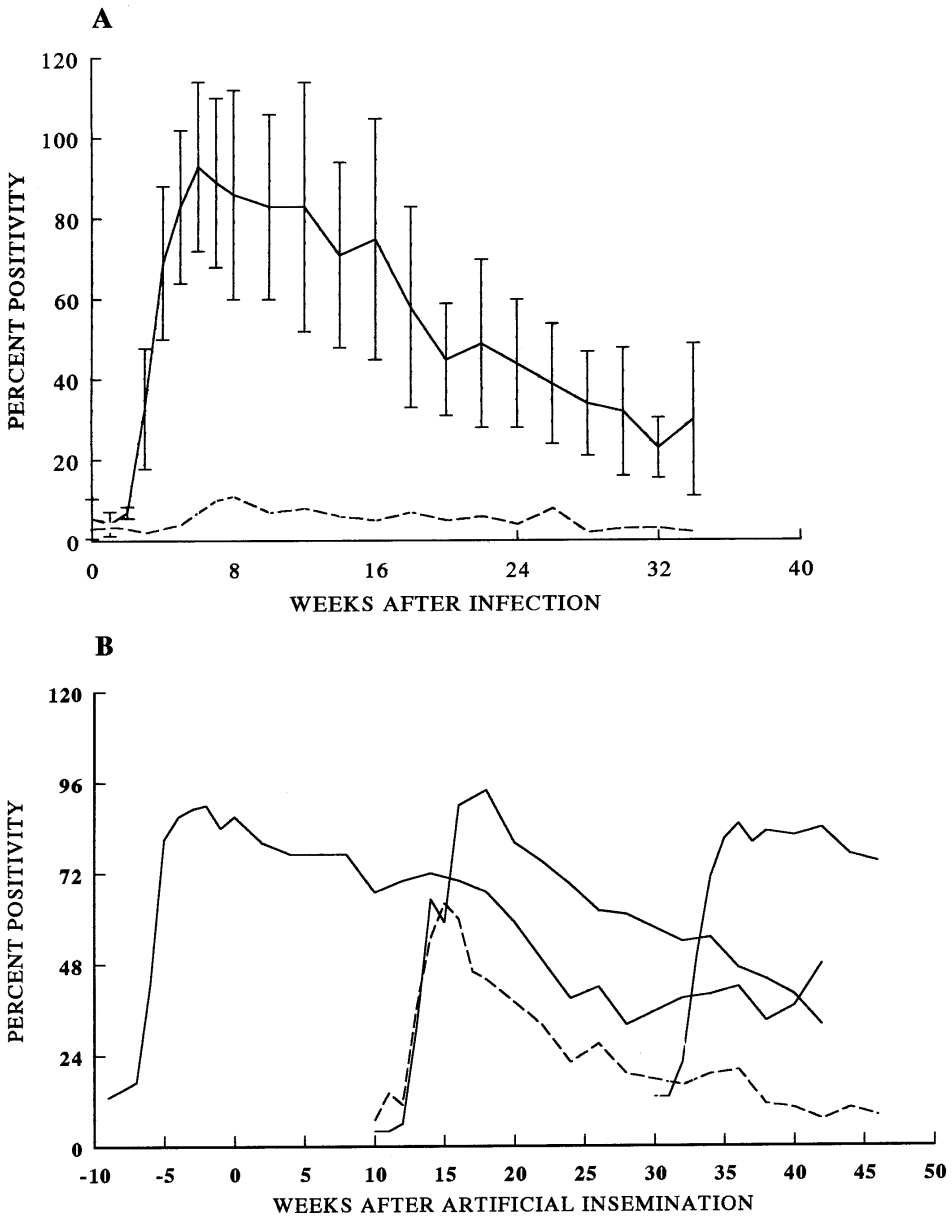

Fig. 3. *Neospora caninum*-specific IgG response in serum from cattle following intravenous inoculation with 10^7 tachyzoites. Serum was collected at weekly intervals and antibodies measured using the MASTAZYME ELISA. Results are expressed as percent positivity (PP), that is the optical density of the test sample expressed as a percentage of the mean optical density of the high positive control. (A) Changes in antibody after infection in the infected, non-pregnant Group IV (mean of 6 cows; ——) and the uninfected, pregnant Group V (mean of 3 cows; - - - -). (B) Mean responses in Group I ($n = 6$; ——) infected 9 weeks before artificial insemination, the 5 cows in Group II (-) infected 10 weeks after artificial insemination and whose foetuses were estimated to have died by 13 weeks of gestation, and Group III ($n = 6$; ——) infected 30 weeks after artificial insemination. The antibody response in the sixth cow in Group II which went on to produce a live calf at full term is also shown (- - - -). Error bars in (B) omitted for clarity.

