Immunity to

Neospora caninum

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To John and Martha Marks
and Jonathan Steers

Abstract

Neospora caninum is an intracellular coccidian parasite which has recently been recognised as a major cause of abortion and congenital defects in cattle and dogs worldwide. Infection with *N.caninum* is linked to 4-10% of bovine abortions in England and Wales, 4-10% in Northern Ireland and 16% in Scotland and has been estimated to cost Scottish agriculture approximately £2.5 million per year. The cost to the rural economy due to neosporosis is therefore significant.

The main route of disease transmission is by transplacental infection, but as the definitive host of *Neospora* was only recently identified as the dog (McAllister *et al*, 1998) an oral route of transmission by ingestion of *Neospora* oocysts is also possible. The disease is often linked with abortion storms in dairy herds and the repeat abortion rate due to neosporosis has been estimated at approximately 5% of affected animals. Congenitally infected calves may appear clinically normal but the effect of neurological deficits on production is unknown. The full host range of the parasite or it's zoonotic potential is also unknown, and currently there is no recommended method of control.

This thesis covers three main areas of research. The first is a longitudinal sero-epidemiological study of a dairy herd which suffered an abortion storm linked to infection with *N.caninum* during August/September 1995. The main aims were to study the long term antibody response in cattle which have suffered *N.caninum* associated abortion, and to assess the rates of congenital infection, abortion and repeat abortion on the farm during the subsequent 3 year period. Serum antibody titres in cattle which had aborted during the abortion storm of 1995 dropped to levels considered negative within 3-4 months post abortion. The reason for this is unknown but clearly has implications for diagnosis, suggesting that current diagnostic tests such as IFAT and ELISA which use whole antigen preparations are not sensitive enough for diagnosis in individual animals. Antibody levels increased in these animals during the 4th-5th month of gestation the following year when the majority of control animals remained sero-negative suggesting that they were persistently infected with *N.caninum*. One of the control animals did

however sero-convert during gestation and gave birth to a congenitally infected calf indicating that low levels of infectious material may have been present on the pasture. Pre-colostral serum samples could only be obtained from a small number of new-born calves therefore it was not possible to calculate the rate of congenital infection, but one suspected and three proven cases were shown. Only one of these calves had neurological abnormalities, therefore it is likely that the majority of congenitally infected animals are clinically normal. Aborted foetuses sero-positive for *N.caninum* continued to be produced periodically, and a total of 29 abortions or resorptions were linked to infection with *N.caninum* from early 1995 to December 1997. This had a significant financial impact on this small dairy herd of approximately 130 cattle. Repeat abortion due to neosporosis may have occurred in 2-5 animals, with the repeat abortion rate estimated at between 10-21%, at least double the rate reported by Dubey and Lindsay (1988a). The calving index for the herd was particularly high suggesting that infection with *N.caninum* may cause fertility problems.

The second area of study investigated *Neospora* antigens recognised by *Neospora* antibody positive sera using western blot. Diagnosis of infection with *N.caninum* depends on detection of anti-*N.caninum* antibody in serum, but animals which have previously aborted due to neosporosis can become sero-negative by *Neospora* IFAT and ELISA several months post-abortion. Some cross reactivity with antigens of closely related coccidian parasites has also been observed. Development of a diagnostic test using *N.caninum* specific antigens widely recognised by naturally infected cattle may improve sensitivity and specificity of diagnosis. This study showed that a *N.caninum* antigen of approximately 65kDa separated by SDS PAGE was widely recognised by sera from naturally infected cattle and aborted foetuses from around Scotland. This antigen was parasite specific as no cross reaction with serum antibody positive for *Toxoplasma gondii, Sarcocystis tenella, Cryptosporidium parvum and Babesia spp* was observed. An antigen of approximately 18kDa and another at the base of the western blot did however cross react with *Babesia* and/or *Sarcocystis* positive sera suggesting that develoment of an antigen specific test could improve diagnosis. Intensity of staining of individual bands varied between animals and could affect the sensitivity of

diagnosis if too few antigens were incorporated into a test of this kind. Differences were seen in the antibody profile of seropositive animals in Scotland and Northern Ireland, possibly due to strain variation, which again suggests that a combination of several antigens could be a better source of antigen for diagnosis.

The third area investigates cell mediated immune responses to *N.caninum* and the antigens involved in induction of T cell responses. *N.caninum* can induce repeat abortion in some individuals unlike the closely related coccidian parasite *Toxoplasma gondii* which induces life long protective immunity after primary infection. Cellular immune responses are important in the development of immunity to *T.gondii* and therefore are likely to be important in preventing repeat abortions in 95% of the cattle which abort due to neosporosis. This study showed that experimental infection of calves with *N.caninum* NC1 tachyzoites stimulated a cell mediated response detectable in peripheral blood using a simple proliferation assay. This response was characterised by the production of the T cell cytokine IFNγ which is produced by CD4+, CD8+ and natural killer cells and is known to be important for protection against other intracellular parasites. However PBM from infected cattle which were sero-negative for *T.gondii* also proliferated in response to *T.gondii* water soluble antigen *in vitro* and stimulated production of IFNγ from these cells. The response of CD4+ T cells from *Neospora* infected cattle were tested using various antigen fractions separated by SDS PAGE or by HPLC. Antigen fractions of ≤30kDa consistently induced proliferation of CD4+ T cells and production of IFNγ. Interestingly, despite the cross reactive response observed in PBM with *Neospora* and *Toxoplasma* crude lysate antigen *Toxoplasma* antigen fractions did not stimulate proliferation of CD4+ T cells from *Neospora* infected cattle. Further work is needed to characterise these important antigens and to identify the immune mechanisms which will protect against clinical neosporosis.

Declaration

I hereby declare that I composed this thesis entirely by myself and that it describes my own research.

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Abbreviations

APC	antigen presenting cell
BSA	bovine serum albumin
Ci	Curie
CNS	central nervous system
Con A	concanavalin A
CPE	cytopathic effect
CPM	counts per minute
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FITC	fluorescein isothyocianate conjugate
g	relative centrifugal force
HBSS	Hanks balanced salt solution
IFAT	indirect fluorescence antibody test
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
Iscom	immunostimulating complex
kDa	kilo Dalton
Min	minute
Mab	monoclonal antibody
NBT	nitro blue tetrazolium
N.caninum	*Neospora caninum*
OD	optical density
OPD	ortho-phenylenediamine
PAGE	polyacrylamide gel electrophoresis

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pw	primary wall
r	recombinant
s	second
SDS	sodium dodecyl sulphate
se	standard error
SI	stimulation index
T.gondii	*Toxoplasma gondii*
Th	T helper
wsf	water soluble factor
v/v	volume per volume

Chapter 1

Introduction

1. Introduction

Neospora caninum (Dubey *et al*, 1988a) is a recently recognised coccidian parasite first identified as a distinct organism in 1984 (Bjerkas *et al*) and isolated into tissue culture in 1988 (Dubey *et al*, 1988b). Although many studies have been carried out since we still do not fully understand the epidemiology of this disease or the pathology and immune response to infection with *N.caninum*.

Neospora was first reported as a cause of abortion in British cattle in 1994 (Trees *et al*). The parasite has a wide host range and can cause abortion and congenital defects in cattle (Thilstead & Dubey, 1989), goats (Dubey *et al*, 1992), horses (Dubey & Porterfield, 1990), and dogs (Dubey *et al*, 1988a). It has been identified as an important cause of abortion in cattle, implicated in 43% of bovine abortions in California (Anderson *et al*, 1995), 4-10% in Northern Ireland (McNamee *et al*, 1996) and 16% in Scotland (Buxton *et al*, 1997a). The parasite is found worldwide and has been reported to be associated with bovine abortions in the USA (Yeager, 1994), Canada (Duivenvoorden and Lusis, 1995), UK (Otter *et al*, 1993; McNamee and Jeffrey, 1994), Ireland (Collery, 1995), Australia (Boulton *et al*, 1995), Japan (Ogino *et al*, 1992) and Zimbabwe (Jardine and Wells, 1995) among others. The cost to the Californian dairy industry alone is thought to be US$34.5m each year (Ellis, pers comm), therefore the parasite is economically significant.

At present there are no control strategies for neosporosis. The majority of infected dams produce congenitally infected calves which are often clinically normal and may provide a reservoir of infection within the herd, although *Neospora* can cause repeat abortion in a minority of animals. Accurate diagnosis of infected individuals to be culled or treated may be important for control of this disease. Diagnosis of neosporosis is largely achieved using the indirect fluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA) which detect *Neospora* specific antibody in sera from

infected animals. The parasite antigens used in these tests may cross react with antibody produced in response to antigens shared with closely related coccidian parasites leading to false positive diagnoses. ELISA and IFAT are therefore not accurate enough for diagnosis of individual cases but will provide an indication of the herd infection status. Development of a serological test using selected *Neospora* specific proteins widely recognised by naturally infected animals may improve diagnosis. One aim of this thesis is to identify *Neospora* specific antigens widely recognised by naturally and experimentally infected cattle but not recognised by sera from cattle infected with other coccidian parasites for use in the development of a more specific and reliable diagnostic test for neosporosis

Very little is known about the host immune response to *N.caninum* and the precise cause of abortion is unknown. Cell mediated responses have previously been shown to be important in the development of protective immunity to the closely related parasite *Toxoplasma gondii* (Suzuki and Remington, 1988; Parker *et al*, 1991; Innes and Wastling, 1995). IFNγ, an important T cell cytokine produced mainly by CD4+ T cells, is a major mediator of resistance to *Toxoplasma* in sheep (Innes *et al*, 1995c). As *N.caninum* is also an obligate intracellular parasite it is likely that CD4+ T cells may be a critical component of the cell mediated response to *N.caninum.* An aim of this thesis is to characterise the immune response to the parasite in naturally and experimentally infected cattle, and to identify *Neospora* specific antigens which may stimulate a potentially protective cell mediated immune response. These studies would provide important information for the development of an effective vaccine against neosporosis in the future.

Very few longitudinal studies have been undertaken to investigate disease epidemiology on farms suffering losses due to neosporosis. The main cost of natural infection with *Neospora* is loss of income to the farmer through abortion, lower milk yield and production of weak calves. Few long term studies on persistence of serum antibody levels in naturally infected cattle or the rate of repeat abortion and congenital infection have been published. A better understanding of the sero-epidemiology of this disease could be important for the development of control strategies and improvement in

diagnosis. This study details a 3 year investigation of neosporosis in a small dairy herd in Northern Ireland which has suffered abortions due to neosporosis since 1995.

In summary, this thesis is comprised of three areas of study;

- The longitudinal study of neosporosis in a dairy herd in Northern Ireland
- The humoral immune response to natural and experimental infection with *N.caninum* and detection of *Neospora* specific antigens
- The cell mediated immune response to experimental infection with *N.caninum* and the detection of parasite specific antigens which stimulate this response

It is hoped that these studies will lead to a better understanding of the humoral and cell mediated immune responses to *N.caninum* in cattle which may contribute to the future development of effective diagnostic tests and control measures against this widespread and economically important pathogen.

Chapter 2

Literature Review

2. Literature Review

2.1 *Neospora caninum*: historical background

2.1.1 Discovery of a new pathogen

Neospora caninum is an obligate intracellular coccidian parasite. It was first described in 1984 by Bjerkas, Mohn and Presthus who reported neurologic disorders 2-6 months after birth in five of six boxer pups which were all offspring of the same dam. Post mortem examination of all 6 animals identified lesions in the central nervous system and skeletal muscles associated with cysts containing a parasite. Under light microscopy the parasites present in these lesions resembled *Toxoplasma gondii,* a protozoan which can cause abortion and congenital defects in humans and sheep, but sera taken from the infected animals lacked specific antibodies to *Toxoplasma.* It was not until 1988 that the parasite associated with these lesions was first isolated into tissue culture and mice by Dubey *et al* who proposed a name for the newly discovered protozoan parasite *Neospora caninum* (Dubey *et al*, 1988a).

2.1.2 Neosporosis in dogs

In 1984 Bjerkas *et al* reported an unidentified cyst forming sporozoon which caused fatal disease in dogs. The parasite was found in association with necrosis and severe inflammatory lesions in all parts of the CNS. Although the organisms looked similar to *T.gondii* tachyzoites infected animals lacked antibodies to *Toxoplasma.* A further study by Dubey *et al* (1988a) reviewed tissues from 23 cases of *Toxoplasma*-like illness in dogs and again found parasite associated with lesions in the CNS which did not cross react with anti-*T.gondii* antibody positive serum. Dubey named the newly recognised parasite *Neospora caninum* using the same terminology as that

used for *T.gondii.*

Koch's postulates were fulfilled by Dubey *et al* (1988b) when *N.caninum* was isolated from homogenates of canine tissues into tissue culture and experimental inoculation reproduced the disease in a dog. Dubey was also the first to suggest that *N.caninum* was congenitally transmitted in dogs after observing infection in 2 successive litters from the same bitch indicating reactivation of subclinical infection. Vertical transmission in dogs was confirmed in 1990 (Dubey *et al*, 1990b) by a case study in which successive litters of German shorthaired pointers from the same clinically normal bitch developed limb paralysis. The other 8 littermates of this bitch all had hind limb paralysis and died before 6 months of age. Four of her pups were found to have *N.caninum* tachyzoites, tissue cysts or both present in extraocular muscles. Transplacental transmission was later experimentally induced in dogs by Cole *et al* (1995). Inoculation on day 21 of gestation induced abortion in 5 pregnant bitches, and fullterm pups from another bitch were born with clinical symptoms of neosporosis including proprioception deficits and spasticity in pelvic limbs.

There have been very few surveys on the incidence of neosporosis or *Neospora* antibody in the dog population and there is some debate over what level of antibody is considered positive for neosporosis in dogs. A study by Trees *et al* (1993) showed 13% of 163 randomly selected and tested dogs at the Liverpool Small Animal Hospital had antibody titres of ≥1:200, with no correlation between antibody titre and breed, sex, age, type of feeding or presence of other dogs in the household. Again there was no association between *Toxoplasma* and *Neospora* antibody in these samples, suggesting minimal serological cross reactitvity between the parasites.

None of the animals tested in the British study had clinical symptoms of neosporosis. This indicates a high level of subclinical infection in British dogs when compared to a study in Kansas by Lindsay *et al* (1990b) where only 2.2% of dogs had titres >1:50, although a small scale survey of feral coyotes in Texas found 10% had low antibody titres to *N.caninum* of ≤1:100 with 4% ≥1:25. A retrospective analysis of histopathology cases has however shown that *Neospora caninum* infection in dogs has occurred since 1957 in the USA.

The dog has very recently been identified as a definitive host of *Neospora caninum* (McAllister *et al*, 1998). Oocysts morphologically similar to *T.gondii,*

Hammondia hammondi and *Hammondia heydorni* were found in faeces of 3 beagles fed mouse tissue infected with *N.caninum* tissue cysts. Mice had been inoculated with the canine NC2 (Hay *et al,* 1990) and NC Liv (Barber *et al*, 1995) and bovine NC Beef (McAllister *et al*, 1998) strains of the parasite. *Neospora* tachyzoites were isolated from mice orally inoculated with infected faeces confirming that the oocysts were infective and caused clinical neosporosis. This finding clearly has implications for the development of disease control strategies and further studies on the survival of oocysts, levels of contamination on pasture and shedding of oocysts by infected dogs will be important in the development of these strategies.

Figure 2.1 The dog is the definitive host of *N.caninum*

Photograph courtesy of Dr.D.Buxton

2.1.3 Neosporosis in cattle

In California as early as 1985 it was becoming clear that some previously unknown *Toxoplasma* like infectious agent was responsible for numerous abortions in Californian dairies (Barr *et al,* 1996). A two year retrospective study begun in 1988 indicated the disease was the largest single identifiable cause of bovine abortion in California. Development of an immunohistochemical test to identify *Neospora* organisms in tissues associated with these lesions (Lindsay and Dubey 1989) led to the discovery of the parasite in aborted bovine foetuses. Neosporosis has since been reported in cattle worldwide including the USA (Yaegar *et al,* 1994), Australia (Boulton *et al*, 1995), New Zealand (Thornton *et al,* 1994), Ireland (McNamee and Jeffrey, 1994), Canada (Duivenvoorden and Lusis, 1995), Zimbabwe (Jardine and Wells, 1995), Japan (Ogino *et al*, 1992) and the UK (Otter *et al*, 1993). Surveys of bovine neosporosis using IFAT (Conrad *et al*, 1993b) and ELISA (Paré, 1995) tests for antibody to *Neospora* in sera linked the disease to high rates of bovine abortion in Scotland, 16% (Buxton, 1997a), Ireland, 4-10% (McNamee *et al*, 1997), England and Wales, 4.2-10.5% (Otter *et al*, 1995), and California, 43%, (Anderson *et al,*1995).

Transplacental transmission was found to occur in naturally infected cattle (Barr *et al*, 1993; Anderson *et al*, 1997) and unlike the closely related protozoan *Toxoplasma,* which stimulates life long immunity in the host after primary infection (Innes, 1997), abortion due to *N.caninum* infection can occur repeatedly in some animals. The rate of repeat abortion is not yet known but has been quoted at approximately 5% of cattle affected (Dubey and Lindsay, 1996). Transplacental transmission has been experimentally reproduced in cattle (Barr *et al*, 1994a) providing a model for the study of infection in the natural host.

Although the full extent of the problem is not yet known bovine abortion due to infection with *N.caninum* is clearly of significant economic importance.

2.1.4 Neosporosis in other domestic animals

Natural infection with *N.caninum* has been reported in a wide range of animals and can cause abortion in goats (Dubey *et al*, 1992). *Neospora* has been diagnosed in a fullterm stillborn deer, (Dubey *et al,* 1996) and has been reported to cause a range of symptoms in horses including blindness, rear limb paralysis and anaemia (Lindsay *et al*, 1996c; Gray *et al*, 1996; Daft *et al*, 1996).

Transplacental transmission has been experimentally induced in sheep (McAllister *et al,* 1996, Buxton *et al*, 1997b). However a survey by Otter *et al* (1995) did not find antibodies to *Neospora* in tissue samples from 281 aborted ovine foetuses submitted from veterinary investigation centres around England and Wales suggesting *Neospora* is not a major cause of abortion in sheep. Transplacental transmission has also been experimentally induced in cats (Dubey and Lindsay, 1989a) but there have been no reports of the disease occurring naturally in this species. Further studies are needed as the full host range for this parasite is not yet known.

2.1.5 Neosporosis in humans

Neosporosis has not yet been reported in humans, but to date no large scale surveys have been undertaken. Non-human primates (rhesus macaque) experimentally infected were susceptible to transplacental *Neospora* infection (Barr *et al*, 1994a). Foetal lesions induced were found to be similar to those caused by transplacental *Toxoplasma* infections in primates and infection was confirmed by reisolation of the parasite *in vitro*.

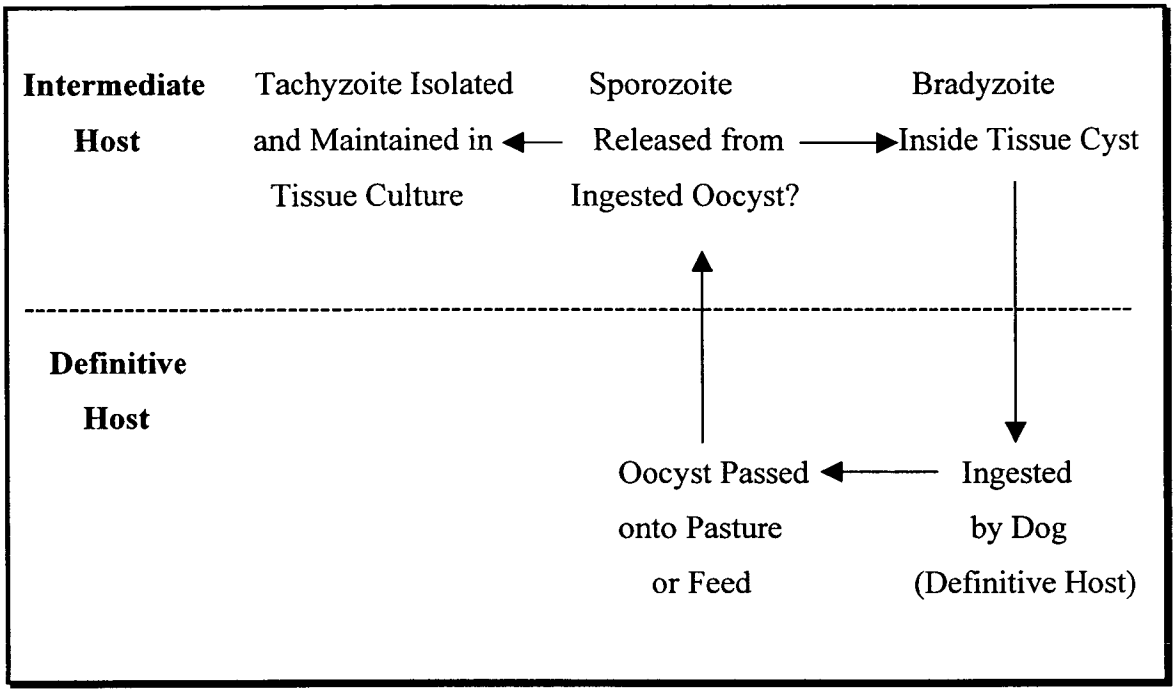
2.2 Transmission and life cycle

2.2.1 Life cycle of *N.caninum*

The complete life cycle of *N.caninum* is not yet known. A speculative life cycle based on *T.gondii* is illustrated in figure 1. Tissue cysts containing bradyzoites (slow replicating form) are largely confined to the tissues of the CNS of both dogs and cattle. The tachyzoite or fast replicating form of the parasite has been isolated in tissue culture from both bovine and canine neural tissue. Most research has therefore been conducted using this stage of the life cycle. *Neospora* infects the foetus during gestation and can cause abortion and congenital infection in cattle. It is also an important cause of neuromuscular paralysis in dogs.

The dog has only recently been identified as the definitive host of *N.caninum* (McAllister *et al*, 1998). Three beagle puppies fed tissues from mice inoculated with both bovine and canine strains of the parasite shed oocysts morphologically similar to those of closely related coccidian parasites. Experimental infections of other carnivorous potential hosts including cats (Cuddon *et al*, 1992), coyotes (Lindsay *et al*, 1996b) and raccoons (Dubey *et al*, 1993) have so far been unsuccessful in producing oocysts in the faeces of these species, but these studies have used only a maximum of 3 animals each. Infected coyotes and racoons did however sero-convert after infection. Another study to evaluate raptoral birds as the definitive host for *N.caninum* (Baker *et al*, 1995) used 2-3 mostly adult birds of 4 different species which had been living in the wild before being submitted to public or wildlife agencies. No oocysts were found in the faeces of these animals but it is possible that they could have ingested the parasite previously and were now immune to it.

Figure 2.2 Life cycle of *Neospora caninum*



2.2.2 Ultrastructural characteristics of *Neospora*

Several *Neospora* isolates have been grown in tissue culture from naturally infected bovine and canine tissues. A recent study by Jardine (1996) studied the ultrastructure of bradyzoites and tissue cysts of these isolates and concluded that there are no distinct morphological differences between bovine and canine derived parasites at each stage of the parasite life cycle (Jardine, 1996).

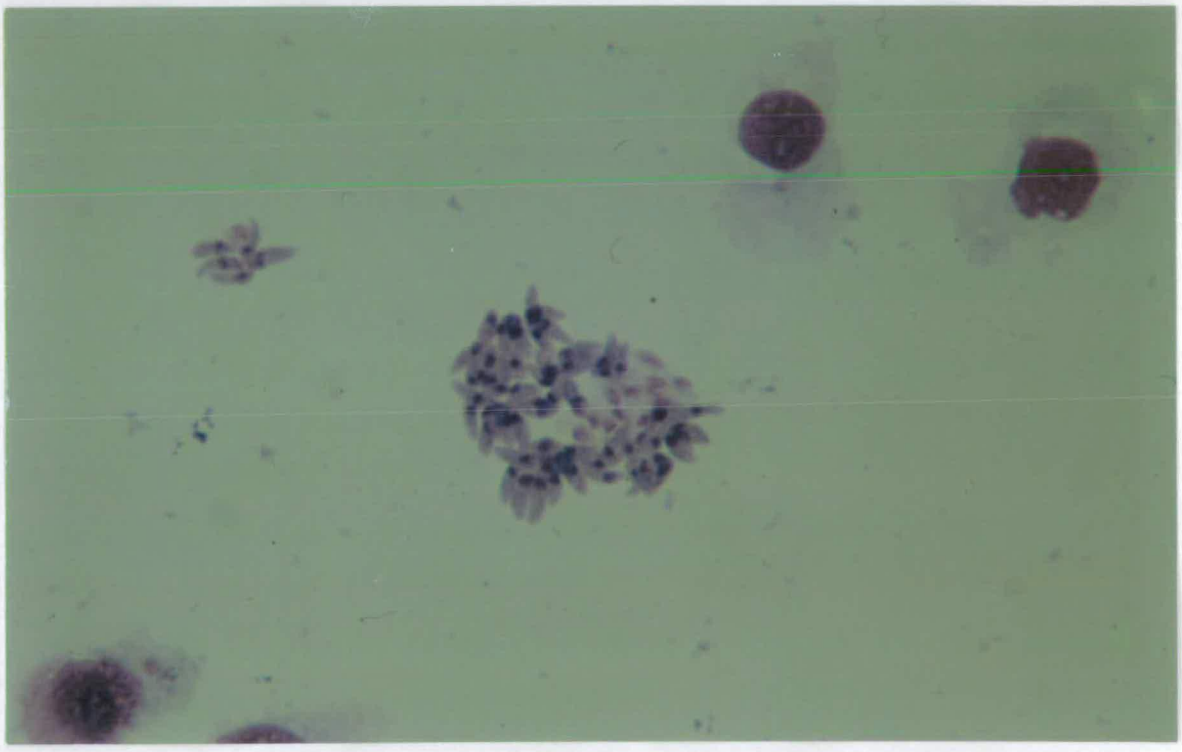
The following description of *N.caninum* tachyzoite, bradyzoite and tissue cyst morphology and ultrastructure are derived from studies of tissues taken from naturally infected dogs by Speer and Dubey (1989), Lindsay *et al,* (1993) and Dubey *et al,* (1988a).

2.2.2.1 *N.caninum* tachyzoites

The tachyzoite is the rapidly dividing stage of the parasite. It is crescent shaped, 4-7μm x 1.5-5μm in size with a single vesicular nucleus and has all the ultrastructural features that are characteristic of other closely related cyst forming

coccidia. This stage in the parasite's life cycle replicates by endodyogeny and has a pellicle consisting of a plasmalemma and a single inner membrane. Twenty two subpellicular microtubules were observed beneath the inner membrane complex and 2 elongated branched tubular mitochondria were present, 1 anterior and 1 posterior to the nucleus. Tachyzoites have several unusual characteristics including 8-12 anterior rhopteries, electron dense posterior rhopteries, many anterior and few posterior micronemes and lack micropores. As many as 50-100 tachyzoites may occur free or within a parasitophorous vacuole within a range of host cells including macrophages, neutrophils, neural cells, hepatocytes, fibroblasts and blood vascular endothelial cells. Tachyzoites of *N.caninum* are virtually indistinguishable from those of *T.gondii* by light microscopy.

Figure 2.3 *N.caninum* tachyzoites in tissue culture



Photograph courtesy of Miss.W.Panton

2.2.2.2 *N.caninum* tissue cysts and bradyzoites

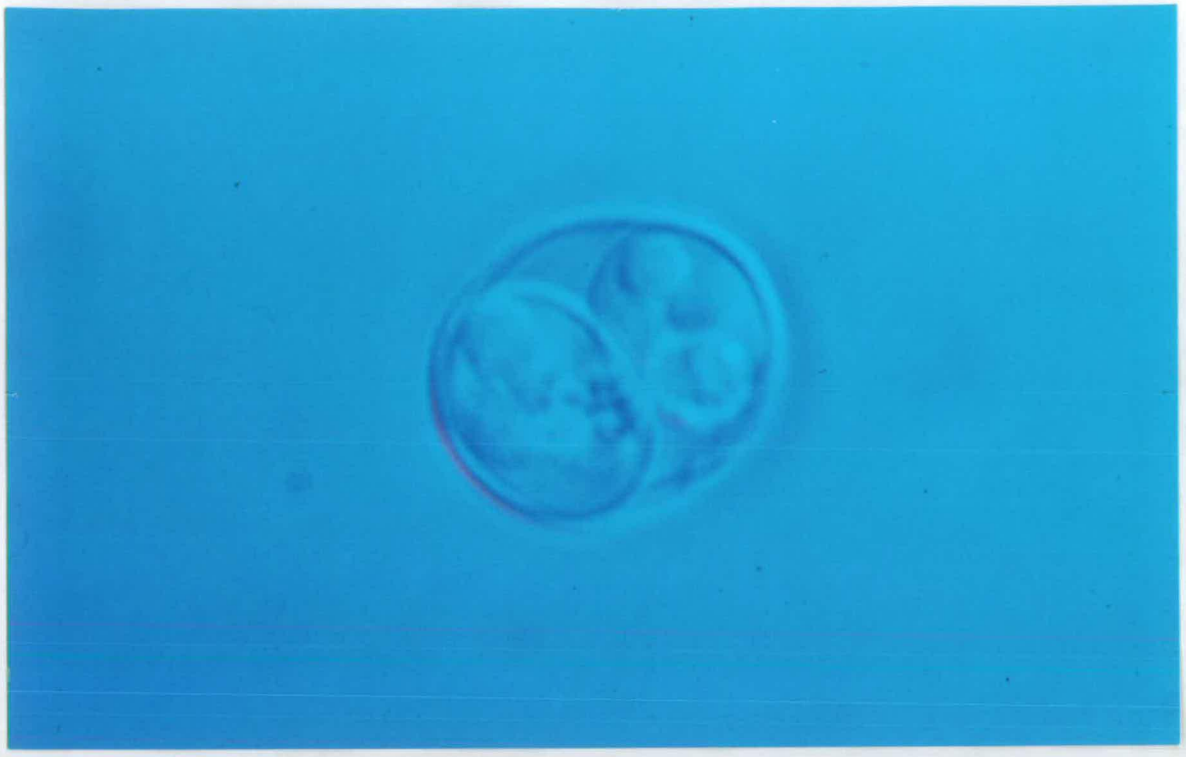
Tissue cysts are found inside neural cells of naturally infected cattle, dogs and other infected hosts. The cyst wall is 1-4μm thick, slightly thicker than that of *T.gondii*, and consists of a single primary wall (pw) which completely surrounds the cyst, and a thick granular layer containing electron dense vesicles embedded in a matrix of fine granules. In contrast the pw of the *T.gondii* cyst is highly convoluted and does not have an electron dense matrix.

N.caninum bradyzoites found inside tissue cysts are approximately 7.3x1.5μm in size. In addition to organelles and inclusion bodies typical of other coccidian parasites they contain numerous micronemes and amylopectin granules, and 6-12 anterior rhopteries. They are therefore similar in size and ultrastructure to those of *T.gondii.*

2.2.2.3 *N.caninum* oocysts

Neospora caninum oocysts have only recently been identified in faeces from dogs fed *N.caninum* tissue cysts present in neural tissue of mice experimentally infected with NC2 (Hay *et al,* 1990), NC Liv (Barber *et al,* 1995) and NC beef strains of the parasite (McAllister *et al,* 1998). Oocysts produced were unsporulated, spherical to subspherical in shape, measured 10-11μm in diameter and contained a central sporont. The oocysts sporulated within 3 days, contained 2 sporocysts, each with 4 sporozoites, and were morphologically similar to *T.gondii, Hammondia hammondi* and *Hammondia heydorni* (figure 1.3).

Figure 2.4 A *Neospora caninum* oocyst



Photograph courtesy of Dr.M.McAllister

2.2.2.4 Ultrastructural differences between *T.gondii* and *N.caninum* tachyzoites

Neospora caninum is structurally similar to *T.gondii* and has previously been misdiagnosed as *Toxoplasma* (Dubey *et al,* 1988a). A study by Lindsay *et al* (1993) identified differences between 3 canine isolates of *N.caninum,* NC1, NC2 and NC3, and 2 *T.gondii* strains, the RH isolate and a temperature sensitive mutant, TS-4, of the RH isolate. When examined by transmission electron microscopy, tachyzoites of the two species were found to have numerous differences. Those of *T.gondii* had fewer anterior rhopteries (4-8), anterior micronemes and anterior electron dense bodies than *N.caninum* tachyzoites. Posterior rhopteries and micronemes were absent, but unlike *N.caninum* tachyzoites, micropores were common.

2.2.3 Isolates of *N.caninum*

Many bovine and canine isolates of *N.caninum* are in use in studies around the world but very little work has been done to characterise them. Marsh *et al* (1995) compared the nuclear small subunit ribosomal (nss-r) RNA sequences of bovine and canine *Neospora* strains to that of *T.gondii, Cryptosporidium parvum* and *Sarcocystis muris,* three closely related coccidians. Although this sequence of approximately 400 nucleotides of the 5' region has been identified as evolutionarily unstable, no differences were found between 4 different bovine *Neospora* isolates, BPA 1-4, isolated from aborted bovine foetuses and congenitally infected calves. Holmdahl *et al* (1997) also compared the internal transcribed spacer 1 (ITS1) sequence of a bovine *Neospora* strain isolated from a stillborn calf in Sweden, named NC1-SweB1 (Stenlund *et al*, 1997), with *N.caninum* NC1 canine isolate. This sequence is considered to be conserved within species but to be more variable than individual rRNA genes between species (Hyde, 1990). No differences were recorded between NC SweB1 and NC1 isolates, in contrast to a large number of sequence differences between *N.caninum* and *T.gondii.* Comparison of the ITS1 region of *N.caninum* NC1 and NC Liv (both canine isolates) by Barber *et al* (1995) also did not detect any differences in this region. A high degree of homology between bovine *Neospora* sp. and *N.caninum* canine isolates supports close taxonomic placement but more information is needed before it can be determined if they are the same species. Therefore, as bovine and canine *Neospora* sp. are so closely related, the use of canine isolates of *N.caninum* in diagnostic tests is justified.

2.2.4 Transmission and life cycle in cattle

Neosporosis has been found in both beef and dairy cattle, but has been reported more frequently in dairy cattle, particularly those in drylot dairies (Anderson *et al*, 1994). This may be due to the dense population of cattle in dairies allowing infection to pass between animals in close proximity although there is currently no evidence for this. Another possibility is faecal contamination of harvested feeds stored on or around the farm, often open to contamination, by farm

dogs and possibly rodents or other local wildlife with the oocyst stage of the parasite. Infected feed is then fed to the entire herd. This may explain why neosporosis induces clusters of abortions or 'abortion storms' (Anderson *et al,* 1991; Dannat *et al,* 1995). Both of these factors would be conducive to the spread of disease among a large number of animals.

Basic epidemiologic information on seasonal patterns of neosporosis may help direct studies identifying events that may contribute to transmission of disease or trigger abortion in infected cows. A study by Thurmond *et al* (1995) of dairy cattle over a 6 year period indicated that there does seem to be an increased risk of abortion due to *Neospora* in the months of November, December, January and February. But as there is only 16% difference between expected and observed figures a substantial number of abortions may not be influenced by seasonally related factors.

Neospora was confirmed as a cause of abortion in cattle when Barr *et al* (1994b) reproduced foetal infection and death by experimentally infecting pregnant cattle with a bovine isolate, BPA1, and re-isolating parasite from foetal tissues. Infection was found to be similar to that caused by natural infection as it did not cause clinical symptoms in the dams and immunohistochemical analysis of foetal neural tissue revealed clusters of tachyzoites which reacted positively to anti-*Neospora* sera. As with the closely related coccidian parasite *Toxoplasma gondii* the time of infection and therefore the immune competence of the foetus may be important in determining the outcome of infection. A mummified foetus was expelled at 67 days post infection from a dam infected at 95 days gestation. A second animal infected at 115 days gestation, when some foetal immune competence would have been attained, carried a clinically normal calf to full term. This calf had raised pre-colostral antibody titre to *Neospora* and mild encephalomyelitis, although no parasites were found in tissues.

Neosporosis has also been reported to cause repeat infection of foetuses by the same dam, although how often this occurs is not known. This was first reported in a study by Barr *et al* (1993) who followed four dairy cows which had previously aborted due to neosporosis. All of the cows were kept on their respective dairies, successfully rebred, were monitored throughout pregnancy and all produced full term calves. Two of the calves had marked neurologic deficits including decreased

patellar reflexes and ataxia. Both were found to have mild non-suppurative encephalomyelitis, and thick walled protozoal cysts were found randomly distributed in brain tissue and spinal cord. Tachyzoites and tissue cysts were found in the CNS of one other calf, although it was clinically normal. Exposure of cows to *Neospora* prior to pregnancy does not therefore prevent transplacental infection during subsequent pregnancy. This contrasts with the closely related protozoan parasite *T.gondii* which stimulates a strong immune response in the host which provides life long protection against repeat abortion.

It has not been determined whether repeat foetal infection is due to recrudesence of infection or re-infection in the dam, but as repeat congenital infection is not characterised by widespread infection in other previously uninfected animals on the farms, the former is likely to be true.

A large scale study by Paré *et al* (1996), which lasted over 2 years and monitored over 400 calves born on 2 dairies, showed that although a majority of seropositive cows (81%) produced infected offspring, 5% of seronegative cows also produced congenitally infected calves. Therefore the serological status of the dam will not always predict infection in the calf. Congenital infection was not associated with dam age, lactation number, history of abortion, calf gender or length of gestation. High dam Neospora antibody levels at calving were significantly associated ($P<0.001$) with probability of congenital infection in the calf. From this study it was concluded that congenital transmission was likely to be the major mode of transmission of *N.caninum*.

Surprisingly this study found that calves which had been congenitally infected but clinically normal had a marginally but consistently higher suvivorship than non-infected animals. Reasons for this are unknown but may be due to antibodies against antigens which are shared by *N.caninum* and other closely related coccidian parasites and which may be cross protective.

2.2.5 Transmission and life cycle in dogs

Congenital transmission of *N.caninum* has been shown to occur in naturally infected puppies (Barber and Trees, 1998; Dubey *et al,* 1988b; Dubey *et al*, 1990b)

and typically the first clinical signs appear 5-8 weeks after birth. Neosporosis is therefore often thought of as a disease of puppies although infection has been reported in dogs of all ages (Barber and Trees, 1996). The disease may affect several littermates but more often than not involves an isolated case (Barber and Trees, 1996). Transplacental transmission may also occur repeatedly in litters from the same bitch (Barber and Trees, 1998; Dubey *et al*, 1990b). A study by Barber and Trees (1998) reports that the transmission rate to pups is low, as 80% of pups born to seropositive dams were not infected as determined serologically. The frequency of vertical transmission was also found to vary between litters and may be too low to sustain infection alone. These findings suggest that postnatal infection must occur to maintain infection at seroprevalence rates reported in dog populations.

Vertical transmission of *N.caninum* has been reproduced in a canine model of infection by inoculation with NC1 tachyzoites ($5x10^6$) on day 21 of pregnancy (Cole *et al*, 1995). Of 6 bitches infected 5 aborted, and *N.caninum* tachyzoites were isolated from pups in 2 of the aborted litters and from 4 infected bitches. A previous study by Dubey and Lindsay (1989b) reported that experimental infection on day 35 of pregnancy caused only a mild illness, although this may have been due to a lower dose ($1.5x10^6$) of tachyzoites. Experimental infection with *Neospora caninum* during pregnancy can therefore lead to fetal death, but whether *N.caninum* causes fetal deaths, abortion and sterility in naturally infected dogs is as yet unknown and requires further investigation.

As previously discussed the dog has recently been identified as the definitive host of *N.caninum* (McAllister *et al*, 1998). Oocysts morphologically similar to those of *T.gondii, Hammondia hammondi* and *Hammondia heydorni* were found in faeces of 3 beagles fed mouse tissue infected with *N.caninum* tissue cysts. Faecal contamination by dogs may therefore be a major route of transmission of this disease.

2.2.6 Transmission in primates

As previously discussed it is not yet known if *Neospora* can cause disease in humans, but experimental infection can induce transplacental infection in the non-

human primate. *Neospora* tachyzoites were found in association with lesions in foetal tissues similar to those induced by the closely related protozoan *Toxoplasma gondii,* which is known to infect and cause abortion and congenital defects in humans.

2.3 Epidemiology of neosporosis

2.3.1 Sero-epidemiology in cattle

The main diagnostic techniques used to detect infection with *N.caninum* are based on binding parasite specific antibody present in serum using ELISA or IFAT. It is important therefore to understand the kinetics of antibody production and maintenance of antibody titres in an infected animal to determine what a *Neospora* positive antibody titre in cattle means for production and disease control.

It has been proposed that congenital infection may be the main means of persistence and spread of the disease in cattle (Anderson *et al,* 1997; Bjorkman *et al,* 1996; Paré *et al*, 1996). In a study by Bjorkman *et al,* (1996) all of the serpositive cattle in a dairy herd which had suffered sporadic abortions since 1980 were descendants of 2 cattle bought in 1980, indicating that *Neospora* has the ability to be transmitted from dam to offspring for several generations. All of the abortions which occurred on this farm from 1991-1993 occurred in animals which were descendants of these two cattle. Repeat abortion has been reported in about 5% of cattle who have previously aborted due to neosporosis (Dubey and Lindsay, 1996). Identification of cows which are likely to abort or produce congenitally infected offspring could be important for the implementation of control strategies. The risk of abortion in cows that are seropositive for *N.caninum* throughout pregnancy has been found to be twice that for seronegative cows and calves born to cows seropositive at 240 days gestation are more likely to be congenitally infected (Paré *et al*, 1997). *Neospora* post-natal antibody titres do however drop dramatically from immediate post abortion levels and cows which have aborted *Neospora* infected foetuses can become apparently

seronegative within 150 days of giving birth (Conrad *et al*, 1993b). Antibody levels increased again during subsequent pregnancies in these animals and the calves born were found to be congenitally infected. These results indicate that congenital infection and abortion in cattle may be caused by infection with *N.caninum* acquired before pregnancy which then persists in the adult.

It is clear however that calves born with high pre-colostral serum *N.caninum* antibody titres are congenitally infected with the parasite which persists in tissue cysts in the brain and spinal cord of these animals (Conrad *et al,* 1993a). A study by Paré *et al* (1996) on the prevalence of infection in calves from 2 large Californian dairies indicated that congenital infection does not necessarily have a detrimental effect on the health of the calf which is born without clinical symptoms. Survival analyses of female calves indicated a consistently greater suvivorship of infected calves compared to non-infected calves. The rate of congenital infection was found to be high with 81% of seropositive cows producing infected calves. Another study by Thurmond *et al* (1997) found an association between seropositivity and abortion in only 4 of 15 herds, but in this case animals were tested for antibody to *N.caninum* up to 6 months after an abortion epidemic had occurred. As previously discussed the rapid fall in post-natal antibody titre to the parasite could have greatly affected these results. It is likely therefore that congenital infection is the major mode of transmission of the disease.

The potential role of concurrent infection in animals already infected with *N.caninum* is not known. Suppression of the immune response caused by increased pressure on the immune system could allow a chronic sub-clinical infection to become apparent by increasing blood parasitaemia levels reflected in an increase in anti-parasite antibody titres. This opportunistic route of infection could trigger abortion and may explain why disease onset is often linked with abortion storms and why *Neospora* antibody levels fluctuate in infected individuals. Clearly the role of concurrent infections could be important to disease outcome and should be investigated.

A proposed method of control is the selection of animals for culling. It is important that clinicians who may embark on this method are aware that fluctuation in antibody titres occurs in naturally infected animals and that accurate diagnosis

clearly depends on targeting the time of sampling to when *Neospora* antibody titres are likely to be high, for example during gestation for maternal sera and immediately after birth in congenitally infected calves.

2.3.2 Repeat abortion in Cattle

Neospora caninum can cause repeat abortion in cattle (Anderson *et al*, 1995; Obendorf *et al*, 1995) although the rate of repeat abortion is unknown as no large scale studies have been carried out to investigate how often this occurs. An accurate assessment of the problem would be difficult to achieve as cows which have aborted once may be more likely to be sold on or culled. Also, confirmation that abortion was due to infection with *N.caninum* depends on identifying *Neospora* antigen in aborted foetal tissue, and abortion occuring in the field during a long period of grazing may result in foetal tissue being lost. Individual reports of repeat abortion in single herds indicate that it occurs relatively infrequently with <5% of animals aborting two successive pregnancies (Anderson *et al*, 1995; Obendorf *et al*, 1995; Thornton *et al*, 1994).

2.3.3 Non-clinical congenital infection in cattle

Congenital infection was first reported in puppies which suffered clinical symptoms of neosporosis such as progressive hind-limb paralysis (Dubey and Lindsay, 1990). Clinical neosporosis in post natal calves was reported but found to be rare (Collery, 1996). A study by Barr *et al* (1993) investigating transmission of *N.caninum* in successive pregnancies found that cows which had previously aborted due to neosporosis produced transplacentally infected calves the following season. Infected calves, which were identified by their high pre-colostral *Neospora* antibody titres, were all born fullterm after uncomplicated pregnancies. It is not known if congenital infection in offspring was due to recrudesence of infection in the dam or reinfection, but as animals which produced congenitally infected offspring did so in the absence of *Neospora* induced abortion storms on their respective dairies it is likely that it is due to recrudesence of infection within individual animals.

Figure 2.5 Cow with healthy calf

Photograph courtesy of Dr.D.Buxton

Other studies have also shown that acquisition of infection with *N.caninum* during pregnancy is not a pre requisite for foetal infection or abortion to occur (Thurmond *et al*, 1997; Pare *et al*, 1997). In Barr's study only 2 of 5 infected calves showed marked neurologic deficits which included slight loss of conscious proprioception. The remaining 3 calves were clinically normal and would otherwise have gone undiagnosed. Although it is not yet known if these congenitally infected offspring can abort due to recrudesence of *N.caninum* in later life it is possible that these animals provide a reservoir for infection which persists in the herd undetected.