TNFα levels were measured in sera from Groups I–IV. No TNFα was detected at any point. No TNFα was detected in culture supernatants of PBMC from Group II cattle between 0 and 6 weeks following infection (data not shown).

Antibody responses

The anti-*N. caninum* IgG response following infection is shown in Fig. 3. A PP of $\geqslant 20$ is considered to be a positive result (Williams *et al.* 1999). The antibody response increased in all infected cattle 4–6 weeks after infection and reached a peak around 7 weeks, irrespective of when the cattle were infected. From 10 weeks after infection the antibody response declined from these peak levels but remained positive until the end of the experiment.

The *N. caninum* specific IgG_1 and IgG_2 responses following infection are shown in Fig. 4. The sub-isotype-specific responses mirrored the IgG

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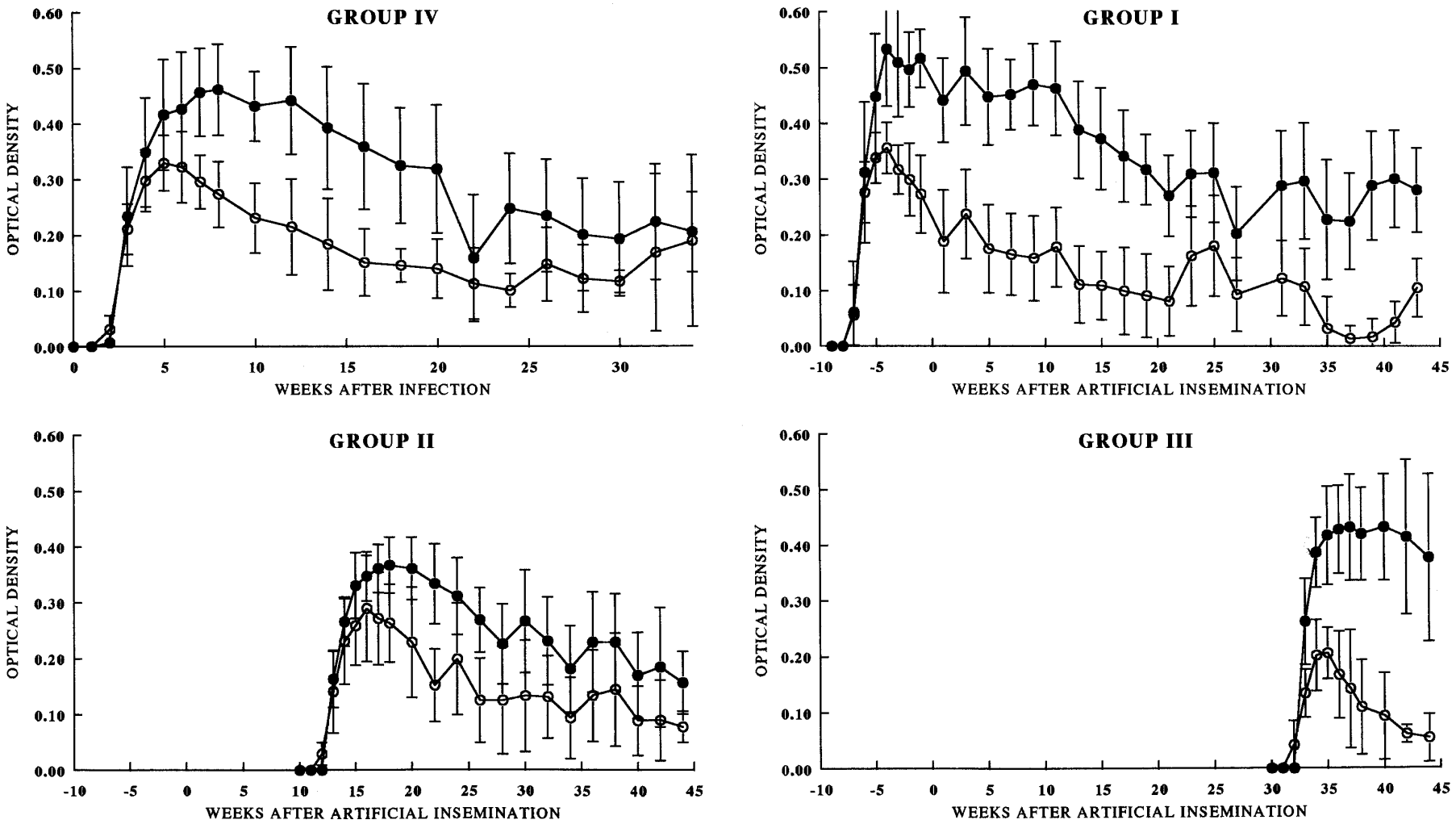