Current information suggests that congenital infection is the major mode of transmission of *N.caninum* (Pare *et al*, 1996). Seropositive cows have been shown to produce less milk and have a shortened production life compared with seronegative animals (Thurmond and Hietala, 1996). Removal of infected animals from the herd may reduce the rate of infection and abortion over time and improve production,

therefore identification of clinically normal congenitally infected calves may become important in the development of a control strategy for this disease. However seronegative replacements may have an increased risk of acquiring neosporosis from the environment, possibly leading to an increase in abortions. Further investigation on the effect of introducing seronegative animals into a potentially infected environment is needed before selective culling of seropositive animals can be recommended as a method of disease prevention.

2.3.4 Search for definitive hosts

As previously discussed *Neospora caninum* has been found in a wide range of hosts including cattle, dogs, goats, horses and deer. The dog has recently been identified as a definitive host of *N.caninum* (McAllister *et al*, 1998) but experimental infections of other potential carnivorous hosts including cats (Cuddon *et al*, 1992), coyotes (Lindsay *et al*, 1996b), racoons (Dubey *et al*, 1993) and raptoral birds (Baker *et al*, 1995) have so far been unsuccessful in producing oocysts in the faeces of these animals. Very few animals were used in these studies and in some cases were adult and had previously lived wild. These animals may have been infected before experimental inoculation and thus been resistant to secondary infection. Further studies using larger numbers of naïve animals are needed to investigate the possibility of other definitive hosts, and in particular using wild canids and raptors.

2.4 Pathogenesis of *N.caninum* infection

2.4.1 Naturally infected cattle

Since 1985 diagnosticians at the California Veterinary Diagnostic Laboratory System (CVDLS) have observed a distinct pattern of inflammatory lesions consisting of focal nonsuppurative necrotising encephalitis and myositis in many aborted bovine foetuses submitted for diagnosis (Barr *et al*, 1990). The pattern of lesions, particularly in the brain, were similar to those seen with *Toxoplasma gondii* infection in sheep (Buxton and Finlayson, 1986) and were found to be caused by *N.caninum.*

2.4.2 Experimentally infected cattle

Bovine foetal neosporosis has been induced by experimental infection of bovine foetuses and pregnant cattle with the BPA1 bovine *Neospora* isolate (Barr *et al*, 1994b). Cattle were infected between 85 and 161 days gestation. Two foetuses were inoculated intramuscularly and the remaining pregnant cattle were inoculated intramuscularly and intravenously. Histologic examination of foetal tissues showed systemic foetal infections identical to those reported for spontaneous *Neospora* abortions (Barr *et al*, 1990; Ogino *et al,* 1992). Parasites were found associated with characteristic focal mononuclear inflammatory cell infiltrates in brain, kidney, lung and placentome tissues of the foetus. Transplacental infection was therefore successfully induced by experimental inoculation, and as with natural infection caused no clinical signs in the dams.

2.4.3 Naturally infected dogs

Lesions associated with tachyzoites and tissue cysts of *N.caninum* are usually found in the central nervous system (CNS) and skeletal muscle of infected dogs but lesions and tachyzoites have also been reported in heart, lungs, liver, kidney, adrenal glands, uterus, sciatic nerve, muscularis of the oesophagus, extraocular muscles and

in the eye (Dubey *et al*,1988b; Dubey *et al*, 1990b; Barber *et al*, 1996; Barber and Trees, 1996; Sheahan *et al*, 1993). Clinical signs of neosporosis are associated with rapid intracellular multiplication of the parasite causing cell death and stimulating an inflammatory response. Dogs often suffer from generalised nonsuppurative encephalomyelitis characterised by necrosis, neovascularisation, perivascular infiltration of mononuclear cells, demyelination, gliosis and multifocal inflammation with focal infiltration of mononuclear cells. Organisms are rarely found even when there is inflammation. Skeletal myositis is characterised by mild necrosis, vasculitis, basophilia and infiltrations of mononuclear cells, all found particularly in the hindlimbs. Lesions in the eyes and extraocular muscles can lead to ocular abnormalities such as a slow pupillary light reflex and may cause focal retinitis of the inner retinal layers. In general there is a widespread dissemination of tachyzoites in the acute phase of infection. As tachyzoites were found to be common in the quadriceps muscle a muscle biopsy taken in this area may aid diagnosis.

Static tissue cysts do not stimulate a response from the host and have been described only within the CNS (Dubey and Lindsay, 1993). A study by Barber *et al* (1996) reported that the distribution and number of parasites varies markedly between individual animals and is not related to the age of the dog. Tachyzoites or cysts occur at all levels of the CNS and are found in all areas of the brain, more often in the grey matter, therefore clinical signs are complex and vary in each case

2.4.4 Experimental model – sheep

The study of neosporosis in cattle, the natural host, is expensive therefore the development of an experimental model in a small ruminant would be beneficial. Very few cases of neosporosis have been reported in sheep, but as they suffer from abortion due to infection with *T.gondii* which is closely related to *N.caninum,* the development of an ovine experimental model of neosporosis has been poposed.

Several studies on the effect of experimentally induced neosporosis in pregnant sheep have been carried out using canine isolates of *N.caninum* (McAllister *et al,* 1996; Buxton *et al*, 1997b). Experimental infection early in gestation (on day 65) with $1.7x10^5$ or $1.7x10^6$ tachyzoites resulted in abortion in all cases. Infection

mid-gestation (day 90) caused abortion/mummification in some foetuses while other lambs, both weak and normal, were born fullterm. All ewes infected late gestation (day 120) produced clinically normal congenitally infected offspring. These studies indicate that the time of infection of the foetus is important and therefore the immune response of the foetus may be important in the outcome of infection.

The most common condition seen in both fullterm lambs and aborted foetuses was encephalitis. Lesions ranged from mild perivascular cuffing to severe infiltration of cells, necrotising encephalitis and meningitis and were most frequently seen in the cerebral cortex, thalamus, mid-brain and medulla oblongata. Cell infiltrations consisted of lymphocytes or microglia and in post natal lambs contained additional plasma cells and astrocytes. Protozoal cysts were found in the brains of 38% of aborted foetuses and 39% of clinically normal congenitally infected lambs in a study by McAllister *et al* (1996). As in natural infection cysts were not seen outside the CNS. Buxton *et al* (1997b) did not report on the number of cysts in fetal neural tissue but did find lesions consistent with *Neospora* infection in neural tissue from all foetuses from dams infected at 90 days gestation. Other common symptoms of disease included placentitis and necrosis of placental villi. Clusters of tachyzoites were seen in some placentas examined. Myositis of the tongue and diaphragm occurred frequently characterised by leucocyte infiltration and oedema. Similar lesions and inflammation were seen in heart muscle (myocarditis), but occurred infrequently.

In both studies maternal neural and extraneural tissues were sampled and tested for the presence of parasite antigen. There was no evidence of infection in the ewes from either experiment.

Experimental infection with *N.caninum* in sheep can induce abortion, stillbirth and birth of weak and congenitally infected clinically normal lambs similar to natural bovine infection. The distribution of cysts and lesions is also similar to those caused by natural infection in cattle. Experimentally induced neosporosis in sheep therefore appears to be a good model of neosporosis in cattle, although further studies on natural and experimental infection of cattle have yet to be carried out for direct comparison with this model.

2.4.5 Experimental model – goats

Abortion caused by natural infection with *Neospora caninum* has been reported in pygmy goats (Barr *et al*, 1992; Dubey *et al*, 1992). Lesions in aborted kids were found to be similar to those found in naturally infected aborted bovine foetuses. For this reason, and their small size and short gestation period, pygmy goats would provide a useful model for infection in cattle if experimental infection was found to reproduce the disease in this species.

A study by Lindsay *et al* (1995b) investigated the effects of experimental infection of pygmy goats with NC1 strain *N.caninum* tachyzoites and compared the pathology with that caused by natural infection in cattle. Abortions and stillbirths were induced and were the only clinical signs of infection with *Neospora*. Inoculation early in gestation resulted in abortion of severely autolysed foetuses and tachyzoites were identified in the brain spinal cord and heart of these offspring. Infection mid gestation did not clinically affect most kids but did cause one abortion. *Neospora* tachyzoites were identified only in the aborted foetus and could not be isolated from clinically normal kids. Microscopic lesions and the gestational age of aborted kids were similar to those of *Neospora* infected bovine foetuses, and closely resembled naturally acquired *N.caninum* induced abortions in cattle. The main difference in this model was that infection of kids was not observed when the does were rebred, although this may have been due to attenuation of the parasite strain in tissue culture or resistance of the host to infection.

2.4.6 Experimental model – Mice

Outbred strains of laboratory mice are resistant to clinical neosporosis when inoculated with currently available strains (Lindsay *et al,* 1990, 1992). Treatment of mice with an immunosuppressant such as methylprednisolone (MPA) is needed to overcome natural resistance (Lindsay *et al,* 1990). Development of a rodent model that was susceptible to clinical infection was important to further knowledge of immunity, pathogenesis and treatment of *N.caninum* infections.

Studies by Lindsay *et al* (1995a) and Long *et al* (1998) found that inbred BALB/c mice are susceptible to infection with the NC1 strain of *N.caninum* without

the use of immunosuppressants. Clinical signs of illness included development of rough coats 5 days PI followed by the development of head tilts and pelvic limb paralysis/weakness by 14-28 days PI. Deaths due to infection occurred from 26-70 days PI. Brain lesions were randomly scattered, and infection induced multifocal meningoencephalitis characterised by multifocal necrosis with macrophage infiltration and lymphocytic perivascular cuffing. Tachyzoites were observed within macrophages and associated with brain lesions. Tissue cysts were not found in brain tissue. Lesions observed were similar to those reported for mice given MPA suggesting the BALB/c model will be valuable for examining the pathogenesis and treatment of neosporosis.

2.5 Diagnosis of *N.caninum* infection

Clearly it is important to be able to identify animals infected with *Neospora* and several diagnostic techniques are currently in use. As *N.caninum* tachyzoites, present in smears of cells taken from cerebro-spinal fluid (CSF), bronchial lavage and dermal sores, are not distinguishable morphologically from *T.gondii* tachyzoites by light microscopy, more specific tests are essential for accurate diagnosis.

2.5.1 Indirect fluorescent antibody test

The indirect fluorescent antibody test (IFAT) was the first diagnostic test developed for detection of *N.caninum* infection and measures the *Neospora* specific antibody titre in serum of infected animals (Conrad *et al*, 1993b). This test is not accurate enough for diagnosis of individual cases as clinically normal animals have been found to have titres greater than 1:800 (Dubey and Lindsay, 1993), but it can give a good indication of herd status. As IFAT results are read manually the process is time consuming and subjective. Antibody titres in infected animals can also decrease significantly over a prolonged period of time (Conrad *et al*, 1993b). As whole *Neospora* tachyzoites are used in this test there is also some possibility of

cross reaction with antibody to closely related coccidian parasites. In all cases care must be taken in the interpretation of serology results as we cannot predict the relationship between a high antibody titre and the outcome of pregnancy. For example, it is not yet clear if seropositive cows are more likely to abort or produce congenitally infected calves in the future and therefore whether or not they should be maintained in the herd or culled (Conrad *et al*, 1993b). Further studies are needed before we can answer these important questions.

2.5.2 Enzyme linked immunosorbant assay

Enzyme linked immunosorbant assays (ELISA) can provide consistent, objective and rapid results (Seefekdt *et al*, 1989), often with increased sensitivity and specificity when compared to IFAT (Kuby, 1992). ELISA kits are currently commercially available for detection of *Neospora* specific antibody in cattle (Williams *et al*, 1997; IDEXX UK). The kit developed by Williams *et al* used whole tachyzoites and very little cross reaction was observed with sera from cattle experimentally infected with other closely related coccidian parasites. Several ELISA's have been developed using sonicated tachyzoites of bovine and canine *Neospora* tachyzoites as antigen (Osawa *et al*, 1998; Paré *et al*, 1995). Incorporation of a range of antigens may overcome the potential problem of antigenic diversity of *Neospora* isolates giving greater sensitivity (Paré *et al*, 1995).

More specific ELISA's using extracted tachyzoite proteins of *Neospora caninum* NC1 isolate incorporated into immunostimulating complexes have been developed by Björkman *et al* for diagnosis in dogs (1994) and cattle (1997). The major antigens incorporated into iscoms are amphipathic molecules such as membrane proteins (L□vgren, 1987). However, the immunodominant antigens of *N.caninum* identified by Western blot are located in rhopteries, dense granules, micronemes and the parasitophorous vacuole (Bjerkas *et al*, 1994; Barta and Dubey, 1992). It is currently unclear which antigens are present in the *Neospora* iscoms and whether these antigens will be recognised consistently by the majority of infected animals, but this is being investigated. Another ELISA developed by Lally *et al* (1996) using recombinant antigen fractions of 30 and 35kDa was found to be able to

distinguish between *Neospora* infected (naturally and experimentally) and uninfected cattle but very few samples were used and no sera from aborted foetuses were tested.

Bjerkas *et al* (1994) showed consistent recognition of several dominant antigens of the *Neospora* NC1 dog isolate with immune sera from domestic and wild animals, therefore a dog isolate can be used for diagnosis of natural infection in cattle. There is a possibility that antigenic differences between strains isolated from the tissues of dogs, such as NC Liv (Barber *et al*, 1993) and NC1 (Dubey *et al*, 1988b), which are widely used for diagnostic tests, and the parasite strains which cause natural infection in cattle may result in misdiagnosis.

2.5.3 Histopathology

As cattle which have previously aborted due to neosporosis can become antibody negative diagnosis should not be based on IFAT or ELISA results alone but also on detection of the parasite in the tissues of the animal and the pathology of the disease in the aborted foetus. Diagnosis is usually confirmed where possible using immunohistochemical techniques to examine paraffin embedded tissue sections taken from the aborted foetus for the presence of *Neospora* tachyzoites (Barr *et al*, 1994b). However it should be noted that polyclonal *Neospora* serum from experimentally infected rabbits can occasionally cross react weakly with *T.gondii* antigen (Bjerkas *et al*, 1994), cross reactions have occurred at dilutions of less than 1:50 (Dubey and Lindsay, 1993), and that each laboratory produces its own polyclonal serum which may also cause a variation in results.

2.5.4 Polymerase chain reaction

As previously discussed, serology based on diagnostic IFAT and ELISA tests is not accurate enough for diagnosis of neosporosis in individual animals. Definitive diagnosis depends on the identification of parasite antigen in aborted foetal tissues, amniotic fluid or blood. One method used to identify parasite antigen is by amplification of parasite specific DNA in host tissues using the polymerase chain reaction (PCR) (Zhi-Gang Guo and Johnson, 1995; Holmdahl and Mattsson, 1996;

Ho *et al*, 1996). PCR is a highly sensitive and specific method for the detection of *Neospora* parasites but as *N.caninum* tachyzoites and tissue cysts are found infrequently in maternal and foetal tissues sample size could greatly affect diagnosis using this method. In addition PCR testing is a difficult and expensive procedure which is not yet commercially available. PCR should therefore be used as a supplementary method of diagnosis for confirming *Neospora* infection in a herd, and would be more usefully employed in epidemiological studies to identify the host range of *N.caninum.*

This method has also been used to determine the relationship of *Neospora* to protozoa classified in the family Sarcocystidae of the phylum apicomplexa. Ho *et al* (1996) used the random amplified polymorphic DNA (RAPD) PCR technique to compare the genomic DNA of *N.caninum* with that of *T.gondii* and 3 *Sarcocystis* species, all coccidia which were though to be closely related to *Neospora.* The tests showed that *N.caninum* is an independent species of protozoan parasite.

2.5.5 Clinical symptoms

2.5.5.1 Clinical symptoms of neosporosis in dogs

Neosporosis is generally manifested as an often fatal ascending paralysis of puppies (Duncan, 1995) although the disease occurs in dogs of all ages (Barber and Trees, 1996). The most common clinical signs include progressive hindlimb paresis or ataxia which may vary from mild, where the animal shows a reluctance to jump and cannot stand correctly, to complete hindlimb paralysis leading to tetraplegia (Barber and Trees, 1996; Sheahan *et al*, 1993). At this stage of the disease the dog is often euthanised. All cases show some defects in proprioception, often in the hindlimbs. Other symptoms include muscle atrophy, signs of pain in the lumbar and quadriceps muscles, head tilt, ocular abnormalities, rigid hyperextension of limbs and dysphagia (Barber and Trees, 1996; Knowler and Wheeler, 1995). Neosporosis can also cause pyogranulomatous dematitis forming multiple draining nodules on the skin of the head and thorax (Dubey *et al*, 1995), pneumonia (Grieg *et al*, 1995), pancreatitis and myocarditis (Dubey *et al*, 1988a) which can cause sudden collapse

and death due to heart failure.

2.5.5.2 Clinical symptoms of neosporosis in cattle

Abortion is the only clinical sign of neosporosis in cattle. The foetus may be resorbed, mummified, autolysed or stillborn, or may be born weak with little coordination. Calves may also be born congenitally infected but clinically normal (Dubey and Lindsay, 1996). Why this range of effects on the foetus occurs is not yet known but may be due to the point of gestation at which the foetus becomes infected, and therefore could be altered by the immune response of the foetus.

2.6 Treatment and control of neosporosis

2.6.1 Drug treatment

There is no recommended drug for the treatment of neosporosis but some case studies have reported success using anti-coccidial drugs effective against toxoplasmosis and intestinal coccidiosis.

Lindsay *et al* (1996a) examined the effect of a range of sulphonamides and dihydrofolate reductase/thymidylate synthase (DHFR/TS) inhibitors against *N.caninum* NC1 tachyzoites in cultured cells. Combinations of these drugs have been used successfully for the treatment of toxoplasmosis in humans (Luft and Remington, 1992) and intestinal coccidiosis in animals (Long, 1993). This study showed that combinations of sulphonamides and DHFR/TS inhibitors were also found to work synergistically to reduce proliferation of *N.caninum in vitro* in a cell culture flask lesion based assay, although a mutant strain resistant to pyrimethamine (a DHFR/TS inhibitor) was easily induced by culturing the parasite in permissive amounts of the drug after 20 passages at varying concentrations. Several case studies have been reported where treatment of dogs showing clinical signs of neosporosis including mild hind limb paresis and proprioceptive defects with a combination of trimethoprim (a DHFR/TS inhibitor), sulphadiazine (a sulphonamide) and

pyrimethamine have resulted in resolution of the disease (McGlennon *et al,* 1990; Mayhew *et al,* 1991). Success depends upon treatment at a very early stage in disease progression. Treatment is most successful if given at the first appearance of clinical symptoms. Another tissue culture based lesion assay using *N.caninum* NC1 strain tachyzoites identified decoquinate, a quinolone anticoccidial used in cattle, sheep and goats, as a possible therapeutic drug for the treatment of neosporosis (Lindsay *et al,* 1997). Intracellular stages of the parasite were killed within 5 minutes of treatment with 0.1μg/ml decoquinate although this drug was not effective against extracellular stages at this or higher concentrations. As the amount needed to kill parasite was low and did not affect host cell viability it is possible that this drug may be of value for effective treatment of neosporosis in cattle.

Dogs often respond to treatment although this may depend upon the severity of clinical signs and the speed of the disease progression. A review of neosporosis in dogs by Barber and Trees (1996) reported that an early start to treatment with clindamycin, sulphonamides and/or pyrimethamine resulted in 5 of 16 dogs with clinical neosporosis recovering fully and a further 5 responding favourably. Animals with rigid hyperextension were found to be the least likely to respond to drug treatment (Barber and Trees, 1996; Hay *et al,* 1990).

Neosporosis can also in some cases cause pyogranulomatous dermatitis (Dubey *et al,* 1995) caused by tachyzoites invading the dermis and epidermis and appearing as nodules on the skin of the head and thorax. Treatment with clindamycin hydrochloride was found to dramatically improve the clinical symptoms, with all dermal lesions resolved within 14 days of initiation of treatment.

As yet drug treatment of neosporosis in cattle has not been reported. All the evidence on effectiveness of drug therapy for the treatment of dermal and visceral neosporosis is at this stage only anecdotal referring to individual cases in dogs or to tissue culture tests. Drug trials need to be carried out to evaluate the effectiveness of the range of drugs currently being used and also to determine a treatment regime which allows optimal dosing. This should avoid production of drug resistant strains and reduce unnecessary side effects due to administration of high doses.

2.6.2 Disease control

Non-clinical congenital infection with *Neospora caninum* in cattle has been shown to occur in the majority of calves born to seropositive cows. In an extensive study by Paré *et al* (1996) 81% of seropositive cows and 5% of seronegative cows gave birth to congenitally infected offspring. This provides a reservoir of infection which allows the parasite to persist in the herd. One method of reducing levels of infection may be selective culling of calves with high pre-colostral *Neospora* antibody levels and replacing stock only with sero-negative females. There is some evidence that animals infected with *N.caninum* have a higher risk of being culled as these animals have a lower rate of milk production than sero-negative cows (Thurmond and Hietala, 1997). Although congenitally infected calves may appear clinically normal it is possible that *N.caninum* induced necrosis of the brain, myocardium, liver and kidney could compromise physiologic efficiency and subsequent milk production. Risk of culling also increases for cows that have a history of abortion (Thurmond and Hietala, 1996). This method of control may however prove too expensive if prevalence is high, as has been previously demonstrated.

Neospora caninum can also be transmitted via feacal contamination of the environment by dogs, the definitive host. Transmission of disease could be controlled by minimising the exposure of cattle and cattle feed to contamination by dogs and other carnivores. Specific measures would include maximising rodent control, minimising the number of cats and dogs co-habiting with the herd and covering feed. Farm dogs in particular may also become infected by eating *Neospora* infected aborted bovine tissues, completing the life cycle and persisting contamination in the environment. In this case it is important to dispose of aborted material promptly to prevent dogs from consuming it, although this maybe difficult to achieve if an abortion occurs when the cattle are out on the pasture.

Ingestion of undercooked meat containing *T.gondii* tissue cysts can cause disease in humans. It is not known if *Neospora* can infect humans, but the disease has been reproduced in experimentally infected rhesus macaque monkeys (Barr *et al*, 1994a). *Neospora* tachyzoites injected during gestation resulted in transplacental infection and foetal lesions similar to those induced by transplacental *Toxoplasma*

infection in primates. To date no surveys have been published to assess seroprevalence of *Neospora* in humans. Further studies are needed to assess the zoonotic potential of neosporosis.

As discussed in section 2.9.2 a vaccine to prevent clinical toxoplasmosis is currently commercially available. The vaccine called Toxovax®, which consists of the live S48 strain of *T.gondii,* protects against *T.gondii* induced abortion in sheep but does not prevent chronic infection. As *T.gondii* is very closely related to *N.caninum* and causes a disease similar to neosporosis it is possible that vaccination may be an option for the prevention of neosporosis with the target being to prevent abortion not infection.

2.7 Humoral immune response to infection with *Neospora caninum* in cattle

2.7.1 Kinetics of the humoral immune response

Few long term studies of neosporosis on farms have been undertaken and we do not fully understand the implications of high antibody titres in cattle. The risk of abortion for *N.caninum* sero-positive dams at the time of pregnancy diagnosis and during gestation has been shown to be twice that for sero-negative cattle (Paré *et al*, 1997). The risk of congenital infection is also high in sero-positive cows (Paré *et al*, 1996) but it has been reported that antibody levels can fluctuate in naturally infected animals, unlike *T.gondii* infection in sheep which induces and maintains high anti *Toxoplasma* titres. This is important for accurate diagnosis and has implications for application of control measures. Further studies on the kinetics of the humoral immune response to *N.caninum* in naturally infected cattle are therefore vital.

2.7.2 Identification of *Neospora* specific antigens

Neospora specific antibody titres can be detected and quantified by a

commercially available indirect fluorescence antibody test (IFAT) (VMRD, Pullman, USA) and more recently by ELISA (Bjorkman *et al*, 1994; Pare *et al*, 1995; Lally *et al*, 1996; Bjorkman *et al*, 1997; Williams *et al,* 1997; Osawa *et al*, 1998). Diagnostic ELISA tests developed for detection of neosporosis have been shown to be more sensitive and specific than IFAT for serodiagnosis in cattle (Williams *et al*, 1997) and although they are not accurate enough for identification of individual animals affected they can give an indication of disease prevalence in the herd. Antibodies to whole *Neospora* tachyzoite antigen can cross react with conserved antigens of closely related coccidian parasites. Western blot analysis of *Neospora* tachyzoite antigen has shown cross reaction with sera from cattle infected with *T.gondii* (Conrad *et al*, 1993b; Bjerkas *et al*, 1994) or *Sarcocystis spp.* (Baszler *et al*, 1996). The development of a diagnostic test using *Neospora* specific antigens which are not recognised by antibody to closely related coccidian parasites may provide a more specific and reliable method of diagnosis of individual animals infected with *N.caninum.*

Previous studies have identified a range of immunodominant *Neospora* antigens. The predominant antigens recognised by *Neospora* positive dog and rabbit serum in ELISA tests using extracted *Neospora* NC1 tachyzoite proteins incorporated into immunostimulating complexes (iscoms) (Bjorkman *et al*, 1994) were approximately 30-45 and 17-19kDa. Iscoms are composed of tachyzoite surface proteins bound to an adjuvant (Bj□rkman *et al*, 1994) and therefore are designed to incorporate external parasite antigens. Monoclonal antibodies raised against iscom antigens of 30-32, 18 and 41 kDa bound to the tachyzoite surface indicating these antigens were surface antigens. However on further investigation using immunogold on section labelling the 30-32kDa doublet was present on both the surface and within the dense granules of the parasite, suggesting these proteins could play an important role during the initial interaction with the host cell surface membrane (Bjorkman and Hemphill, 1998). Other surface antigens of *N.caninum* NC1 isolate identified include 3 antigens of molecular weight 17, 42 and 43kDa, and another at 35kDa was directed against a dense granule antigen (Schares *et al*, 1997). All antigens were immunoprecipitated using sera from *N.caninum* infected cattle.

Hemphill *et al* (1996) attempted to identify tachyzoite surface antigens

involved in physical interaction with host cells used detergent to incorporate membrane components. Two antigens, Nc-p43 and Nc-p36, were identified as major surface antigens which did not cross react with *T.gondii*. Nc-p43 was also found to be expressed on both the tachyzoite and bradyzoite stage of the parasite, whereas Nc-p36 which has over 75% sequence homology with p30 (SAG1), the major *T.gondii* tachyzoite surface protein, was found only in the tachyzoite stage (Fuchs *et al*, 1998; Sonda *et al,* 1998). Both are implicated in parasite adhesion to the host cell surface membrane, and Nc-p43 has been shown to inhibit host cell invasion in vitro, identifying it as an important ligand for the mediation of contact between the parasite and host cell (Hemphill, 1996).

Another study by Paré *et al*, 1995, analysed the immune response of a naturally infected cow to crude lysates of NC1 and bovine BPA1 antigens. Very few antigens were recognised under reducing conditions and all were below 45kDa. The many differences between these studies including species used for production of antibody positive sera, antigen preparation and parasite strains used to produce antigen for inoculation and detection make direct comparison of results difficult.

A study of specific antigens recognised by naturally infected cattle, congenitally infected calves and fluids from aborted foetuses submitted to the Washington State diagnostic laboratory identified several *Neospora* NC1 immunodominant antigens of approximately 116, 65 and 25kDa which did not cross react with sera from cattle experimentally infected with *Sarcocystis* spp and *T.gondii* (Baszler *et al*, 1996). The epitope recognised on the 65kDa antigen was found to be a carbohydrate molecule bound to the surface of the protein.

Another approach which lead to the identification of two dense granule proteins, NCDG1 and NCDG2, in *N.caninum* tachyzoites used immunoscreening of a cDNA library with sera from *N.caninum* infected cows. Expression of these antigens in recombinant form using *E.coli* as a vector allowed their use in an ELISA. Both antigens were identified as useful candidates for detection of anti- *N.caninum* antibodies (Lally *et al*, 1997). Further sequence analysis showed that NCDG1 was structurally similar to dense granule proteins from *T.gondii* (Cesbron-Delauw, 1994). NCDG2 appears to be closely related to the *T.gondii* dense granule protein GRA6 (Liddell *et al*, 1998).

Further studies are needed to identify which of the *N.caninum* antigens described here are widely recognised and specific to *Neospora.* It is possible that due to strain differences a combination of several antigens may be required for the development of an effective diagnostic test.

2.8 Cell mediated immune response to *N.caninum*

Very little is known about the cell mediated immune response to *N.caninum.* Although Koch's postulates for the parasite have been fulfilled (Barr *et al*, 1994b) no studies on the immune response to experimental or natural infection in cattle, the natural host, have been published. Unlike toxoplasmosis in sheep, which stimulates protective immunity following primary infection (Innes, 1997), neosporosis can cause repeat abortion in cattle (Anderson *et al*, 1995, Moen *et al*, 1995). But the rate of repeat abortion is estimated to be less than 5% (Anderson *et al*, 1995; Moen *et al*, 1995), therefore it is probable that many animals do develop some form of protective immunity. As *Neospora caninum*, like *T.gondii,* is an obligate intracellular parasite, it is likely that cell mediated immune mechanisms are an important component of the immune response. Several models of infection including the tissue culture model and the mouse model have been used to investigate the role of the cell mediated immune response in host immunity to *N.caninum.*

2.8.1 Tissue culture model of infection

Neospora caninum tachyzoites have been successfully isolated and cultured *in vitro* from bovine and canine tissue. It has previously been shown that pre-treatment of fibroblast cells inoculated with *N.caninum* NC1 strain tachyzoites with recombinant interferon gamma (rIFNγ) *in vitro* causes significant inhibition of intracellular multiplication of the parasite compared to untreated control cells (Innes *et al*, 1995a). As IFNγ is an important cytokine of the cell mediated immune

response the T cell response may be important in resistance to neosporosis. IFNγ has previously been shown to be important in host immunity to other intracellular coccidian parasites (Nacy *et al*, 1985; Reed, 1988) including protective immunity to *T.gondii* in sheep (Innes *et* al, 1995b; Subauste and Remington, 1991).

The tissue culture model has also been useful in the identification of chemotherapeutic agents which can prevent the growth and replication of *Neospora in vitro* and may be of use for the treatment of the disease *in vivo*. In particular this model allows differentiation between drugs which are coccidiocidal and coccidiostatic (Lindsay *et al*, 1994).

Although the applications of a tissue culture model are limited it provides us with a means to investigate the role of individual cytokines, chemicals and cell populations on the growth of the parasite. For this reason the tissue culture model is a useful tool for the investigation of neosporosis.

2.8.2 Mouse model of infection

Outbred strains of mice are resistant to clinical infection with *N.caninum* parasite strains presently grown in tissue culture and treatment with methylprednisolone acetate (MPA), which suppresses the immune system, is required to overcome natural resistance to the disease. Chronic infection with tissue cysts in the murine CNS can be induced after initial treatment with MPA although this may depend on the strain of the parasite used for inoculation (Lindsay *et al*, 1990a; Lindsay *et al*, 1991; Lindsay *et al*, 1992). Immune suppression of outbred strains therefore provides a good source of tissue cysts for infection experiments but is not ideal for studying the immune response to *N.caninum*. These animals are also more likely to develop bacterial septicaemia and therefore have to be kept in a sterile environment making them difficult to work with.

A mouse model of infection which does not rely on immunosuppression was developed by Lindsay *et al* (1995a) using an inbred BALB/c mouse model infected with *N.caninum* NC1 strain tachyzoites. Animals showed clinical signs of infection, remained chronically infected in the absence of MPA treatment and lesions were restricted to neural tissue. As these animals show clinical signs of disease this model

may be useful for evaluating effects of potential vaccines or treatment regimes against the parasite. This model will therefore be a useful tool in the study of the immune response to *N.caninum.*

Another inbred mouse model developed by Khan *et al* (1997) has been used to investigate the role of cytokines in host immunity. This study evaluated cytokine production and cellular immune response of inbred A/J mice inoculated with a high dose inoculum of NC1 *N.caninum* tachyzoites ($1x10^6$). A/J mice do not develop clinical signs of infection and sustain few lesions after inoculation with *Neospora.*

The cellular response to the parasite in this model was characterised by the proliferation of splenocytes from infected animals to parasite antigen *in vitro.* This response was not however parasite specific and could also be stimulated using *T.gondii* antigen. Levels of IL12 were found to increase dramatically *in vivo* almost immediately after infection followed by a rapid decrease by 24 hours pi. At this point production of IFNγ began to increase and continued climbing until day 10 pi. IFNγ production by unstimulated splenocytes from infected mice was high on all days pi up to day 14 (end of study). The role of IL12 *in vivo* was investigated by treating mice daily with antibody to IL 12 which severely reduced immunity to the parasite. Mice treated with anti-IFNγ Mabs were also rendered suseptible to disease. This study therefore shows that *N.caninum* stimulates a protective T cell response in this experimental murine model which is principly mediated via IL 12 and IFNγ which have previously been shown to be important in the induction of protective immunity to *T.gondii* (Gazzinelli *et al,* 1993a; Innes and Wastling, 1995). These studies also identified IL 12 as the principle stimulator of IFNγ. It is possible that the mechanism of induction of the immune response in this model is similar to that shown for *T.gondii* as neutralisation of either of these cytokines results in the loss of innate resistance to the parasite.

The study by Kahn *et al* (1997) also showed that infection with *Neospora* may confer some cross-protection against toxoplasmosis as splenocytes from mice infected with *N.caninum* also reacted to *T.gondii* antigen. CD8+ve T cells have been shown to be the major effector cells responsible for protection against *T.gondii* (Kasper and Boothroyd, 1993). These cells act in two ways; by destroying cells infected with parasites and also by secreting IFNγ. A recent study by Kasper and

Khan (1998) showed that vaccination of mice with *N.caninum* protected against a lethal challenge with *T.gondii* and that adoptive transfer of CD8+ve T cells from mice infected with *N.caninum* into naive recipients can confer protection against a normally lethal *T.gondii* challenge. CD8+ve T cells from vaccinated mice were also found to proliferate in response to both *Neospora* and *Toxoplasma* specific antigen fractions suggesting the immune response is stimulated by shared immunoreactive epitopes. These observations are in contrast to results of a similar study by Lindsay *et al* (1990a) where vaccination with *N.caninum* did not protect against lethal infection with *T.gondii*. Differences in results may be due to the known difference in virulence of the parasite strains used in the two studies. Lindsay used the RH strain of *T.gondii* which is known to be more lethal than the PLK strain used by Kasper. A later study by Lindsay *et al* (1998) confirmed that vaccination of mice with *N.caninum* can provide some protection against a moderately pathogenic (TS-4) strain of *T.gondii* but had no effect on tissue cyst formation. Therefore protection is dependant on the strain of parasite used.

It is clear that the cellular immune response mediated by the T cell cytokines IFNγ and IL 12, and CD8+ve effector T cells, previously shown to be important in protective immunity to the closely related coccidian *T.gondii*, are important in the development of protective immunity to *N.caninum* in the mouse model. As previously discussed no studies on the immune response to *N.caninum* in cattle, the natural host, have yet been published. The relevance of the mouse as a model of infection in cattle can only be achieved by comparison of the immune response mechanisms in mice to those stimulated by natural and experimental infection in cattle.

2.9 *Toxoplasma gondii* and Toxoplasmosis

Toxoplasma gondii is an obligate intracellular coccidian parasite which is closely related to *Neospora caninum*. The parasite was first isolated from a rodent by

Nicolle and Manceaux in 1908, and since then has been found to affect most warm-blooded animals.

Toxoplasma has a wide host range and the consequences of infection vary in different species (Innes *et al*, 1997). Marsupials and new world monkeys are very susceptible to toxoplasmosis which can prove fatal in these animals. In contrast infection in more resistant species such as humans and sheep causes only mild clinical symptoms. If toxoplasmosis is acquired during pregnancy however it can cause congenital defects in the foetus and may lead to abortion. Once infected with the parasite *Toxoplasma* tissue cysts persist in the host for life. Infection confers life long immunity to reinfection and under normal circumstances bradyzoites will remain within cysts in the CNS and other infected tissues. Recrudesence of the disease can occur when the immune system of the host is suppressed. Therefore Toxoplasmosis can be a problem in patients infected with the human immunodeficiency virus (HIV) or transplant patients who undergo long-term treatment with immunosuppressive drugs.

2.9.1 Life Cycle and Transmission

The life cycle of *Toxoplasma* is comprised of the sexual and asexual stages of development. The sexual stage occurs in the epithelium of the small intestine of the cat, the definitive host, after ingestion of tissues containing *Toxoplasma* tissue cysts (Frenkel *et al*, 1970; Dubey *et al*, 1970). The walls of the cysts are disrupted by the host's digestive enzymes releasing bradyzoites (slow replicating form) into the intestinal lumen which then invade the enteroepithelial cells where differentiation into male and female gametocytes occurs. Penetration of the (female) macrogamont with the (male) microgamont forms a zygote which is then surrounded by a protective wall forming an oocyst. Rupture of the intestinal epithelium releases oocysts into the lumen of the gut. Following excretion into the environment sporulation occurs (1-5 days). At this stage the oocyst, which contains 8 sporozoites, is infective.

The asexual cycle occurs after ingestion of oocysts by the intermediate or secondary host. As *Toxoplasma* can infect a wide range of species most warm-

blooded animals can be included as potential hosts. Digestive enzymes again break down the walls of ingested oocysts or tissue cysts in the lumen of the gut releasing sporozoites or bradyzoites. Rapid asexual multiplication of the parasite by endodyogeny (Joiner *et al*, 1990) in surrounding cells produces large numbers of tachyzoites which disseminate via the blood stream and lymphatic system. *Toxoplasma* tachyzoites can invade virtually all cells and tissues of the body (Krahenbuhl and Remington, 1982) and survive inside an intracellular parasitophorous vacuole which does not fuse with host lysosomes (Joiner and Dubremetz, 1993). The host eventually develops an immune response which clears extracellular parasite antigen but intracellular *Toxoplasma* tachyzoites evade the immune system and establish persistent infection. Intracellular multiplication slows and *T.gondii* tachyzoites transform into slowly replicating bradyzoites. The parasitophorous vacuole containing bradyzoites converts into a cyst which is derived from components of both the host cell and parasite and does not elicit an inflammatory response. Cysts are most frequently found in brain, heart and skeletal muscle and persist for life (Jacobs *et al*, 1960; Remington and Cavanaugh, 1965; Conley and Jenkins, 1981; Dubey and Thulliez, 1993)

2.9.2 Toxoplasmosis in Sheep

Toxoplasmosis causes few clinical symptoms in adult sheep but can cause congenital defects and abortion if a primary infection is contracted during pregnancy. Timing of infection can greatly affect the outcome as sheep exposed to *T.gondii* in early to mid gestation are more likely to abort or produce weak lambs. Infection with *T.gondii* does however induce a life long immunity to reinfection and recrudesence, and a live vaccine against toxoplasmosis is currently available. This vaccine is comprised of an incomplete strain of the parasite which stimulates a protective immune response but does not persist in tissues of the host (O'Connell *et al*, 1988). Although it does not prevent infection and persistence of wild type strains it does prevent abortion in sheep.

Figure 2.6 Ewe with stillborn lamb and weak live lamb following primary infection with *T.gondii* in mid pregnancy.

Photograph courtesy of Dr.D.Buxton.

2.9.3 Toxoplasmosis in cattle

There is very little published information on toxoplasmosis in cattle as infection with *T.gondii* does not cause clinical disease in this species (Dubey, 1986). In a study comparing experimental infection of sheep and cattle with *T.gondii* cattle orally infected with *T.gondii* oocysts were found to be chronically infected with

T.gondii cysts present in brain and cardiac muscle. However these animals were able to control blood parasitaemia levels more effectively than sheep during primary experimental infection (Esteban Redondo, 1997). IFNγ was more frequently detected in bovine plasma and activated cell supernatants when compared with ovine samples. This cytokine has previously been shown to play an important role in the protective immunity to toxoplasmosis conferred to sheep following vaccination with the S48 *T.gondii* strain. Resistance in cattle may be due to the ability to control *T.gondii* multiplication through a combination of immune mechanisms including early production and higher levels of IFNγ resulting in a reduction in the number of parasites disseminated into tissues (Esteban Redondo, 1997).

2.9.4 Immunity to *T.gondii*

Most information about host immune responses to *T.gondii* results from experimental infections in mice. These studies have shown that protective immunity depends on cell-mediated immune mechanisms (Subauste and Remington, 1993). Adoptive transfer of CD8+ve T cells from mice infected with *T.gondii* confers protective immunity in naïve recipients challenged with the parasite (similar to the mouse model for *N.caninum*). This protection is dependant on the production of IFNγ as treatment of mice with anti-IFNγ Mabs at the time of transfer ablates any protection previously observed (Suzuki and Remington, 1990). Depletion of IFNγ in mice chronically infected with *T.gondii* can also lead to the reactivation of dormant tissue cysts in the brain causing inflammation similar to that seen in AIDS patients with toxoplasmic encephalitis (Suzuki *et al*, 1989). As protective immunity can be induced by transfer of T cells but not by transfer of serum the role of antibody production in immunity is thought to be minimal.

Toxoplasmosis has also been studied in sheep using the technique of lymphatic cannulation to study lymphoid cell traffic and function *in vivo* produced in response to infection with the *T.gondii* S48 vaccine strain (Innes and Wastling, 1995). This parasite strain is currently commercially available for vaccination of sheep to prevent abortion due to toxoplasmosis. S48 is an incomplete strain of *T.gondii* which does not form tissue cysts and therefore cannot persist in the host, but

does stimulate life long protective immunity. The majority of lymphoblasts in cell output from the lymph node immediately after infection were CD4+ T cells but at the time of peak output at 10 days pi this switched to CD8+ T cells which were found to inhibit tachyzoite multiplication in a tissue culture model of infection. From day 6 pi CD4+ cells were able to proliferate in response to parasite antigen *in vitro*. These cells also produced IFNγ in response to stimulation. Examination of efferent lymph showed that IFNγ was produced by all sheep 2-5 days after a primary infection and levels remained high for a further 6-7 days (Innes *et al*, 1995c). Production of IFNγ was also accelerated and reached higher concentrations after a secondary challenge with *T.gondii*. Lymph plasma samples were found to significantly inhibit the intracellular multiplication of *T.gondii in vitro* (Innes and Wastling, 1995).

This model has clearly shown the importance of CD8+ T cells in protective immunity to an intracellular pathogen. CD4+ cells were found to precede CD8+ cells in efferent lymph in sheep infected with the S48 vaccine strain suggesting that CD4+ cells are required for induction of CD8+ cells. Other studies have shown that both CD4+ and CD8+ cells are required for an optimal response to infection with *T.gondii* (Gazzinelli *et al*, 1991). IFNγ is produced mainly by CD4+, CD8+ and natural killer cells. Recombinant IFNγ has previously been shown to inhibit intracellular multiplication of *T.gondii* tachyzoites within ovine fibroblasts and macrophages (Oura *et al*, 1993) and is an important factor in protective immunity to *T.gondii* in the mouse model (Suzuki and Remington, 1990). Cell mediated immune mechanisms are therefore protective against the intracellular coccidian parasite *T.gondii*.

Chapter 3

Materials and methods

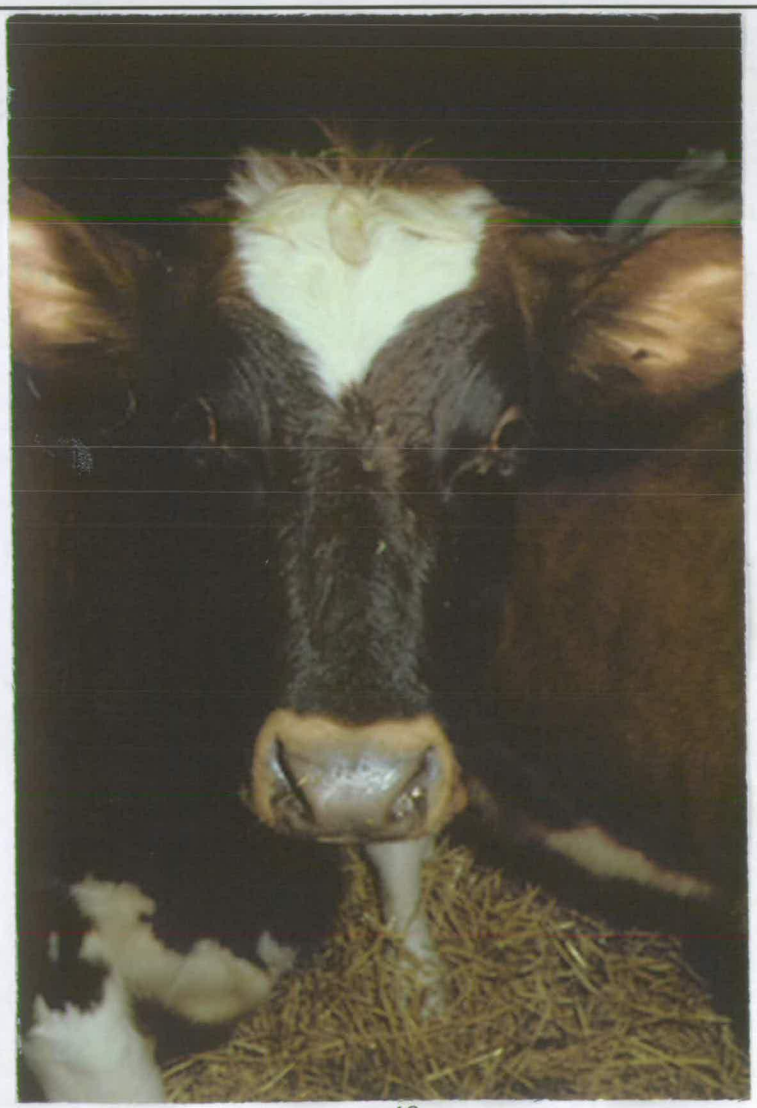
3. Materials and methods

3.1 Animals

3.1.1 Cattle (*Bos taurus)*

All cattle experimentally infected with *N.caninum* were housed in straw bedded pens. Disinfectant footbaths were used to minimise any possible spread of infection. Animals experimentally infected with *Neospora* were penned separately from controls.

Figure 3.1 Holstein Friesian cow (*Bos taurus*) (photograph courtesy of Brian Easter)



3.1.1.1 Experimental infection

Twelve holstein friesian calves, approximately 6 months old, were chosen for a study examining the immune response to experimental infection with *Neospora caninum* tachyzoites cultured *in vitro.* All calves were seronegative for *Neospora caninum* by ELISA and for *Toxoplasma gondii* by IFAT pre-infection. Details of experimental design are given in tables 6.1, 7.1a and 7.1b.