Fig. 4. *Neospora caninum*-specific IgG_1 (-○-) and IgG_2 (-●-) antibody responses in 4 groups of cattle after intravenous infection with 10^7 tachyzoites. Group I was infected 9 weeks before artificial insemination. Group II was infected 10 weeks after artificial insemination. Group III was infected 30 weeks after artificial insemination. Group IV was infected but not pregnant. Results are presented as the mean for all the cows in each group, except for Group II which shows the means for the 5 cows whose foetuses were estimated to have died by 13 weeks of gestation.

Table 3. Endpoint titres of *Neospora caninum*-specific IgG_1 and IgG_2 antibody in cattle taken 10 weeks after infection with *N. caninum* tachyzoites before or during pregnancy, showing 95% confidence intervals (CIs)

Group	IgG_1 endpoint titre $[\log_2 \times 10^3]$ (95% CIs)	IgG_2 endpoint titre $[\log_2 \times 10^3]$ (95% CIs)
Group I	11·8 (0, 24)	29·3 (23, 36)*
Group II	12·3 (6, 19)	32·0 (32, 32)
Group III	1·5 (0·9, 2)	23·3 (9, 37)†
Group IV	9·7 (0, 21)	29·3 (23, 36)‡

* $P = 0{\cdot}016$; † $P = 0{\cdot}011$; ‡ $P = 0{\cdot}006$ for within-group comparisons.

responses in the pattern of increase and decline. There was a decrease in IgG_1 in Groups I and III from 33 weeks of gestation which lasted until parturition and which was probably associated with the shunt of serum IgG_1 to colostrum prior to calving. There was no corresponding decrease in the 5 Group II cows who had lost their foetuses at 13 weeks gestation (Fig. 4). The sixth cow in that group that calved normally had a similar drop in her serum IgG_1 levels (data not shown).

The end-point titres of *N. caninum*-specific IgG_1 and IgG_2 were calculated for serum samples from each animal in Groups I–IV collected 10 weeks post-infection. The results are shown in Table 3. Titres of IgG_2 were significantly higher than the titres of IgG_1 in Groups I–IV ($P < 0{\cdot}02$).

DISCUSSION

This experiment demonstrates unequivocally that intravenous inoculation of cattle with tachyzoites of *N. caninum*, at 10 weeks of gestation, induces foetal death. No clinical signs were noted in the dams after infection, other than a transient rise in the body temperature of 0·5–1·0 °C, which was detected in 17 of the 24 cows, 4–7 days after infection (data not shown). There was no evidence of infection in foetuses of the 6 cows infected before pregnancy and cattle infected in late pregnancy gave birth to asymptomatic but seropositive calves. Infection in all the adult cows, irrespective of stage of gestation, was accompanied by a profound cellular proliferation response, γIFN production and a parasite-specific IgG_2 response that was greater than the IgG_1 response.