3.1.1.2 Farm study

The sero-epidemiology of neosporosis was studied in a dairy herd which suffered losses due to infection with *N.caninum.* The herd consisted of a maximum of 130 cows at any one time which were grazed on pasture in summer and over-wintered indoors. All of the cattle were holstein friesian and were fed silage with Bioferm added, a lactobacillus strain used for fermentation. This diet was supplemented with Bibby's dairy meal.

3.2 Maintenance of parasite cultures

3.2.1 *Neospora caninum* NC1 strain

The NC 1 strain of *Neospora caninum* was first isolated from neural tissue of a dog into tissue culture and mice by Dubey *et al* (1988b).

3.2.1.1 Maintenance of NC1 in tissue culture

Neospora caninum NC1 tachyzoites were maintained in tissue culture by twice weekly passage in African green monkey kidney (VERO) cells (3.2.4.1). Cells and parasite were grown in Iscove's modified dulbecco's medium (IMDM) supplemented with 50U/ml penicillin, 50μg/ml streptomycin and 2% horse serum (Advanced Protein Products, Brockmoor, UK). Parasite was harvested from tissue culture flasks by scraping

off the cell monolayer using a sterile cell scraper (Corning Costar, High Wycombe, UK). Tachyzoites were then counted and added to $25cm^2$ vented tissue culture flasks (Corning Costar, High Wycombe, UK) seeded 24 hours previously with $1x10^5$ cells. Tachyzoites were added to the cell monolayer at a ratio of 2 tachyzoites per cell. All cultures are incubated at 37°C in a 5% CO_2 humidified incubator.

3.2.2 *Neospora caninum* NC Liverpool strain

NC Liverpool (NC Liv) strain tachyzoites were first cultured from infected canine neural tissue by Barber *et al* (1993).

3.2.2.1 Maintenance of NC Liv in tissue culture

N.caninum NC Liv tachyzoites were maintained in tissue culture by weekly passage in ovine ST6 fibroblasts (3.2.4.2). Cells and parasite were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 50U/ml penicillin, 50μg/ml streptomycin and 2% horse serum (Advanced Protein Products, Brockmoor, UK). Tachyzoites were harvested from tissue culture flasks by scraping off the cell monolayer using a sterile cell scraper (Corning Costar, High Wycombe, UK), then counted and added to $25cm^2$ vented tissue culture flasks (Corning Costar, High Wycombe, UK) seeded 24 hours before with $1x10^5$ cells. Tachyzoites are added to the cell monolayer at a ratio of 2 tachyzoites per cell. All cultures are incubated at 37°C in a 5% CO_2 humidified incubator.

3.2.3 *Toxoplasma gondii* S48 strain

Toxoplasma gondii S48 strain was originally isolated at Wallaceville, New Zealand, from an aborted ovine foetus (Hartley and Marshall, 1957). This strain was passaged twice weekly in laboratory mice about 3000 times before being shown to have lost the ability to develop bradyzoites (Wilkins *et al*, 1987). The S48 strain has lost its

capacity to form oocysts in cats and is therefore referred to as an incomplete strain (Wilkins *et al*, 1987; Frenkel *et al*, 1986).

3.2.3.1 Maintenance of S48 in mice

Toxoplasma gondii tachyzoites of the S48 strain were maintained by passage in female Swiss White mice. Mice were killed with carbon dioxide (CO2) and parasites were harvested with a 26 G needle and syringe from the peritoneal cavities of mice that had been infected three days earlier with $1x10^6$ tachyzoites by intra-peritoneal injection.

3.2.3.1 Maintenance of S48 in tissue culture

Toxoplasma gondii S48 tachyzoites were maintained in tissue culture by twice weekly passage in African green monkey kidney (VERO) cells (3.2.4.1). Cells and parasite were grown in Iscove's modified dulbecco's medium (IMDM) supplemented with 50U/ml penicillin, 50μg/ml streptomycin and 2% foetal bovine serum (Sigma, Poole, UK). Tachyzoites harvested from tissue culture flasks by scraping off the cell monolayer using a sterile cell scraper (Corning Costar, High Wycombe, UK) were then counted and added to $25cm^2$ vented tissue culture flasks (Corning Costar, High Wycombe, UK) seeded 24 hours before with $1x10^5$ cells. Tachyzoites are added to the cell monolayer at a ratio of 2 tachyzoites per cell. All cultures are incubated at 37°C in a 5% CO_2 humidified incubator.

3.2.4 Maintenance of cell lines

3.2.4.1 African green monkey kidney (VERO) cells

VERO cells were maintained in tissue culture by twice weekly passage. Cells were grown in Iscove's modified dulbecco's medium (IMDM) supplemented with 50U/ml penicillin, 50μg/ml streptomycin (Sigma, Poole, UK) and 5% horse serum (Advanced Protein Products, Brockmoor, UK) (5% horse serum medium). Cells were harvested from tissue culture by washing the monolayer with PBS and adding 5ml of

trypsin/versene solution (4ml of 0.25% trypsin solution in tris saline mixed with 16ml of 0.02% solution of versene in PBS) to a T75cm^2 flask (Corning Costar, High Wycombe,UK) containing a confluent layer of cells. After 2-3min incubation at 37^o the cells were collected and added to an equal volume of 5% horse serum medium. Cells were then counted and 1x10^5 added to each T25cm^2 for parasite culture. 2.5mls of cell suspension was seeded into each new T75cm^2 stock flask.

3.2.4.2 Ovine fibroblasts (ST6 cells)

ST6 cells were maintained in tissue culture by once weekly passage. Cells were grown in Iscove's modified dulbecco's medium (IMDM) supplemented with 50U/ml penicillin, 50μg/ml streptomycin and 5% horse serum (Sigma, Poole, UK) (5% horse serum medium). Cells were harvested from tissue culture by washing the monolayer with PBS and adding 5ml of trypsin/versene solution (4ml of 0.25% trypsin solution in tris saline mixed with 16ml of 0.02% solution of versene in PBS) to a T75cm^2 flask (Corning Costar, High Wycombe,UK) containing a confluent layer of cells. After 2-3min incubation at 37^o the cells were collected and added to an equal volume of 5% horse serum medium. Cells were then counted and 1x10^5 added to each T25cm^2 for parasite culture. 2.5mls of cell suspension was seeded into each new T75cm^2 stock flask.

3.3 Sample collection

3.3.1 Collection of serum samples

Blood samples were taken from the jugular vein of cattle into preservative free evacuated blood collection tubes, Vacutainer®™ (Becton Dickinson Ltd., Cowley, UK) and allowed to clot. Following removal of the clot the serum was centrifuged at 500*g* for 15 minutes and stored at -20°C.

3.3.2 Collection of whole blood for separation of peripheral blood mononuclear cells

Blood was collected from the jugular vein of cattle into preservative free heparinised evacuated blood collection tubes, Vacutainer®™ (Becton Dickinson Ltd., Cowley, UK). Peripheral blood mononuclear cells (PBMC) were then separated from whole blood as described in section 3.7.1.1.

3.3.3 Supernatant collection for cytokine analysis

Cell free supernatant from cultured PBM (3.7.1) and CD4+ T cells (3.7.2) was collected after 4 days incubation with antigen and stored at -20°C until used for cytokine analysis.

3.4 Preparation of parasite antigen

3.4.1 Preparation of antigen for IFAT

Tachyzoites were washed 3 times by suspension in PBS and centrifugation at 900g. Parasite was then resuspended in PBS at a concentration of $1x10^7$ tachyzoites per ml and fixed in a 0.2% solution of formaldehyde. 1ml aliquots were then stored at -20°C until required for IFAT.

3.4.2 Preparation of water soluble fraction antigen

Neospora caninum and *Toxoplasma gondii* and VERO cell water soluble fraction (wsf) antigen was prepared by 3x freeze/thaw cycle of tachyzoites/cells suspended in distilled water followed by 7x15 s cycles of sonication on ice to form a crude lysate and centrifuged at 10,000g for 30 minutes. The wsf antigen was stored in aliquots of 100µl

at -20 °C and used as antigen in proliferation assays (Innes *et al*, 1995b). Protein concentration was assayed using BCA reagent (Pierce, Rockford, Il, USA) and read using a Monarch 2000 spectrophotometer (Instrumentation Laboratories, Lexington, MA, USA).

3.4.3 Preparation of SDS soluble antigen

To prepare sodium dodecyl sulphate (SDS) (Sigma, Poole, UK) soluble *N.caninum* antigen SDS solution was added to the crude parasite lysate (ref. 3.4.2) at a final concentration of 1% prior to centrifugation. Protein concentration was assayed using BCA reagent (Pierce, Rockford, Il, USA) and read using a Monarch 2000 spectrophotometer (Instrumentation Laboratories, Lexington, MA, USA).

3.5 Antibody assays

3.5.1 Indirect fluorescent antibody test (IFAT)

IFAT was employed to detect anti-*Neospora* and *Toxoplasma* IgG antibodies in bovine sera. $5x10^4$ formalin fixed tachyzoites (ref 3.4.1) were added to each well of a 15 well multitest slide (ICN Biomedicals, Thame, UK) and air dried overnight. Directly before use the tachyzoites were fixed in methanol for 10 minutes and washed gently with a 0.05% solution of Tween 20 in PBS. 5µl aliquots of serum titrated in PBS in doubling dilutions from 1:32 - 1:2048 were added to each well and incubated for 30 minutes in a humidity chamber at room temperature. Slides were again gently washed twice with a solution of Tween 20 in PBS. Fluorescein isothiocyanate (FITC) conjugated to rabbit anti-bovine IgG (Sigma, Poole, UK) was used to detect bound primary antibodies, the reagent being diluted in 0.2% Evans blue and a 5µl aliquot added to each well. After incubation in a humidity chamber for a further 30 mins at room temperature slides were washed twice as before and coverslips mounted using Citifluor

(glycerol/PBS solution) (Citifluor Ltd, Caterbury, UK). Slides were then viewed with UV light under an Olympus BX50 microscope using a U-MNB filter cube with a x40 objective. Known positive and negative bovine sera were examined concurrently with each test as controls. Bovine *Neospora* antibody titres ≥ 1:512 for adults and ≥ 1:64 for foetal fluids and *Toxoplasma* antibody titres ≥ 1:256 for adults were considered positive.

3.5.2 Enzyme linked immuno-sorbent assay (ELISA)

An ELISA to quantify IgG antibody to *N.caninum* in bovine serum samples was used as described by Osawa *et al*, 1998. In brief, polystyrene 96-well microtitre plates (F-Form of Immunolon, M-129A, Dynex Technologies, Billinghurst, UK) were coated with 150µl per well of water soluble *N.caninum* NC1 antigen (3.4.2) diluted 1:6000 in 0.05M carbonate-bicarbonate buffer (pH9.6) and incubated for 24 h at 4°C. Plates were given three washes in PBS containing 0.05% Tween 20 (pH7.4) and were shaken dry. A final volume of 150µl of serum diluted 1:500 in PBS/0.05% Tween 20 with 5% Marvel dried skimmed milk (Premier Beverages, Stafford, UK) was added to each well. Plates were then placed in a humidified chamber and incubated for 2 h at 37°C and again washed and dried as before. 150µl of peroxidase-conjugated rabbit anti-bovine whole molecule IgG (Sigma, Poole, UK) diluted 1:8000 in a solution of PBS/1% BSA/0.05% Tween 20 was added to each well and again incubated for 2 h at 37°C. Plates were washed and dried and 150µl of OPD (*o*-phenylenediamine dihydrochloride) enzyme substrate (Sigma, Poole, UK) [OPD was diluted to a final concentration of 0.4mg/ml in phosphate citrate buffer containing 0.04% of 30% (v/v) hydrogen peroxide, pH5.0] added to each well. After incubation for 30 mins in the dark the reaction was stopped by adding 50µl of 2.5M sulphuric acid. The optical density was read at 492nm in a microplate reader (Titertek® Multiscan Type 312B, ICN Biomedical, Thame, UK). Serum obtained from a 6 month old calf experimentally infected with $2.5x10^8$ *N.caninum* NC1 tachyzoites and collected twelve weeks after inoculation which had a *Neospora* IFAT titre of 1:8192 was used as a positive control. A negative control serum

was collected from a cow that had no history of abortion and had a *Neospora* IFAT titre of 1:64. Both the positive and negative control sera described here were also used by Osawa *et al* (1998) for the development of this *N.caninum* ELISA.

All samples were tested in duplicate. The percentage positivity (PP) for each serum sample was calculated by dividing the average serum OD value by the average positive control serum value and multiplying the result by 100. Sera with ≥30% PP were considered seropositive. This test was optimised using sera from recently aborting cattle naturally infected with *N.caninum* (Osawa *et al*, 1998). Sera were collected through the Scottish Agricultural College Veterinary Service (SACVS) from farms throughout Scotland as part of a Scottish serological survey of neosporosis (Buxton *et al*, 1997a). When used to test bovine maternal sera the percentage sensitivity and specificity of the *Neospora* ELISA was were 97% and 100% respectively when compared with the *Neospora* IFAT.

3.6 Protein separation

3.6.1 SDS PAGE: mini protean

NC1 *N.caninum* proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions as described by Laemmli (1970). 100µl of SDS soluble antigen was mixed with an equal volume of reducing buffer and boiled for approximately 5 minutes in a water bath. The mini Protean system (Biorad, Hemel Hempstead, UK) was used to separate antigen on a 10% polyacrylamide gel and a combination of molecular weight markers in the range of 12,000-78,000 kDa was used to determine molecular mass (Merck, Lutterworth, UK). Proteins were transferred from the gel to a nitrocellulose membrane (Schleicher & Shuell, London, UK) by electroblotting with a trans-blot electrophoretic transfer cell (Biorad, Hemel Hempstead, UK) at a constant voltage of 100v for 45mins to 1 hour (current not exceeding 0.7

amps).

3.6.2 SDS PAGE: protean II

Proteins present in SDS soluble NC Liv and NC1 *N.caninum* antigen fraction were separated by SDS polyacrylamide gel electrophoresis (PAGE) using the Protean II system (Biorad, Hemel Hempstead, UK) on a large 12% polyacrylamide gel under reducing conditions as described by Laemmli (1970). Proteins were then blotted from the gel onto a nylon membrane (Immobilon) (Millipore, Bedford MA, USA) by electroblotting with a trans-blot electrophoretic transfer cell (Biorad, Hemel Hempstead, UK) at a constant voltage of 100v for 45mins to 1 hour (current not exceeding 0.7 amps). The blot was then air dried, treated with a solution of 5% Marvel in tris buffered saline tween (TBST) to reduce background staining and probed with serum antibodies positive for a range of coccidian parasites.

3.6.3 Western blot

Neospora specific protein fractions were separated from a combination of equal volumes of SDS and wsf *Neospora* antigen under reducing conditions by SDS PAGE and proteins transferred to nitrocellulose membrane as previously described. The membrane was then cut into 0.5cm wide strips and whole protein bound to the membrane visualised by staining one strip with Ponceau dye (Sigma, Poole, UK). Strips were treated with 5% Marvel dried skimmed milk in TBST for 1 hour to block non-specific background staining before incubation for 90 mins with a 1:150 dilution of bovine serum or a 1:500 dilution of ovine serum in TBST. After several washes and a further 90 min incubation with secondary antibody (alkaline phosphatase conjugated rabbit anti-bovine IgG, whole molecule) (Sigma Immuno-Chemicals, Poole, UK) the strips were developed using a freshly prepared mixture of nitro blue tetrazolium (NBT) (Sigma, Poole, UK) and bromochloroindolyl phosphate (BCIP) (Sigma, Poole, UK) in alkaline phosphatase buffer (Sigma, Poole, UK).

3.6.4 Periodate treatment of membrane bound antigen

Neospora antigen separated by SDS PAGE and bound to a nitrocellulose membrane was oxidised to remove carbohydrate epitopes. The membrane was washed to remove Ponceau stain (Sigma, Poole, UK), rinsed with acetate buffer and incubated in the dark for 60 minutes at 23°C with sodium periodate (50mM $NaIO_4$) in sodium acetate solution (50mM NaAc) (pH4.5). The blot was then rinsed x2 with sodium acetate, 2x with TBST and incubated for 30 mins with 50mM $NaBH_4$/TNTT. After 3x10 min rinses with TNTT the blot was blocked with 5% Marvel in TBST and developed as before in section 3.6.3.

3.6.5 Preparation of nitrocellulose bound antigen fractions for CD4+ T cell assays

Water soluble fraction parasite antigen (3.4.2) separated by SDS PAGE and transferred to a nitrocellulose membrane (3.6.1) was air dried and cut into 20 strips 2-3mm wide, each strip containing antigen of the same molecular weight. Fraction number 1 had the highest molecular weight and number 20 the lowest. Strips were then cut into pieces of approximately $2mm^2$ and placed for 1 hour in Hanks balanced salt solution supplemented with 2% foetal calf serum (Sigma, Poole, UK), 100U/ml penicillin, 100μg/ml streptomycin (Gibco, Paisley, UK) and 0.01% sodium azide (Sigma, Poole, UK) (FACS medium) followed by 4 washes with sterile PBS. Nitrocellulose bound antigen fractions were stored at 4°C in PBS until used in the proliferation assay.

3.6.6 High performance liquid chromatography (HPLC)

Water soluble fraction antigen (section 3.4.2) was separated into antigen fractions using a Vydac 4 carbon chain (C4) reverse phase column (Millipore, Bedford, MA, USA). Antigens are eluted according to their hydrophobicity using 2 solutions:

Solvent A – 97.5% water
2.5% isopropanol (IPA)
0.04% trifluoroacetic acid

Solvent B - 10% water
90% isopropanol (IPA)
0.04% trifluoroacetic acid

Antigen at a concentration of 1mg/ml was added to the column and bound to the carbon molecules. The column was then washed with 100% solution A and 0% solution B (table 3.1). Gradually over 70 minutes the concentration of solutions was changed inversely to 0% solution A and 100% solution B. Samples were collected at 1 minute intervals for seventy minutes and assayed for protein concentration. The first 10 samples contained no protein and were not used in cell proliferation experiments. Samples 11-70 were condensed by combining each 3 consecutive minutes of samples resulting in fractions 1-20 which were then diluted to a final concentration of 2μg/ml. The final 5 samples collected during 70-75 minutes are washes. These 5 samples were combined to make fraction 21 and contain small amounts of fractions 1-20.

3.6.7 Silver Stain

N.caninum NC1 antigen was run on a 10% polyacrylamide gel as described in section 3.6.1. The gel was then fixed in 200ml of trichloroacetic acid (TCA) solution (20% w/v) for 1 hour at room temperature, and then placed in 40% (v/v) acetic acid in water and soaked for 1 hour. The gel was washed thoroughly (2 x 20 minutes) to facilitate rehydration of the gel and removal of methanol. After a further soak in 10% (w/v) glutaraldehyde solution for 20 minutes at room temperature the gel was again washed in water (3 x 20 minutes). Protein present in the gel was stained by soaking the gel in silver diamine solution for 30 minutes followed by several washes in water (3 x 5 minutes). The gel was then placed developing solution for approximately 5 minutes (2.5ml of 1%(w/v) citric acid, 0.26ml of 36% (w/v) formaldehyde made up to 500ml with water). Proteins are visualised as dark brown stains on the gel. Development was

terminated by placing the gel in stopping solution (40% (v/v) ethanol, 10% (v/v) acetic acid in water).

3.7 Cellular immune response

3.7.1 Antigen induced proliferation assay on peripheral blood mononuclear cells

3.7.1.1 PBM cell preparation

Blood was collected from the jugular vein of cattle into preservative free heparinised evacuated blood collection tubes, Vacutainer®™ (Becton Dickinson Ltd., Oxford, UK). Each sample was diluted 1:2 with sterile PBS and centrifuged at 450*g* for 20mins at 12 °C with the brake off in a GS-6R Beckman®™ centrifuge. The buffy coat was removed and diluted 1:2 in Hanks balanced salt solution (HBSS) supplemented with 2%FCS, 100U/ml heparin (Sigma, Poole, UK), 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Paisley, UK) (wash medium). The cell suspension was layered over lymphoprep (Robbins Scientific, Solihull, UK) and centrifuged at 550g for 30 minutes with the brake off. Peripheral blood mononuclear cells (PBMC) were collected from the interface, centrifuged at 550g and washed x3 in wash medium and resuspended in Iscove's modified Dulbecco's medium supplemented with 10%FCS (Sigma, Poole, UK), 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Paisley, UK) (T cell medium) at a concentration of $2x10^6$/ml.

3.7.1.2 WSF antigen preparation

Water soluble extract of *N.caninum* and *T.gondii* was prepared as described in section 3.4.2.

3.7.1.3 Nitrocellulose bound *N.caninum* antigen fractions

Preparation of nitrocellulose bound antigen fractions for use in CD4+ T cell assays is described in section 3.6.5.

3.7.1.4 Concanavalin A

Concanavalin A (Con A) (Sigma, Poole, UK), the lectin of the jack bean (*Canavalia ensiformis*) was used as a positive control for the test as it has been described to selectively stimulate T lymphocytes (Janossy and Greaves, 1971). Untreated cells were used as a negative control for comparison.

3.7.1.5 Radiolabelling

All cultures were pulsed with 18.5kBq [^{3}H]-thymidine (Amersham, Little Chalfont, UK), which is selectively incorporated into replicating cells, for the final 18h of incubation with antigen.

3.7.1.6 Assay procedure

100µl of PBMC and 100µl of diluted antigen were cultured in 96 well round bottom tissue culture plates (Gibco, Paisley, UK) in triplicate or quadruplicate for 5 days at 37°C in a humidified 5% CO2 incubator. Cells were pulsed for the final 18h with 0.5µCi (18.5kBq) per well of [^{3}H]-thymidine (Amersham, Little Chalfont, UK) before harvesting onto fiberglass filters (Canberra Packard, Pangbourne, UK). Cell associated radioactivity was then quantified in a gas proportional counter (Canberra Packard, USA).

3.7.1.7 Determination of stimulation index

Results reported as counts per minute (cpm) are the mean value of triplicate or quadruplicate cultures. The differential incorporation of [^{3}H]-thymidine between treated and untreated cultures was used as a measure of proliferation expressed as stimulation index (SI):

$$SI = \frac{\text{CPM of test culture}}{\text{CPM of negative control culture}}$$

3.7.2 Phenotypic analysis of peripheral blood mononuclear cells

An indirect immunofluorescence test was used to stain PBM cells from cattle as described by Innes *et al* (1995b).

3.7.2.1 PBM cell preparation

Peripheral blood mononuclear cells were separated from whole blood as described in section 3.7.1.1.

3.7.2.2 Monoclonal antibodies

A range of monoclonal antibodies (Mab) at pre-determined optimal dilutions were used to stain the different populations of cells which make up PBM. The relevant antibodies used are given in the results chapters where appropriate. The antibody preparations specific for cattle with the prefix CC were kindly donated by the Institute for Animal Health, Compton, UK, with the pre-fix ILA were donated by the International Laboratory for Research on Animal Diseases, Kenya, and with the pre-fix VPM65 by Dr.John Hopkins of the Dick Veterinary College, Edinburgh University.

3.7.2.3 Conjugate

Fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin (Dako, Glostrup, Denmark) was used to stain mouse anti-bovine Mab bound to bovine cells.

3.7.2.4 Assay procedure

100µl aliquots of PBM at a concentration of $2x10^7$ cells/ml were resuspended in 50µl of fluorescence activated cell sorter (FACS) medium (HBSS supplemented with 2% FBS and 0.1% sodium azide) and mixed with 50µl of the appropriate Mab at a pre-determined optimal dilution. Cells and Mabs were incubated at 4°C for 30 min and washed 3 times by repeated re-suspension and centrifugation at 300*g* for 5 min. Cells were then resuspended in 50µl of a 1:50 dilution of fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin (Dako, Glostrup, Denmark) and incubated in the dark for a further 30 min. Cells were then washed 3 times with FACS medium and finally fixed in FACS medium containing 1% paraformaldehyde.

3.7.2.5 Determination of stained cells

The percentage of cells stained with FITC for each Mab was determined using flow cytometry (FACScan, Becton Dickinson, Oxford, UK). Ten thousand cells were analysed per sample. A sample stained with FITC conjugate only was used as a control.

3.7.3 Cytokine analysis

The concentration of IFNγ in samples in supernatants from short term PBM cell cultures and assays was quantified using an ELISA kit produced by CSL Veterinary, Melbourne, Australia, and also by bioassay measuring the IFN controlled inhibition of the cytopathic effect of Semliki Forest virus in fibroblast cells (Entrican *et al,* 1992). Recombinant ovine IFNγ (rovIFNγ) (Dr.G.Entrican, MRI) was used as a control to calibrate results. Samples were analysed in duplicate.

3.7.3.1 Supernatants from short term PBM cell cultures

Supernatant was collected from PBM cells cultured in medium or with *Neospora* and *Toxoplasma* wsf antigens at a final concentration of 10µg/ml. Each culture was set up with 24 replicates and after 96hrs supernatant was collected, pooled and stored at -

20°C until analysed for IFN activity

3.7.3.2 Bioassay for detection of biologically active IFN

The presence of biologically active interferons was determined using the method described by Entrican *et al* (1992). The cytopathic effect of Semliki forest virus which is inhibited by interferon is measured in this test.

3.7.3.2.1 Ovine fibroblast cells

ST6 cells cultured from an ovine adenocarcinoma of the small intestine by repeated passage *in vitro* were used as the target cell in the assay. The derivation of and characterisation of these cells have already been described by Norval *et al*, 1981.

3.7.3.2.2 Semliki forest virus

Semliki forest virus (kindly provided by Dr.Gary Entrican, MRI) was added to the cultures in order to measure the cytopathic effect (CPE) provoked by the virus in comparison with protection provided by biologically active interferons.

3.7.3.2.3 Assay procedure

Two neutralising monoclonal antibodies to recombinant bovine IFNγ (rBovIFNγ) were used to distinguish between IFNγ and other anti-viral factors. Samples were pre-incubated with and without a mixture of the Mab's on confluent monolayers of an ovine fibroblast cell line (ST6) for 24h. Supernatants were then removed and 100 tissue culture infective dose (TCID) of Semliki forest virus in IMDM with 2% FBS added and incubated for a further 48h. The assay was then read visually. IFN titres were expressed as the inverse of the highest dilution resulting in at least 50% protection against viral cell destruction. Recombinant ovine IFNγ (rOvIFNγ) was used as a control to detect variations between tests. In this study the Mab's were used at a concentration that consistently neutralised rOvIFNγ with a titre of 256.

3.7.3.3 ELISA for detection of IFNγ

The concentration of bovine IFNγ in supernatant collected from cells stimulated with antigen was quantified using an ELISA kit produced by CSL Veterinary, Melbourne, Australia. Recombinant bovine IFNγ (rBovIFNγ) (Ciba Geigy, St.Aubin, Switzerland) was used as a control to calibrate results and the concentration of IFNγ was measured in international units (u), with 1 unit approximately equivalent to 50pg/ml. The assay was performed as described by the manufacturers. Samples were analysed in duplicate.

3.7.4 Antigen induced proliferation of CD4+ T cell lines

3.7.4.1 Generation of CD4+ T cell lines

Infected and control animals were bled 4-6 weeks post infection and PBM were separated from whole blood as previously described. Equal volumes of PBM at $2x10^5$ cells/well and NC1 wsf antigen at 5μg/ml were cultured in 96 well round bottom tissue culture plates (Gibco, Paisley, UK) at 37 °C in a humidified 5% CO_2 incubator. After 7 days the cells were harvested and diluted 1:3 in T cell medium supplemented with 10U/ml of human recombinant IL2 (hrIL2) (Cetus Labs, UK). Cells were cultured for a further 7 days prior to harvesting when they were used in proliferation assays.

3.7.4.2 Antigen preparation

3.7.4.2.1 Preparation of WSF antigen

Water and sodium dodecyl sulphate soluble fraction antigens were prepared from *Neospora caninum* NC1 strain and *Toxoplasma gondii* S48 strain tachyzoites as described in sections 3.4.2 and 3.4.3 respectively.

3.7.4.2.2 Preparation of antigen fractions for T cell assays

Parasite antigen fractions were separated by SDS PAGE and transferred to a

nitrocellulose membrane as described in section 3.6.1. Fractions were then separated and sterilised as described in section 3.6.5. Antigen was also separated by HPLC as described in section 3.6.6 and resuspended in T cell medium at a concentraion of 1μg/ml.

3.7.4.2.3 Western blot analysis

Parasite antigens separated by SDS PAGE (3.6.1) were probed with antibody positive serum to identify antigens recognised by the humoral immune response. The complete method is described in section 3.6.3.

3.7.4.3 Concanavalin A

Concanavalin A (Con A) is used as a positive control for proliferation assays as described in section 3.7.1.4.

3.7.4.4 Radiolabelling

All cultures were pulsed with [^{3}H]-thymidine, which is selectively incorporated into replicating cells, for the final 18h of incubation with antigen.

3.7.4.5 Proliferation assay procedure

Proliferation assays with the CD4+ T cells were performed in the presence of autologous PBM irradiated with 3000 rad from a ^{37}Cs source as antigen presenting cells (APC). The ratio of APC:CD4 T cell was 10:1. Cells cultured in 5μg/ml Concanavalin A (Con A) (Sigma, Poole, UK) and in medium alone were used as positive and negative controls respectively. Unfractionated NC1 wsf antigen was dot blotted directly onto nitrocellulose and used as a positive control, and cells were also tested for non-specific stimulation by nitrocellulose membrane alone. In proliferation assays cells from the control animals were set up in quadruplicate, and assays measuring responses of cells from the infected animals to the nitrocellulose bound antigens were set up in triplicate.

3.7.4.6 Determination of stimulation index

Results reported as counts per minute (cpm) are the mean value of triplicate or quadruplicate cultures. The differential incorporation of [^{3}H]-thymidine between treated and untreated cultures was used as a measure of proliferation expressed as stimulation index (SI) as described in section 3.7.1.7.

3.7.5 Phenotypic analysis of cell lines

Cellular composition of the CD4+ T cells was analysed using a panel of monoclonal antibodies (Mab) recognising specific bovine leukocyte populations (descriptions of the releavant antibodies are given in chapter 7, table 7.3). The method used to stain the cells is largely described by Innes *et al*, 1995b and in section 3.7.2.

3.8 Clinical response to infection

3.8.1 Rectal temperatures

Rectal temperatures of experimentally infected animals were recorded daily for 3 days pre-infection and 14 days post infection with a clinical electronic thermometer (Solex International, Broughton Astley, UK).

3.9 Statistical analysis

Student's T test was calculated on Minitab version 9.2 using the mean values in each group to determine significant differences, and the data were tested at a $P<0.05$ significance level.

Chapter 4

Longitudinal sero-epidemiological study of a dairy herd in Northern Ireland infected with *Neospora caninum*

Aims

- To study the antibody response in cattle which have suffered *Neospora caninum* associated abortion and age matched controls over a three year period.

- To assess the rate of congenital infection with *Neospora caninum* in newborn calves.

- To assess the rate of abortion and repeat abortion linked with infection with *Neospora caninum* in a small dairy herd.

4. Longitudinal sero-epidemiological study of a dairy herd infected with *Neospora caninum* in Northern Ireland

4.1 Introduction

Neosporosis is predominantly recognised as a disease of cattle (Barr *et al*, 1991) and dogs (Dubey *et al*, 1988a). The parasite has been found worldwide and is linked to 14.7% of bovine abortions in New Zealand (Dubey & Lindsay, 1993), 19% in California (Anderson *et al*, 1991), 10% in England and Wales (Otter *et al,* 1995), and 12-17% in Scotland (Buxton *et al*, 1997). The normal abortion rate on pasture based dairy farms in Britain has been estimated at 2% (Murray, 1990), and previously in England and Wales only 6-10% of investigated bovine abortions were found to have a definite cause with no single infectious agent had been identified as causing more than 4% of losses (VIDA, 1993). Neosporosis was first reported in Northern Ireland in 1994 (McNamee and Jeffrey, 1994) and has since been recognized as a major cause of bovine abortion in the province. A survey carried out in 1996 estimated the incidence of *Neospora* associated abortion to be between 4-10% (McNamee *et al*, 1996) indicating that neosporosis may be of significant importance to the dairy industry in Ireland.

Onset of neosporosis in dairy herds has been linked to 'abortion storms' where a large number of animals abort within a short period of time followed periodically by single cases of abortion (Anderson *et al*, 1994; Yaeger *et al*, 1994). Incidence of abortion does not appear to be linked to seasonal variations (Anderson *et al,* 1991; Thurmond *et al*, 1995). Naturally infected cattle show no clinical symptoms immediately prior to abortion which may occur from around 3 months gestation to term (Dubey and Lindsay, 1996). Diagnostic tests such as IFAT and ELISA which quantify *Neospora* specific antibody titres in sera from dams and aborted foetuses have been found to be unreliable for diagnosis of infected individuals as sero-negative dams can abort sero-

positive foetuses (Buxton *et al*, 1997a) and cattle which have aborted *Neospora* infected foetuses can become seronegative by 150 days post abortion (Conrad *et al*, 1993b). Infection can also lead to the birth of congenitally infected clinically normal calves but how often this occurs is unclear. A study by Paré (1996) reported that a high proportion (81%) of sero-positive cattle gave birth to congenitally infected clinically normal calves which have a consistently greater suvivorship than non-infected calves. These seemingly healthy animals used to replace stock may be chronically infected and act as a reservoir for infection in the herd. Some congenitally infected calves suffer damage to the central nervous system resulting in neurologic defects, paralysis (Dubey *et al*, 1989) and ataxia (Barr *et al*, 1993), but again the number of calves that are likely to be affected and the subsequent financial cost to an individual herd has not yet been evaluated.

Neospora caninum is very closely related to the coccidian parasite *Toxoplasma gondii* which causes abortion and congenital defects in sheep but stimulates a cell mediated immune response which provides the animal with life long protection from repeat abortion (Innes, 1997). This is not always the case in cattle infected with *Neospora* which can induce repeat abortion (Anderson *et al,* 1995), although how often this occurs and whether it is due to reinfection or recrudesence of the parasite in chronically infected animals is also unknown. Analysis of antibody titres to *Neospora* over a long period of time may indicate whether the parasite is persisting and recrudescing, or if it is being effectively cleared by the immune system but not providing a protective secondary immune response.

This study focuses on a dairy farm in Carrowdore on the Ards Peninsula, NI, which has encountered losses linked to infection with *Neospora.* The parasite was first identified on the farm when an abortion storm resulted in the loss of 14 calves in 2 weeks. *Neospora* tachyzoites were identified in aborted foetal tissues at the diagnostic pathology department of the Veterinary Science Department, Stormont, NI. The aims of this study are to determine the prevalence and effects of infection on the farm from August 1995 to March 1998 and in particular to assess the rate of repeat abortion and congenital infection associated with *N.caninum* infection or recrudesence, and correlate this with antibody response to *N.caninum* in individual animals and in the herd.

4.1.1 Description of the study farm

This farm was chosen for the sero epidemiological study of neosporosis after an abortion storm in August/September 1995 confirmed to be associated with *N.caninum* infection by the diagnostic service at the Veterinary Science Division, Stormont, NI. At the beginning of the study the farmer filled in a questionnaire detailing farm practices, herd health and information about the area where the farm is situated. A copy of the questionnaire and answers is given in appendix chapter 4.

The farm is situated 50ft above sea level on the flat land of the Ards Peninsula near the coast with the Irish Sea. The area is therefore quite exposed to the elements and gets about 30 inches of rainfall each year, but the sea provides insulation preventing frost and snow during the winter. The farmer manages 147 acres (117 owned, 30 rented) and produces most of the silage used. The farm stocks only dairy cattle, mostly Holstein Friesians, and is semi-closed, therefore the farmer replaces most of his own stock and buys in very few animals. At the beginning of the study there was a total of 127 cattle on the farm. The only other animals kept on the farm were 2 adult farm dogs, 1 male and 1 female which at the beginning of this study in September 1995 had never produced a litter.

Herd health

There were no clinical symptoms in the dams before or at the time of the abortion storm in August/September 1995. The whole herd had been vaccinated for leptospirosis for 5-6 years up to 1993 but treatment had lapsed in 1994. The farmer did not cull all the cattle which aborted on the farm during the abortion storm but some were sent to slaughter as they were more than 5 years old.

Insemination

Cows and heifers are artificially inseminated commencing in January and continuing until April, with about 20 cows inseminated each month. Cattle are then put out to pasture with the bull in May which acts as a 'sweeper' to get into calf any cows which have not been successfully inseminated. Calving then begins in November and continues until February.

Feed and water

Bioferm, live lactobacillus strain which ferments and breaks down cellulose into sugars is added to silage. The farm dogs, rodents and wild animals have access to the silage which is stored open to the environment under a large open fronted barn and covered by black plastic sheeting. The dogs also had access to the cow's water supply. The cow's diet was supplemented with Bibby's dairy meal stored in a concrete built block bin.

Milking

Cattle are milked in a herringbone system. During milking up to 100 cows are fed out of 10 troughs to which the dogs have access.

Rodents and wildlife

Rats were reported to be a problem from time to time but were usually dealt with quickly, within 7-10 days. Local wildlife is mixed including numerous hares and a small number of badgers and foxes.

Local tourism

A caravan park is situated on the border of the farm near the farmhouse, milking parlour and animal housing. A regular visitor to the park also uses the farm land to walk his dog during August.

Control methods

No control methods were advised by the vet associated with this herd

4.2 Materials and methods

4.2.1 Study farm

The sero-epidemiology of a naturally occuring *Neospora caninum* infection in a dairy herd in Carrowdore, NI was studied. An abortion storm occurred on the farm in August/September 1995 when 14 cows, all friesians, aborted their foetuses in the third trimester of pregnancy. Immediately prior to abortion the cows were clinically normal. *Neospora* was confirmed to be associated with abortion when parasite was identified in aborted foetal tissue by immunohistochemistry. Cattle which had aborted also had high anti-*N.caninum* antibody titres. Diagnosis was made at the diagnostic department of the Veterinary Science Division, Stormont. No other diseases were reported to be associated with the abortions which occurred at this time.

4.2.2 Collection of serum samples

Serum was collected approximately every two to three months beginning September 1995 until June 1996, and 5-6 months from June 1996 – March 1998 from seven cattle which aborted in August/September 1995 and from seven age matched control cattle which did not abort at this time (table 4.1). Cow 45 was culled in June 1996, leaving 6 cows which were monitered for the duration of the study. The entire herd of 130 holstein friesian dairy cows were also tested for antibody to *Neospora* annually in November/December 1995 – 1998, around the time of calving. Pre-colostral serum samples were also collected from new born calves where possible. All blood samples were collected from the jugular vein into preservative free evacuated blood collection tubes and allowed to clot. Following retraction of the clot and centrifugation at 500*g* serum was removed stored at -20°C until required.

Table 4.1 Animal numbers of cattle which aborted during the abortion storm of August/September 1995 and age matched controls. Antibody levels in theses cattle were monitored over a three year period.

Cow No.'s Previously Aborted	Controls
4	127
7	145
17	36
38	108
45	32
63	120
102	100

4.2.3 *Neospora caninum* ELISA

The anti-*Neospora* antibody response in cattle was evaluated by ELISA based on the method described by Osawa *et al* (1998) and described in brief in section 3.5.2 of materials and methods. Samples were analysed in duplicate. Cattle sera are considered seropositive for *N.caninum* at □30% positive.

4.2.4 *Neospora caninum* IFAT

Serum anti-*Neospora* antibody levels in cattle were also quantified using IFAT based on the method described by Conrad *et al*, 1993b, and described in brief in section 3.5.1 of materials and methods. Cattle with antibody titres □1:512 were considered positive for neosporosis.

4.2.5 Parasite detection

Neospora tachyzoites and tissue cysts were identified in aborted foetal tissue. Parasite antigen in paraffin embedded tissue sections was stained using a rabbit anti-*Neospora* polyclonal antibody prior to microscopic examination. This work was carried out by the diagnostic pathology department at VSD, Stormont.

4.2.6 Calving index

The calving index gives the average time in days for each cow between production of calves, therefore a high calving index reflects problems with fertility. It is calculated as the sum of days between calvings for all cows in the herd divided by the number of cows in the herd. The value should ideally be 365.

4.3 Results

4.3.1 Abortion

4.3.1.1 Summary of abortions

A summary of all the abortions that occurred on the study farm during this study is given in table 4.2. The farmer reported cow 408 was bought in early 1995, served, diagnosed pregnant and then lost her calf by 6 weeks gestation in mid April 1995. About this time three other calves numbered 17, 81 and 93 were diagnosed pregnant and then aborted or resorbed the foetus. No blood samples were taken from these animals at this time and no tissues were found, therefore the cause of foetal death is unknown.

Within a 2 week period in August/September 1995 an abortion storm resulted in the loss of 14 calves in 2 weeks. One of these cows, no.17, had lost its calf the previous April but it is not clear if this was a repeat abortion caused by neosporosis. The aborting cattle tested positive by IFAT for antibody to *Neospora*, and tachyzoites were identified by immunocytochemistry in tissue samples from 6 aborted foetuses. This work was carried out by the diagnostic pathology department at VSD, Stormont, NI.

During the course of this study 5 animals aborted more than once;

1) In February1996 cow no.408, which had been pregnancy diagnosed positive and then negative early in 1995 before this study began aborted twin calves three weeks early. *Neospora* tachyzoites present in the brain and spinal cord of one of the calves were identified by histological and immuno-cytochemical examination.
2) Cow no.200 aborted twice in August 1995 and November 1997. The dam was sero-positive at the time of both abortions. Foetal serum was not available for testing.
3) Cow 93 also aborted twice once in early 1995 (no serum samples at this time) and in August 1997 when the dam was strongly sero-positive for *N.caninum.* Foetal serum was not available for testing.

4) Cow 103 aborted 3 times during this study in August/September 1995, December 1996 (twins, one born dead) and in November 1997. Maternal sera was positive for *Neospora* antibody after each of the abortions. Fluid collected from the third aborted foetus was sero-negative. Foetal serum was not available for testing.
5) Cow number 114 aborted twice, during the abortion storm of August/September 1995 and in September 1997. Maternal serum was positive at the time of both abortions. Foetal serum was not available for testing.

Cow 7 aborted in Aug 1996 and cow 106 aborted mid-September 1997 at 5 months gestation. Both were the first and only abortions to occur in these cows and both foetuses had brain lesions consistent with *Neospora* infection and sera from cow 106 was strongly positive for *Neospora* antibody (PP 142%).

Four animals, numbers 38, 102, 140, 141, which aborted during the abortion storm did not abort again and produced antibody negative uninfected calves in November 1997.

A total 27 abortions occurred on this farm from early 1995 to December 1997. The rate of abortion during the first year of this study when the abortion storm occurred was high at 15% of breeding cattle. During the following year that dropped to 3.6% of the herd and rose slightly to 4.2% in 1997.

Of this total of 27 reported abortions and resorptions which occurred between early 1995 and December 1997 (approximately 3 years) 5 animals aborted for a second time. In 2 cases in early 1995 the first abortion/resorption could not be directly associated with *N.caninum* infection as the dams were not tested at this time, but were sero positive at the time of second abortion. For the remaining 3 cattle, maternal sera was positive at the time of both abortions. The rate of repeat abortion on this study farm associated with infection with *N.caninum* is therefore 10-21%.

4.3.1.2 Observations on abortions

The farmer reported that many of the cattle which aborted did so 3-4 weeks after being turned out onto pasture in Spring (May) at around 4 months gestation. The diet

changed at this point as the cattle only are grass fed during the summer months and receive no feed supplements incorporating extra protein (Bibby's dairy meal). The farmer noted that prior to aborting the cow's posture was affected, appearing as though about to give birth. At this time the farmer took animals off the pasture and into a covered area on hay. Abortion occurred in some cases but in others the womb receded and the cows went on to produce full term calves. When an abortion did occur there were no clinical signs in the dam, and the aborted foetus was expelled quickly. The dam was then 'clean' one day later with no persisting infections or production of fluids.

4.3.1.3 Calving index

Monthly milk records from 15th August 1995 to 14th March 1996 show that the calving index during this period fluctuated between 370 and 377, and was still at approximately 375 at the end of the study in early 1998 (pers comm, farmer).

Cow and Abortion No.	Date of Abortion	Foetal Neospora Ab (PP / IFAT)	Maternal Neospora Ab (PP / IFAT)	Foetal Lesions
1995				
408 (first)	Early '95	ND	ND	ND
17 (first)	Early '95	ND	ND	ND
81	Early '95	ND	ND	ND
93 (first)	Early '95	ND	ND	ND
4	Aug/Sept '95	ND	Positive @1:640	ND
9	Aug/Sept '95	ND	Positive @1:640	ND
17 (second)	Aug/Sept '95	ND	Positive @1:640	ND
38	Aug/Sept '95	ND	Positive @1:640	ND
42	Aug/Sept '95	ND	Positive @1:640	ND
45	Aug/Sept '95	ND	Positive @1:640	ND
63	Aug/Sept '95	ND	Positive @1:640	ND
94	Aug/Sept '95	ND	Positive @1:640	ND
101	Aug/Sept '95	ND	Positive @1:640	ND
102	Aug/Sept '95	ND	Positive @1:640	ND
103 (first)	Aug/Sept '95	ND	Positive @1:640	ND
114 (first)	Aug/Sept '95	ND	Positive @1:640	ND
125	Aug/Sept '95	ND	Positive @1:640	ND
140	Aug/Sept '95	ND	Positive @1:640	ND
141	Aug/Sept '95	ND	Positive @1:640	ND
200 (first)	Aug/Sept '95	ND	Positive @1:640	ND
1996				
408 (second)	Feb '96	ND	66%	IPX positive brain and spinal cord
119	April '96	ND	18%	
7	Aug '96	Negative	ND	Brain lesions similar to Neospora
103 (second)	Dec '96	ND	79.5%	ND
?	June '96	ND	145.8%	ND
1997				
21	May '97	ND	179.1%	ND
153	July '97	ND	138.7%	ND
93 (second)	Aug '97	ND	148.7%	ND
106	Sept '97	Negative	142%	Brain lesions similar to Neospora
114 (second)	Sept '97	ND	Positive @1:640	ND
131	Nov '97	Negative	137.8%	ND
103 (third)	Dec '97	18.3%	Positive	Neospora negative
200 (second)	Dec '97	ND	Positive	Neospora negative

Table 4.2 Details of abortions which occurred between early 1995 and December 1997 on the study farm. Bovine serum samples are considered antibody positive for *Neospora* at titres of ≥1:512 by IFAT and ≥30% positive by ELISA. Most of the maternal serum samples tested were positive for Neospora antibody at the time of abortion. Foetal fluids are positive for Neospora at ≥1:80 by IFAT and ≥15% positive by ELISA. All foetal fluids collected were antibody negative for N.caninum . (ND – not done). First, second and third refer to the number of abortion that occurred in an individual cow during this study for those which had more than one abortion.

4.3.2 Sero-epidemiology of aborting cattle and controls

The serum antibody levels to *N.caninum* in the seven cattle remaining on the farm which aborted during the abortion storm of September 1995 (figure 4.1a) and seven age matched controls (figure 4.1b) from September 1995 to November 1997 are shown in figure 4.1 (ELISA results are shown). See appendix chapter 4 tables 1-10 for ELISA and IFAT values for all sera tested. The other 7 cattle which had aborted were sent to slaughter as they were all more than 5 years old. All of the cattle which aborted were strongly sero-positive for *N.caninum* by IFAT at the time of abortion with titres of between 1:2048-1:4096. Antibody levels then dropped dramatically with all cattle in both groups testing sero-negative by both IFAT and ELISA two months later. Over the next 7 months until June1996 antibody levels fluctuated but most animals remained sero-negative. The herd was then tested around the time of calving in November 1996 when it was found that antibody levels had again risen and all of the animals which had aborted the previous year were again strongly seropositive. Most of the controls remained sero-negative but one animal sero-converted and became strongly positive for *N.caninum.* Subsequent testing in January and May 1997 revealed continuing high antibody levels in the aborting group. Animals in the control group remained antibody negative (<30% positive) although a small but consistent rise in titres was observed.

Figure 4.1a *Neospora* antibody levels in 7 animals which aborted due to natural infection with *N.caninum* on the farm in Carrowdore in August/September 1995. All animals were kept on the same farm in Carrowdore. Bovine serum samples were tested for antibody by ELISA and are considered antibody positive for *Neospora* at ≥30% positive (represented by the broken line). Dates in brackets beside the key show the time of any further abortions after the abortion storm of 1995. Cow 45 was culled in June 1996.

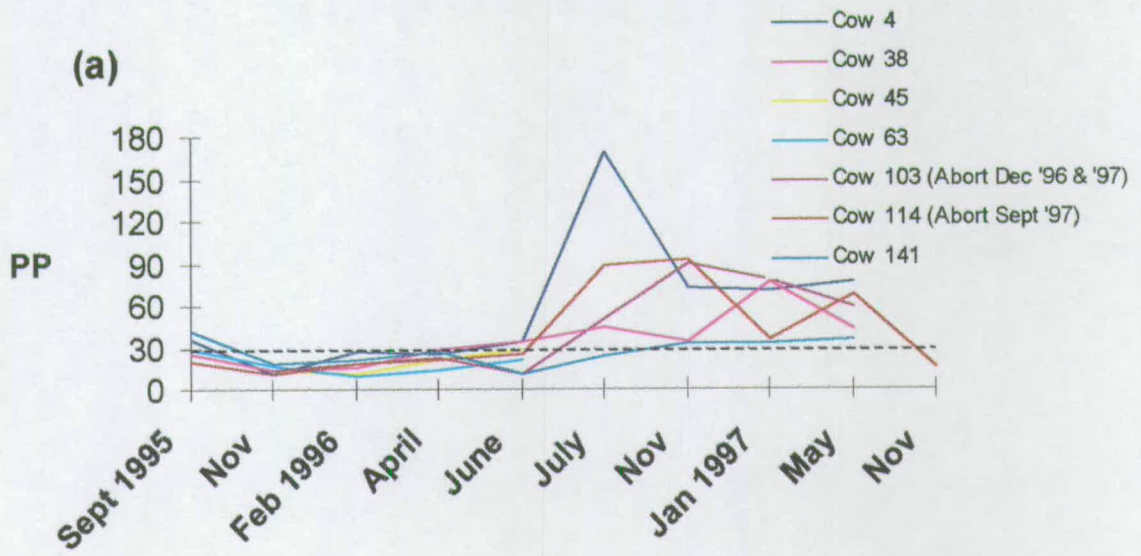
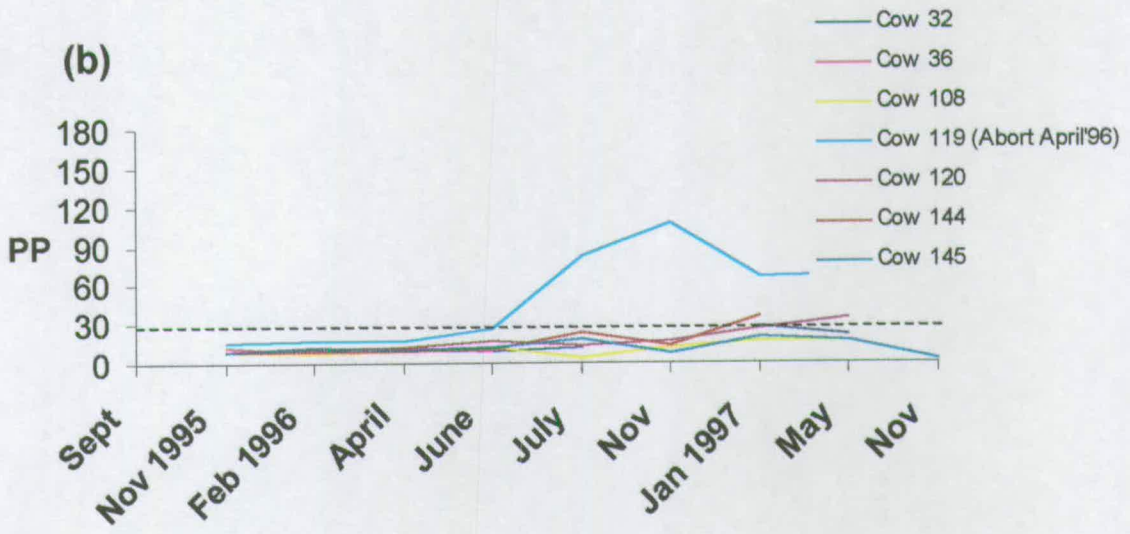


Figure 4.1b *Neospora* antibody levels in 7 age matched control animals which did not abort during the abortion storm of August/September 1995, however Cow 119 did abort in April 1996. All animals were kept on the study farm. Bovine serum samples were tested for antibody by ELISA and are considered antibody positive for *Neospora* at ≥30% positive (PP) (represented by the broken line).



4.3.3 Congenital infection

A summary of all the cases of congenital infection diagnosed on the study farm is given in table 4.3.

Pre-colostral sera from calves born on the farm and fluid from aborted foetuses were collected when possible. Of 4 calves born in November 1995 one calf from cow 170 was found to be strongly positive for *Neospora* with an PP value of 118%. The calf did not show any clinical signs of infection.

In early April 1996 a calf born to a heifer (cow number not known) was born with contracted tendons, was unable to rise and appeared to have neurological defects. The calf was sero-positive for *Neospora* but had been stomach tubed with colostrum before the blood sample was taken. Neosporosis was confirmed by histological examination at post mortem.

A clinically normal congenitally infected full term calf was born in September 1996 to cow number 114 which had aborted on two other occasions, the first time during the abortion storm of August/September 1995 and the second in September 1997. Maternal serum was positive at the time of birth of the clinically normal calf and immediately after both abortions.

Twin calves born to cow no.68 which had no history of abortion, were tested for pre-colostral antibody to *N.caninum* but were found to be negative. A further 20 pre-colostral samples submitted to VSD during late October and November 1997 were tested for antibody to *N.caninum* but were negative.

An insufficient number of pre-colostral serum samples were collected from new born calves to estimate the rate of congenital infection on this farm.

Table 4.3 Details of congenitally infected calves diagnosed on the farm. Serum samples were collected from the calves immediately after birth, before ingestion of colostrum. Calf *Neospora* antibody titres ≥30%OD by ELISA and ≥1:512 by IFAT were considered positive. ND, not done.

Cow No.	Date of Birth of Calf	Calf Neospora Ab (PP / IFAT)	Maternal Neospora Ab (PP / IFAT)	Clinical Status	Foetal Lesions
170	November '95	118%	ND	Normal	ND
?	April '96	1 :8192	ND	Contracted tendons	Neospora IPX negative
114	September'96	146%	94.3%	Normal	ND
119	September'96	101%	107.7%	Normal	ND
119	November '97	95%	ND	Normal	ND

4.3.4 Herd status

In order to assess the number of animals infected/sero-positive for *N.caninum* the herd was bled and tested for antibody to *Neospora* annually.

Herd Bleed 1:

February 1996. IFAT and ELISA values are given in appendix chapter 4, table11. Only the animals that were milking at the time were included. Of the 93 heifers tested from a total of approximately 130 heifers on the farm, 5 months after the abortion storm none of the animals were sero-positive for *N.caninum* by ELISA.

Herd Bleed 2:

November 1996. ELISA values are given in appendix chapter 4, table 12. All of the cattle on the farm at this time, 137 in total, were tested for antibody to *N.caninum* of which 16% were positive for *Neospora.*

Herd Bleed 3:

November 1997. ELISA values for the whole herd are given in appendix chapter 4, table 13, and those samples which could be matched to calf number are given in appendix chapter 4, table 14. All of the cattle on the farm at this time, a total of 190, were tested for antibody to *N.caninum* of which 21% were positive.

4.3.5 Other reported health problems

An out break of *Salmonella* infection occurred in the herd during the winter of 1996-1997. One cow died and the herd have been vaccinated annually since then.

A problem with scour in calves has been present in the herd for the last 2 years (early 1997-1998). Calves were tested and treated with Rotavec paste but this had no effect and the cause has not yet been identified.

The whole herd has also been vaccinated annually against leptospirosis with the exception of 1995.

4.4 Discussion

There are several possible sources of environmental contamination with *Neospora caninum* parasite. The definitive host for *N.caninum* has been identified as the dog therefore faecal contamination of the environment with *Neospora* oocysts is likely to be a source of infection. Exposure to aborted foetal material possibly containing viable *N.caninum* tachyzoites or bradyzoites could be infective. There may also be an as yet unidentified population of wild animal hosts of *N.caninum* acting as a reservoir of infection, for example other canids such as foxes may be able to produce oocysts. Neosporosis was first seen on the study farm when an 'abortion storm' occurred. The large number of abortions in a short period of time suggests infection came from a point source which all of these animals were exposed to. The farm dogs had free access to feed and feeding troughs and occasionally dogs were walked across the land by a visitor to the caravan park bordering the site. Also, four cows previously pregnancy diagnosed positive either aborted or resorbed their foetuses early in 1995 after being put onto pasture. No foetal material was found but cows have been reported to eat aborted foetal tissue (pers comm, David Graham, VSD, Stormont). One of the control cows (no.119) which did not abort during the abortion storm of 1995 and was sero-negative for *N.caninum* at that time sero converted during June/July 1996 suggesting the animal became infected around the time it was put out to pasture in May. This cow went on to produce a congenitally infected calf in each of the following 2 breeding seasons. Since the other control cattle did not seroconvert contamination of the environment must have been limited. The source of contamination therefore is not clear, but preventing dogs from crossing farm land where possible and removing aborted tissues from the environment could reduce levels of infection in the herd. Identification of possible wild animal resevoirs of infection is necessary if successful methods of disease control are to be developed. The sensitive and specific polymerase chain reaction (PCR) which amplifies *Neospora* DNA present in host tissues would be a useful tool to determine the role of wildlife reservoirs in neosporosis.

The reason for the dramatic drop in *Neospora* antibody titres several months post abortion is unknown. *T.gondii* cysts in heart tissue, skeletal muscle and the CNS slowly replicate occasionally releasing bradyzoites into the circulation which periodically primes the immune system and maintains high specific antibody titres (Wong and Remington, 1993; Gazzinelli *et al,* 1993a). *Neospora* tissue cysts have only been found in the CNS of congenitally infected calves (Barr *et al,* 1993), an immunologically privileged site. Periodically low antibody titres may therefore be due to parasite antigen being hidden from the immune system and not being released to maintain antibody levels. It is unlikely that fluctuation of antibody titre is due to parasite being cleared from tissues and then reinfected the following year as repeat abortions and congenital infection occurred throughout the study without repeated widespread abortions. The closely related coccidian parasite *Toxoplasma gondii* persists in ovine tissues in the form of tissue cysts. Hartley and Moyle (1974) isolated *T.gondii* tachyzoites from 14 of 15 sheep which had persistent or fluctuating titres but could not recover organisms from 8 animals which were consistently seronegative. High or frequently fluctuating levels of parasite specific antibodies in serum are therefore likely to be associated with persistent parasite infections. Repeat transplacental infection with *N.caninum* in cattle has previously been reported in individual animals on their respective farms with no evidence of widespread abortion (Barr *et al,* 1993) which also suggests that recrudesence of infection and not re-infection may be the cause of repeat abortion/foetal infection. High *Neospora* titres in naturally infected cattle in this study immediately after abortion and again during gestation the following year, leading to production of congenitally infected calves in some cases, suggests that the parasite does persist in tissues of infected cattle, and that recrudesence of infection may in some cases cause abortion. It is clear however that antibody titre is not a reliable indication of infection, and therefore diagnosis should not depend on IFAT or ELISA alone but should be supported by identification of parasite antigen in aborted foetal tissues.

Neospora antibody levels began to increase between June and July 1996 in the cattle which had aborted the previous year. It is likely that all of these cattle were pregnant since the end of April. The farmer also observed that many of the abortions

occurred at approximately 4 months gestation. During gestation the immune system of the host is altered to accommodate a foetus which is essentially foreign tissue (Raghupathy, 1997) and it is possible that down-regulation of the cell mediated immune response in pregnancy could reduce immune pressure, allowing *N.caninum* bradyzoites to replicate and escape into circulation causing the foetus to become infected and antibody titres to rise. More information on the effect of gestation on the internal environment and how that affects parasite replication and release, particularly around the 4^{th}-5^{th} month of gestation, is needed before we can understand what actually causes foetal death and abortion in cattle infected with *N.caninum.*

Anti-*Neospora* antibody levels in the herd were estimated annually. All of the animals in the herd were sero-negative for *N.caninum* 5 months after the onset of the abortion storm in February 1996. Herd bleeds carried out in November 1996 and November 1997 showed that 16% and 21% respectively of animals were sero-positive. Again this may be linked to gestation as the first herd bleed was completed when most of the animals had already given birth. The remaining tests were carried out around the time of late gestation or parturition when the immune system may still have been suppressed. These results suggest that an accurate measure of sero prevalence in the herd may depend on when the animals are tested and indicate that serum antibodies may be at their highest levels during mid to late gestation.

Transplacental infection has been proposed in previous studies as the main means of infection in cattle (Paré *et al*, 1996). Unfortunately very few pre-colostral sera were collected from new born calves in this study, but analysis of samples collected has shown that 1 suspected and 3 proven cases of congenital infection occurred on the case study farm between November 1995 and April 1996. Three of the calves were clinically normal but one had contracted tendons and was unable to rise. No other weak or uncoordinated calves were born on the farm during this period. It is therefore clear that the majority of congenitally infected calves are likely to be clinically normal. All of 20 pre-colostral sera from new born calves collected during October/November 1997 were negative suggesting the rates of congenital infection on this farm were low, but as an insufficient number of sera were collected the rate of congenital infection in this case

cannot be assessed. Obvious differences in frequency of congenital infection compared to the study of 2 large dairy herds in California by Paré may be due to differences in climate, farm practices or virulence of parasite strains.

After the initial abortion storm the abortion rate fell dramatically and remained low for the duration of the study. Serum samples collected at the time of or soon after abortion from three dams which had aborted more than once were antibody positive for *N.caninum*. It is possible that repeat abortion may have occurred in a total of 5 animals as 3 dams had previously either reabsorbed or aborted a foetus in early 1995 (no foetal tissues were found) but as we have no serum samples from the cattle at this time the cause of these abortions is unknown. These results indicate that abortion and repeat abortion correlate with high *N.caninum* antibody titres in the dam, but that the rate of repeat abortion is between 10-21%, at least double the estimated incidence of repeat abortion (Dubey and Lindsay, 1996). Variation may be due to differences in virulence of parasite strains although at present very little is known about *N.caninum* strains currently sustained in tissue culture. Clearly further work needs to be done to identify and characterise parasite strains which may cause the most damage to infected individuals.

The farmer observed that many abortions and resorptions (not all of which are reported here) occurred 3-4 weeks after the herd was put out to graze on the pasture at approximately 4 months gestation. Cattle put into the field experience higher levels of stress as their diet changes from easily digestible silage and dairy feed supplements containing extra protein to grass without extra feed. By removing any animal from the field which showed signs that it was about to abort (the womb became raised), and keeping it in farm accommodation bedded on hay the farmer effectively reduced environmental stress and in some cases these animals did not abort, the womb fell again and the cow produced a full term calf. This anecdotal information suggests that stress may be an important factor in determining the outcome of infection. If this is the case alleviating stress by improving feeding around this time or changing the time of release onto pasture may reduce the number of abortions due to neosporosis.

The calving index for this herd was high at between 370-377 at the beginning and end of this study which suggests that infection with *N.caninum* may affect fertility

although this cannot be confirmed from the results shown here. The fecundity of the herd was affected as approximately 29 abortions or resorptions linked to infection with *N.caninum* occurred on the farm between early 1995 and December 1997. In a small dairy herd this accounts for relatively large financial losses for the farmer. Further studies are urgently needed to identify possible control strategies as at present there is little official guidance for farmers coping with this problem.

Papers and Publications Arising from this Work:

Marks J., Graham D., McNamee P., Lunden A. and Innes E.A. (1997). Sero-epidemiological studies of a dairy herd infected with *Neospora caninum* in Northern Ireland. VII International Coccidiosis Conference and European Union COST820 Workshop, Keble College, Oxford, 1-5th September.

Chapter 5

Identification of *Neospora caninum* specific antigens recognised by the humoral immune response in infected cattle

Aims

- To identify specific *Neospora caninum* antigens which are commonly recognised by the humoral immune response in experimentally and naturally infected cattle and do not cross react with antibodies to closely related coccidian parasites.

- To compare antigens recognised by bovine maternal and foetal *Neospora* antibody positive natural infection sera submitted to 8 veterinary investigation centres around Scotland.

- To compare the humoral immune response to *Neospora* in cattle naturally and experimentally infected with *N.caninum.*

- To identify antigenic variation between NC Liverpool and NC1 isolates of *N.caninum* tachyzoites recognised by immune sera.

5. Identification of *Neospora caninum* specific antigens recognised by the humoral immune response in infected cattle

5.1 Introduction

Neospora caninum is a protozoan parasite that has been shown to be a major cause of abortion in cattle. Barr *et al* (1991) reported that *Neospora* was responsible for approximately 19% of bovine abortions in California, and recent studies in Scotland and Northern Ireland have suggested a foetal infection rate of approximately 12-17% (Buxton *et al,* 1997a) and 4-10% (McNamee *et al,* 1996) respectively.

Definitive diagnosis of neosporosis is achieved by identification of parasite in tissues (Barr *et al*, 1994b). This is not always possible as parasite may not be present in aborted foetal tissue and in many cases the aborted foetus may not be found. *Neospora* specific antibody titres can be detected and quantified by a commercially available indirect fluorescence antibody test (IFAT) (VMRD, Pullman, USA) and more recently by ELISA (Bjorkman *et al*, 1994; Pare *et al*, 1995; Lally *et al*, 1996; Bjorkman *et al*, 1997; Williams *et al,* 1997; Osawa *et al*, 1998) all of which use either whole tachyzoites or a combination of many parasite antigen fractions. Diagnostic ELISA tests developed for detection of neosporosis have been shown to be more sensitive and specific than IFAT for serodiagnosis in cattle (Williams *et al*, 1997). But antibodies to *Neospora* tachyzoite antigen can cross react with conserved antigens of closely related coccidian parasites. In some cases sera from cattle with low *Neospora* antibody IFAT titres (1:160-1:620) were found to react with the apical end of *Toxoplasma* RH tachyzoites (Conrad *et al,* 1993b). This may be due to the presence of common highly conserved apical

complex antigens recognised by antibody to a previous infection with *T.gondii* or another closely related coccidian parasite (Conrad *et al*, 1993b). Western blot analysis of *Neospora* water soluble fraction (wsf) tachyzoite antigen, which incorporates a large number of antigen fractions, with sera from cattle experimentally infected with *T.gondii* and *Sarcocystis spp.* has shown cross reaction occurs (Baszler *et al*, 1996). *Neospora* antibody titres in naturally infected cattle may also fall dramatically with some cattle becoming antibody negative for *Neospora* several months after abortion (chapter 4), and a survey of neosporosis in Scotland has shown that cattle which are sero-negative to *Neospora* by IFAT can produce infected calves (Buxton *et al,* 1997a). The existing forms of diagnosis which use serum antibody levels as an indicator of infection may not therefore be a reliable indicator of infection or abortion.

Some of these problems may be overcome by developing an ELISA using *N.caninum* antigen fractions which are consistently and strongly recognised by naturally infected animals but which do not cross react with those of closely related organisms. Experimental infection in rabbits with *N.caninum* NC1 tachyzoites stimulated a strong antibody response to 20 antigen fractions, the most immunodominant of which were 16/17, 29, 37, and 46kDa on a non-reduced SDS PAGE gel (Barta and Dubey, 1992). A similar range of antigens including one at 30kDa were found to be recognised by sera from a goat, a pig, a sheep, a rabbit, a cow, a naturally infected cow and a calf experimentally infected with *Neospora* NC1 tachyzoites (Bjerkas *et al*, 1994) although in this study only one serum sample from each species was tested for specific antibody response to *N.caninum* experimental infection, and the antigens recognised were not tested for cross reaction with other coccidian parasites. Clarification of which antigen fractions are the most specific and would give the greatest sensitivity in a diagnostic test is essential if a more effective method of diagnosis is to be developed.

Neospora caninum recombinant antigen fractions of approximately 30 and 35kDa have been expressed in cDNA clones and used to develop two ELISA tests (Lally *et al*, 1996). These antigens were found to be able to distinguish between sera from cows naturally or experimentally infected with *Neospora* and uninfected control cows and did not cross react with serum from cattle experimentally inoculated with *T.gondii,*

Sarcocystis cruzi, Sarcocystis hirsuta or *Sarcocystis hominis.* The ELISA's were also found to complement each other with each recognising *Neospora* sero-positive animals which the other did not. This suggests that development of this kind of ELISA should include a combination of antigen fractions for optimum diagnosis, though only a few serum samples were used in each case and no sera or fluids from foetuses infected with *N.caninum* were tested.

Previous studies clearly have not tested large numbers of sera from naturally infected cattle and aborted foetal tissues for widespread recognition of *Neospora* antigens, and in general cross reaction has been tested using serum from experimentally infected animals. The main aims of this study were:

- to identify *N.caninum* antigens which do not cross react with sera from cattle and sheep naturally and experimentally infected with *Babesia, Sarcocystis, Cryptosporidium* or *Toxoplasma,* and
- to identify *N.caninum* antigens which are widely recognised by *Neospora* positive bovine sera from naturally and experimentally infected cattle and aborted foetal tissues from around Scotland and from a farm in Northern Ireland which has suffered losses due to neosporosis (chapter 4).

The NC Liverpool (NC Liv) strain of *Neospora* which has been cultured *in vitro* for a relatively short period of time compared to NC1 which has been continuously in culture since it was first isolated in 1988 (Dubey *et al*, 1988b) was used in this study and compared with the NC1 strain. As the long term aim of this project is to develop a specific diagnostic assay it is important to identify whether a strain which is less adapted to tissue culture contains a wider range of antigens.

5.2 Materials and methods

5.2.1 Parasites

5.2.1.1 *Neospora caninum*

Neospora caninum NC1 and NC Liv strain tachyzoites were cultured within VERO cell monolayers in IMDM supplemented with 2% horse serum, as described in chapter 3, section 3.2.1.1 and 3.2.2.1.

5.2.2 Experimental design

Neospora caninum SDS soluble fraction tachyzoite antigen was separated by SDS PAGE and probed with sera from cattle and sheep experimentally and naturally infected with *N.caninum, T.gondii* and other closely related coccidian parasites (table 5.1). Serum from naturally infected animals and post infection from experimentally infected animals were all strongly antibody positive. Post inoculation sera were collected 3-4 weeks post infection. Pre-infection sera were antibody negative. (See chapter 5 appendix for serum *Neospora* IFAT and ELISA values).

Table 5.1 Anti-parasite antibody specificity of sera used to probe NC1 *N.caninum* SDS soluble antigen separated by SDS PAGE. Pre- (control) and post infection sera from experimentally inoculated animals were used. IFP, Institut f$_{s}$ r Parasitologie; MRI, Moredun Research Institute; NI, Northern Ireland; NIVR, Nationaal Instituut voor Diergeneeskundig Onderzoek (Brussels); VSD, Veterinary Science Division (Stormont)

Antigen	Parasite Specificity of Antibody Positive Sera	Species	Infection Type	No. of Serum Samples	Source of Sera
N.caninum	*Neospora*	Bovine	Natural	3 + Control	NI Study Farm
NC Liv	*Neospora*	Bovine	Experimental	3 + Control	MRI
	Toxoplasma	Bovine	Experimental	3 + Control	MRI
	Toxoplasma	Ovine	Natural	3 + Control	MRI
	Neospora	Bovine	Natural	20	NI Study Farm
N.caninum	*Neospora*	Bovine	Natural	3 + Control	NI Study Farm
NC1	*Neospora*	Bovine	Experimental	3 + Control	MRI
	Toxoplasma	Bovine	Experimental	3 + Control	MRI
	Toxoplasma	Ovine	Natural	3 + Control	MRI
	Neospora	Bovine	Natural	50	Scottish Survey
	Sarcocystis	Ovine	Experimental	5 + Controls	IFP, Hannover
	Babesia divergens	Bovine	Natural	2	VSD, Belfast
	Babesia divergens	Bovine	Experimental	2 + Controls	VSD, Belfast
	Babesia bovis	Bovine	Experimental	2 + Controls	VSD, Belfast
	Babesia bovis/bigemina	Bovine	Experimental	1 + Control	VSD, Belfast
	Babesia bigemina	Bovine	Experimental	2	VSD, Belfast
	Cryptosporidium parvum	Bovine	Experimental	6	NIVR, Brussels

5.2.3 Production of SDS soluble fraction antigen

Neospora caninum SDS soluble tachyzoite antigen was produced as previously described in chapter 3, section 3.4.3.

5.2.4 SDS PAGE and Western blot

N.caninum SDS soluble proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions as described by Laemmli (1970) and in section 3.6.1, 3.6.2 and 3.6.3 of this thesis. In brief, the Protean II system (Biorad, Hemel Hempstead, UK) was used to separate. Approximately 100µl of *N.caninum* NC1 SDS soluble antigen at a concentration of 1mg/ml was diluted in 100µl of sample reducing buffer (incorporating mercaptoethanol) and boiled for 5 minutes. The reduced antigen was then loaded onto a large 10% polyacrylamide gel, and a combination of molecular weight markers in the range of 12,000-78,000 was used to determine molecular mass (Merck, Lutterworth, UK). Proteins were transferred from the gel to a nylon membrane (Immobilon) (Millipore, Bedford, MA, USA) by electroblotting with a trans-blot electrophoretic transfer cell (Biorad, Hemel Hempstead, UK) at a constant voltage of 100v. Membrane bound antigen was treated with a 5% solution of dried skimmed milk (Marvel) (Premier Beverages, Stafford, UK) (Kachani *et al*, 1992) to block non-specific background staining before incubation with bovine sera at a dilution of 1:150. After a further incubation with alkaline phosphatase conjugated rabbit anti-bovine IgG (Sigma, Poole, Dorset, UK), strips were developed using nitro blue tetrazolium (NBT) (Sigma, Poole, Dorset, UK) and bromochloroindolyl phosphate (BCIP) (Sigma, Poole, Dorset, UK) in alkaline phosphatase buffer (Sigma, Poole, Dorset, UK). Serum dilution and concentration of NC1 antigen loaded onto 10% polyacrylamide gels were optimised as described in Harkins *et al*, 1998.

5.2.5 Periodate treatment of blots

Neospora NC Liv antigen separated by SDS PAGE and bound to a nitrocellulose

membrane was oxidised to remove carbohydrate epitopes. The method is given in methods section 3.6.4. In brief, the blot was washed to remove Ponceau stain (Sigma, Poole, Dorset, UK), rinsed with acetate buffer and incubated in the dark for 60 minutes at 23°C with sodium periodate (50mM $NaIO_4$) in sodium acetate solution (50mM NaAc) (pH4.5). The blot was then rinsed x2 with sodium acetate soulution, 2x with buffer solution (TBST) and incubated for 30 mins with 50mM $NaBH_4$/TNTT. After 3 x 10 min rinses with TNTT the blot was blocked with 5% Marvel in TBST and developed as before in section 5.2.4.

5.2.6 Sera

Neospora caninum antigen separated by SDS PAGE was probed with sera from cattle naturally and experimentally infected with a range of coccidian parasites (IFAT values of all serum samples used are given in appendix chapter 5.

5.2.6.1 *Neospora caninum*

Serum samples from cattle naturally and experimentally infected with *N.caninum* were used to probe NC Liv antigen (figure 5.1) and NC1 antigen (figure 5.2) separated by SDS PAGE. Pre-infection sera from experimentally infected animals were used as negative controls (serum IFAT values given in appendix chapter 5, table 5.1).

Approximately 50 bovine *Neospora* positive sera from naturally infected animals, both maternal (30 samples) and foetal (21 samples), collected and tested for the Scottish survey of neosporosis in cattle were kindly donated by Dr David Buxton, Moredun Research Institute (serum IFAT values given in appendix chapter 5, table 5.3). A further 20 sera from naturally infected cattle (all adult females) which had suffered abortions due to *Neospora* (figure 5.4) and sera from 2 calves congenitally infected with *N.caninum* (figure 5.5), all from the study farm in Antrim, NI (chapter 4), were kindly donated by David Graham, Veterinary Science Department, Stormont, Belfast (serum IFAT values given in appendix chapter 5, tables 5.4 and 5.5). All sera were tested by IFAT with *Neospora* titres ≥1:512 for maternal sera and ≥1:64 for foetal fluids being

regarded as positive. Sera from cattle experimentally infected with *N.caninum* NC1 strain tachyzoites at Moredun Research Institute were used as positive controls, and pre infection sera from the same animals were used as negative controls.

Neospora NC Liv antigen, periodate treated to remove carbohydrate epitopes (figure 5.6), was probed with serum samples from cattle naturally and experimentally infected with *N.caninum* as before (serum IFAT values given in appendix chapter 5, table 5.6).

5.2.6.2 *Toxoplasma gondii*

Antigen was probed with sera from 3 sheep naturally infected with *T.gondii*, from 3 cattle orally infected with 10,000 M3 strain oocysts and one bovine pre-infection serum sample. This sera was kindly donated by Dr.Irma Esteban, Moredun Research Institute (serum IFAT values given in appendix chapter 5, tables 5.1 and 5.2).

5.2.6.3 *Babesia divergens, Babesia bovis* and *Babesia bigemina*

Serum from 2 cattle naturally infected with *B.divergens,* and pre and post inoculation sera from 7 cattle experimentally infected with *B.divergens, B.bovis* and *B.bigemina* were kindly donated by Hilary Edgar, Veterinary Science Department, Stormont NI. All post infection sera were collected 4 weeks post infection and are strongly antibody positive (antibody titres are not available for these samples but details are given in appendix chapter 5, table 5.7).

5.2.6.4 *Cryptosporidium parvum*

Pre and post infection sera from 3 calves orally infected with 10,000 *C.parvum* oocysts was kindly donated by Dr.Dirk de Graaf, National Institute of Veterinary Research, Brussels, Belgium (serum IFAT values given in appendix chapter 5, table 5.8).

5.2.6.5 *Sarcocystis tenella*

Pre and post inoculation sera from 5 sheep experimentally infected with *S.tenella*

was kindly given by Dr.Anja Heckeroth, Institut für Parasitologie, Hannover, Germany (serum IFAT values given in appendix chapter 5, table 5.9)

5.3 Results

5.3.1 Identification of commonly recognised *Neospora caninum* antigens

Both *Neospora* NC1 and NC Liv blots (figures 5.1 and 5.2 respectively) were probed with the same serum samples detailed in table 5.2. *Neospora* NC1 and NC Liv antigens of approximately 16kDa and a group of bands at 66, 68 and 71kDa were consistently recognised by cattle naturally or experimentally infected with *Neospora* (figures 5.1 and 5.2) (serum antibody values are given in appendix chapter 5, tables 5.1 and 5.2 respectively). Another band at 90kDa was stained on the NC1 blot only by these sera. Other bands at 21-24 and 38kDa were consistently and strongly recognised by serum from the Scottish survey (figure 5.3) (serum antibody values are given in appendix chapter 5, table 5.3) but recognised only by some samples from experimentally infected animals. (Figure 5.3 shows a Western blot using SDS PAGE separated NC Liv antigen probed with approximately half of the Scottish survey samples, the rest are shown in appendix 5, figure 5.1).

Table 5.2 Antibody titres of sera used to probe SDS PAGE separated NC Liv and NC1 *N.caninum* antigen, shown in figures 5.1 and 5.2.

Strip No.'s	Serum
1→3	*Neospora* Natural Infection (Cattle)
4	Control for 1-3
5→7	*Neospora* Experimental Infection (Cattle)
8	Control for 5-7(pre-infection)
9→11	*Toxoplasma* Experimental Infection (Cattle)
12	Control for 9-11 (pre-infection)
13→15	*Toxoplasma* Natural Infection (Sheep)
16	Control for 13-15
17	*Sarcocystis cruzi* Infection (Cattle) (titre not known)

Strip No.	***Animal No.***	***Infection Status/IFAT***	
1	38 (Batch 1)	+ve	1:4096
2	63 (Batch 1)	+ve	1:4096
3	170 (Batch 2)	+ve	1:16,384
4	6 (Batch 2)	-ve	0
5	525	+ve	1:4096
6	3679	+ve	1:2048
7	619	+ve	1:4096
8	525	-ve	Pre-Infection
9	2716	+ve	1:1024
10	2713	+ve	1:1024
11	2401	+ve	1:1024
12	2401	-ve	1:128
13	5227/8	+ve	1:1024
14	580/8	+ve	1:1024
15	5227/5	+ve	1:1024
16	5226	-ve	negative
17	NA	+ve	

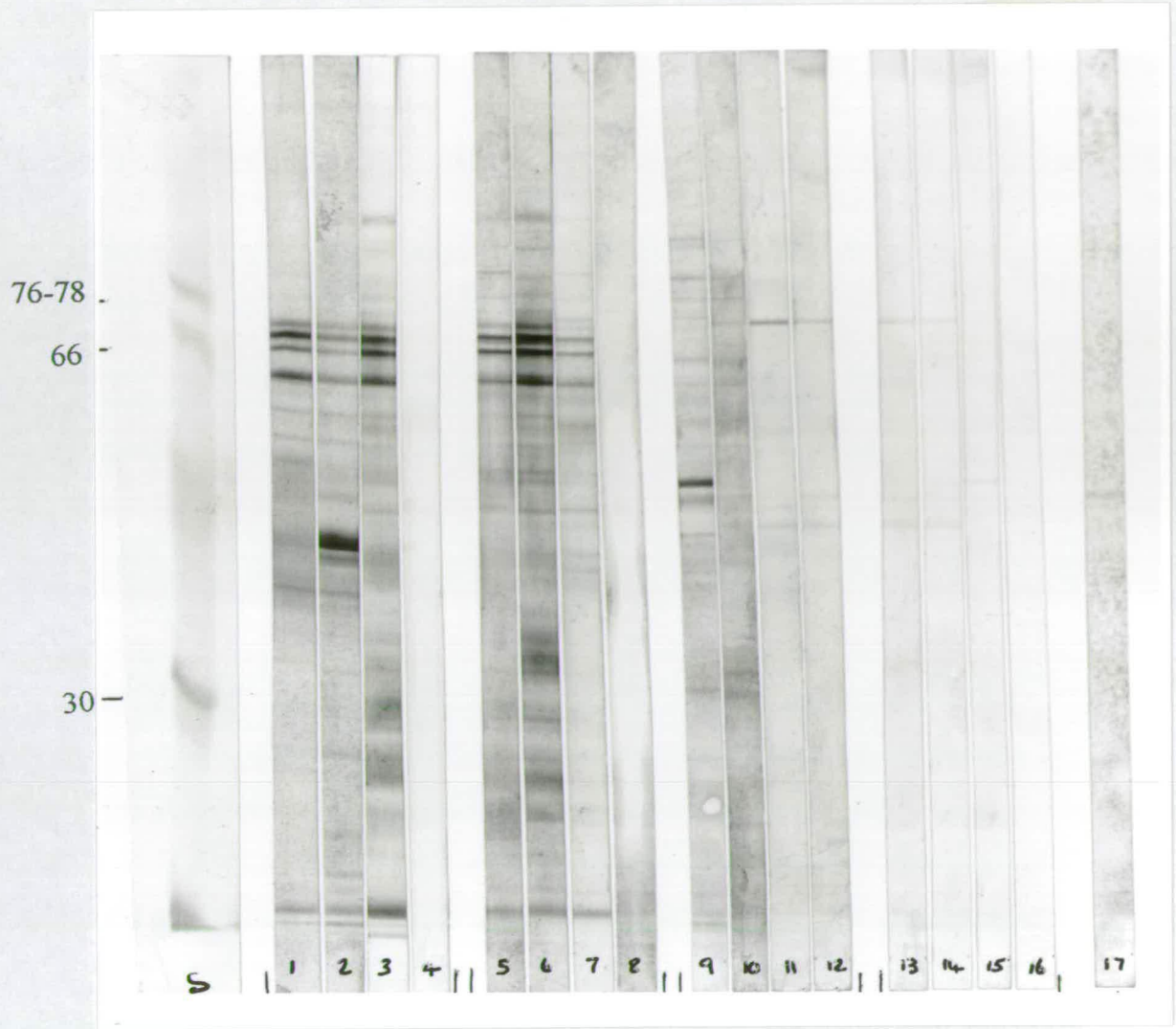


Figure 5.1 *Neospora caninum* NC Liv antigen probed with *Neospora, Toxoplasma* and *Sarcocystis cruzi* antibody positive sera, (natural and experimental infection antibody titres given in table 5.2) (sera also used to probe NC1 antigen in figure 5.2). Strips 1-3: sera from natural bovine *Neospora* infection (IgG antibody titre >1:4096). Strips 5-7: sera from experimental bovine *Neospora* NC1 infection (IgG antibody titre >1:2048). Strips 9-11: sera from experimental bovine *Toxoplasma* M3 infection. Strips 13-15: sera from natural ovine *Toxoplasma* infection (IgG antibody titre >1:1024). Strip 17: sera from experimental bovine *Sarcocystis cruzi* infection. Strips 4, 8, 12, 16: antibody negative controls. S = standards, MW range 12,300 - 76-78,000kDa, BDH Laboratories Supplies.

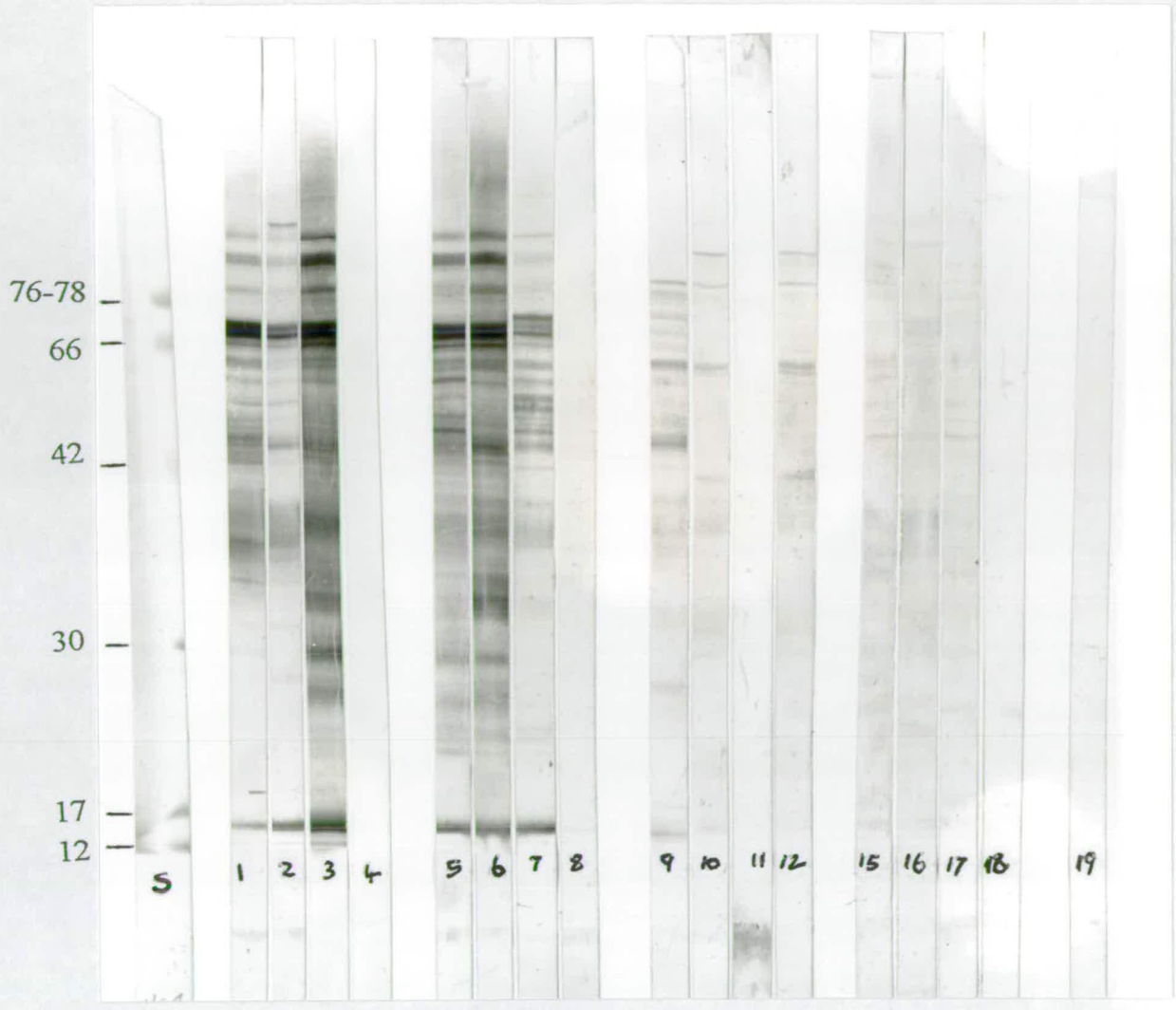


Figure 5.2 *Neospora caninum* NC1 antigen probed with *Neospora, Toxoplasma* and *Sarcocystis cruzi* antibody positive sera (natural and experimental infection, antibody titres given in table 5.2) (sera also used to probe NC Liv antigen in figure 5.1). Strips 1-3: sera from natural bovine *Neospora* infection (IgG antibody titre >1:4096). Strips 5-7: sera from experimental bovine *Neospora* NC1 infection (IgG antibody titre >1:2048). Strips 9-11: sera from experimental bovine *Toxoplasma* M3 infection. Strips 13-15: sera from natural ovine *Toxoplasma* infection (IgG antibody titre >1:1024). Strip 17: sera from experimental bovine *Sarcocystis cruzi* infection. Strips 4, 8, 12, 16: antibody negative controls. S = standards, MW range 12,300 - 76-78,000kDa, BDH Laboratories Supplies.

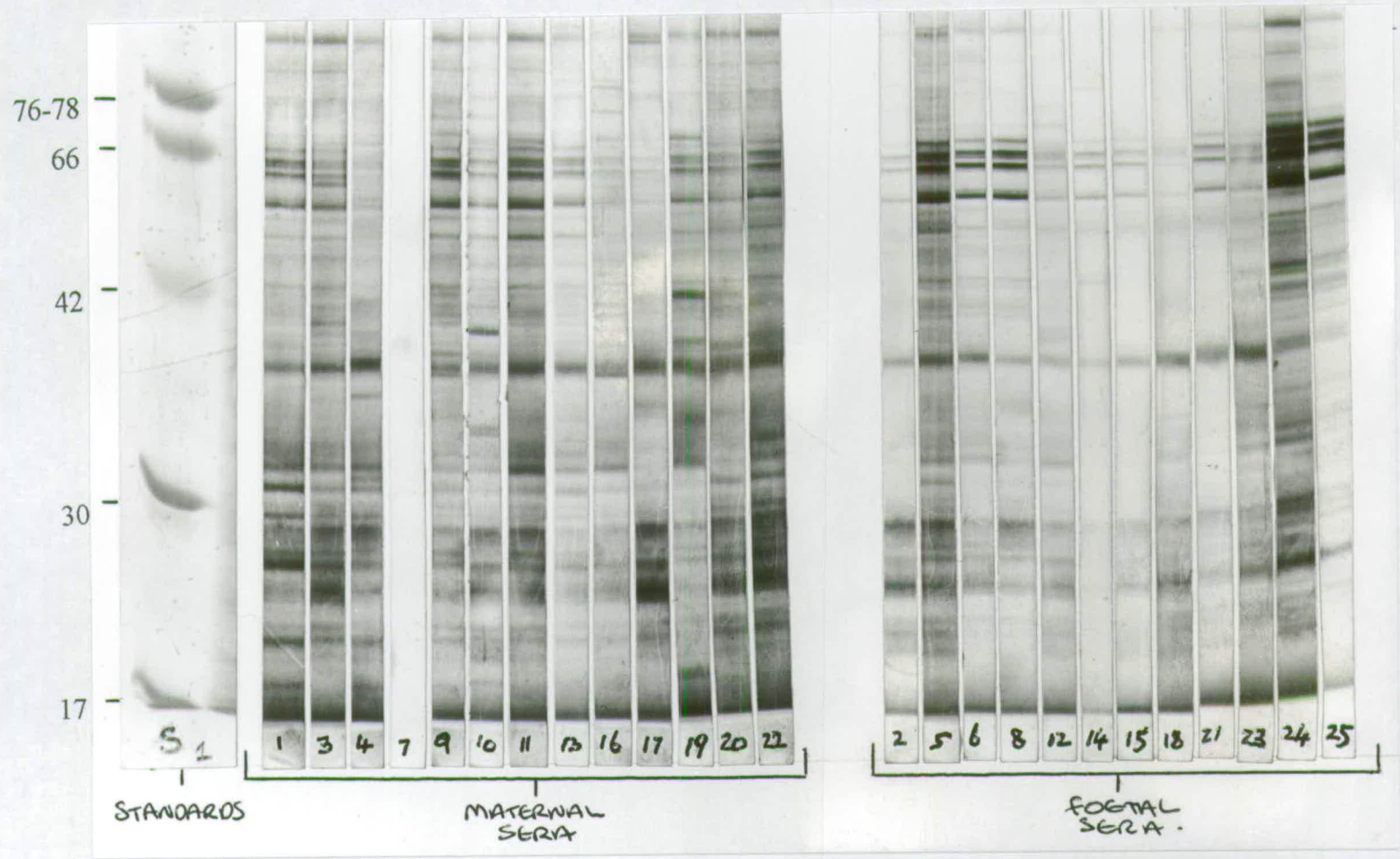


Figure 5.3 *Neospora caninum* NC Liv antigen probed with *Neospora* positive cattle sera (IgG antibody titre >1:512) from 8 veterinary investigation centres around Scotland. The number on each strip correlates to an animal number, details of which are given in appendix chapter 5, table 5.3. S = standards, MW range 12,300 - 76-78,000kDa, BDH Laboratories Supplies.