Infection with *T. gondii* elicits IL12 and γIFN production in mice, characteristic of a Type 1 helper T cell response (Gazzinelli *et al*. 1994). It has been predicted that the immune response to *N. caninum* will resemble that described for *T. gondii* since these two parasites are so closely related and similar in their biology. Lunden *et al*. (1998) and Marks *et al*. (1998) demonstrated that high levels of γIFN and proliferation were observed following *N. caninum* infection in non-pregnant cattle. In the study described here, *ex vivo* antigen-specific cellular proliferation, γIFN and IgG_2 production were detected in both pregnant and non-pregnant cattle following infection with *N. caninum*. These are all indicative of a Type 1 helper T cell response. The polarization of helper T cell function in cattle is less-well defined than in mice, but γIFN and IgG_2 have been associated with T_H1 responses described in other parasitic infections in cattle (Brown & Estes 1997). In 1993, Wegmann and colleagues (Wegmann *et al*. 1993) proposed an hypothesis to explain, in immunological terms, how a foetus survives within the maternal environment. They suggested that the balance of helper T cell responses in the mother is affected by Type 2 cytokines derived from foetal tissues resulting in a dominant T_H2 cell response during pregnancy. In contrast, a T_H1 cell response may be detrimental to the survival of the foetus. This hypothesis has been supported by studies of *Leishmania major* infection in resistant C57/Bl mice which, when administered during pregnancy, resulted in foetal death (Krishnan *et al*. 1996). The precise mechanism whereby a polarization of the response in favour of Type 1 cytokines affects the viability of the foetus is not fully understood although γIFN appears to be associated with damage to foetal tissues and NK cells have been shown to kill trophoblast cells (Drake & Head, 1989; Hunt, Atherton & Pace, 1990). A similar mechanism may have occurred in the cattle infected with *N. caninum* at 10 weeks of gestation, when the production of substantial levels of γIFN may have had a pathogenic effect on the foetuses. However, high levels of γIFN were also observed after infection at 30 weeks of gestation, but no foetal death occurred. It has been suggested that the temporal positioning of a T_H1 response during pregnancy is critical to foetal survival (Raghupathy, 1997) and thus the γIFN response induced by infection with *N. caninum* during late pregnancy may have occurred too late to affect an existing, well-established T_H2 response at the maternal–foetal interface. In this experiment, the peak *in vitro* proliferation response was significantly delayed in Group II cattle. T_H1 helper cells are thought to proliferate more strongly in *in vitro* culture than T_H2 helper cells, but whether there was a predominance of T_H2 helper cells, in the cultures derived from these cows is not clear. All 4 groups of infected cattle produced similar levels of γIFN.

As well as the nature of the maternal immune response to *N. caninum* infection affecting foetal viability, it is also possible that foetal immuno-competence at the time of infection may play a vital role in its survival. Immunocompetence in the bovine foetus is thought to develop at around 120 days or 17 weeks of gestation (Swift & Kennedy,

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1972). The infection administered at 10 weeks of gestation may therefore have given rise to a fulminating parasitaemia and sufficient placental and/or foetal pathology to kill the conceptus. Because no foetuses were recovered from these cattle it was not possible to determine if there were any parasite-induced lesions in the placentae or foetal tissues. The majority of *N. caninum*-associated abortions (NAAs) are reported to occur between 4 and 7 months gestation (Anderson *et al*. 1991, 1995; Barr *et al*. 1991*a*), i.e. after the foetal immune system has developed. Nevertheless it is likely that a substantial number of NAAs occur earlier than 4 months but are not reported as no foetal tissues are recovered. Many herds with a history of NAA also report fertility problems and a high incidence of return to service. Our data support the view that *N. caninum* infection early in gestation can lead to foetal death and resorption.

The Group III cattle, that were infected late in pregnancy, all gave birth to live, healthy calves. These calves had high pre-colostral anti-*N. caninum* antibody titres indicating that they had been exposed to the parasite *in utero* since there is no transplacental transfer of maternal immunoglobulin across the ruminant placenta (Husband, 1998). However, we were only able to demonstrate parasitological evidence of infection in 1 calf by PCR. It is likely that this is due to the low density of parasites in the tissues of the remaining 5 calves in Group III, a situation which has been described for field cases (Otter *et al*. 1995). Although several samples of tissue were taken for both histological examination and PCR, it was still only possible to screen a small proportion of the whole brain in this way. A positive result was obtained solely with the second PCR (Uggla *et al*. 1998), which used a different set of primers and more cycles than the original PCR described by McMillan *et al*. (1997). Again, this is probably a reflection of the limited amount of parasite DNA present in the tissues.