5.3.2 *Neospora* antigens commonly recognised by maternal and foetal natural infection sera.

Neospora NC Liv tachyzoite antigen was probed with sera from naturally infected cattle and aborted foetuses strongly antibody positive for *N.caninum* (figure 5.3) (serum antibody values are given in appendix chapter 5, table 5.3). This sera was collected for the Scottish survey on neosporosis in cattle and tested by IFAT. The antigen bands recognised were consistent for both maternal and foetal sera.

Many antigens were recognised including a group of bands at 65-68kDa, a broad area between 22-25kDa which showed diffuse staining, and single bands at 90, 60, 38 and at 15kDa at the base of the blot.

Neospora NC Liv tachyzoite antigen was also probed with *N.caninum* antibody positive sera from naturally infected cattle from the study farm in Northern Ireland which has suffered abortions due to neosporosis (figure 5.4) (serum antibody values are given in appendix chapter 5, table 5.4). The antibody response in these animals recognised a wide range of antigens and there was no clear banding pattern. Antigens of 16 and 60kDa and a group of antigens from 66-70kDa were the most frequently recognised antigens.

Pre-colostral sera was collected from 2 congenitally infected calves from the same farm in Northern Ireland (figure 5.5) (serum antibody values are given in appendix chapter 5, table 5.5). Sera from these animals recognised NC1 antigens of 19, 20, 65, 68, 74, 78 and 85kDa and three groups of bands at 22-25, 30-33 and 36-38kd. The 15kDa band stained by serum from naturally infected Scottish cattle was not present in these Western blots.

5.3.3 Periodate treatment of *Neospora caninum* NC Liv antigen

Carbohydrate epitopes bound to parasite antigen which may be recognised by the immune response to the parasite were removed from *N.caninum* SDS PAGE separated NC Liv antigen by periodate treatment (figure 5.6) (serum antibody values are given in appendix chapter 5, table 5.5). Four antigens of approximately 60, 65, 66 and 67kDa

were still strongly recognised by sera from experimentally and naturally infected cattle. These antigens were not recognised by animals naturally and experimentally infected with *T.gondii* or experimentally infected with *S.cruzi.*

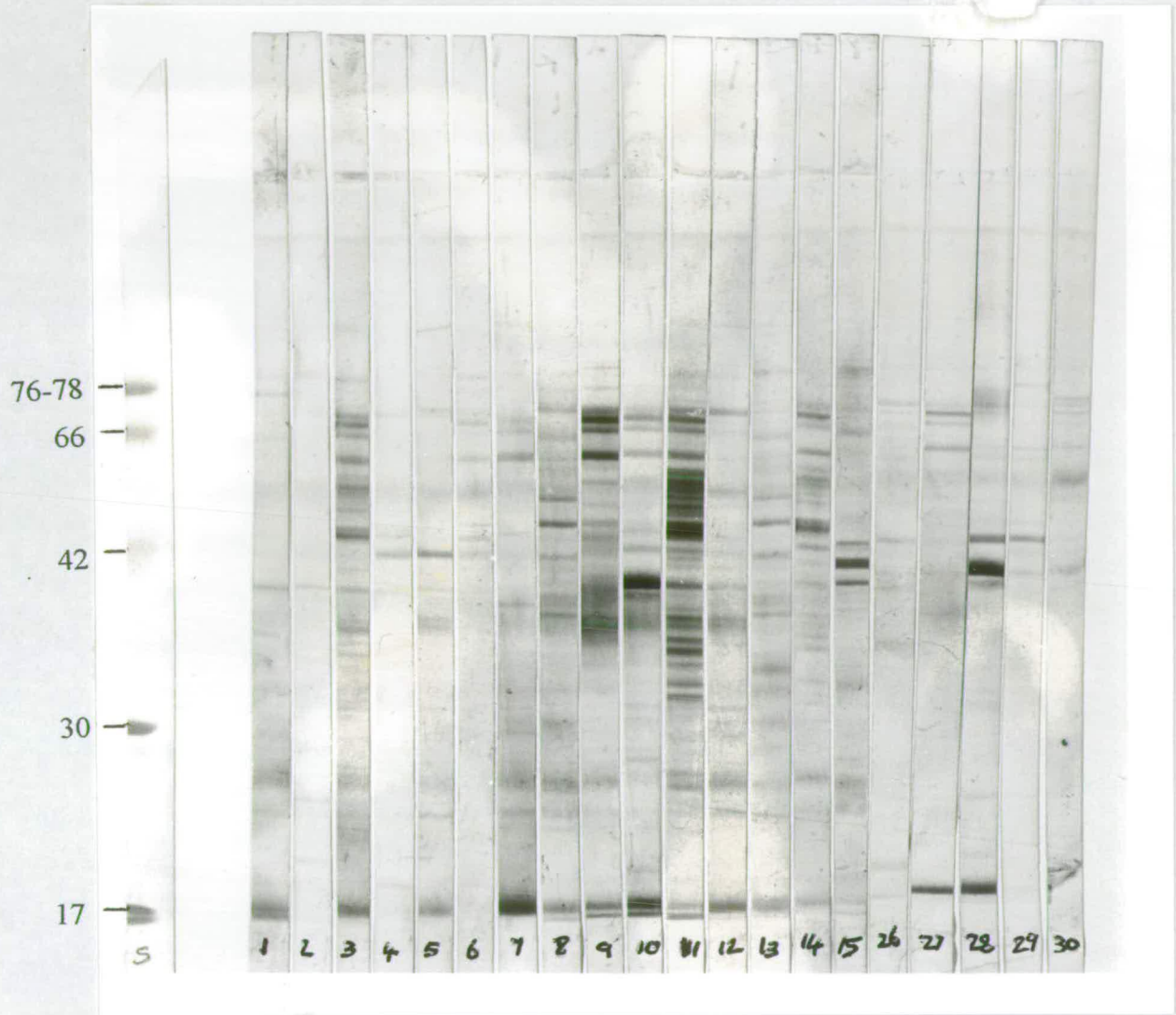


Figure 5.4 *Neospora caninum* NC Liv antigen probed with sera from 30 naturally infected *Neospora* positive cattle (IgG antibody titre >1:512) from Carrowdore, NI. The number on each strip correlates to an animal number, details of which are given in appendix chapter 5, table 5.4. S = standards, MW range 12,300 - 76-78,000kDa, BDH Laboratories Supplies.

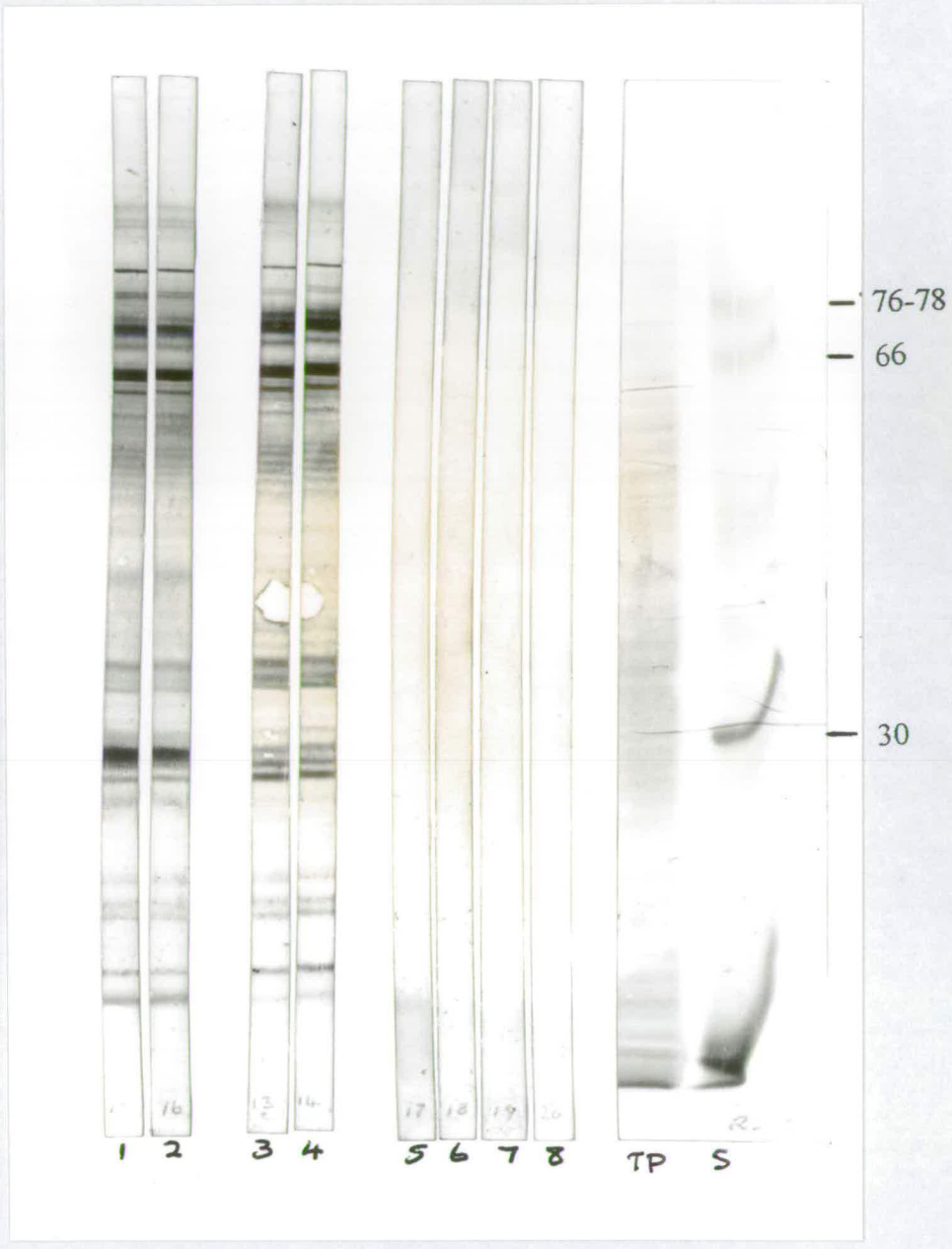


Figure 5.5 *Neospora caninum* NC1 antigen probed with sera from congenitally infected *Neospora* positive calves D (strips 1 & 2) and E (3 & 4) and control calves H (5 & 6) and U (7 & 8). All sera from calves D and E ≥97% OD by *Neospora* ELISA (≥30% OD is considered antibody positive). S = standards, MW range 12,300 - 76-78,000kDa, BDH Laboratories Supplies. TP = total protein stain.

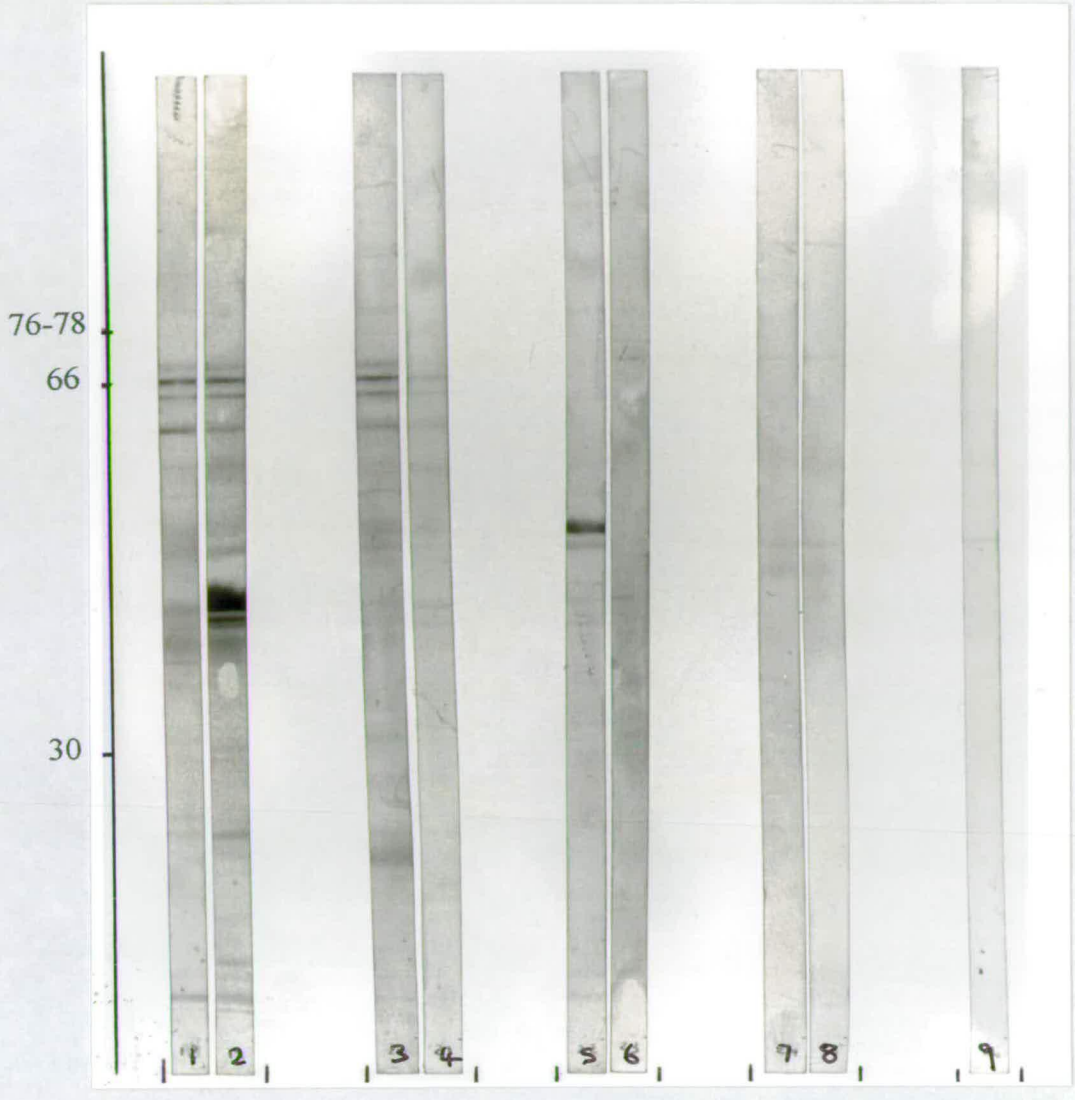


Figure 5.6 Periodate treated *Neospora caninum* NC Liv antigen probed with *Neospora, Toxoplasma* and *Sarcocystis cruzi* positive sera from naturally and experimentally infected animals as used in figures 5.1 and 5.2. Strips 1: sera from natural bovine *Neospora* infection (IgG antibody titre >1:4096). Strip 3: sera from experimental bovine *Neospora* NC1 infection (IgG antibody titre >1:2048). Strip 5: sera from experimental bovine *Toxoplasma* M3 infection. Strip 7: sera from natural ovine *Toxoplasma* infection (IgG antibody titre >1:1024). Strip 9: sera from experimental bovine *Sarcocystis cruzi* infection. Strips 2, 4, 6, 8: antibody negative controls. S = protein standards, MW range 12,300 - 76-78,000kDa, BDH Laboratories Supplies.

5.3.4 Identification of *N.caninum* specific antigens

5.3.4.1 *Toxoplasma gondii* positive sera

There was very little cross reaction of *Toxoplasma* positive sera from naturally or experimentally infected animals with *Neospora* NC Liv (figure 5.1) and NC1 (figure 5.2) antigen. Only a few faint random bands showed up and none corresponded to those recognised by *Neospora* natural or experimental infection sera.

5.3.4.2 *Babesia divergens, Babesia bigemina and Babesia bovis* positive sera

Neospora NC1 antigen was probed with *B.divergens, B.bovis* and *B.bigemina* positive serum from naturally and experimentally infected animals (figure 5.7) (serum antibody values are given in appendix chapter 5, table 5.7). Only one *Neospora* antigen of approximately 18kDa cross reacted with 2 samples of *B.bovis* and *B.bigemina* positive serum. A single band of antigen of the same weight was also consistently recognised by serum from sheep experimentally infected with *S.tenella*.

5.3.4.3 *Cryptosporidium parvum* positive sera

No *N.caninum* SDS soluble antigens cross reacted with sera from cattle experimentally infected with *C.parvum* (figure 5.8) (serum antibody values are given in appendix chapter 5, table 5.8).

5.3.4.4 *Sarcocystis tenella* positive sera

Only one *N.caninum* antigen of approximately 18kDa was recognised by *S.tenella* positive ovine sera (figure 5.9) (serum antibody values are given in appendix chapter 5, table 5.9). *N.caninum* antibody positive serum samples from sheep experimentally infected with NC1 strain tachyzoites also recognised this antigen.

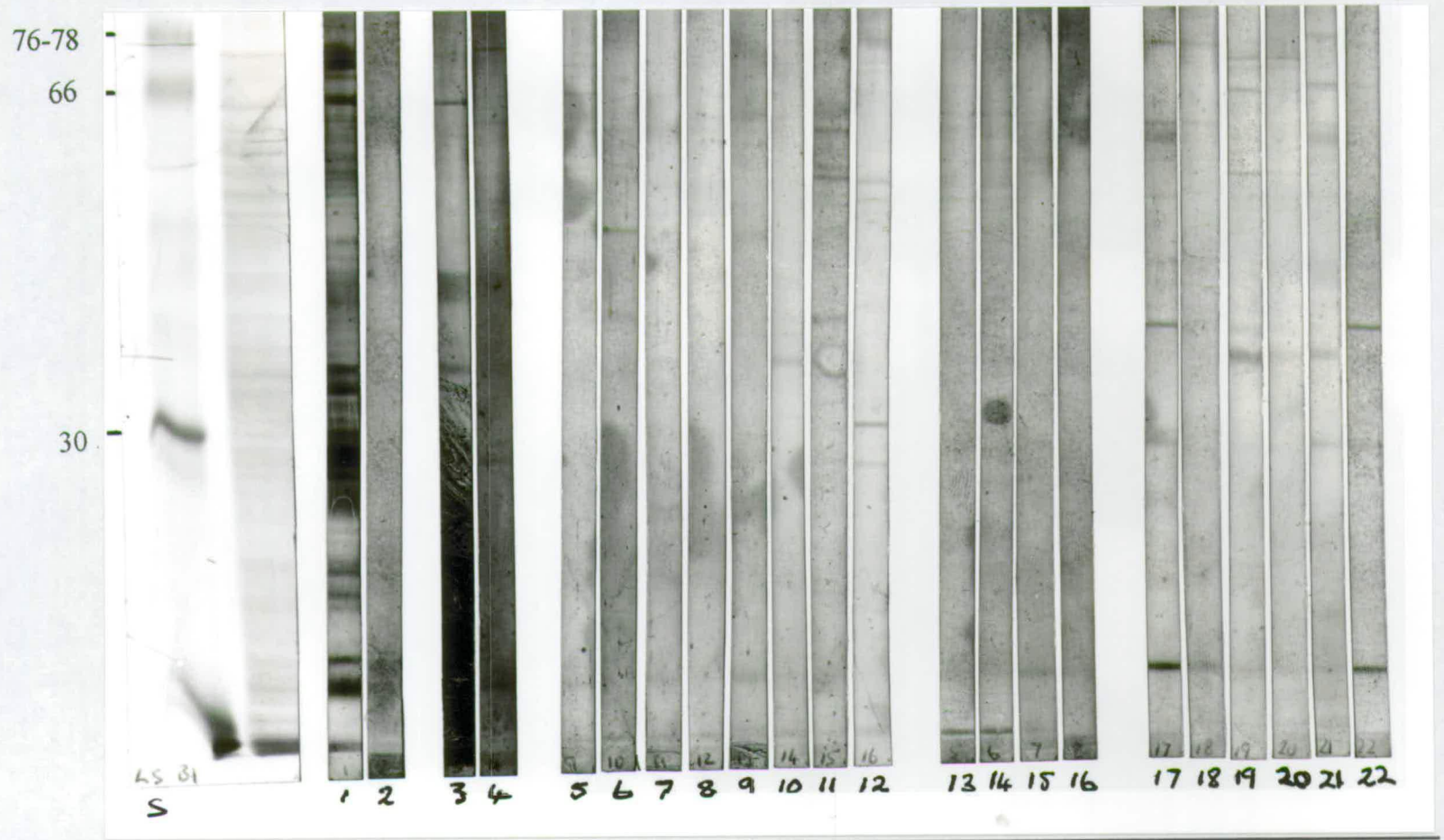


Figure 5.7 *Neospora caninum* NC1 antigen probed with *Babesia divergens* (strips 3-12), *B.bovis* (strips 13-16) and *B.bigemina* (strips 17-22) positive bovine sera collected pre (strips 6, 8, 10, 12, 14, 16, 18, 20) and post (strips 5, 7, 9, 11, 13, 15, 17, 19) experimental infection and after natural infection (strips 3, 4, 21, 22). Positive control: *Neospora* natural infection, foetal sample (scottish survey) (strip 1), IgG 1:16,384. Negative control (strip 2): pre infection bovine serum. S = protein standards, MW range 12,300 - 76-78,000kDa.

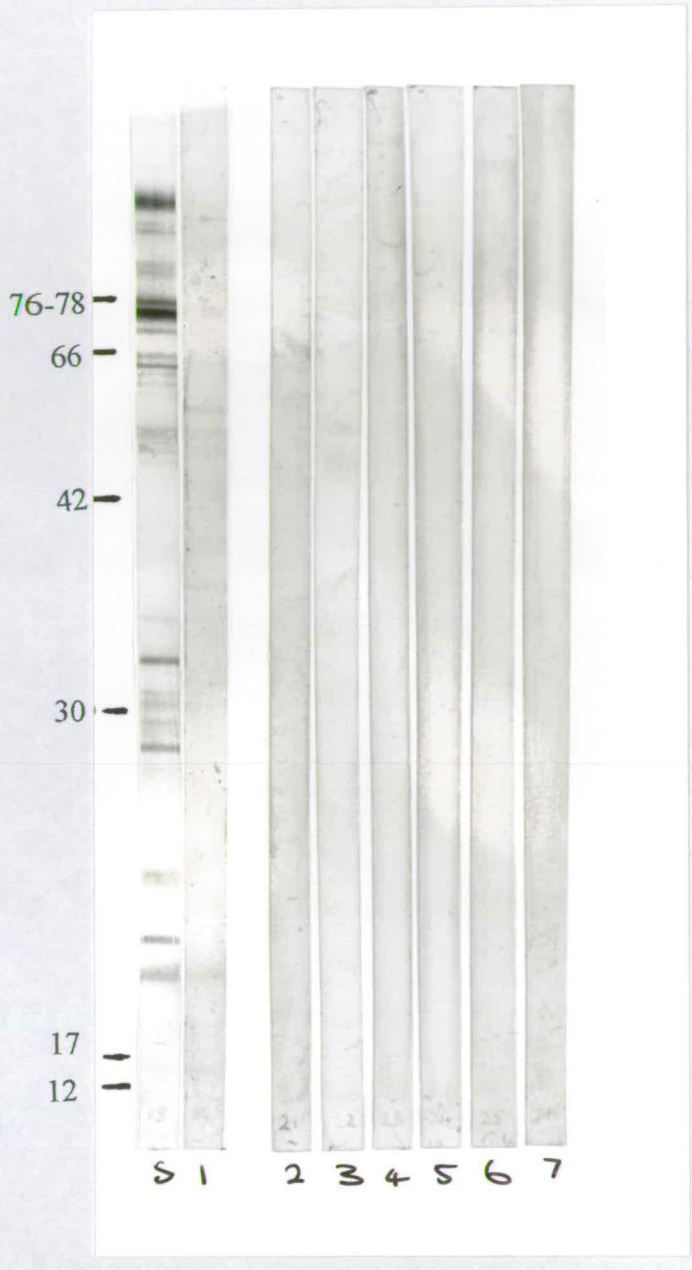


Figure 5.8 *Neospora caninum* NC1 antigen probed with *Cryptosporidium parvum* positive bovine sera collected 3 weeks post infection from calves 2 (strips 3 & 4), 3 (5 & 6) and 4 (7 & 8). All samples are positive at IgM >1:640. Positive control: (strip 1) NC1 bovine experimental infection, IgG 1:4096. Negative control: (strip 2) pre infection sera from the same cow. S = protein standards, MW range 12,300 - 76-78,000kDa, BDH Laboratories Supplies.

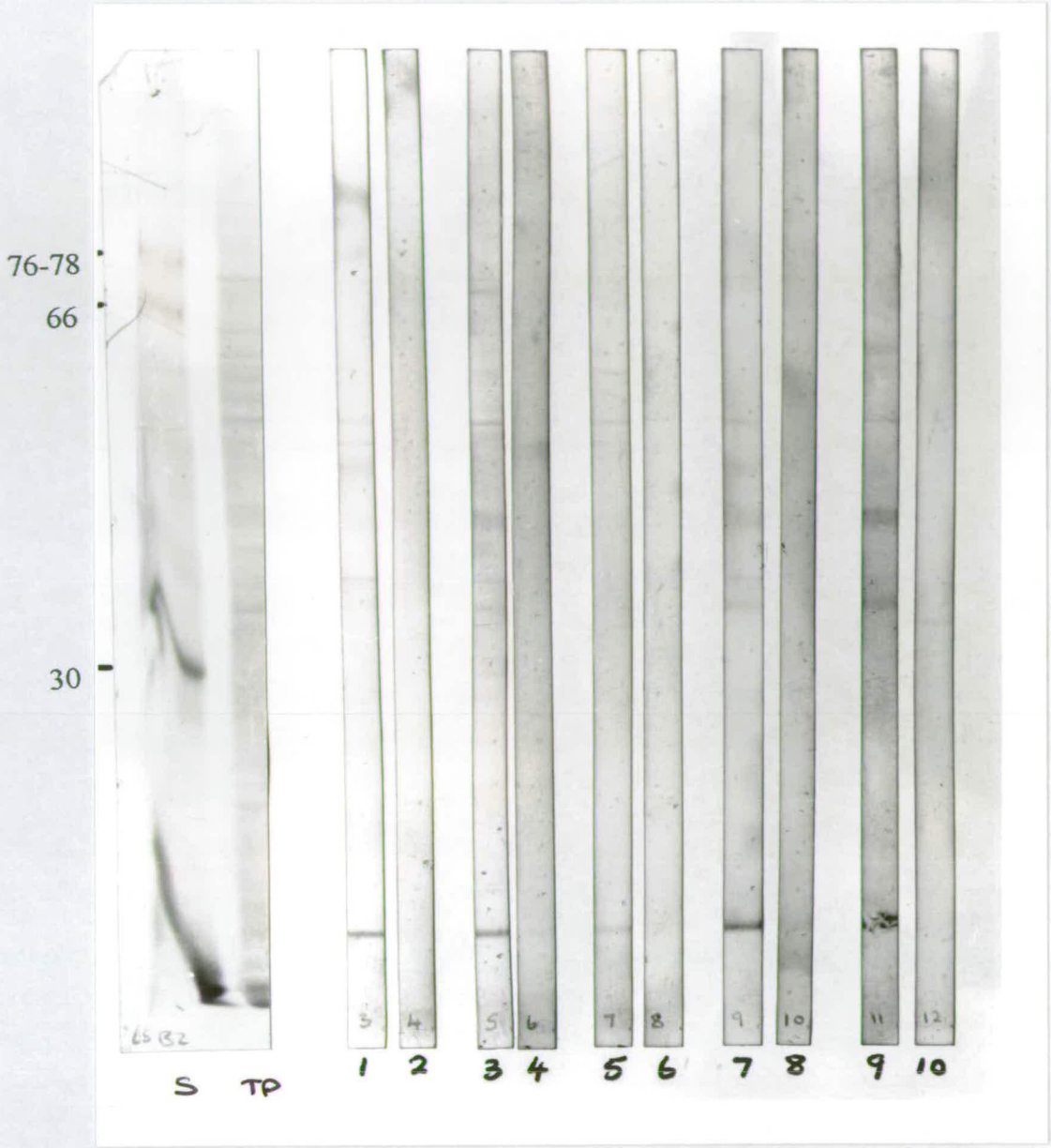


Figure 5.9 *Neospora caninum* NC1 antigen probed with *Sarcocystis tenella* positive sera collected pre (strips 2, 4, 6, 8 and 10) and post (strips 1, 3, 5, 7 and 9) infection from 5 sheep. All pre infection sera were negative, and all post infection sera were positive for IgG by IFAT at 1:81 920. Positive control: strip 11, ovine NC1 experimental infection, IgG 1:2048. Negative control: strip 12, pre infection sera from same animal. S = protein standards, MW range 12,300 - 76-78,000kDa, BDH Laboratories Supplies. TP = total protein stain.

5.4 Discussion

Development of an ELISA using *Neospora* specific antigens may lead to a more accurate diagnostic test with increased sensitivity and specificity as any serological assay with whole *Neospora* tachyzoites as a source of antigen has the potential to cross react with highly conserved antigens from closely related organisms. Several *Neospora* specific antigens were identified in this study which did not cross react with serum from animals strongly antibody positive for *Sarcocystis, Babesia, Toxoplasma* and *Cryptosporidium* (some naturally infected) and were generally consistently recognised by animals naturally and experimentally infected with *N.caninum*. SDS is a detergent and is likely to contain a combination of antigens present in the surface membrane of the parasite. A group of antigens of approximately 65-70kDa which are present in SDS soluble antigen preparations made from tachyzoites of both NC1 and NC Liv isolates were recognised by maternal and foetal sera collected from the 8 veterinary investigation centers around Scotland, by sera from cattle experimentally infected with *Neospora* and from naturally infected cattle on the case study farm in Carrowdore, NI. In general very little cross reaction with serum antibody positive for the closely related coccidian parasites was identified in this study. One antigen at 18kDa was however recognised by both *Babesia* and *Sarcocystis* positive sera, *Babesia* positive sera also recognised an antigen at the base of the blot, and *T.gondii* positive sera did faintly recognise some bands. The possibility for some cross reaction does therefore exist.

Interestingly, although the two congenitally infected animals recognised the same antigen bands the intensity of staining at each band, which indicates the concentration of antibody, varied greatly between the two animals. Sensitivity of an antigen fraction ELISA could be greatly affected by this variation in intensity of response, and should be taken into account in the event of development of a test of this kind.

Another antigen band at approximately 15kDa present in both NC1 and NC Liv

isolates was also recognised by sera from naturally infected cattle in Ireland and Scotland (maternal and foetal) and experimentally infected cattle, but was not recognised by antibody positive sera from congenitally infected calves on the study farm in Ireland. It is possible that infection on this farm may be caused by several strains of the parasite and that the change in antibody profiles may be due to parasite strain variation. Evidence for differences in phenotype possibly reflecting differences in genotype can be seen when comparing the NC Liv and NC1 isolate blots in this study which were probed with the same serum samples (figures 5.1 and 5.2). *Neospora* NC1 antigen appears to have a higher number of faintly stained minor antigen bands, particularly in the region between the 42 and 66kDa protein markers. Conversely a band at approximately 60kDa is recognised on the NC Liv blot only. Comparison of NC Liv and NC1 isolates using rabbit anti-*N.caninum* polyclonal sera (Barber *et al*, 1995) also identified some minor differences in antigenic profile with only one antigen at 28kDa uniquely detected by anti NC Liv antiserum. This suggests that the range of antigens expressed in different isolates varies, possibly caused by prolonged growth in tissue culture, or that the isolates may be comprised of different parasite strains. Until the isolates currently in tissue culture are typed and characterised a comparison of studies identifying immunodominant antigens will be difficult. Standardisation of antigens is important if we are to identify which ones are potentially the most useful for diagnosis and can be used for the development of better diagnostic tests.

Previous studies have identified a range of immunodominant *Neospora* antigens but few have been directly compared with antigen recognition by antibody positive sera from animals infected with closely related coccidian parasites. The predominant antigens recognised by *Neospora* positive dog and rabbit serum in ELISA tests using extracted *Neospora* NC1 tachyzoite proteins incorporated into immunostimulating complexes (iscoms) (Bjorkman *et al*, 1994) were approximately 30-45 and 17-19kDa. Iscoms are composed of parasite surface proteins bound to an adjuvant and the absence of internal antigens in these preparations may explain the difference in range of antigens recognised in this study. Another study by Paré *et al*, 1995, analysed the immune response of a naturally infected cow which aborted a *Neospora* infected foetus to the water soluble

fraction of NC1 and bovine BPA1 antigen. Very few antigens were recognised under reducing conditions and all were below 45kDa. There are many differences between these studies including species used for production of antibody positive sera, antigen preparation and parasite strains used to produce antigen for inoculation and detection. These differences make direct comparisons with this study difficult.

A study by Baszler *et al*, (1996) identified several *Neospora* NC1 immunodominant antigens of approximately 116, 65 and 25kDa which were recognised by naturally infected cattle, congenitally infected calves and fluids from aborted foetuses collected by Washington State diagnostic laboratory (antigen was also reduced and separated by SDS Page). A monoclonal antibody raised against *N.caninum* NC1 tachyzoite antigen, 4A4-2, was found to bind to a carbohydrate epitope bound to the 65kDa NC1 antigen successfully blocking antibody from naturally infected cattle binding to the 65kDa antigen in a competitive inhibition ELISA. In this study recognition of the 65kDa antigen of the NC Liv strain by a wide range of naturally and experimentally infected cattle was not inhibited by periodate treatment to remove carbohydrate epitopes. This difference and the recognition of two other protein bands not present on blots used in this study may be a result of antigenic differences between strains as previously discussed. Further work needs to be done to characterise this important antigen.

This study clearly shows that antigenic differences exist between isolates of *N.caninum,* both between NC1 and NC Liv used in this study, and also shown in the difference in results shown here when compared to studies done previously by Baszler *et al* (1996), Barta and Dubey (1992), and Bjerkas *et al* (1994). This study identified antigens present in an SDS soluble preparation of antigen. As seen with the antigen recognition profile of the congenitally infected calves strength of recognition of individual antigens also varies between individual animals which could affect diagnosis. This supports previous work by Lally *et al* (1996) which showed that an ELISA based on one recombinant antigen could not diagnose infection in two animals from the same herd which had previously been tested and found sero-positive for *N.caninum* by IFAT. Also, false negatives may arise due to genetic restriction of cattle in recognition of

specific antigens. It is likely therefore that development of a diagnostic test which could be used worldwide may need to use a combination of proteins to overcome the possibility of false negatives due to strain variation

It is possible that stage specific antigens such as those expressed by the bradyzoite stage of parasite development could be more useful for diagnosis of chronically infected animals as they may be more likely to be exposed to bradyzoite antigen periodically. Previous studies have shown that bradyzoites can be periodically released from *T.gondii* tissue cysts into the bloodstream maintaining a high serum antibody level (Wong and Remington, 1993). Very little work has been published on *Neospora* bradyzoite specific antigens probably due to difficulties in obtaining large enough quantities of antigen. Another possibility for the development of an effective diagnostic test could be based on an antibody avidity assay, previously developed for *T.gondii,* which measures the strength of the antigen-antibody bond which weakens over time. As the outcome of infection in toxoplasmosis can depend on when infection occurred, with heaviest losses occurring after primary infection during the first trimester of pregnancy, this test can give an indication of the likely effects of a high antibody titre during pregnancy. Further studies to examine the effects of infection with *N.caninum* during each trimester in pregnancy are also needed before we can interpret maternal antibody titres.

Lally *et al* (1996) showed that the development of ELISA tests based on recombinant antigens could provide a more specific and sensitive diagnostic test for neosporosis but that a combination of antigens could increase sensitivity. This study shows that the NC1 and NC Liv antigens between 65-70kDa are likely candidates for use in such a test and that the likelihood of cross reaction of these antigens with sera antibody positive for closely related coccidian parasites is low. Differences in intensity of humoral immune response in individual animals naturally infected with *N.caninum* shown here also support the case for the combination of several antigen fractions. Clearly further work on the identification of other stage specific antigens could greatly increase the specificity and sensitivity of such a test.

Acknowledgements;

The author would like to thank David Graham, Dr.Dirk de Graaf, Dr.David Buxton, Anya Heckeroth, Dr.Stuart Taylor and Dr.Irma Esteban for supplying serum samples.

Chapter 6

Cellular immune response to *Neospora caninum* in experimentally infected cattle

Aims

- To monitor cell proliferation responses in calves experimentally infected with *Neospora caninum* tachyzoites.

- To assay production of IFNγ by peripheral blood mononuclear cells from experimentally infected animals incubated with *Neospora* antigen *in vitro*.

- To monitor the cell populations in peripheral blood before and during the acute phase of experimental infection with *N.caninum*.

- To describe the humoral immune response to experimental infection with *N.caninum* NC1 strain tachyzoites in calves.

6. Cellular immune response to *Neospora caninum* in experimentally infected cattle

6.1 Introduction

Neospora caninum is an obligate intracellular parasite which can cause abortion in cattle, dogs, goats and horses. Very little is known about the host immune response to *N.caninum* and the precise cause of abortion is unknown. *Neospora* is closely related to *Toxoplasma gondii* which can cause abortion in sheep. As a consequence of infection with *Toxoplasma* the host develops life long immunity which protects against subsequent challenge. Cell mediated responses have previously been shown to be important in the development of this protective immunity (Suzuki and Remington, 1988; Parker *et al*, 1991; Innes *et al,* 1995b). In contrast infection with *Neospora* does not always confer protection against clinical neosporosis as a number of cases of repeat abortion in cattle have been reported (Anderson *et al*, 1995; Moen *et al*, 1995). The rate of repeat abortion is estimated at <5%, therefore it is likely that many animals do develop some form of protective immunity.

Macrophages are phagocytic cells which play an important role in innate and adaptive immunity. They act as scavengers engulfing and destroying blood borne extracellular microorganisms and can also be targeted by the adaptive immune response to the site of an infection. Chronically infected macrophages are activated by the T cell cytokine IFNγ, secreted by activated Th1 cells, to kill invading intracellular pathogens. IFNγ is therefore a major activator of macrophages (Suzuki and Remington, 1990), and is important for protection against intracellular parasites. This cytokine, produced by CD4+ and CD8+ T cells and natural killer (NK) cells, has been shown to suppress the

growth of S48 strain *Toxoplasma gondii* (Oura *et* al, 1993) and NC1 strain *N.caninum* tachyzoites (Innes *et al*, 1995a) cultured in fibroblasts *in vitro* and is one of the main mediators of resistance to *Toxoplasma* in sheep (Innes *et al*, 1995c). Khan *et al* (1997) has also shown that *in vivo* depletion of IFNγ in inbred A/J mice, which under normal conditions develop no clinical symptoms when infected with *N.caninum,* renders the mice suseptible to infection. This evidence suggests that the cell mediated immune response to *N.caninum* in cattle may be important in protection against clinical neosporosis.

Neospora and *Toxoplasma* are closely related but antigenically distinct (Bjerkas *et al*, 1984) although clearly the two parasites share some common antigens. The implications of this for diagnosis, cell mediated immune response and possible cross protection is unknown.

In the wider context of the work carried out at Moredun Research Institute the long term aim of investigations into the cellular immune response to *N.caninum* is to examine the feasibility of developing a vaccine against neosporosis. This depends on the identification of relevant protective immune responses and of the antigens responsible for inducing these responses. As repeat abortion due to neosporosis occurs in <5% of cases it is possible that the immune response to the parasite in a majority of infected cattle may be protective against abortion. Clearly there is no evidence from the field that animals develop protection against infection as the rate of congenital transmission is high at approximately 80% of sero positive cattle (Paré *et al*, 1996).

The aim of this study is to examine cell proliferation, production of IFNγ and humoral immune response in cattle experimentally infected with *N.caninum* NC1 strain tachyzoites cultured *in vitro*. Peripheral blood mononuclear cells were tested for their ability to respond to *Neospora* and *Toxoplasma* water soluble fraction (wsf) antigen and supernatants from these cells were analysed for IFNγ production. The cellular composition of peripheral blood was also analysed during acute infection.

6.2 Materials and methods

6.2.1 Calves

Six calves approximately 6 months old which were seronegative for *N.caninum* and *T.gondii* by indirect fluorescent antibody test (IFAT) were used in this study. Antibody titres measured by IFAT of <1:512 for *Neospora* and <1:256 for *Toxoplasma* (Esteban Redondo, 1997) were considered negative. Calves were monitored for any sign of clinical disease and rectal temperatures were recorded daily from the day before inoculation until 14 days after inoculation. Control and infected calves were penned separately throughout the experiment.

6.2.2 Parasites

6.2.2.1 *Neospora caninum*

Neospora caninum NC1 strain tachyzoites were cultured within VERO cell monolayers in IMDM supplemented with 2% horse serum, as described in chapter 3, section 3.2.1.

6.2.2.2 *Toxoplasma gondii*

Toxoplasma gondii S48 tachyzoites were also cultured within VERO cell monolayers in IMDM supplemented with 2% horse serum as described in chapter 3, section 3.2.3.

6.2.3 Experimental design and inoculum

Neospora caninum NC1 strain tachyzoites were grown in IMDM supplemented with 2% horse serum, as described in chapter 3 section 3.2.1, and harvested by scraping

the VERO cell monolayers containing parasites off the tissue culture flasks. Parasite and cells were then centrifuged at 500*g* for 10 mins and resuspended at a concentration of $1.25x10^8$ tachyzoites per ml in IMDM containing 2% horse serum, 50U/ml penicillin and 50μg/ml streptomycin. The inoculum was grown and prepared immediately before inoculation and a record was made of the number of VERO cells injected with the parasite.

The calves were allocated into 2 groups (table 6.1). Suspensions containing $2.5x10^8$ NC1 tachyzoites were administered to the animals in group 1 by sub cutaneous injection in the left hind quarter. Control animals (group 2) received the same number of VERO cells administered to the animals in group 1 also by sc injection in the left hind quarter.

Table 6.1 Experimental design.

Group	Status	Animal Numbers	Sex	NC1 Tachyzoites	VERO Cells
1	Infected	13	Female	$2.5x10^8$	$6.5x10^6$
		14	Female	$2.5x10^8$	$6.5x10^6$
		20	Female	$2.5x10^8$	$6.5x10^6$
		21	Female	$2.5x10^8$	$6.5x10^6$
2	Control	15	Female	0	$6.5x10^6$
		25	Male	0	$6.5x10^6$

6.2.4 Serology

Blood samples were collected from the jugular vein into preservative free evacuated blood collection tubes and allowed to clot. Following retraction of the clot and centrifugation at 500*g* serum was removed and stored at -20°C until required.

N.caninum and *T.gondii* antibody titres were then analysed by ELISA and IFAT as described in chapter 3, sections 3.5.1 and 3.5.2.

6.2.5 Collection of samples

Blood samples were collected for preparation of PBM cells and serum from the 4 infected calves on day -3, -2 and -1 prior to inoculation and then every second day from day 2 to day 20 post inoculation. Blood samples were collected on three occasions from the two control calves.

6.2.6 Isolation of peripheral blood mononuclear cells

Blood was collected from the jugular vein into preservative free heparinised evacuated tubes containing 10U/ml heparin. Peripheral blood mononuclear cells were then separated from whole blood over Lymphoprep (Nycomed, Robbins Scientific, Solihull, UK) as described in chapter 3, section 3.7.1.1.

6.2.7 Production of water soluble fraction antigen

Neospora caninum and *Toxoplasma gondii* wsf antigen was produced as described in chapter 3, section 3.4.2.

6.2.8 Lymphocyte proliferation assay

Peripheral blood mononuclear cells separated from whole blood were resuspended in IMDM supplemented with 10% foetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin at a concentration of $2x10^6$ cells/ml. Cells were cultured with 10μg/ml of *N.caninum* and *T.gondii* wsf antigen, 10μg/ml concanavalin A (Con A) and in medium alone in 96 well round bottomed microtitre plates at a concentration of $2x10^5$ cells/well (see methods chapter 3, section 3.7.1). A final concentration of 10μg/ml

of *N.caninum* antigen was selected for use in proliferation assays as higher concentrations of antigen (up to 20μg/ml) did not significantly increase proliferation. Each concentration of antigen was set up in quadruplicate. After 96 h incubation at 37°C and 5% CO_2, cells were pulsed with 18.5 kBq [H^3] thymidine (Amersham, Little Chalfont, UK) for a further 18 h before harvesting onto fibreglass filters (Canberra Packard, Pangbourne, UK). Cell associated radioactivity was quantified in a gas proportional counter (Canberra Packard, Pangbourne, UK).

The proliferative response of PBM cells from infected and control calves to VERO cell wsf antigen was measured in a similar experiment outlined in chapter 7.

6.2.9 IFNγ analysis

Supernatant was collected from PBM cells cultured in medium alone, with con A or with *Neospora* or *Toxoplasma* wsf antigens at a final concentration of 10μg/ml as described above. Each culture was set up with 24 replicates and after 96hrs supernatant was collected, pooled and stored at -20°C until analysed for IFN activity. The presence of biologically active interferons present in supernatant was determined using the method described by Entrican *et al*, (1992), and described in chapter 3, section 3.7.3.2. In this assay samples were tested for their ability to inhibit the replication of Semliki Forest virus (SFV) in fibroblasts and measured as the cytpathic effect of the virus (CPE). A sample was considered negative for interferon if the fibroblast cells rounded up due to viral replication, and cells were protected if biologically active interferon was present.

6.2.10 Phenotypic analysis of PBM cells

Peripheral blood mononuclear cells collected before inoculation and every 2 days until day 20 post infection was analysed using a panel of monoclonal antibodies (table 6.2). The method used to stain the cells was carried out as described by Innes *et al*, 1995b, and also described in detail in chapter 3 section 3.7.2. A total of 5,000 cells from each sample of PBMC were counted. Cells from each sample were labelled with FITC

alone (Dako, Glostrup, Denmark) as a control to determine background staining with the flourescent label.

Table 6.2 Antibodies used in FACS analysis of peripheral blood mononuclear cells during acute infection. (IAH, Institute of Animal Health; ILRI, International Livestock Research Institute; MRI, Moredun Research Institute).

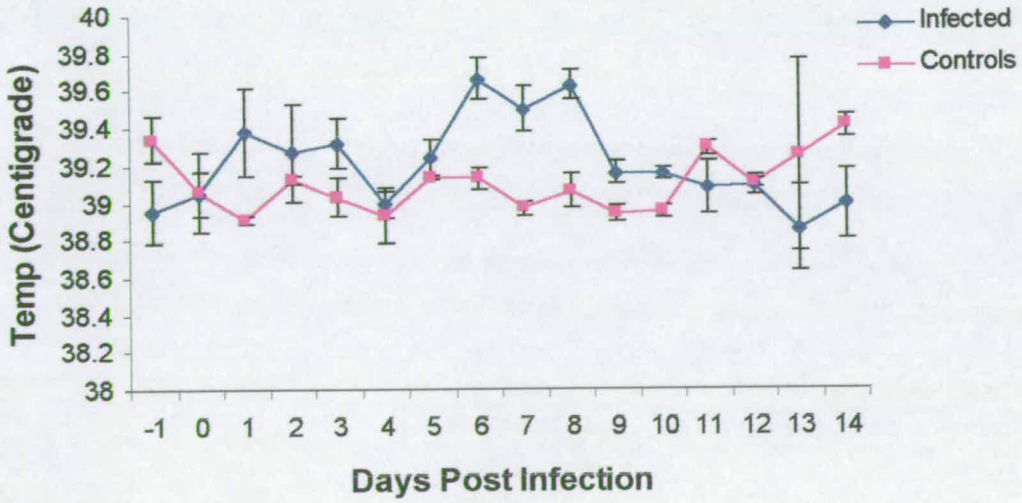
Antibody	Specificity	Location	Source
None	Control	Background staining with FITC	
CC42	CD2	T Cells, thymocytes, natural killer cells	IAH, Compton, UK
CC8	CD4	"Helper" T Cells Subset	IAH, Compton, UK
CC63	CD8	"Cytotoxic" T Cells Subset	IAH, Compton, UK
CC15	T19 (γδ)	γδ T Cell	IAH, Compton, UK
VPM30		B Cell	Dr.J.Hopkins, Dick Vet College, Edinburgh University
CC21	CD21	Mature B Cell	IAH, Compton, UK
ILA111	IL2 Receptor	T Cell	ILRI, Kenya
CC76A	CD45 RB	Resting and activated T cells, B Cells, monocytes, macrophages and granulocytes	IAH, Compton, UK
ILA24		Monocyte/Macrophage	ILRI, Kenya
MHCII	Class II	B Cells, monocytes, macrophages and Activated T Cells	D.Deane, MRI

6.3 Results

6.3.1 Rectal Temperatures

Body temperatures of inoculated calves were significantly higher than controls on days 6 and 8 post inoculation ($P<0.01$) when compared with the base line value of 39.1° (figure 6.1). The base line value was calculated as the mean temperature for all the animals prior to inoculation. Temperatures higher than 39.5°C in indicate a febrile response therefore inoculated animals were considered to have shown a febrile response to experimental infection on days 6 and 8 post inoculation. Temperatures in the control calves remained low throughout the experiment. Full details of temperatures of individual animals and P values are given in appendix chapter 6, tables 6.1 and 6.2.

Figure 6.1 Average temperatures of the experimentally infected calves (n=4) pre and post inoculation with *N.caninum* and controls (n=2). Values are based on a mean value for each group, and bars represent the standard error of the mean (SE ± 0.01-0.51)

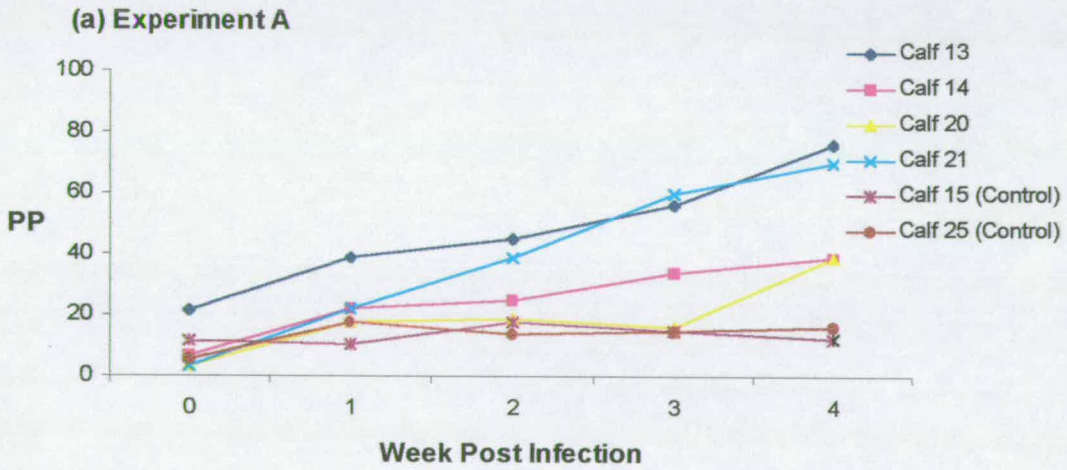


6.3.2 Serology

6.3.2.1 *Neospora caninum* antibody response

Calves were tested for antibody to *N.caninum* by ELISA (figure 6.2). All calves were negative pre-infection. Antibody titres in infected animals showed a significant increase from one week post infection to the end of the period monitored ($P<0.05$). Animals were considered sero-positive when percentage positivity (PP) values were greater than 30% (Osawa *et al*, 1998). Control calves remained negative throughout the experiment. Full details of serum antibody levels and P values for individual animals are given in appendix chapter 6, tables 6.3 and 6.4.

Figure 6.2 *Neospora* antibody levels in serum from experimentally infected calves pre and post inoculation with *N.caninum* measured by ELISA; OD >30% positivity is recognised as seropositive.



6.3.2.2 *Toxoplasma gondii* antibody response

Before inoculation and throughout the experiment all calves were tested by IFAT and ELISA for antibody to *T.gondii.* Serum antibody titres ≥1:256 measured by IFAT were considered sero-positive (Esteban-Redondo, 1997). When tested by IFAT two of the infected animals (13 and 14) showed a one-fold increase in the level of antibody (1:256 to 1:512 and 1:64 to 1:128 respectively). One of the control animals (25) showed a one fold increase (1:128 to 1:256).

Animals were also tested for specific *T.gondii* antibody by ELISA (recently developed at MRI by Claire Lamb). A minimal increase in percentage positive (PP) values was detected in the 2 infected calves and one control calf (25) which had increased *T.gondii* antibody titres measured by IFAT (table 6.3).

Table 6.3 *Toxoplasma gondii* titres measured by IFAT and ELISA (%PP) in calves pre-infection and week 3 post infection.

		IFAT		ELISA	
Animal No.	**NC1 Infection Status**	**Pre-Infection**	**Post-Infection**	**Pre-Infection**	**Post-Infection**
13	Infected	1:256	1:512	18.8	35.7
14	Infected	1:512	1:512	18.3	16.1
20	Infected	1:64	1:128	9.4	6.7
21	Infected	1:128	1:128	6.7	8.5
15	Control	1:256	1:256	9.4	6.7
25	Control	1:128	1:256	7.1	12.1

6.3.3 Cell proliferation

Figure 6.3 and 6.4 illustrate the proliferative response of PBM cells from all four infected animals at 2 day intervals to *N.caninum* and *T.gondii* antigen and to the non specific mitogen Con A until day 20 pi. The background proliferative response to medium control varied between animals therefore to allow comparison of the magnitude of response to parasite antigen values are presented as stimulation index (SI) (CPM values are presented in appendix chapter 6, table 6.5). Peripheral blood mononuclear cells taken from the animals before inoculation did not proliferate when cultured with NC1 or S48 antigen *in vitro* except for calf 20 (figure 6.3, graph c) which did respond to S48 antigen on day –3 and calf 13 (figure 6.3, graph c) which responded to S48 *T.gondii* antigen on day –3 and -2, although by day 2 post infection the response had dropped back to negative. An increase in proliferation of PBM cells to both *Neospora* and *Toxoplasma* wsf antigen (final concentration of 10μg/ml) is first seen around day 6-8 post-inoculation when compared with pre-inoculation values. This response remained high throughout the course of the experiment (for 20 days pi). However high post infection SI values for animals 14 and 20 are a result of low background proliferation and are not due to exceptionally high proliferative responses of these animals to parasite antigen. The proliferative responses of PBM cells from the 4 infected animals to the non-specific mitogen Con A are shown in figure 6.4. The response to Con A in all calves fluctuated both pre and post infection.

Figure 6.5 shown that cells from control animals did not proliferate in response to NC1 or S48 wsf antigen. The non-specific mitogen Con A at a final concentration of 5μg/ml strongly stimulated a proliferative response in cells from control and infected animals.

The bovine proliferative response to VERO cell wsf antigen was not tested in this experiment but was tested in a parallel experiment explained in chapter 7 (experiment B). Inoculation of control and infected calves with $2.3x10^7$ VERO cells (almost twice the dose given to calves in the experiment described in this chapter) did not induce proliferation of PBM to VERO cell wsf antigen in any of the animals.

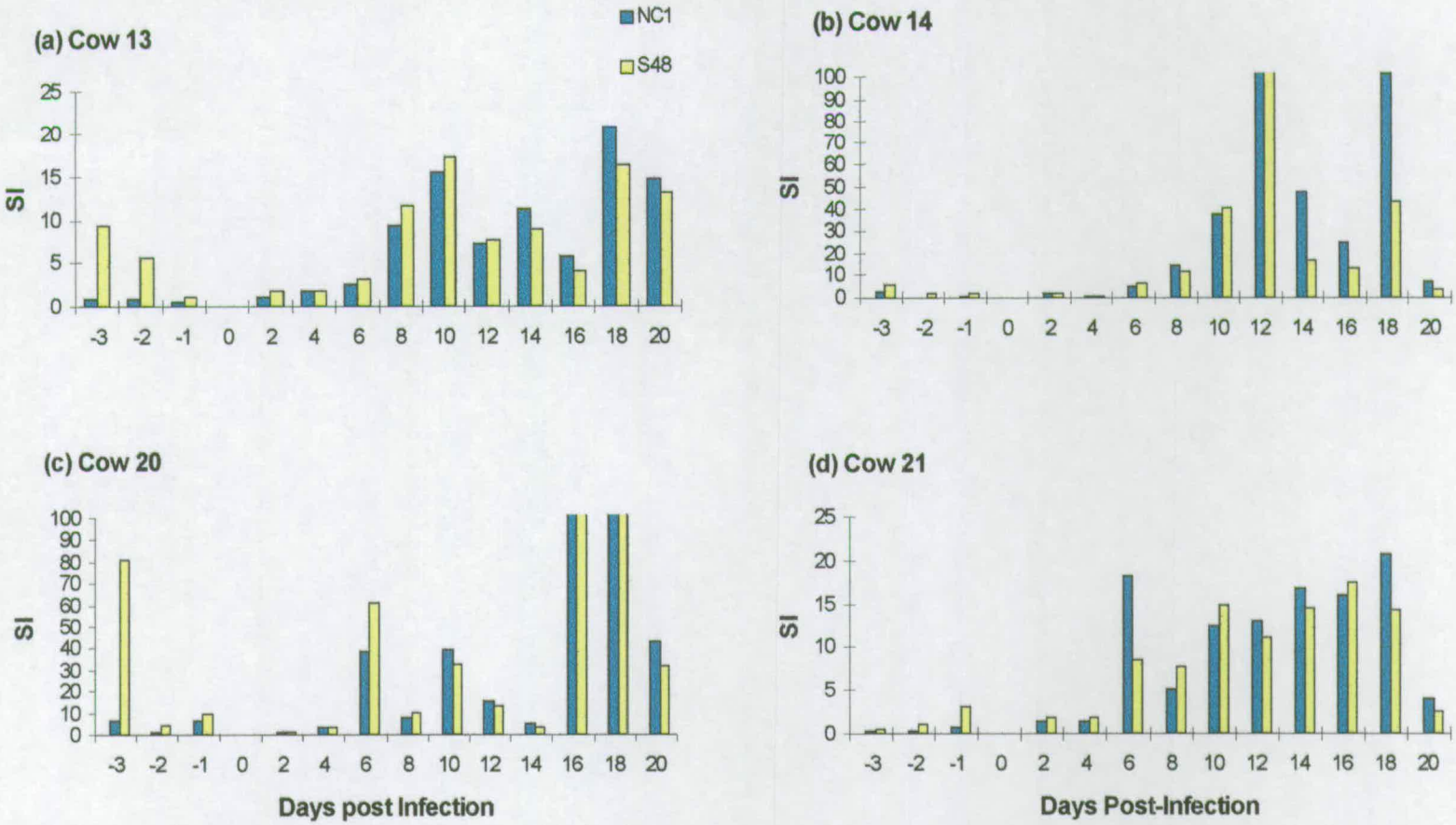


Figure 6.3 Proliferative responses to *Neospora* NC1 and *Toxoplasma* S48 wsf antigen of PBM from 4 calves (a-d) experimentally infected with *Neospora caninum* tachyzoites. Calves were inoculated subcutaneously with $2.5x10^8$ NC1 strain *N.caninum* tachyzoites on day 0. Cell growth was measured pre-infection and at 2 day intervals post infection and is represented above as stimulation index (SI units=mean CPM cells stimulated by antigen/mean CPM medium control, samples tested in quadruplicate (n=4)).

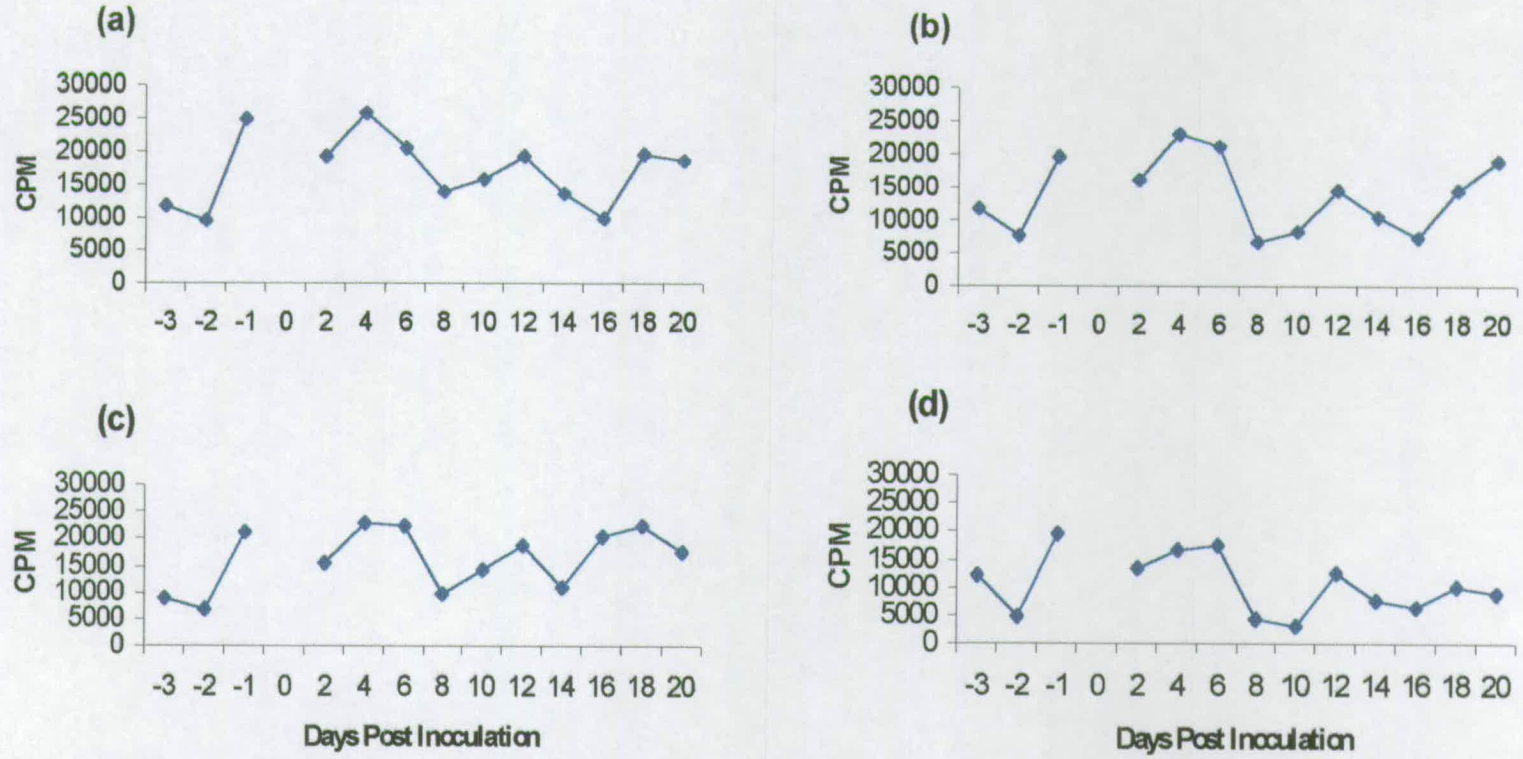
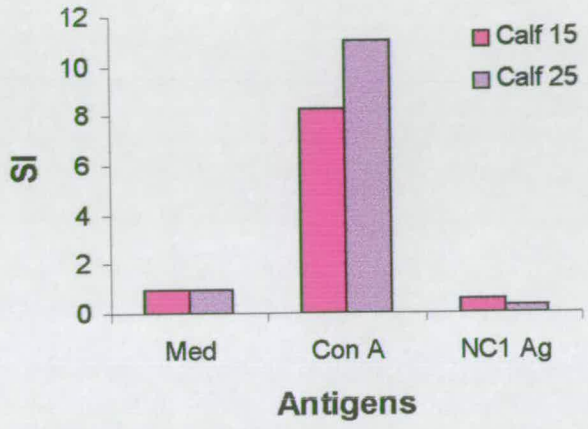


Figure 6.4 Proliferative responses of PBM from the 4 experimentally infected calves, (a) 13, (b) 14, (c) 20 and (d) 21, to the non-specific mitogen Con A. Cell growth was measured in counts per minute (CPM) of ^{3}H thymidine selectively incorporated into proliferating cells (n=4).

Figure 6.5 Proliferative responses of PBM from 2 control calves (15 and 25). Calves were inoculated with the same number of VERO cells as the infected calves received ($6.5x10^6$). Cell growth was measured in CPM as before and represented below as SI values (n=4).



6.3.4 IFNγ production

The concentration of biologically active IFNγ in supernatant was calculated by inhibition assay using anti-IFNγ monoclonal antibodies. High levels of IFNγ were detected in supernatants from cells incubated *in vitro* with *N.caninum* and *T.gondii* antigen from infected calves which were collected from day 8 pi (figure 6.6) (appendix chapter 6, table 6). IFNγ was detected in pre-infection cultures of cells from calves 20 and 21 incubated with *N.caninum* and *T.gondii* antigen although in both animals PBM cells incubated with antigen 2 days post infection produced negligible amounts of this cytokine. When results for calf 21 were excluded IFNγ levels in supernatants on days 10, 12 and 14 pi from PBM cultured with *N.caninum,* and on days 8, 10 and 14 pi from PBM cultured with *T.gondii* were significantly higher than pre inoculation levels ($P<0.05$) (appendix chapter 6, tables 6.7a and 6.7b). Occasional anti-viral activity remained after neutralisation of IFNγ indicating the presence of other antiviral factors. Cells from the two control calves incubated with *N.caninum* and *T.gondii* antigens did not produce any detectable interferons.

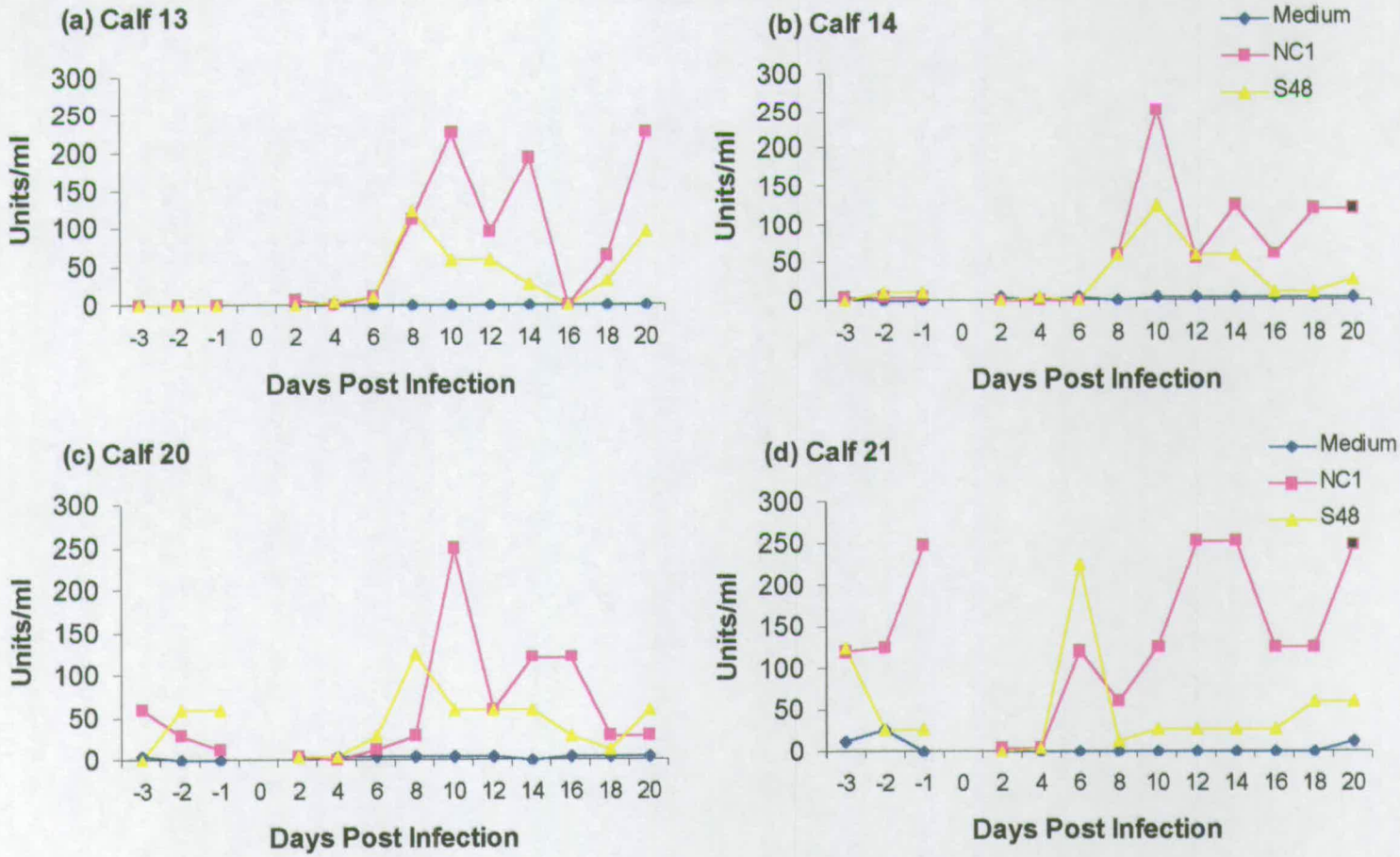


Figure 6.6 Active IFNγ produced by PBM from *Neospora* infected calves in response to *Neospora* and *Toxoplasma* wsf antigen in vitro. The concentration of active IFNγ (units per ml) was measured using a bioassay.

6.3.5 FACS analysis

Figure 6.7 shows a FACS profile of PBMC from calf 20 collected on day 14 post infection with *N.caninum*. Gates shown on the profile delineate the cell population analysed. Figure 6.8 illustrates the mean change in percentage (relative to base line values) of peripheral blood mononuclear cells of CD4+ (CC8) and CD8+ (CC42) T cell subsets and the IL2 receptor (ILA111) following sub cutaneous infection with *N.caninum* NC1 tachyzoites. From day 12 post infection the percentage of cells recognised by Mab ILA111 which binds to the IL2 receptor increased from 10% below base line to approximately 30% above (figure 6.7). The percentage of cells expressing IL2R remained high for the duration of the experiment (18 days pi). At the same time point the number of cells expressing the CD2 cell surface cluster decreased dramatically from approximately 10% above base line to 30% below and remained low.

Minor and transient changes were seen around day 12-14 pi with an increase in the number of CD4+ and CD8+ cells. In both cases the relative percentage of stained cells dropped to previous levels by day 16 pi. Consistently few cells stained with FITC (control) throughout this study. Graphs showing the relative percentage of cells stained by B cells, mature B cells, γδ T cells and monocytes/macrophages are given in appendix chapter 6, figure 6.1.

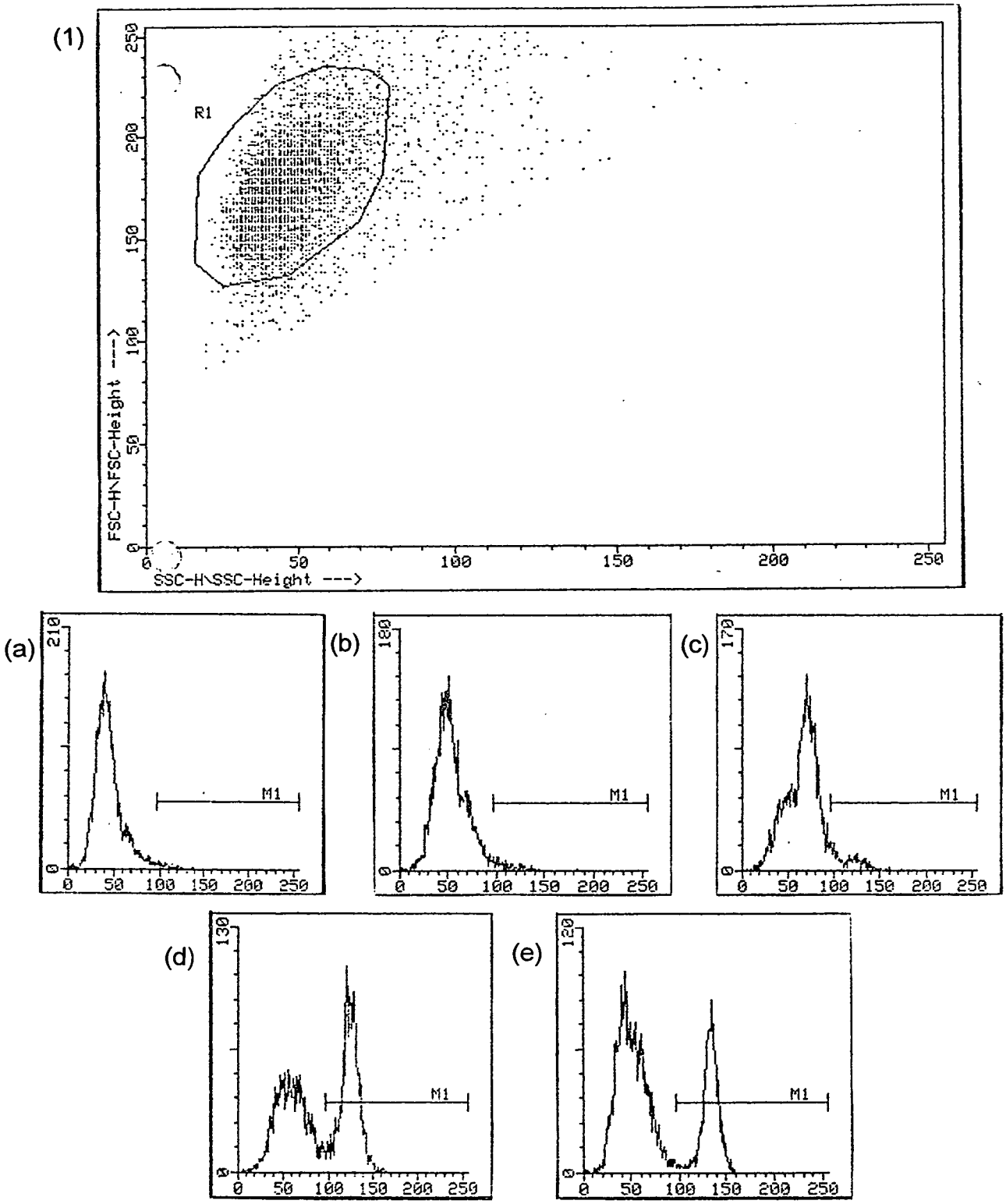


Figure 6.7 FACS profile (1) of 5,000 PBMC from calf 20 on day 14 post infection showing gate settings used to delineate the population analysed; graphs show the percentage of PBMC with FITC labeled antibody against (a) FITC control (no antibody) (1%), (b) CD2 (3%), (c) IL2R (7%), (d) CD4 (50%) and (e)CD8 (31%) (Bar M1 on each graph shows population of cells labelled).

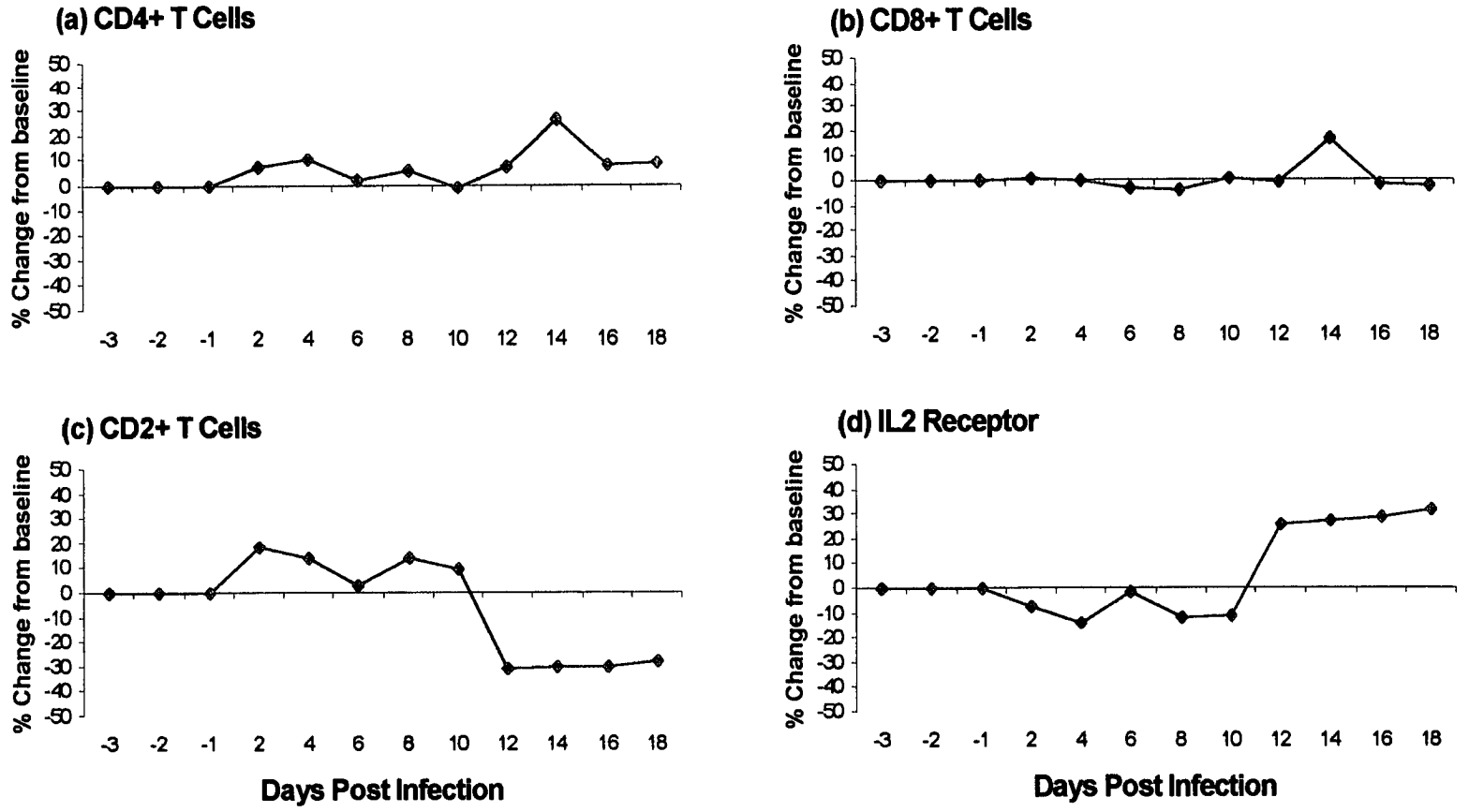


Figure 6.8 Mean percentage change from base line values of bovine peripheral blood lymphocyte from 4 calves pre and post infection with *N.caninum* tachyzoites (calves were experimentally infected with $2.5x10^8$ NC1 N.caninum tachyzoites on day 0). Cell populations were identified by FACS analysis. A table showing the baseline percentages is given in appendix chapter 6, table 6.8.

Discussion

Very little is known about the immune response to *Neospora caninum in vivo.* Khan *et al* (1997) showed that the cell mediated immune response to the parasite, mediated by the T cell cytokines IFNγ and IL12, is important in protection against clinical disease in experimentally infected mice. No work has been published describing the immune response to the parasite in cattle, a natural host. The results of the present study show that experimental infection of cattle with *N.caninum* tachyzoites induces a measurable cell mediated immune response in peripheral blood. This immune response is characterised by strong proliferation of PBM cells to a crude lysate of parasite antigen and production of IFNγ, which is likely to be important in protective immunity (Innes *et al*, 1995c; Innes and Wastling, 1995; Khan *et al*, 1997). PBM cells from control animals (section 7.3.3.1) and from calves pre infection with *N.caninum* tachyzoites did not proliferate in response to antigen or produce detectable levels of IFNγ. Therefore a simple proliferation assay was able to distinguish infected from uninfected animals.

The *Neospora* infected calves sero-converted and became strongly sero-positive for *Neospora* by week 4 post infection but remained only weakly positive for antibody to *Toxoplasma gondii.* Despite low *Toxoplasma* specific serum antibody levels incubation of PBM cells from the infected animals with *T.gondii* crude lysate antigen *in vitro* induced a strong proliferative response from day 6 post infection. This response was characterised by production of IFNγ, although interestingly the *T.gondii* lysate was less effective than *N.caninum* antigen in stimulating IFNγ production. This cross reactive response of PBM cells to *N.caninum* and *T.gondii* antigen suggests that the parasites share some common antigens which stimulate a cell mediated immune response to both. This has previously been shown in mice experimentally infected with *N.caninum* which also produced significant levels of IFNγ in response to *T.gondii* antigen, although in this study the mice were not cross protected against subsequent

infection with *Toxoplasma* (Lindsay *et al*, 1990a). Lack of cross protection was also seen in a study by Innes *et al* (manuscript in preparation) where experimentally induced abortion in sheep inoculated with *Neospora* tachyzoites was not affected by pre-immunisation with the S48 Toxovax® vaccine strain of *T.gondii.* Again, although PBM cells from *Neospora* infected animals responded to *Toxoplasma* antigen *in vitro* this response did not translate into protection against clinical disease in the host (Innes *et al*, manuscript in preparation). As IFNγ is a major component of the cell mediated immune response it is possible that lack of cross protection shown in the mouse model may be due to diminished levels of IFNγ produced in response to *T.gondii* antigen, as shown in this study.

The kinetics of the cellular immune response to infection with the S48 *T.gondii* vaccine strain in sheep *in vivo* has previously been measured by examining the cell populations in efferent lymph collected from lymph nodes draining the site of infection (Innes *et al*, 1995b). Protective immunity stimulated by vaccination was characterised by production of lymphoblasts 5-6 days after primary infection, initially identified as CD4+ T cells. At the time of peak lymphoblast response, around day 10 pi, the predominant population switched from CD4+ to CD8+ T cells. In this study we analysed the cellular sub-populations in PBM cells to find out if a possible change in cell output from lymph nodes could be detected in peripheral blood. Only a very slight increase in CD4+ T cells was seen up to day 4 pi but no change was seen in the percentage of CD8+ T cells up to day 12 pi. A transient increase in percentage of cells stained on day 14 pi may have been a latent reflection of a peak in lymphoblast response seen in infection with S48 on day 10 pi, but the percentage of cells stained by all the Mab's increased at this point showing that this did not reflect a shift in the balance of sub-populations. Alternatively the consistency in change could have reflected a technical problem as all the samples were processed at the same time. The only consistent change in cell populations seen was an increase in CD2+ T cells (which incorporates CD4+ and CD8+ cells) on day 2 pi which then decreased to approximately 30% below base line levels on day 12 pi and remained at this level until day 18 when the study ended. This is contrary to the response to vaccination with S48 *T.gondii* which stimulated production of

increasing numbers of CD2+ T cells from day 5-day 17 pi in efferent lymph (Innes *et al*, 1995b). Also this study showed that the number of cells expressing the IL2 receptor in PBM cells was reduced until day 12 pi when it increased to approximately 25% post infection which was also maintained until the end of the study. The IL2 receptor (IL2-R) is present on the surface of B cells, T cells and natural killer cells, and as there was no corresponding increase in B cells, CD4+ T cells, and CD8+ T cells the increase may reflect an increase in the percentage of natural killer cells in circulation. Changes seen in cell populations present in efferent lymph after S48 vaccination described by Innes *et al* (1995b) were not reflected in PBM cellular sub-populations from cattle inoculated with *N.caninum*. This could be due to a dilution of the cell output in peripheral blood masking changes in cell populations or could have been altered by cells localising to the site of an infection. Direct examination of the immune response to *N.caninum* in experimentally infected cattle by the method of lymphatic cannulation could improve our understanding of the changes elicited *in vivo* by infection with *N.caninum*.

Toxoplasma gondii has the capacity to stimulate non-immune splenocytes to respond *in vitro* to both whole tachyzoites and soluble tachyzoite antigen (Denkers *et al*, 1994). This superantigen driven expansion of IFNγ secreting CD8+ cells does not require cellular antigen processing and could explain *T.gondii* S48 induced pre-inoculation proliferation of PBM cells from some animals. Another possible cause of pre-infection proliferation of PBM cells to parasite lysate may be due to the crude preparation of parasite antigen that could contain cross reactive antigens as previously discussed. Clearly further work needs to be done to examine antigen specificity of the T cell response to *N.caninum* and the potential for cross reaction with other closely related coccidian parasites.

This study has shown that experimental infection of calves with *Neospora caninum* NC1 tachyzoites induces a cell mediated immune response. It is difficult however to extrapolate these findings to clinical neosporosis as the immune system is modulated during pregnancy to enable the mother to sustain foreign tissue. Immunoregulatory molecules produced during gestation modulate the maternal immune response inducing a shift from cell mediated Th1 type immunity to a humoral Th2 type

response characterised by production of non-cytotoxic IgG1 antibody (Raghupathy, 1997; Bell and Billington, 1980). Soluble factors proposed as candidates for immunomodulation include progesterone induced blocking factor (PIBF), stimulated by the action of progesterone on lymphocytes, trophoblast-cell derived factor (TCDF) and placental suppressor factor (PSF), produced by placental cells, all of which strongly inhibit mitogen induced lymphocyte proliferation and cytotoxic T lymphocyte activity *in vitro* (all human *in vitro* models). Infection with intracellular pathogens during pregnancy may cause this shift in immune response to return to cell mediated mechanisms resulting in expulsion of the foetus or resorption as a byproduct of infection. A previous study by Krishnan *et al* (1996) showed that infection with *Leishmania major* during pregnancy, also an intracellular parasite which stimulates a cell mediated immune response in the host, increased levels of IFNγ and tumor necrosis factor (TNF) in experimentally infected mice which resulted in significantly higher numbers of foetal resporptions compared to uninfected controls. Induction of cell mediated immunity during pregnancy has also been linked with increased rates of abortion caused by infection with *Plasmodium falciparum* in humans (Mendez, 1995). Alternatively immunosuppressive factors produced in the placenta could create a localised low level immune response relative to peripheral blood which may lead to concentration of parasites in those tissues. Subsequent tissue damage caused by parasite replication could therefore be the cause of abortion or foetal death and mummification (Mendez, 1995).

To date no studies examining the immune response to infection and recrudesence of *N.caninum* in the pregnant host have been published, and we do not understand the mechanisms which lead to expulsion of the foetus. Development of a pregnant bovine model of *N.caninum* infection is needed if we are to understand the immune response to primary and secondary infection with *N.caninum* during early, mid and late gestation, and how immunomodulatory factors during pregnancy interact with infection and recrudesence of *N.caninum* tachyzoites in the host.

Chapter 7

Identification of *Neospora* Antigens Recognised by CD4+ T Cells and Immune Sera from Experimentally Infected Cattle

Aims

- To identify *Neospora* antigen fractions recognised by CD4+ T cells from cattle experimentally infected with *Neospora caninum* NC1 tachyzoites.

- To identify *Neospora* antigen fractions which stimulate both a cell mediated and humoral immune response in cattle.

- To examine reactivity of CD4+ T cells from *Neospora* infected cattle to stimulation with *Toxoplasma gondii* antigens.

7. Identification of *Neospora* antigens recognised by CD4+ T cells and immune sera from experimentally infected cattle

7.1 Introduction

Unlike toxoplasmosis in sheep, which stimulates protective immunity following primary infection, neosporosis can cause repeat abortion in cattle (Anderson *et al*, 1995, Moen *et al*, 1995). But the rate of repeat abortion is estimated to be less than 5% (Anderson *et al*, 1995; Moen *et al*, 1995), therefore it is likely that many animals do develop some form of protective immunity. *Neospora caninum*, like *T.gondii,* is an obligate intracellular parasite, therefore it is likely that cell mediated immune mechanisms are an important component of the immune response.

IFNγ is an important T cell cytokine produced mainly by CD4+ T cells and is a major mediator of resistance to *Toxoplasma* in sheep (Innes *et al*, 1995b). Vaccination of sheep with the S48 vaccine strain of *T.gondii* stimulated production of high levels of IFNγ in efferent lymph of sheep 2-5 days after experimental infection which were subsequently protected against abortion after challenge infection (Innes and Wastling, 1995). Phenotypic analysis showed that the majority of lymphoblasts initially produced in efferent lymph following a primary infection were CD4+ T cells which produced IFNγ in response to *T.gondii* antigen *in vitro*. We have previously shown that recombinant IFNγ suppresses the growth of NC1 strain *N.caninum* tachyzoites cultured in fibroblasts *in vitro* (Innes *et al*, 1995a). Also recent data by Khan *et al* (1997) demonstrated a protective cell mediated immune response in 3 inbred strains of mice (C57BL/6, A/J, Balb/c) experimentally infected with NC1 strain *N.caninum* tachyzoites characterised by the induction of IL-12 and upregulation of IFNγ mRNA *in vivo* (Khan *et al,* 1997). Introduction of antibody to IL12 and IFNγ into these mice ablates protection and results in death. IFN γ plays an

important role in the activation of other cell types, in particular macrophages, to destroy tachyzoites and promotes maturation of CD8+ T cells into effector cytotoxic T cells (Zanovello *et al*, 1988). Results in chapter 6 show that experimental infection of calves with NC1 *N.caninum* tachyzoites stimulates a cell mediated response in the host but the role of CD4+ T cells in this response is unknown.

PBM cells from calves previously sero negative for *N.caninum* and *T.gondii* which were experimentally infected with *N.caninum* tachyzoites proliferated in response to both *N.caninum* and *T.gondii* wsf antigens from day 6 post inoculation despite the absence of *Toxoplasma* specific antibodies (chapter 6). The response to both antigens was characterised by production of IFNγ suggesting *N.caninum* and *T.gondii* share some common antigens which stimulate a cell mediated immune response to both. This cross reactive response has previously been shown in mice experimentally infected with *N.caninum* which also produced significant levels of IFNγ in response to *T.gondii* antigen, although in this study the mice were not cross protected against subsequent infection with *Toxoplasma* (Lindsay *et al*, 1990). Lack of cross protection was also seen in a study by Innes where experimentally induced abortion in sheep inoculated with *Neospora* tachyzoites was not affected by pre-immunisation with the S48 Toxovax vaccine strain of *T.gondii* (Innes *et al*, paper in preparation). As IFNγ is a major component of the cell mediated immune response it is possible that lack of cross protection may be due to diminished levels of IFNγ produced in response to *T.gondii* antigen (chapter 6).

The aim of this study was to identify *N.caninum* antigen fractions responsible for stimulating the cell mediated immune response to infection and to investigate the specificity of the cross reactive response to *T.gondii* antigen. The response of PBM cells and CD4+ T cells from calves inoculated with NC1 *N.caninum* tachyzoites to *Neospora* and *Toxoplasma* whole wsf antigen and antigen fractions *in vitro* was tested. Two distinct methods were used to separate antigen fractions (a) by SDS PAGE and transferred to nitrocellulose membrane (Young & Lamb, 1986), and (b) by high performance liquid chromatography (HPLC). The first method separates antigen by molecular mass only and results in production of solid phase antigen bound to a nitrocellulose membrane. The second method separates antigens by

charge producing antigen in liquid phase. By using these two very different methods of fractionating antigen we can confirm that the immune response to a particular antigen is not an artefact of the method of protein separation. Supernatant taken from CD4+ T cells incubated with *Neospora* antigen fractions was also tested for IFNγ production.