It is assumed that in chronically-infected cattle the parasite is spread to the foetus haematogenously as the result of recrudescence of infection (Anderson *et al*. 1997). Cattle can also be infected experimentally with oocysts (De Marez *et al*. 1999) and it is presumed that such *de novo* infection during pregnancy, could result in either abortion or birth of congenitally-infected offspring. Whatever the route of infection of cattle in the field, by inoculating the cattle intravenously, our experiment was designed to mimic the natural haematogenous spread of the parasite. What triggers the production of tachyzoites during pregnancy in congenitally-infected cattle is not known but the timing of this event is crucial since our data indicate that infection of the foetus early in pregnancy results in foetopathy, whereas infection in late pregnancy can give rise to infected, asymptomatic calves.

As yet there is only circumstantial evidence that post-natal infection can occur and cause abortion, whereas congenitally-infected cattle have been shown to have NAA and may abort more than once (Anderson *et al*. 1995). In this respect *N. caninum* is very different from *T. gondii* in sheep and humans where infection only results in abortion or congenital infections if individuals are exposed during pregnancy (Jackson & Hutchinson, 1989).

The 6 Group I cattle, infected 9 weeks before artificial insemination, had uneventful pregnancies and gave birth to 6 healthy, antibody-negative calves. These observations suggest that intravenous inoculation with tachyzoites did not give rise to cyst formation. No evidence of *N. caninum* infection was found in the tissues of any of the infected cattle, but again this may be due to the lack of sensitivity of the techniques used to detect parasites in bovine tissues.

No TNFα was detected in the plasma or culture supernatants of PBMC from infected cattle. High levels of TNFα are associated with acute toxoplasmosis and TNFα has also been shown to be harmful to the maintenance of pregnancy in mice (Chaouat *et al*. 1990; Gazzinelli *et al*. 1996). The limit of detection of TNFα activity in our assay was 3 pg/ml. However, during acute disease, nanomolar concentrations of TNFα are normally detectable in the circulation. Our results suggest that high levels of TNFα are not produced following infection with *N. caninum*.

The results presented here represent an important step forward in our understanding of the mechanisms leading to NAAs. We have demonstrated categorically that an intravenous bolus of *N. caninum* tachyzoites at 10 weeks of gestation resulted in foetal death and resorption and that infection in late pregnancy resulted in the birth of asymptomatic, congenitally-infected calves. These observations suggest that the reason some cows abort is related to the time during gestation when they become infected or an existing infection recrudesces.

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REFERENCES

ADAMS, J. L. & CZUPRYNSKI, C. J. (1994). Mycobacterial cell wall components induce the production of TNFα, IL1 and IL6 by bovine monocytes and the murine macrophage cell line RAW 264.7. *Microbial Pathogenesis* **16**, 401–411.

ANDERSON, M. L., BLANCHARD, P. C., BARR, B. C., DUBEY, J. P., HOFFMAN, R. L. & CONRAD, P. A. (1991). *Neospora-*

like protozoal infection as a major cause of abortion in dairy cattle. *Journal of the American Veterinary Association* **198**, 241–244.

ANDERSON, M. L., PALMER, C. W., THURMOND, M. C., PICANSO, J. P., BLANCHARD, P., BREITMEYER, R. E., LAYTON, A. W., MCALLISTER, M., DAFT, B., KINDE, H., READ, D. H., DUBEY, J. P., CONRAD, P. A. & BARR, B. C. (1995). Evaluation of abortions in cattle attributable to neosporosis in selected dairy herds in California. *Journal of the American Veterinary Association* **207**, 1206–1210.

ANDERSON, M. L., REYNOLDS, J. P., ROWE, J. D., SVERLOW, K. W., PACKHAM, A. E., BARR, B. C. & CONRAD, P. C. (1997). Evidence of vertical transmission of *Neospora* sp. infection in dairy cattle. *Journal of the American Veterinary Association* **210**, 1169–1172.

BARBER, J. S., HOLMDAHL, O. J. M., OWEN, M. R., GUY, F., UGGLA, A. & TREES, A. J. (1997). Characterisation of the first European isolate of *Neospora caninum* (Dubey, Carpenter, Speer, Topper and Uggla). *Parasitology* **111**, 563–568.

BARR, B. C., ANDERSON, M. L., DUBEY, J. P. & CONRAD, P. C. (1991*a*). *Neospora*-like protozoal infections associated with bovine abortions. *Veterinary Pathology* **28**, 110–116.