Where protection to a particular pathogen is mediated by both humoral and cellular immunity the design of an effective vaccine may necessitate identification of molecules able to activate both antibody production and an antigen specific T cell response (Lamb *et al*, 1988). For this reason *Neospora* antigens recognised by the humoral immune response in these experimentally infected animals were also investigated.

7.2 Materials and methods

7.2.1 Calves

7.2.1.1 *Neospora caninum* experimental infection (A)

The same animals used to study the immune response to experimental infection with *Neospora* (chapter 6) were also used to generate T cell lines. In brief, six calves approximately 6 months old which were seronegative for *N.caninum* and *T.gondii* by ELISA and IFAT respectively were selected. Calves were monitored for any sign of clinical disease and rectal temperatures were recorded daily from the day before inoculation until 14 days after inoculation.

7.2.1.2 *Neospora caninum* experimental infection (B)

Six calves approximately 6 months old which were seronegative for *N.caninum* and *T.gondii* by ELISA and IFAT respectively were selected. Calves were monitored for any sign of clinical disease and rectal temperatures were recorded daily from the day before inoculation until 14 days after inoculation.

7.2.2 Parasites

7.2.2.1 *Neospora caninum*

Neospora caninum NC1 strain tachyzoites were cultured within VERO cell monolayers in IMDM supplemented with 2% horse serum, as described in chapter 3, section 3.2.1.

7.2.2.2 *Toxoplasma gondii*

Toxoplasma gondii S48 tachyzoites were also cultured within VERO cell monolayers in IMDM supplemented with 2% foetal bovine serum as described in chapter 3, section 3.2.3.

7.2.3 Experimental design and inoculum

Neospora caninum NC1 strain tachyzoites (Dubey *et al*, 1988) were grown in IMDM supplemented with 2% horse serum, as described in chapter 3 section 3.2.1, and harvested by scraping the VERO cell monolayers containing parasites off the tissue culture flasks. Parasite and cells were then centrifuged at 500*g* for 10 mins and resuspended at a concentration of $1.25x10^8$ tachyzoites per ml in IMDM containing 2% horse serum, 50U/ml penicillin and 50μg/ml streptomycin. The inoculum was grown and prepared immediately before inoculation and a record was made of the number of VERO cells injected with the parasite.

Two bovine *N.caninum* experimental infections, A and B, were carried out approximately one year apart and as outlined in tables 7.1a and b. Suspensions containing $2.5x10^8$ NC1 tachyzoites were administered to the animals in group 1 and 3 by sub cutaneous injection in the left hind quarter. Control animals in groups 2 and 4 received the same number of VERO cells administered to the animals in groups 1 and 3 respectively, also by sub cutaneous injection in the left hind quarter. Control and infected calves were penned separately throughout the experiment. Cell proliferation assays were carried out using:

- pre and post inoculation PBM cells from calves inoculated with *N.caninum* and PBM cells from controls (table 7.1a)
- CD4+ T cell lines cultured from PBM cells collected 3-6 weeks post inoculation (table 7.1b)

Cells were cultured with *N.caninum* NC1 and *T.gondii* S48 whole water soluble fraction (wsf) antigen and *N.caninum* NC1 and *T.gondii* S48 antigen fractions. Water soluble fraction antigen was fractionated by (a) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and transferred to a nitrocellulose membrane (experiment A), and (b) high performance liquid chromatography (HPLC) (experiment B).

				Pre and Post Inoculation PBM + Whole Parasite Antigen				Pre and Post Inoculation PBM +Parasite Antigen Fractions				
				Source of Cells		Whole Antigen		Separation	Source of Cells		Antigen Fractions	
Date	Expt.	Group	Animal No.	Pre-Infection PBM	Post-Infection PBM	NC1 Wsf Ag	S48 Wsf Ag	Nitro or HPLC Fractions	Pre-Infection PBM	Post-Infection PBM	NC1 Antigen Fractions	S48 Antigen Fractions
August	**A**	**1** Infected	13	✓	✓	✓	✓	N	ND	✓	✓	ND
1996		NC1 Tachys	14	✓	✓	✓	✓	N	ND	✓	✓	ND
		$2.5x10^8$	20	✓	✓	✓	✓	N	ND	✓	✓	ND
			21	✓	✓	✓	✓	N	ND	✓	✓	ND
		2 Controls	15	✓	NI	✓	ND	ND	ND	NI	ND	ND
		VERO Cells	25	✓	NI	✓	ND	ND	ND	NI	ND	ND
		$6.5x10^6$										
October	**B**	**3** Infected	18	✓	✓	✓	✓	H	✓	✓	✓	✓
1997		NC1 Tachys	19	✓	✓	✓	✓	H	ND	✓	✓	✓
		$2.5x10^8$	21	✓	✓	✓	✓	H	✓	✓	✓	✓
			25	✓	✓	✓	✓	H	✓	✓	✓	✓
		4 Controls	63	✓	NI	✓	✓	H	✓	NI	✓	✓
		VERO Cells	64	✓	NI	✓	✓	H	✓	NI	✓	✓
		$2.3x10^7$										

Table 7.1a Part 1 of the experimental design for 2 bovine *N.caninum* experimental infection studies A and B. This table details cell proliferation assays carried out using pre and post inoculation PBM from infected animals and PBM from controls incubated with *N.caninum* NC1 strain or *T.gondii* S48 strain whole wsf antigen or antigen fractions separated by (a) SDS PAGE and bound to a nitrocellulose membrane (expt A) or (b) by HPLC (expt B). H, HPLC fractionation of parasite antigen; N, nitrocellulose bound parasite antigen fractions; nitro, nitrocellulose; ND, not done; NI, not infected.

				Post Inoculation CD4+ T Cell Lines + Antigen Fractions			
				Nitrocellulose Bound Antigen Fractions		HPLC Separated Antigen Fractions	
Date	Expt.	Group	Animal	NC1	S48	NC1	S48
August	**A**	**1** Infected	13	✓	✓	ND	ND
1996		NC1 Tachys	14	✓	✓	ND	ND
		$2.5x10^8$	20	✓	✓	ND	ND
			21	✓	✓	ND	ND
		2 Controls	15	NI	NI	ND	ND
		VERO Cells	25	NI	NI	ND	ND
		$6.5x10^6$					
October	**B**	**3** Infected	18	ND	ND	✓	✓
1997		NC1 Tachys	19	ND	ND	✓	✓
		$2.5x10^8$	21	ND	ND	✓	✓
			25	ND	ND	✓	✓
		4 Controls	63	NI	NI	ND	ND
		VERO Cells	64	NI	NI	ND	ND
		$2.3x10^7$					

Table 7.1b Part 2 of the experimental design for 2 bovine *N.caninum* experimental infection studies A and B. This table details cell proliferation assays carried out using CD4+ T cell lines from infected animals incubated with *N.caninum* NC1 strain or *T.gondii* S48 strain antigen fractions. Whole wsf NC1 and S48 antigen was separated by 2 methods; (a) separation by SDS PAGE and bound to a nitrocellulose membrane (experiment A), and (b) HPLC separation (experiment B). ND, not done; NI, not infected.

7.2.4 Serum samples

Blood samples were collected on a weekly basis from the jugular vein into preservative free evacuated blood collection tubes and allowed to clot. Following retraction of the clot and centrifugation at 500*g* serum was removed stored at -20°C until required. Antibodies to *N.caninum* and *T.gondii* were then analysed by IFAT and ELISA as described in chapter 3, section 3.5.1 and section 3.5.2 respectively using a rabbit anti-bovine IgG fluorescein isothiocyanate conjugate (Sigma, Poole, UK).

7.2.5 Preparation of antigen

7.2.5.1 Preparation of water soluble antigen

Water soluble fraction antigen was prepared from *Neospora caninum* NC1 strain and *Toxoplasma gondii* S48 strain tachyzoites and VERO cells as described in chapter 3, section 3.4.2.

7.2.5.2 Preparation of SDS soluble antigen

Sodium dodecyl sulphate soluble fraction antigen were prepared from *Neospora caninum* NC1 strain and *Toxoplasma gondii* S48 strain tachyzoites as described in chapter 3, sections 3.4.3.

7.2.6 SDS PAGE and transfer to nitrocellulose

7.2.6.1 Preparation of antigen for T cell assays

The preparation of nitrocellulose bound *Neospora* and *Toxoplasma* antigens is described in detail in chapter 3, section 3.6.5. In brief, *Neospora* NC1 proteins present in a mixture of 50µl of SDS soluble antigen (2mg/ml of protein) and 50µl of wsf antigen (1mg/ml of protein) were separated by SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions described by Laemmli (1970) on

a 10% polyacrylamide mini gel using the mini Protean system (Biorad, Hemel Hempstead, UK). A combination of molecular weight markers in the range of 12,000-78,000 kDa was used to determine molecular mass (Merck, Lutterworth, UK). Proteins were transferred from the gel to a nitrocellulose membrane (Schleicher & Shuell, London, UK) by electroblotting with a trans-blot electrophoretic transfer cell (Biorad, Hemel Hempstead, UK). The blot was dried and cut into 20 strips 2-3mm wide, each strip containing antigen of the same molecular weight. The highest molecular weight band of antigen is referred to as fraction number 1, and that with the lowest molecular weight is referred to as fraction number 20. Strips were then cut into pieces approximately $2mm^2$ and sterilised in FACS medium for 1 hour followed by 4 x 5min washes with sterile PBS. Nitrocellulose bound antigen fractions were stored at 4°C in PBS until used in the proliferation assay. A band at the edge of the blot was stained with Ponceau stain to assess the total protein present.

7.2.6.2 Western blot analysis

Neospora and *Toxoplasma* protein fractions separated under reducing conditions by SDS PAGE were transferred to nitrocellulose membrane as described in chapter 3, section 3.6.1. The membranes were then cut into strips which were probed with serum from infected and control animals using Western blot technique to identify proteins recognised by the humoral immune response. In brief, strips were treated with 5% Marvel (dried skimmed milk) to block non-specific background staining before incubation with 20µl sera, diluted 1:150 in tris buffered saline tween (TBST), from each of the infected calves collected 4 weeks post inoculation and on day -1 pre-infection. Sera from the control animals was also tested for antibody to *N.caninum*. Bovine anti *Neospora* IgG was detected with alkaline phosphatase conjugated rabbit anti-bovine IgG whole molecule secondary antibody (Sigma Immuno-Chemicals, Poole, UK). Total protein bound to the membrane was shown by staining one strip with Ponceau dye (Sigma, Poole, UK).

7.2.6.3 Silver Stain

Individual HPLC fractions of *N.caninum* NC1 antigen were run on a 10% polyacrylamide gel as described in section 3.6.1. The gel was then fixed in 200ml of trichloroacetic acid (TCA) solution (20% w/v) for 1 hour at room temperature, and then placed in 40% (v/v) acetic acid in water and soaked for 1 hour. The gel was washed thoroughly (2 x 20 minutes) to facilitate rehydration of the gel and removal of methanol. After a further soak in 10% (w/v) glutaraldehyde solution for 20 minutes at room temperature the gel was again washed in water (3 x 20 minutes). Protein present in the gel was stained by soaking the gel in silver diamine solution for 30 minutes followed by several washes in water (3 x 5 minutes). The gel was then placed developing solution for approximately 5 minutes (2.5ml of 1%(w/v) citric acid, 0.26ml of 36% (w/v) formaldehyde made up to 500ml with water). Proteins are visualised as dark brown stains on the gel. Development was terminated by placing the gel in stopping solution (40% (v/v) ethanol, 10% (v/v) acetic acid in water).

7.2.7 Separation of antigen fractions by HPLC

Neospora caninum and *Toxoplasma gondii* water soluble antigens were separated into antigen fractions using a Vydac 4 carbon chain (C4) reverse phase column (Millipore, Bedford, MA, USA). Antigens are eluted according to their hydrophobicity using 2 solutions:

Solvent A – 97.5% water
2.5% isopropanol (IPA)
0.04% trifluoroacetic acid

Solvent B - 10% water
90% isopropanol (IPA)
0.04% trifluoroacetic acid

Water soluble fraction antigen at a concentration of 1mg/ml was added to the column and bound to the carbon molecules. The column was then washed with 100%

solution A and 0% solution B (table 7.2). Gradually over 70 minutes the concentration of solutions was changed inversely to 0% solution A and 100% solution B. Samples were collected at 1 minute intervals for seventy minutes and assayed for protein concentration. The first 10 samples contained no protein and were not used in cell proliferation experiments. Samples 11-70 were condensed by combining each 3 consecutive minutes of samples resulting in fractions 1-20 which were then diluted to a final concentration of 1μg/ml. The final 5 samples collected during 70-75 minutes are washes. These 5 samples were combined to make fraction 21 and contain small amounts of fractions 1-20.

Table 7.2 Gradient table for solutions A and B in HPLC antigen separation.

Time (minutes)	Flow (ml)	Soln A (%)	Soln B (%)
0	1.5	100	0
3	1.5	100	0
25	1.5	70	30
60	1.5	50	50
70	1.5	0	100
75	1.5	100	0

7.2.8 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were separated from whole blood as previously described in chapter 3, section 3.7.1.1.

7.2.9 Generation of CD4+ T cell lines

Infected and control animals were bled 4-6 weeks post infection and PBM cells were separated from whole blood as previously described in chapter 3, section 3.7.1.1. Equal volumes of PBM cells at $2x10^5$ cells per well and NC1 wsf antigen at 5μg/ml were cultured in 96 well round bottom tissue culture plates (Gibco, Paisley, UK) at 37°C in a humidified 5% CO2 incubator. After 7 days the cells were harvested and diluted 1:3 in T cell medium supplemented with 10U/ml of human recombinant IL2 (Proleukin, Eurocetus). Cells were cultured for a further 7 days prior to harvesting when they were used in proliferation assays.

7.2.10 Proliferation assays

7.2.10.1 Peripheral blood mononuclear cells

Peripheral blood mononuclear cells were separated from whole blood as previously described in section 3.7.1.1 and resuspended at $2x10^6$ cells/ml. Equal volumes of PBM cells and NC1 wsf antigen at 10μg/ml (experiment A) or 5μg/ml (experiment B) were cultured in triplicate quadruplicate in 96 well round bottom tissue culture plates. PBM cells at a concentraion of $2x10^6$ cells/ml and $2mm^2$ sterile nitrocellulose bound *Neospora* antigen fractions were cultured in quadruplicate in 96 well flat bottom tissue culture plates (Gibco, Paisley, UK) at 37°C in a humidified 5% CO2 incubator for 5 days. The concentrations of *N.caninum* wsf antigen used was optimised for each experiment. A final concentration of 10μg/ml of *N.caninum* antigen was selected for use in proliferation assays in experiment A as higher concentrations of antigen (up to 20μg/ml) did not significantly increase proliferation. For experiment B a different batch of *N.caninum* wsf antigen was used. When tested concentrations of antigen higher than 5μg/ml (up to 20μg/ml) did not significantly increase proliferation. Cells cultured in 5μg/ml Concanavalin A (Con A) (Sigma, Poole, UK) and in medium alone were used as positive and negative controls respectively. All cells were pulsed for the final 18h with 18.5kBq [^{3}H]-thymidine

(Amersham, Little Chalfont, UK) per well before harvesting onto fibreglass filters (Canberra Packard, Pangbourne, UK). Cell associated radioactivity was quantified in a gas proportional counter (Canberra Packard, Pangbourne, UK).

7.2.10.2 CD4+ T cells

Proliferation assays with CD4+ T cells were cultured in the presence of autologous PBM irradiated with 3000 rad from a ^{57}Cs source as a source of antigen presenting cells (APC). The ratio of APC:CD4 was 10:1. Cells cultured in 5μg/ml Concanavalin A (Con A) (Sigma, Poole, UK) and in medium alone were used as positive and negative controls respectively. Unfractionated NC1 wsf antigen at a concentration of 1mg/ml was dot blotted directly onto nitrocellulose and used as a positive control, and cells were also tested for nonspecific stimulation by nitrocellulose membrane alone with no antigen. In proliferation assays cells from the control animals were set up in quadruplicate, while cells from the infected animals were set up in triplicate.

7.2.11 Phenotypic analysis of CD4+ T cell lines

The cellular composition of the CD4 T cell lines was analysed using a panel of monoclonal antibodies (Mabs) which recognise specific bovine leucocyte populations (table 7.3). The method used to stain the cells is largely described by Innes *et al* (1995b) and in the methods chapter, section 3.7.5.

Table 7.3 Mabs used to identify sub-populations of cells by FACS analysis.

Antibody	Specificity	Location
None	Control	Background staining with FITC
CC42	CD2	αβ T Cells
CC8	CD4	Helper T Cells Subset
CC63	CD8	Cytotoxic T Cells Subset
CC15	T19 (γδ)	γδ T Cell
CC21	CD21	Mature B Cell
ILA111	IL2 Receptor	T Cell
VPM65		Monocyte/Macrophage

7.2.12 Assay for IFNγ

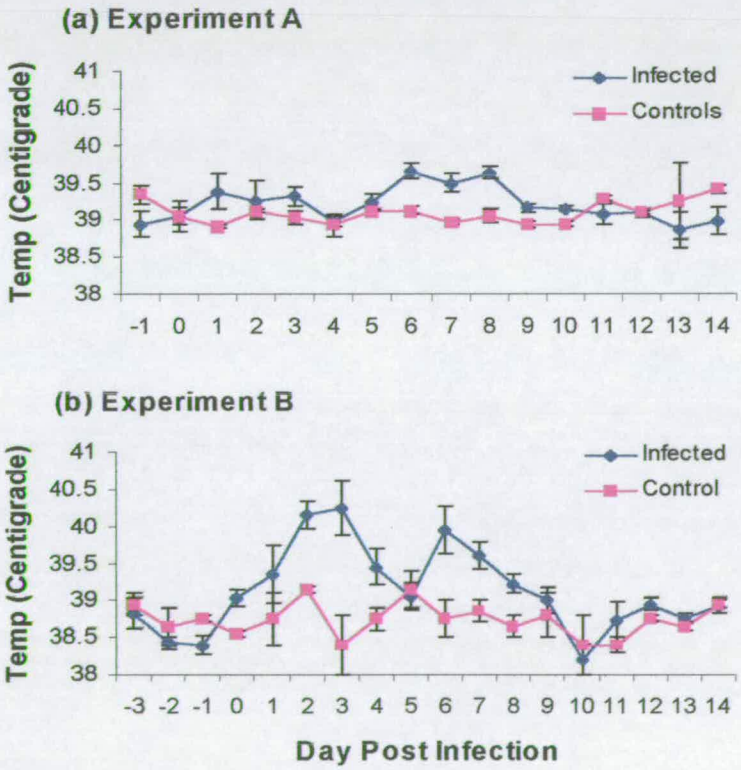
Supernatant was collected from CD4+ T cells stimulated with *Neospora* antigen fractions 14-20 for 4 days. The concentration of bovine IFNγ in these samples was quantified using an ELISA kit produced by CSL Veterinary, Melbourne, Australia. Recombinant ovine IFNγ (rovIFNγ) (Dr.G.Entrican) was used as a control to calibrate results as described in the methods chapter, section 3.7.3.3).

7.3 Results

7.3.1 Rectal Temperatures

Mean rectal temperature for calves infected with *N.caninum* NC1 tachyzoites in experiment A increased on days 6-8 post inoculation and for calves in experiment B on days 2-8 pi (figure 7.1). A temperature of $>39.5^0$ indicates a febrile response therefore infected calves showed a febrile response to infection in experiment A on days 6, 7 and 8 pi and in experiment B on days 2-4 and 6-8 pi (student's T test, $P<0.05$) (appendix chapter 7, tables 7.1 and 7.2). The control calves were clinically normal with no significant change in body temperature.

Figure 7.1 Mean (±se) temperatures of experimentally infected calves (n=4) pre and post inoculation with *N.caninum* NC1 tachyzoites and controls (n=2) for experiments (a) A and (b) B (temperature measured in °C).

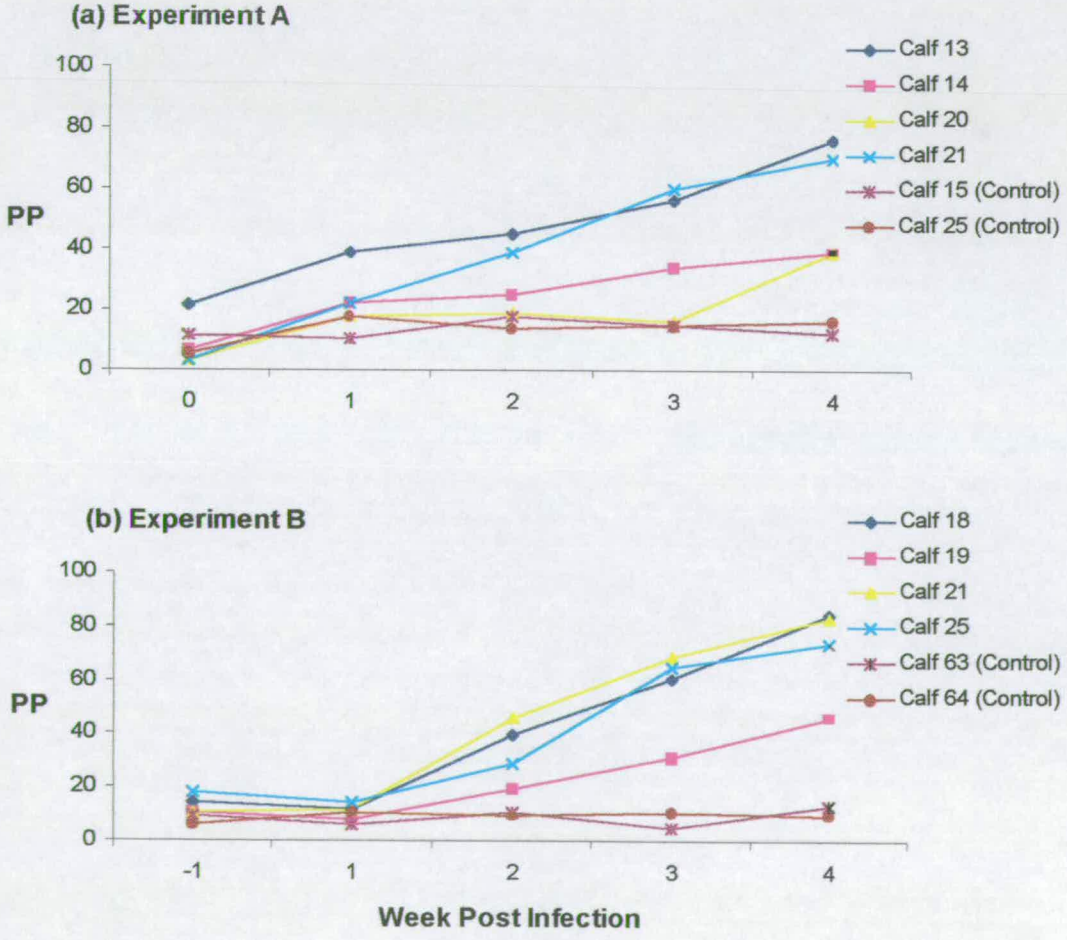


7.3.2 Humoral immune response

7.3.2.1 Anti-*Neospora* antibody response

Calves were tested for antibody to *N.caninum* by ELISA. All calves were antibody negative pre-infection. Animals inoculated with *Neospora* tachyzoites seroconverted and there was a significant increase in *N.caninum* antibody from the mean pre infection value of 11.6%OD from week 2 post infection in both experiments A and B (student T test, $P<0.05$) (appendix chapter 7, tables 7.3 and 7.4). The controls remained negative throughout.

Figure 7.2 *N.caninum* antibody titres in infected and control calves for experiment (a) A and (b) B measured by ELISA. *Neospora* titres >30 percentage positive are considered positive.



7.3.2.2 Anti-*Toxoplasma* antibody response

The calves were also tested for *Toxoplasma gondii* antibody titres by IFAT and ELISA (table 7.4). *Toxoplasma* antibody titres in all calves in experiment B and some infected calves in experiment A were greater post infection with *Neospora* tachyzoites, but titres also increased in most control animals (expt A, no.25 and expt B nos.63 and 64) which only received VERO cell inoculum.

Table 7.4 *Toxoplasma gondii* antibody titres in control calves and calves inoculated with *Neospora* NC1 tachyzoites in experiments A and B. All calves were tested before infection and animals from experiment A 3 weeks post infection and experiment B 4 weeks post infection. A *Toxoplasma* antibody titre of ≥1:256 is considered positive.

Exp.	Animal No.	NC1 Infection Status	Pre-Infection Toxo IFAT	Pre-Infection Toxo ELISA (PP)	Post-Infection Toxo IFAT	Post-Infection Toxo ELISA (PP)
A	13	Infected	1:256	18.8	1:512	35.7
	14	Infected	1:512	18.3	1:512	16.1
	20	Infected	1:64	9.4	1:128	6.7
	21	Infected	1:128	6.7	1:128	8.5
	15	Control	1:256	9.4	1:256	6.7
	25	Control	1:128	7.1	1:256	12.1
B	18	Infected	1:128	22.7	1:512	22.7
	19	Infected	1:64	19.6	1:512	23.2
	21	Infected	1:128	16	1:256	10.8
	25	Infected	1:128	22.2	1:512	33
	63	Control	1:128	20.6	1:256	30.4
	64	Control	1:128	20.1	1:512	22.2

7.3.3 Proliferation of PBM cells to whole wsf antigens

7.3.3.1 Experiment A

Peripheral blood mononuclear cells taken from animals pre-infection did not proliferate to NC1 wsf antigen at a final concentration of 10μg/ml (figure 7.3a). Two animals, calves 13 and 20, did respond to S48 antigen but had returned to baseline levels by day -1 pre-infection. PBM cells from the 4 infected calves began proliferating to both NC1 and S48 antigen *in vitro* on day 6-8 post infection. This response decreased in animals 13, 14 and 20 around day 14-16 and increased again by day 18. PBM from control calves in experiment A (figure 7.3b) did not respond to NC1 wsf antigen. Strong proliferative responses were observed in cells from control and infected animals stimulated by 5μg/ml Con A. Proliferation results of PBM cells from control and infected animals from 3 days pre to 3 weeks post infection are given in appendix chapter 6, table 6.5.

7.3.3.2 Experiment B

Figure 7.4a illustrates the proliferative activity of PBM cells from inoculated calves in experiment B 1 day pre and 2 weeks post infection with NC1 tachyzoites. Pre-infection PBM cells from calf 18 responded to NC1 or S48 whole wsf antigen. Cells taken from inoculated calves 19 and 25 two weeks post infection proliferated in response to NC1 and S48 wsf antigens and proliferated strongly in response to Con A, but cells from calves 18 and 21 did not respond to Con A or to antigen stimulation. PBM cells from control calves did not proliferate in response to NC1 or S48 antigen (figure 7.4b). Cells taken both pre and post infection did not proliferate in response to VERO cell antigen. PBM cells from control animals did not respond to NC1, S48 or VERO wsf antigen but did respond to Con A. Proliferation results of PBM cells from control and infected animals from 3 days pre to 3 weeks post infection are given in appendix chapter 7, table 5.

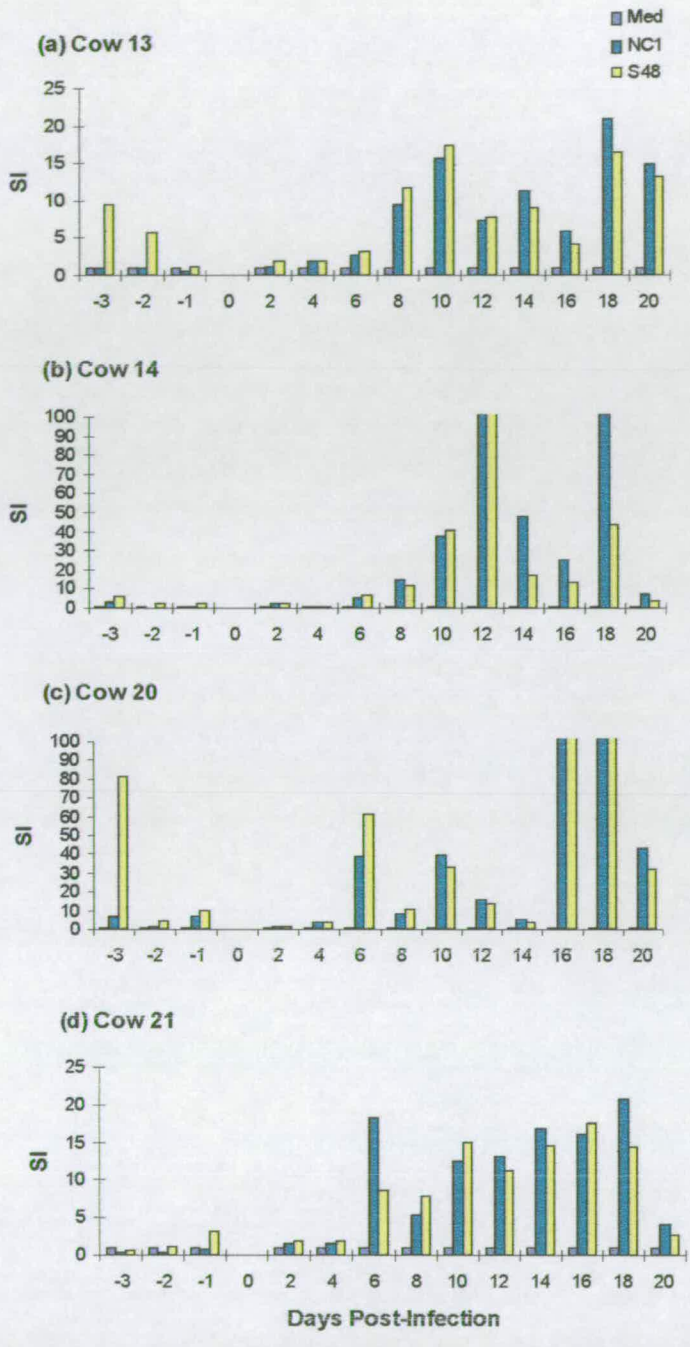


Figure 7.3a Proliferation of PBM from 4 calves (a-d) from experiment A infected with NC1 *Neospora* tachyzoites to *N.caninum* and *T.gondii* wsf antigens at final concentrations of 10μg/ml. Calves were inoculated subcutaneously with $2.5x10^8$ NC1 tachyzoites on day 0. Cell growth was measured pre-infection and at 2 day intervals post infection in counts per minute (CPM) of ^{3}thymidine incorporated into proliferating cells and represented above as stimulation index (SI units = mean CPM of cells stimulated by antigen/mean CPM of medium control, n=4). This graph was previously shown in chapter 6 (graph 6.3). CPM of medium controls on day -1: calf 13, 361; calf 14, 271; calf 20, 906; calf 21, 4011.

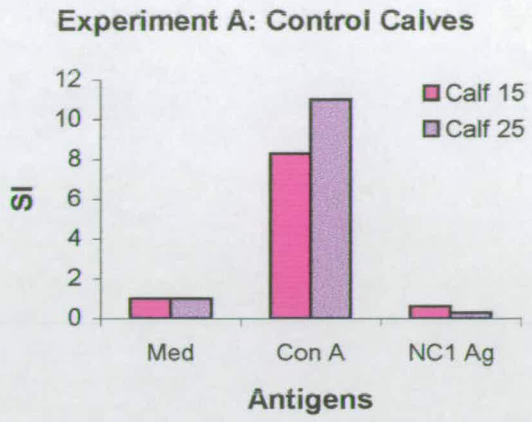


Figure 7.3b Proliferation of PBM from 2 control calves (nos.15 and 25) in experiment A to *N.caninum* wsf antigen at a final concentration of 10μg/ml. Cell growth was measured in counts per minute (CPM) of ^{3}thymidine incorporated into proliferating cells and represented above as stimulation index (SI units = mean CPM of cells stimulated by antigen/mean CPM of medium control, n=4). CPM of medium controls: calf 15, 2733; calf 25, 1687.

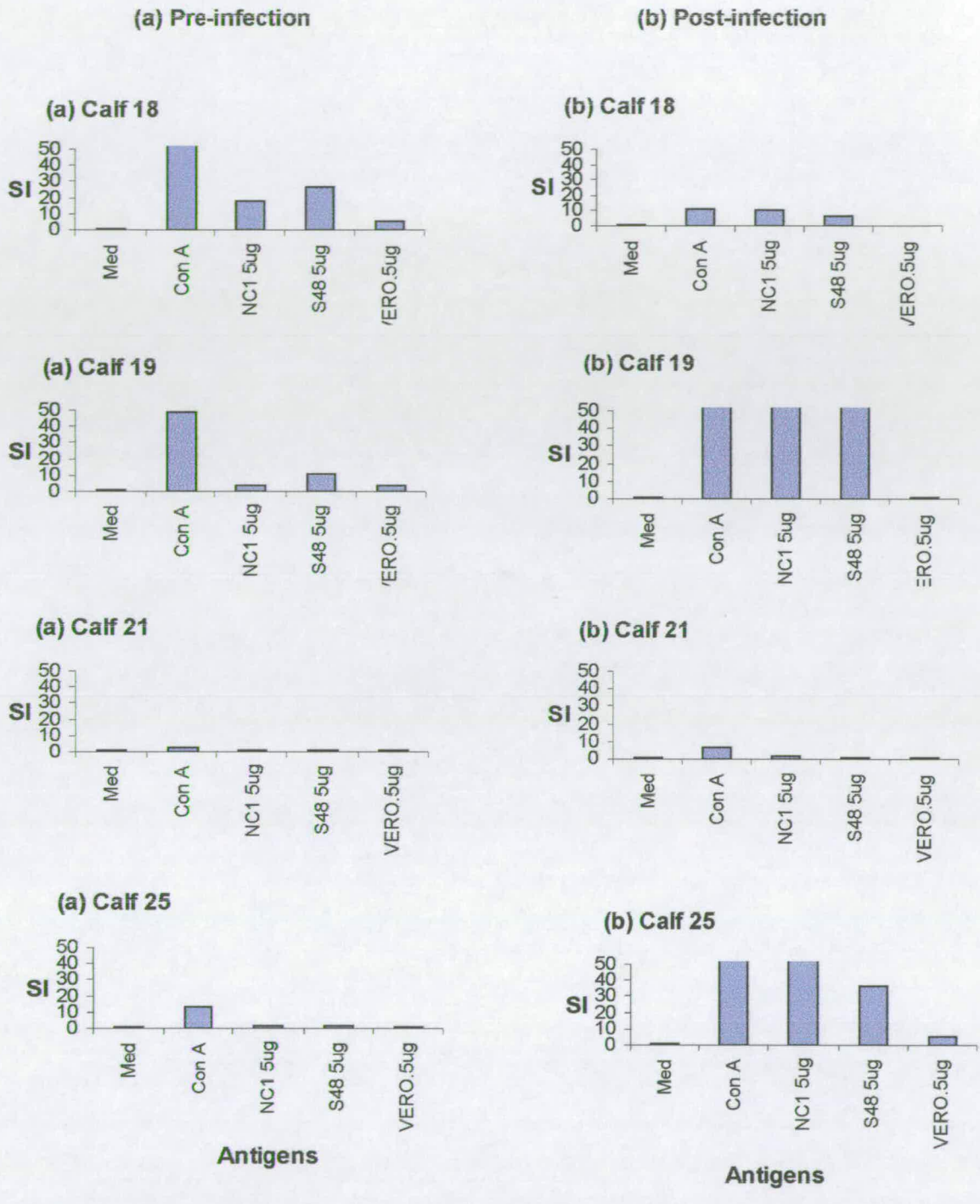


Figure 7.4a Experiment B group 3 calves (numbers 18, 19, 21 and 25). Proliferation of peripheral blood mononuclear cells (a) one day pre infection and (b) 2 weeks post infection with $2.5x10^8$ NC1 tachyzoites to *Neospora* NC1, *Toxoplasma* S48 and VERO cell wsf antigen all at a final concentration of 5μg/ml. Cell growth was measured in CPM ^{3}thymidine incorporated into proliferating cells, represented as before as stimulation index (SI units = mean CPM of cells stimulated by antigen/mean CPM of medium control). CPM of medium controls: (a) calf 18, 105; calf 19, 372; calf 21, 7456; calf 25, 555: (b) calf 18, 1016; calf 19, 40; calf 21, 2598; calf 25,129.

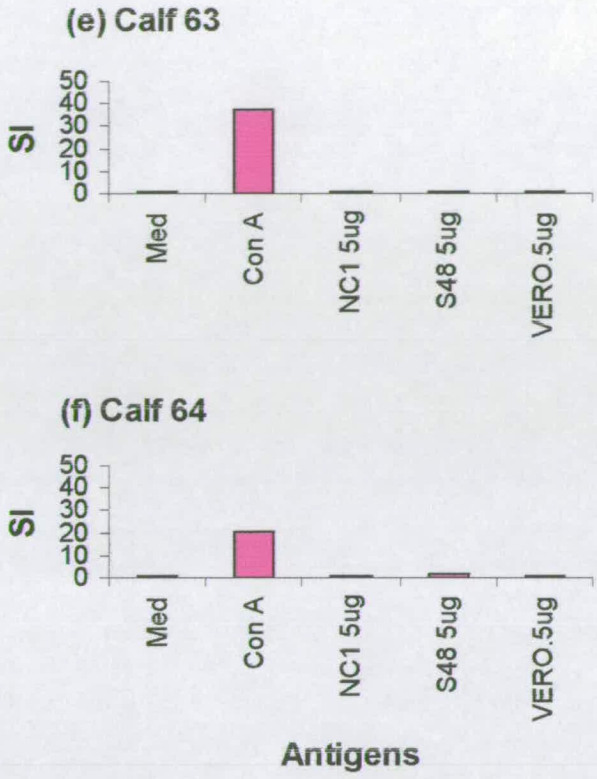


Figure 7.4b Proliferation of peripheral blood mononuclear cells from experiment B group 4 calves (control animals, numbers 63 and 64) to *Neospora* NC1, *Toxoplasma* S48 and VERO cell wsf antigens at final concentrations of 5μg/ml. Cell growth was measured in CPM ^{3}thymidine incorporated into proliferation cells and represented as before as stimulation index (SI units = mean CPM of cells stimulated by antigen/mean CPM of medium control, n=4`). CPM of medium controls: calf 63, 520; calf 64, 794.

7.3.4 Proliferation of pre-inoculation and control PBM to *Neospora caninum* NC1 antigen fractions

7.3.4.1 Experiment B

Pre-inoculation PBM cells from calves 21 and 25 did not respond to NC1 antigen fractions separated by HPLC, but cells from calves 18 and 19 did proliferate in response to antigen fractions 13-21 (figure 7.5a). Cells from the control animals did not respond to any NC1 antigen fractions (figure 7.5b). PBM cells from calves 21 and 64 did not proliferate in response to Con A.

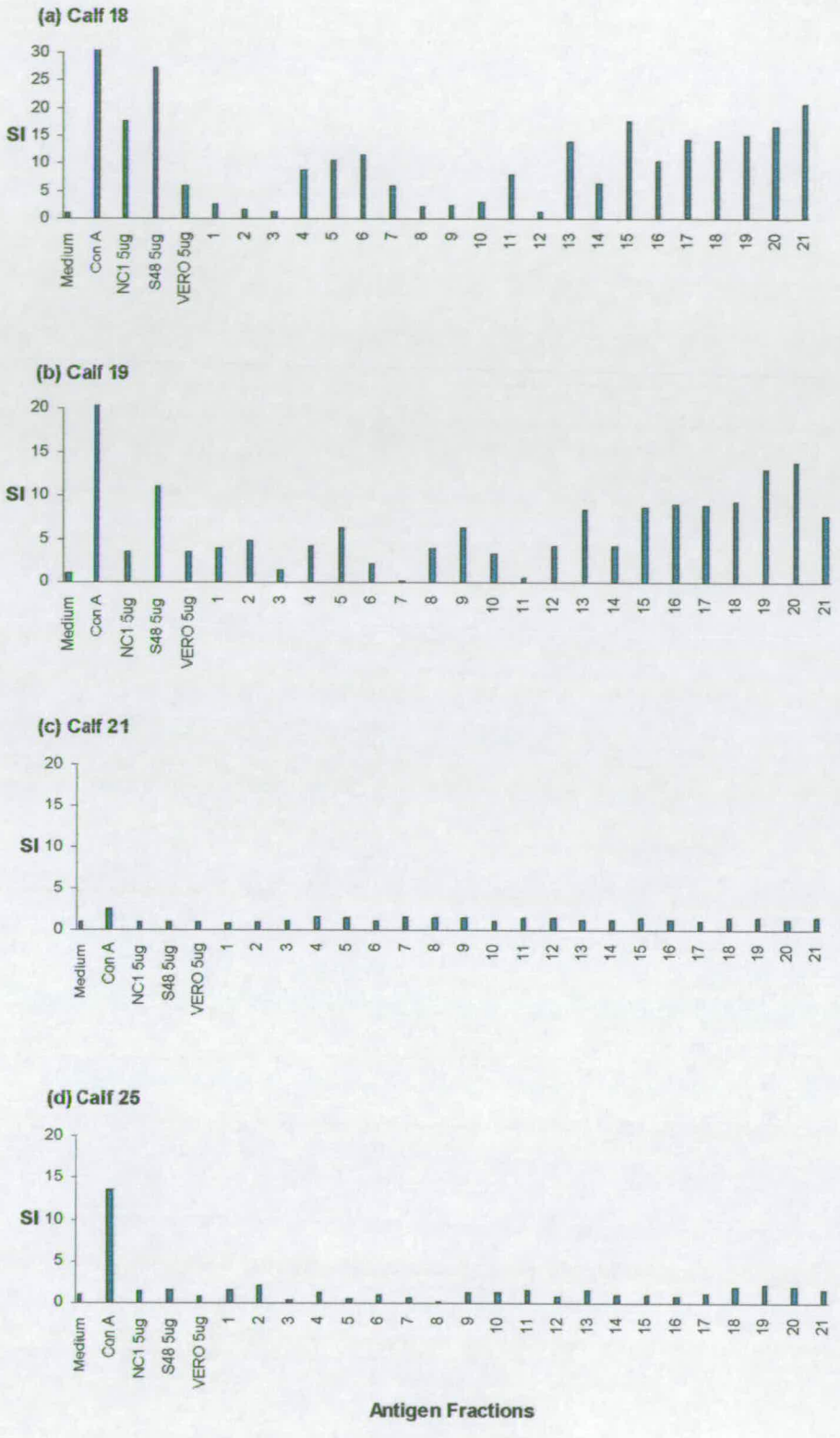
7.3.5 Proliferation of pre-inoculation and control PBM to *Toxoplasma gondii* S48 antigen fractions

7.3.5.1 Experiment B

PBM cells from calves 21 and 25 pre-inoculation (figure 7.6a) and from control calf 64 (figure 7.6b) did not proliferate in response to S48 antigen fractions separated by HPLC. Cells from calf 18 responded to antigen fractions 2, 7 and 12-21, and there was some response from PBM cells from control calf 63 to antigen fractions 11-21. Both of these animals responded to NC1 and S48 wsf antigens. PBM cells from all animals except calf 21 responded to Con A but not to VERO cell antigen. No cells were available for calf 19.

Figure 7.5a

Proliferation of pre-inoculation PBM from group 3 calves in experiment B to *Neospora caninum* NC1 strain antigen fractions separated by HPLC. Cells from calves 18 and 19 were stimulated by Con A and whole *N.caninum* and *T.gondii* water soluble fraction antigen at a final concentration of 5μg/ml, and antigen fractions 1-20, with fraction 1 containing proteins eluted using 100% solvent A (97.5% water/2.5% IPA), and fraction 20 eluted using 100% solvent B (10% water/90% IPA). Fraction 21 is a combination of final washes and contains small amounts of all antigen fractions 1-20. Cells from all animals did not respond to VERO cell antigen. Cell growth was measured in counts per minute (CPM) ^{3}thymidine incorporated into proliferating cells and represented as stimulation index (SI Units = mean CPM cells stimulated by antigen/mean CPM medium control, n=3). CPM of medium controls: Calf 18, 105.4; Calf 19, 372.2; Calf 21, 7456.5; calf 25, 1343.