BARR, B. C., CONRAD, P. A., DUBEY, J. P. & ANDERSON, M. L. (1991*b*). *Neospora*-like encephalomyelitis in a calf: pathology, ultrastructure and immunoreactivity. *Journal of Veterinary Diagnostic Investigations* **3**, 39–46.

BARR, B. C., ROWE, J. D., SVERLOW, K. W., BONDURANT, R. H., ARDANS, A. A., OLIVER, M. N. & CONRAD, P. A. (1994). Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine *Neospora* isolate. *Journal of Veterinary Diagnostic Investigation* **6**, 207–215.

BROWN, W. C. & ESTES, D. M. (1997). Type 1 and Type 2 helper T cell responses in cattle and their regulation. In *Cytokines in Veterinary Medicine*, (ed. Chijins, V. E. C. J. S. & Horzinek, M. C.), pp. 15–33. CAB International, Wallingford, UK.

BUXTON, D., MALEY, S. W., THOMSON, K. M., TREES, A. J. & INNES, E. A. (1997). Experimental infection on non-pregnant and pregnant sheep with *Neospora caninum*. *Journal of Comparative Pathology* **117**, 1–16.

BUXTON, D., MALEY, S. W., WRIGHT, S., THOMSON, K. M., RAE, A. G. & INNES, E. A. (1998). The pathogenesis of experimental neosporosis in pregnant sheep. *Journal of Comparative Pathology* **118**, 267–279.

CHAOUAT, G. E., MENU, D. A., CLARK, M., DY, M., MINKOWSHI, M. & WEGMANN, T. G. (1990). Control of fetal survival in CBAx DBA/2 mice by lymphokine therapy. *Journal of Reproductive Fertility* **89**, 447–458.

DAVISON, H. C., OTTER, A. & TREES, A. J. (1999*a*). Significance of *Neospora caninum* in British dairy cattle determined by estimation of sero-prevalence in normally-calving cattle and aborting cattle. *International Journal for Parasitology* **29**, 1189–1194.

DAVISON, H. C., OTTER, A. & TREES, A. J. (1999*b*). Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. *International Journal for Parasitology* **29**, 1683–1689.

DEMAREZ, T., LIDDEL, S., DUBEY, J. P., JENKINS, M. C. & GASBARRE, L. (1999). Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular immune responses. *International Journal for Parasitology* **29**, 1647–1657.

DRAKE, B. L. & HEAD, J. R. (1989). Murine trophoblast cells can be killed by lymphokine activated killer (LAK) cells. *Journal of Immunology* **143**, 9–14.

DUBEY, J. P., CARPENTER, J. L., SPEER, C. A., TOPPER, M. J. & UGGLA, A. (1988). Newly recognised fatal protozoan disease of dogs. *Journal of the American Veterinary Medicine Association* **192**, 1269–1285.

DUBEY, J. P. & LINDSAY, D. S. (1996). A review of *Neospora caninum* and neosporosis. *Veterinary Parasitology* **67**, 1–59.

DUBEY, J. P., LINDSAY, D. S., ANDERSON, M. L., DAVIS, S. W. & SHEN, S. K. (1992). Induced transplacental transmission of *Neospora caninum* in cattle. *Journal of the American Veterinary Medicine Association* **5**, 709–713.

ELLIS, J. T., MCMILLAN, D., RYCE, C., PAYNE, S., ATKINSON, R. & HARPER, P. A. W. (1999). Development of a single tube nested PCRT for the detection of *Neospora caninum* DNA. *International Journal for Parasitology* **29**, 1589–1596.

GAZZINELLI, R. T., WYSOCKA, A., HAYASHI, S., DENKERS, E., HIENY, S., CASPAR, P., TRINCIERI, G. & SHER, A. (1994). Parasite-induced IL12 stimulates early IFN gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. *Journal of Immunology* **153**, 2533–2543.

GAZZINELLI, R. T., WYSOKA, M., HIENY, S., SCHARTON-KERSTEN, T., CHEEVER, A., KUHN, R., MULLER, W., TRINCHIERI, G. & SHER, A. (1996). In the absence of endogenous IL-10 mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-γ and TNFα. *Journal of Immunology* **157**, 798–805.

HUNT, J. S., ATHERTON, R. A. & PACE, R. L. (1990). Differential responses of rat trophoblast cells and embryonic fibroblasts to cytokines that regulate proliferation and MHC Class I expression. *Journal of Immunology* **145**, 184–190.

HUSBAND, A. (1998). Passive transfer of immunity in cattle. In *Handbook of Vertebrate Immunology*, (ed. Pastoret, P.-P., Griebel, P., Bazin, H. & Govaerts, A.), pp. 464–465. Academic Press, London.

JACKSON, M. H. & HUTCHINSON, W. M. (1989). The prevalence and source of *Toxoplasma* infection in the enviromnent. *Advances in Parasitology* **28**, 55–105.

KHAN, I. A., SWARTZMAN, J. D., FONSEKA, S. & KASPER, L. (1997). *Neospora caninum*: role for immune cytokines in host immunity. *Experimental Parasitology* **85**, 24–34.

KRISHNAN, L., GUILBERT, L. J., WEGMANN, T. G., BELOSEVIC, M. & MOSMANN, T. R. (1996). T helper 1 response against *Leishmania major* in pregnant C57B1/6 mice increases implantation failure and fetal resorptions. *Journal of Immunology* **156**, 653–662.

LUNDEN, A., MARKS, J., MALEY, S. W. & INNES, E. A. (1998). Cellular immune responses in cattle experimentally infected with *Neospora caninum*. *Parasite Immunology* **20**, 519–526.

MARKS, J., LUNDEN, A., HARKINS, D. & INNES, E. (1998). Indentification of *Neospora* antigens recognized by $CD4^+$ T cells and immune sera from experimentally infected cattle. *Parasite Immunology* **20**, 303–309.

MCALLISTER, M. M., DUBEY, J. P., LINDSAY, D. S., JOLLEY, W. R., WILLS, R. A. & MCGUIRE, A. M. (1998). Dogs are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* **28**, 1473–1478.

MOEN, A. R., WOUDA, W., MUL, M. F., GRAAT, E. A. M. & VAN WERVEN, T. (1998). Increased risk of abortion following *Neospora caninum* abortion outbreaks: a retrospective and prospective cohort study in four dairy herds. *Theriogenology* **49**, 1301–1309.

OTTER, A., JEFFREY, M., GRIFFITHS, I. B. & DUBEY, J. P. (1995). A survey to determine the incidence of *Neospora caninum* infection in aborted and stillborn bovine foetuses in England and Wales. *Veterinary Record* **136**, 602–605.

RAGHUPATHY, R. (1997). Th1-type immunity is incompatible with successful pregnancy. *Immunology Today* **18**, 478–482.

SWIFT, B. L. & KENNEDY, P. C. (1972). Experimentally induced infection *in utero* of bovine foetuses with bovine parainfluenza-3 virus. *American Journal of Veterinary Research* **33**, 57–63.

THURMOND, C. M., HIETALA, S. K. & BLANCHARD, P. (1997). Herd-based diagnosis of *Neospora caninum*-induced endemic and epidemic abortion in cows and evidence for congenital and post natal transmission. *Journal of Veterinary Diagnostic Investigation* **9**, 44–49.

TREES, A. J., GUY, F., TENNANT, B. J., BALFOUR, A. H. & DUBEY, J. P. (1993). Prevalence of antibodies to *Neospora caninum* in a population of urban dogs in England. *Veterinary Record* **132**, 125–126.

UGGLA, A., STENLUND, S., HOLMDAHL, O. J. M., JAKUBEK, E. B., THEBO, P., KINDAHL, H. & BJORKMAN, C. (1998). Oral *Neospora caninum* inoculation of neonatal calves. *International Journal for Parasitology* **28**, 1467–1472.

WEGMANN, T. G., LIN, H., GUILBERT, L. & MOSMANN, T. R. (1993). Bi-directional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunology Today* **14**, 353–356.

WILLIAMS, D. J. L., MCGARRY, J., GUY, F., BARBER, J. & TREES, A. J. (1997). A novel ELISA for detection of *Neospora*-specific antibodies in cattle. *Veterinary Record* **140**, 328–331.

WILLIAMS, D. J. L., DAVISON, H. C., HELMICK, B., MCGARRY, J., GUY, F., OTTER, A. & TREES, A. J. (1999). Evaluation of a commercial ELISA to detect serum antibody to *Neospora caninum* in cattle. *Veterinary Record* **145**, 571–575.