(a) Calf 18
(b) Calf 19
(c) Calf 21
(d) Calf 25
SI
Medium
Con A
NC1 5ug
S48 5ug
VERO 5ug
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
Antigen Fractions

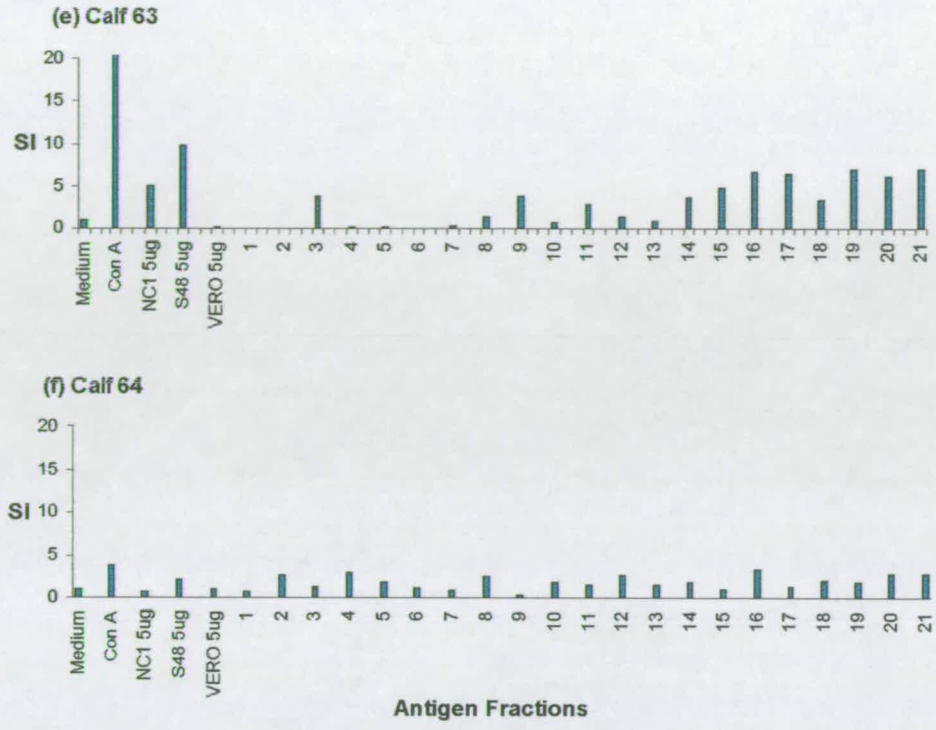
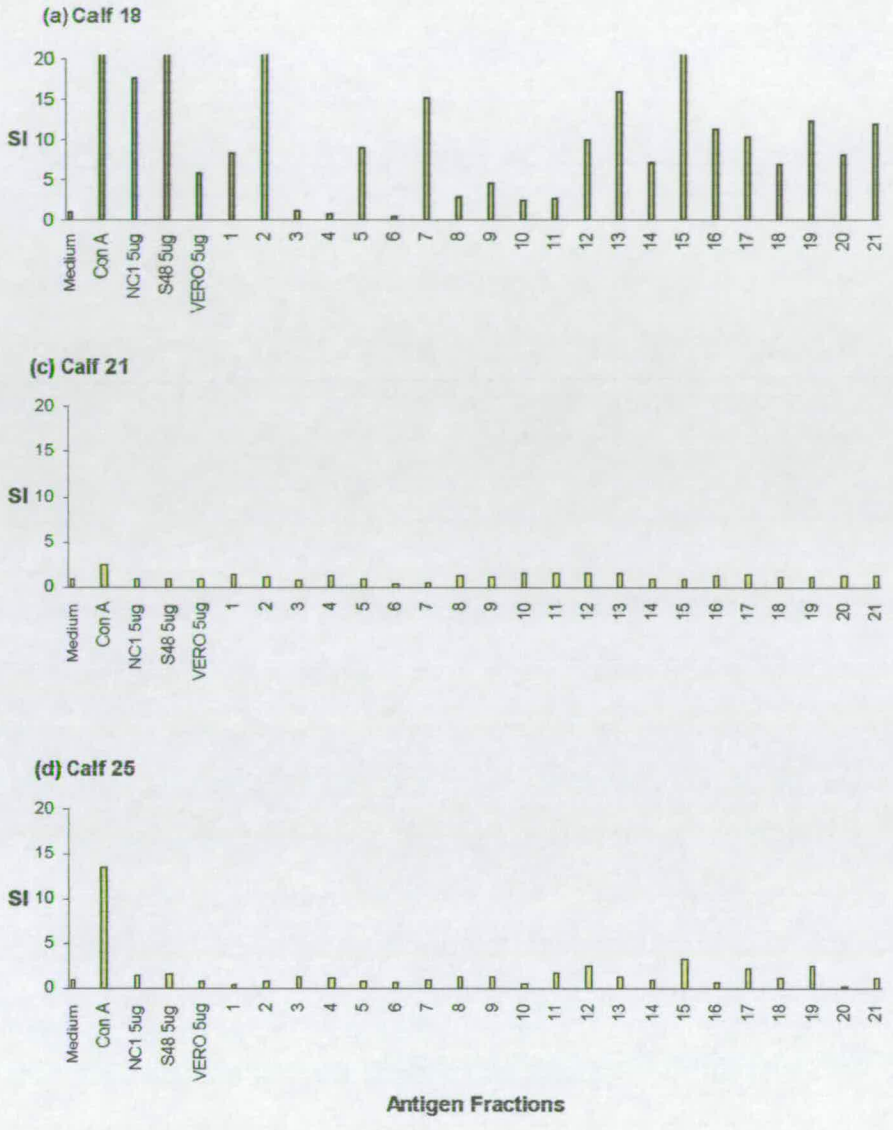


Figure 7.5b Proliferation of PBM from group 2 control calves in experiment B (taken at the same time as pre infection PBM for group 1 calves, fig 7.5) to NC1 antigen fractions separated by HPLC. Antigens are as described in legend for figure 7.5. Cell growth is represented as stimulation index (SI) (n=3). CPM of medium controls: calf 63, 551.3; calf 64, 3702.2.

Figure 7.6a

Proliferation of pre-inoculation PBM from group 3 calves in experiment B to *Toxoplasma gondii* S48 strain antigen fractions separated by HPLC. Cells from calf 18 only were stimulated by Con A and whole *T.gondii* and *N.caninum* water soluble fraction antigens at a final concentration of 5μg/ml, and antigen fractions 1-20, with fraction 1 containing proteins eluted using 100% solvent A (97.5% water/2.5% IPA), and fraction 20 eluted using 100% solvent B (10% water/90% IPA). Fraction 21 is a combination of final washes and contains small amounts of all antigen fractions 1-20. Cells from all animals did not respond to VERO cell antigen.Cell growth was measured in counts per minute (CPM) ^{3}thymidine incorporated into proliferating cells and represented as stimulation index (SI Units = mean CPM cells stimulated by antigen/mean CPM medium control, n=3). CPM of medium controls: Calf 18, 105.4; Calf 21, 7456.5; calf 25, 1343.


(a) Calf 18
SI
20
15
10
5
0
Medium
Con A
NC1 5ug
S48 5ug
VERO 5ug
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
(c) Calf 21
SI
20
15
10
5
0
Medium
Con A
NC1 5ug
S48 5ug
VERO 5ug
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
(d) Calf 25
SI
20
15
10
5
0
Medium
Con A
NC1 5ug
S48 5ug
VERO 5ug
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
Antigen Fractions

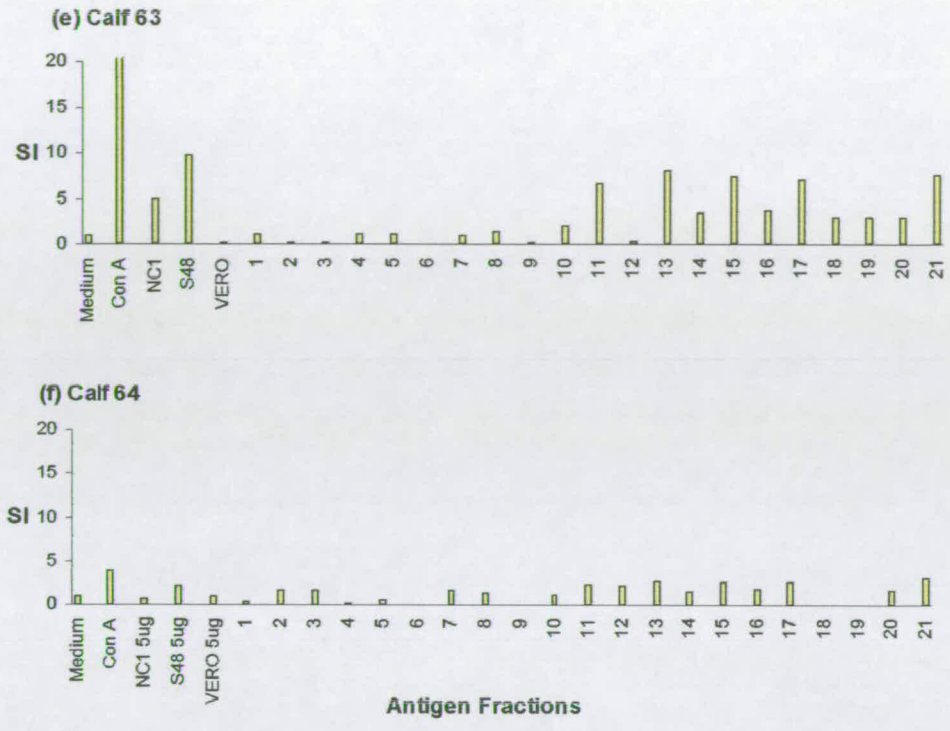


Figure 7.6b Proliferation of PBM from Control animals in experiment B (taken at the same time as for fig 7.6) to S48 antigen fractions separated by HPLC. Antigens are as described in legend for figure 7.6. Cell growth is represented as stimulation index (SI) (n=3). CPM of medium controls: calf 63, 551.3; calf 64, 3702.2.

7.3.6 Proliferation of post inoculation PBM to *Neospora caninum* NC1 antigen fractions

7.3.6.1 Experiment A

Peripheral blood mononuclear cells collected 42 days post inoculation from calves 13 and 14 in group 1 (infected) proliferated in response to whole *Neospora* wsf antigen at final concentrations of 5 and 10μg/ml and to Con A. Calves 20 and 21 responded poorly to con A and NC1 wsf antigen. None of the infected calves responded to the 20 distinct nitrocellulose bound NC1 antigen fractions except for calf 20 which responded to fraction 1 only (figure 7.7).

7.3.6.2 Experiment B

PBM cells collected on day 16 post inoculation from calves 18, 19 and 25 in group 3 (infected) proliferated in response to NC1and S48 wsf antigen and NC1 antigen fractions 10-21 separated by HPLC, however cells from calf 21 did not proliferate any wsf antigens or antigen fractions (figure 7.8). Cells from all animals responded to Con A but not to VERO cell antigen.

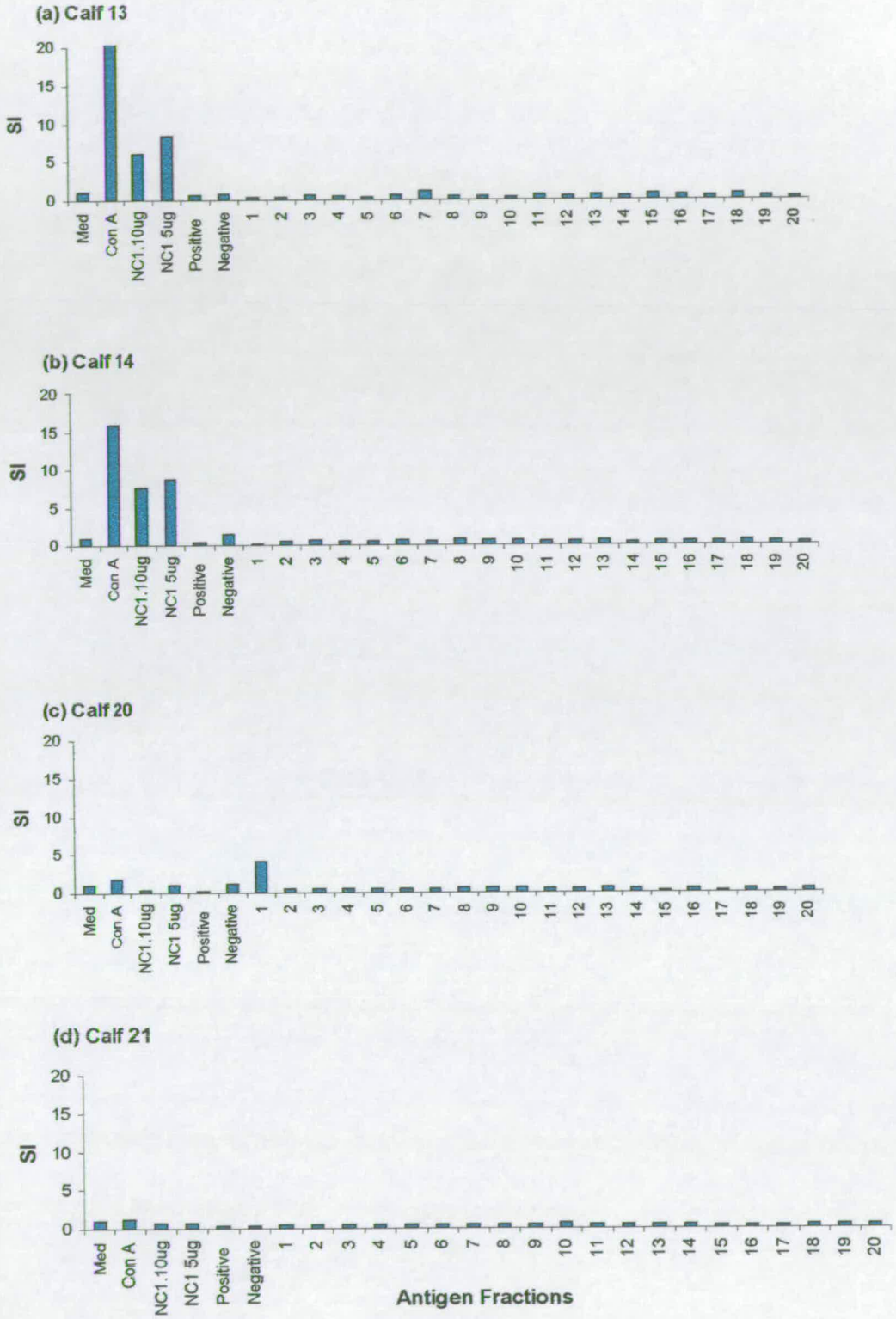
7.3.7 Proliferation of post inoculation PBM to *Toxoplasma gondii* S48 antigen fractions

7.3.7.1 Experiment B

Only calf 25 responded to S48 antigen fractions 10-21 separated by HPLC (figure 7.9). PBM cells taken from all calves responded to both NC1 and S48 wsf antigens. All animals responded strongly to Con A but not to VERO cell antigen.

Figure 7.7

Proliferation of post-inoculation PBM from group 1 calves in experiment A to *Neospora caninum* NC1 strain antigen fractions separated by SDS PAGE. Cells were stimulated by Con A and whole *N.caninum* water soluble fraction antigen at final concentrations of 5 and 10μg/ml, and *N.caninum* specific antigen fractions (numbered 1-20), with fraction 1 the lowest molecular weight, and fraction 20 the highest. The positive control consists of NC1 wsf antigen dot blotted onto nitrocellulose membrane and air dried. The negative control is nitrocellulose membrane alone. Cell growth was measured in counts per minute (CPM) ^{3}thymidine incorporated into proliferating cells and represented as stimulation index (SI Units = mean CPM cells stimulated by antigen/mean CPM medium control, n=3). CPM of medium controls: calf 13,652; calf 14, 1390; calf 20, 11701; calf 21, 11438.


(a) Calf 13
(b) Calf 14
(c) Calf 20
(d) Calf 21
SI
0
5
10
15
20
Med
Con A
NC1.10ug
NC1 5ug
Positive
Negative
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Antigen Fractions

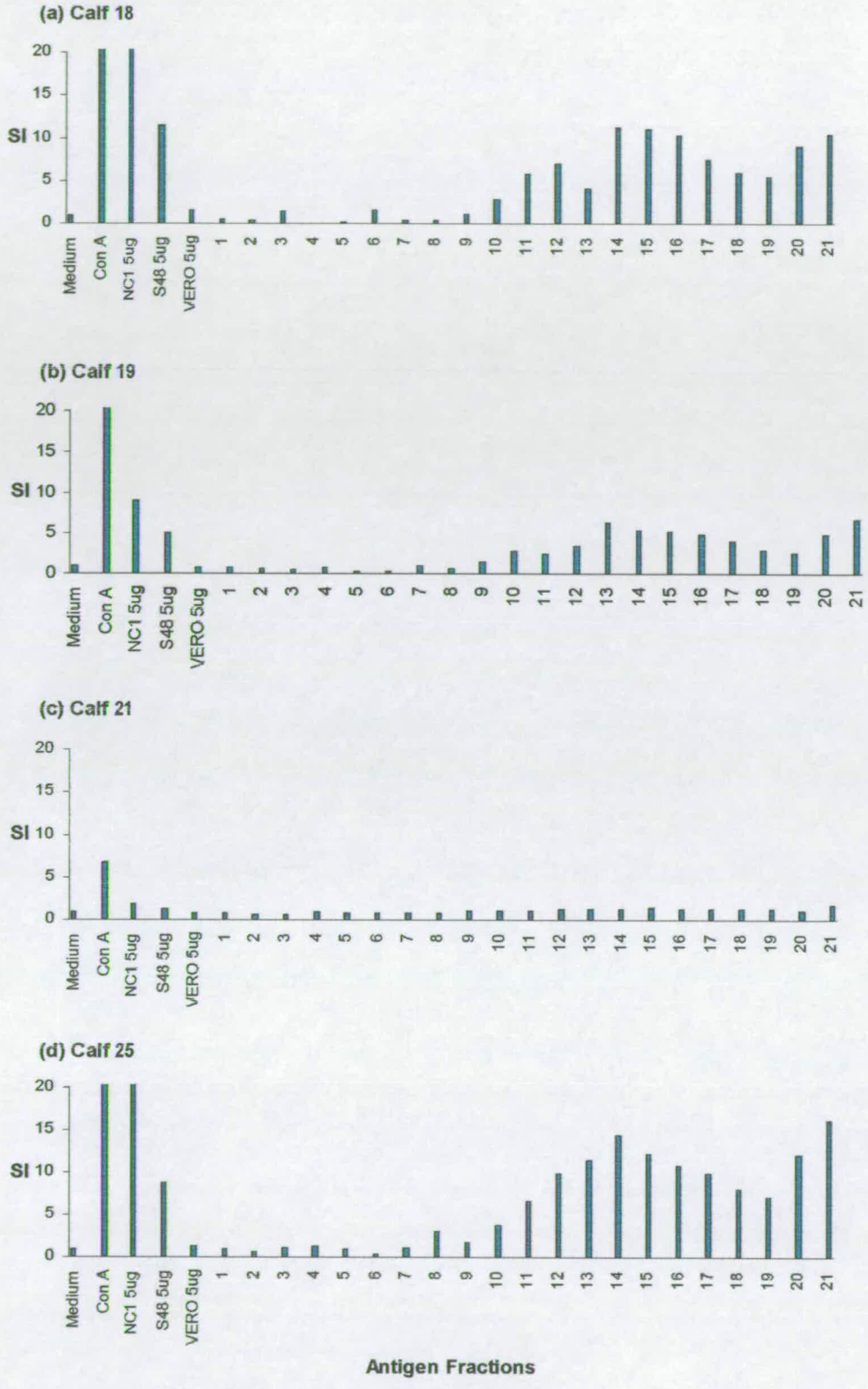


Figure 7.8 Proliferation of post inoculation PBM from group 3 calves in experiment B to NC1 antigen fractions separated by HPLC. Antigens are as described in legend for figure 7.5. Cell growth is represented as stimulation index (SI) (n=3). CPM of medium controls: calf 18, 383; calf 19, 495; calf 21, 2312; calf 25, 356.

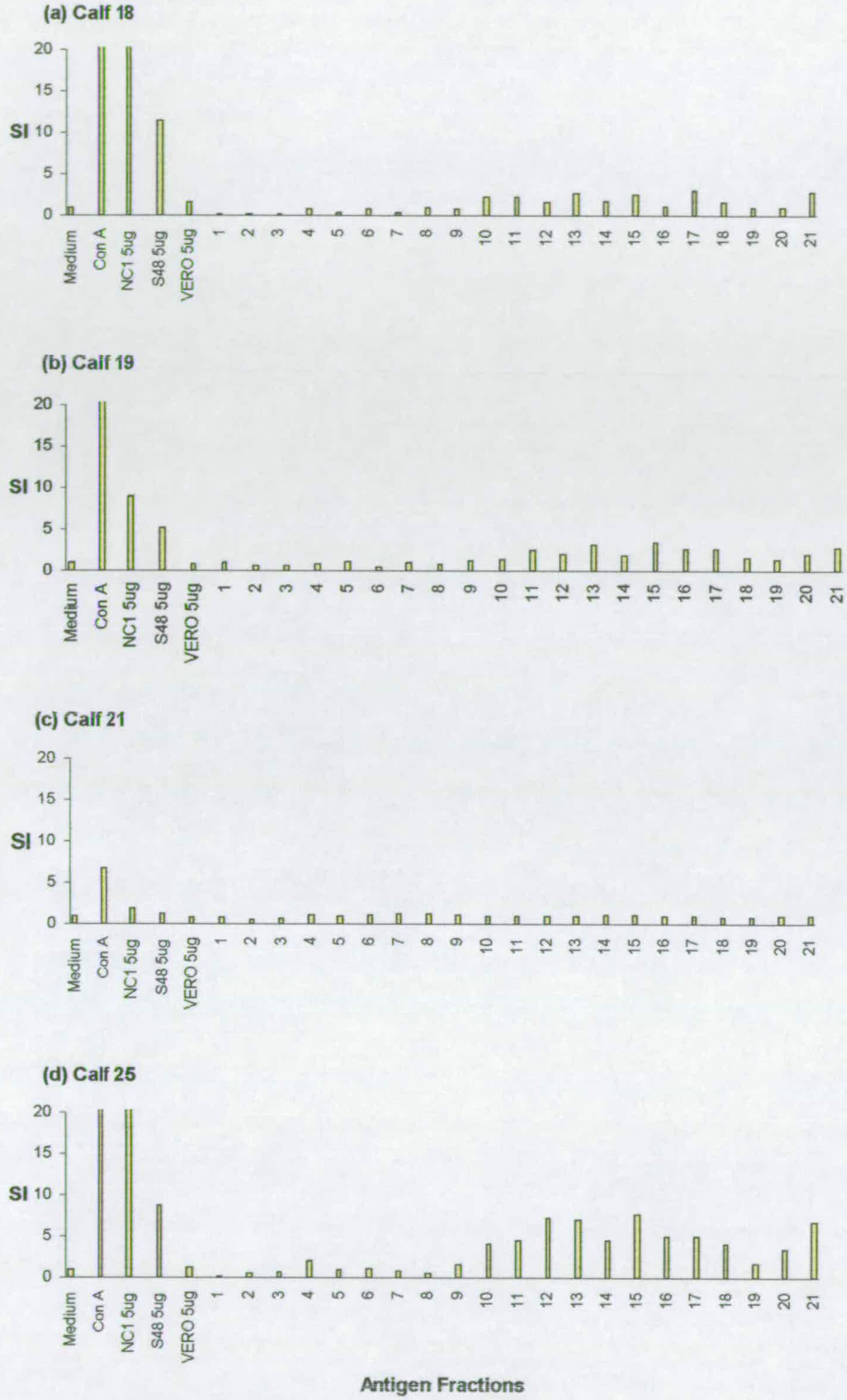


Figure 7.9 Proliferation post inoculation PBM from group 3 calves in experiment B to S48 antigen fractions separated by HPLC. Antigens are as described in legend for figure 7.6. Cell growth is represented as stimulation index (SI) (n=3). CPM of medium controls: calf 18, 387.6; calf 19, 495.3; calf 21, 2311.7; calf 25, 355.6.

7.3.8 Phenotypic analysis of CD4+ T cell lines

7.3.8.1 Experiment A

CD4+ T cell lines were cultured from PBM taken from infected cattle and stimulated with NC1 antigen and IL2 as described in chapter 3, section 3.7.4.1. Phenotypic analysis of the cell lines at the time they were used in proliferation assays with antigen fractions is shown in table 7.5. T cell lines from infected cattle had >97% CD4+ T cells.

Cell lines were also cultured from control cattle. FACS analysis showed these cell lines consisted of fewer CD4+ T cells (≤ 39%) and a greater proportion of CD8+ T cells (≥ 38%) than lines cultured from infected animals (results not shown). The control cell lines were therefore not used in proliferation assays with antigen fractions.

Table 7.5 Phenotypic analysis of cells taken from infected calves in experiment A (no.13, 14, 20 and 21) after stimulation with NC1 *Neospora caninum* wsf antigen for 1 week followed by expansion for a further 7 days with human recombinant IL2. The table illustrates the percentage of cells staining with each of the monoclonal antibodies. Figures are rounded up to the nearest percent. Mab's CC8, CC63 and CC42 were obtained from IAH, Compton and ILA111 from ILRI, Nairobi, Kenya.

Antibody	Specificity	Animal No.			
		13	14	20	21
Control		6	4	5	5
CC8	CD4	99	98	99	98
CC63	CD8	8	11	9	22
CC42	CD2	100	99	100	99
ILA111	IL2R	82	79	100	99

7.3.8.2 Experiment B

CD4+ T cell lines were cultured from PBM cells taken from infected cattle and stimulated with NC1 antigen and IL2 as described in chapter 3, section 3.7.4.1.. Phenotypic analysis of the cell lines at the time they were used in proliferation assays with antigen fractions is shown in table 7.5. T cell lines from infected cattle had >92% CD4+ T cells.

Table 7.6 Phenotypic analysis of cells taken from infected calves in experiment B (no.18, 19, 21 and 25) after stimulation with NC1 *Neospora caninum* wsf antigen for 1 week followed by expansion for a further 7 days with human recombinant IL2. The table illustrates the percentage of cells staining with each of the monoclonal antibodies.

Antibody	Specificity	Animal No.			
		18	**19**	**21**	**25**
Control		11	10	8	9
CC8	CD4	96	94	94	92
CC63	CD8	3	3	3	4
CC42	CD2	92	90	84	89
CC21	B Cell	4	6	5	3
VPM65	Mono/Mϕ	2	2	1	2
CC15	γδ T Cells	2	3	2	4

7.3.9 Response of CD4+ T cell lines to *Neospora caninum* NC1 antigen fractions

7.3.9.1 Experiment A

CD4+ T cell lines generated from the infected cattle showed consistent proliferative activity in response to unfractionated wsf NC1 antigen (figure7.10) (results from a repeat experiment are shown in appendix chapter 7, figure 7.1). When tested with the *Neospora* antigen fractions proliferation of CD4+ T cells was seen in response to a particular group of lower molecular weight antigen fractions (≤30kDa) numbered 14-20. Cells did not proliferate in response to nitrocellulose membrane alone but did proliferate in the presence of whole wsf NC1 antigen dot blotted onto nitrocellulose. All cells proliferated strongly in the presence Con A.

7.3.9.2 Experiment B

CD4+ T cell lines from the infected cattle consistently proliferated in response to S48 whole antigen and NC1 whole antigen and antigen fractions separated by HPLC numbered 9-20 (figure 7.11) (results from a repeat experiment are shown in appendix chapter 7, figure 7.2). Fraction 21 was a combination of washes put through the HPLC column at the end of a run and therefore consisted of a mixture of all of the protein fractions numbered 1-20. This protein mix stimulated a strong proliferative response from all 4 CD4+ T cell lines. Cell lines from calves 18 and 19 responded to Con A but those from calves 21and 25 responded poorly. None of the cell lines responded to VERO cell antigen.

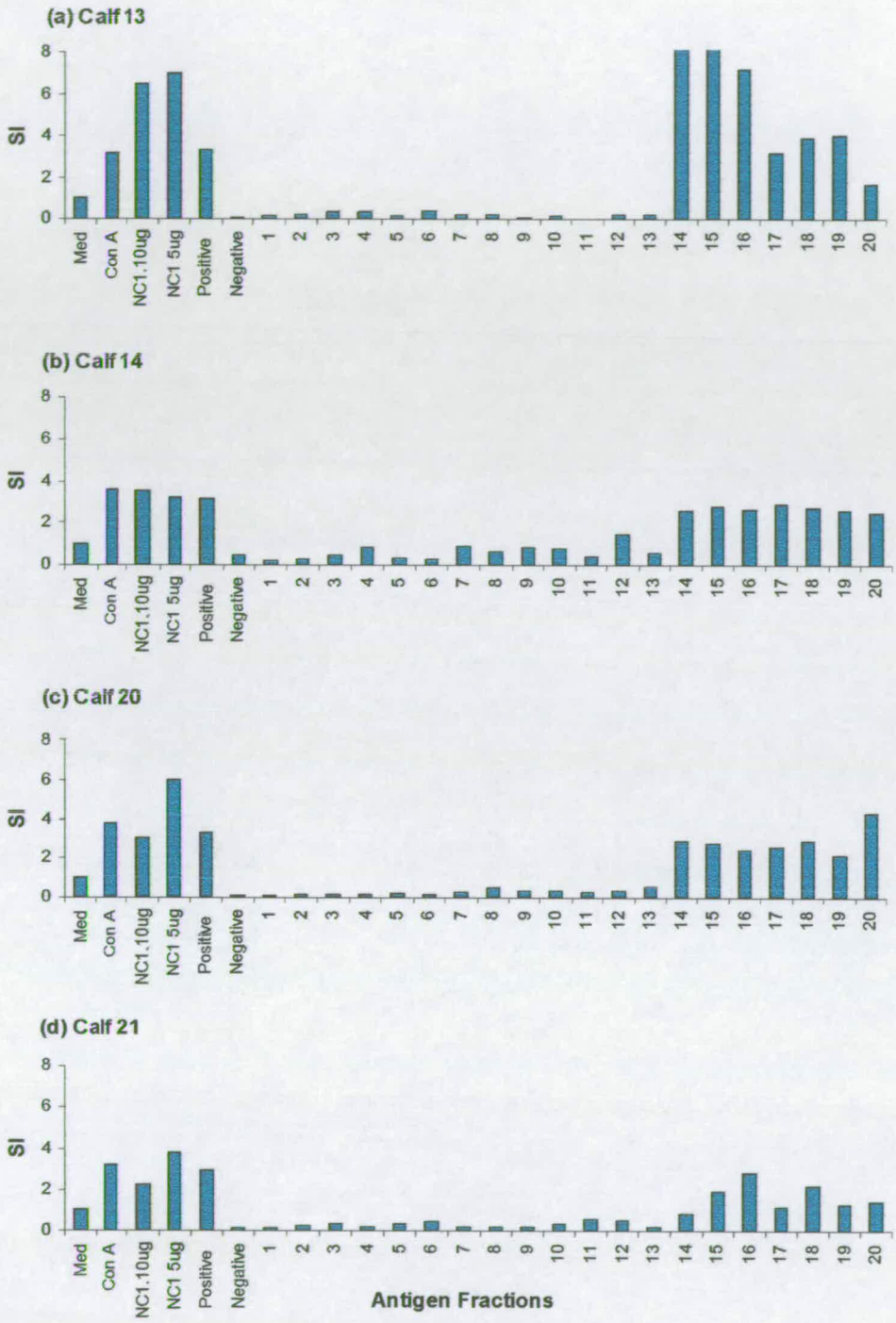


Figure 7.10 Proliferative response of CD4+ T cell lines from group 1 calves in experiment A to *Neospora* antigen fractions separated by SDS PAGE. Antigens are as described in legend for figure 7.7. Cell growth is represented as stimulation index (SI) (n=3). CPM of medium controls: 13, 800; 14,4766; 20, 4306; 21,3769.

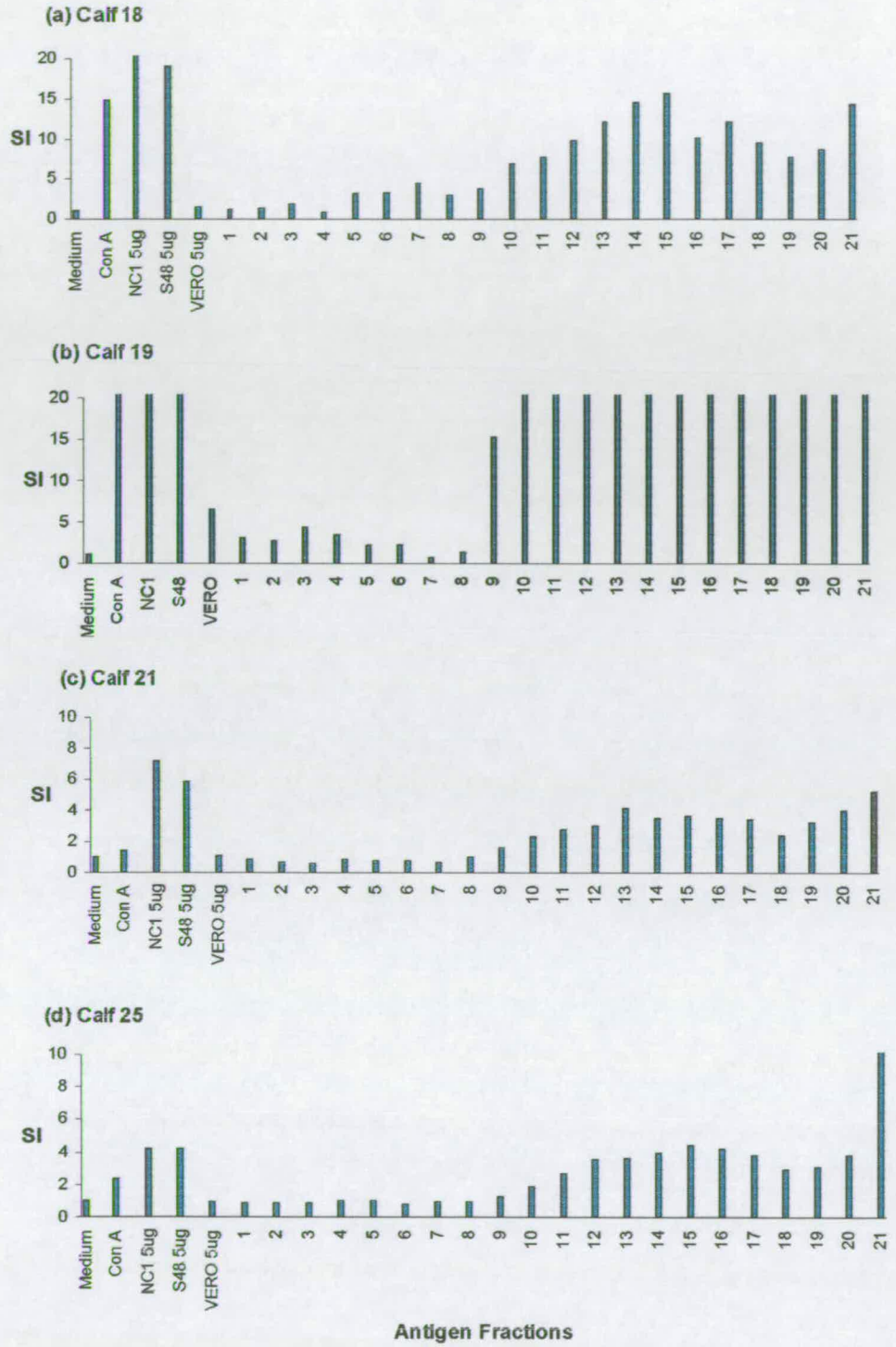


Figure 7.11 Proliferation of CD4+ T cells from group 3 calves in experiment B to NC1 antigen fractions separated by HPLC. Antigens are as described in legend for figure 7.5. Cell growth is represented as stimulation index (SI) (n=3). CPM of medium controls: calf 18, 569; calf 19, 82; calf 21,2452; calf 25, 3504. The SI of cells from calf 19 in response to Con A, wsf antigen and antigen fractions 10-21 was >20.

7.3.10 Response of CD4+ T cell lines to *Toxoplasma gondii* S48 antigen fractions

7.3.10.1 Experiment A

CD4+ T cells from all NC1 infected calves proliferated in response to S48 *Toxoplasma gondii* wsf antigen at final concentrations of 5 and 10μg/ml, but did not proliferate in response to S48 antigen fractions separated by SDS PAGE (figure 7.12). T cell lines from all animals proliferated in response to ConA.

7.3.10.2 Experiment B

CD4+ T cell lines from calves 18 and 19 from group 3 (infected) proliferated in response to NC1 and S48 wsf antigens at a final concentration of 5μg/ml (figure 7.13) (results from a repeat experiment are shown in appendix chapter 7, figure 7.3). Only calf 19 responded strongly to S48 antigen fractions 10-16. Cell lines from animals 21 and 25 responded weakly to Con A and to NC1 and S48 wsf antigens. None of the T cell lines responded to VERO cell antigen at 5μg/ml.

7.3.11 IFNγ Analysis

7.3.11.1 Experiment A

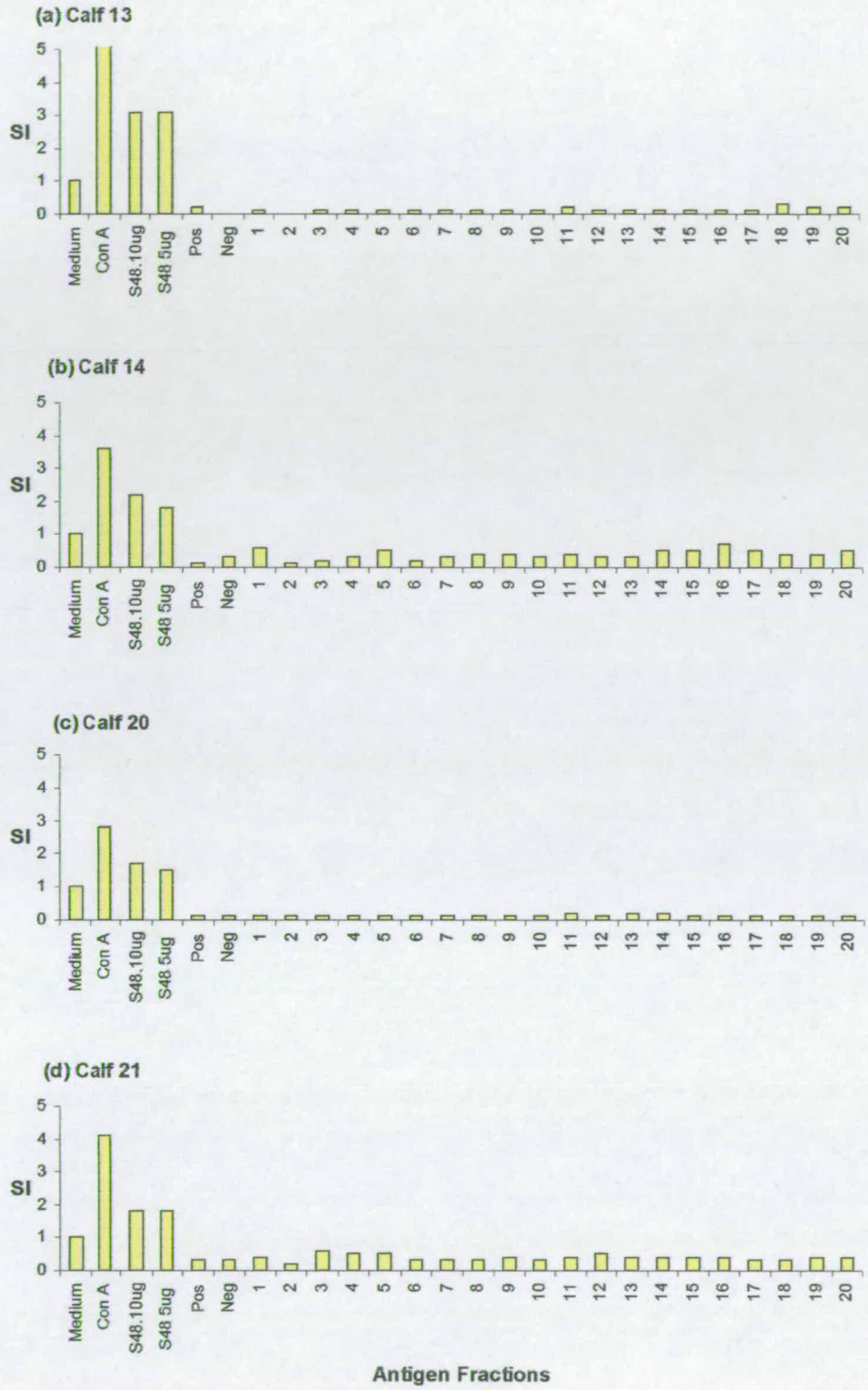
Nitrocellulose bound whole wsf *Neospora* antigen and antigen fractions 14-20 consistently stimulated production of high concentrations of IFNγ from CD4+ T cells generated from the infected animals (table 7.7). Comparatively low levels of this cytokine were detected in cells cultured in medium alone and in cultures of CD4+ T cells incubated with nitrocellulose membrane alone.

Table 7.7 IFNγ produced by CD4+ T cells from group 1 infected calves in experiment A to NC1 antigen fractions 14-20 separated by SDS PAGE. The positive control is nitrocellulose with NC1 wsf antigen dot blotted and air died, and the negative control is nitrocellulose membrane alone.

	IFNγ (U/ml)			
Supernatant	**Calf 13**	**Calf 14**	**Calf 20**	**Calf 21**
Medium	141	74	304	586
Negative	116	118	442	1232
Positive	8256	1312	7136	4864
14	608	416	1888	3488
15	2176	3680	2880	4992
16	2976	2112	4992	6272
17	3904	2624	4608	5856
18	3136	352	4736	4384
19	1920	2176	4416	5152
20	4800	1536	4288	2656

Figure 7.12

Proliferation of CD4+ T cell lines from group 1 calves in experiment A to *Toxoplasma gondii* S48 strain antigen fractions separated by SDS PAGE. Cells were stimulated by Con A and whole *T.gondii* water soluble fraction antigen at final concentrations of 5 and 10μg/ml, and *T.gondii* antigen fractions (numbered 1-20), with fraction 1 the lowest molecular weight, and fraction 20 the highest. The positive control consists of S48 wsf antigen dot blotted onto nitrocellulose membrane and air dried. The negative control is nitrocellulose membrane alone. Cell growth was measured in counts per minute (CPM) ^{3}thymidine incorporated into proliferating cells and represented as stimulation index (SI Units = mean CPM cells stimulated by antigen/mean CPM medium control, n=3). CPM of medium controls: calf 13, 3112, calf 14, 11459, calf 20, 7454.3; calf 21, 4688.3.


(a) Calf 13
(b) Calf 14
(c) Calf 20
(d) Calf 21
SI
0
1
2
3
4
5
Medium
Con A
S48.10ug
S48 5ug
Pos
Neg
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Antigen Fractions

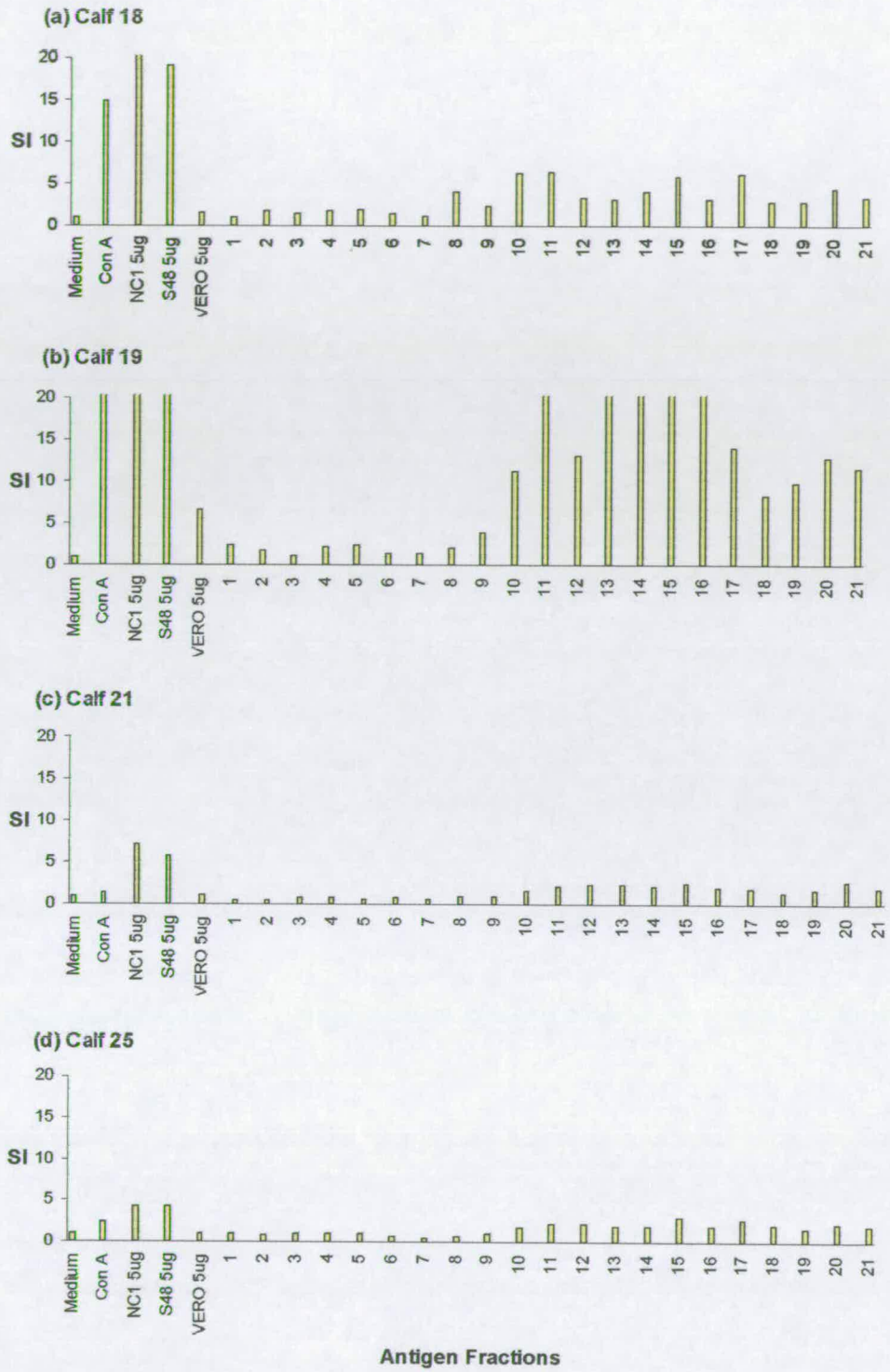


Figure 7.13 Proliferation of CD4+ T cells from group 3 calves in experiment B to S48 antigen fractions separated by HPLC. Antigens are as described in legend for figure 7.6. Cell growth is represented as stimulation index (SI) (n=3). CPM of medium controls: 18, 569; 19, 82; 21, 2452; 25,3504.

7.3.12 Western Blot

7.3.12.1 Experiment A

Antigens recognised by sera from the infected cattle were identified by Western blot (figure 7.15). No *Neospora* specific antibodies were present in sera from the control or pre-infection sera, whereas all four infected cattle showed a strong antibody response at four weeks post infection. *Neospora* specific sera from the infected cattle recognised proteins with molecular weights of approximately 70, 32, 30, 28kDa and also a band at the base of the blot.

7.3.12.2 Experiment B

NC1 and S48 antigen fractions separated by HPLC were run on an SDS PAGE mini gel and stained for total protein using silver stain (section 7.2.6.3) to estimate the molecular weight of each of the fractions. No protein apart from a strongly stained band at approximately 65kDa, estimated to be FBS in the medium in which the fractions were diluted, was present. The molecular weight of the fractions could not therefore be measured (results not shown).

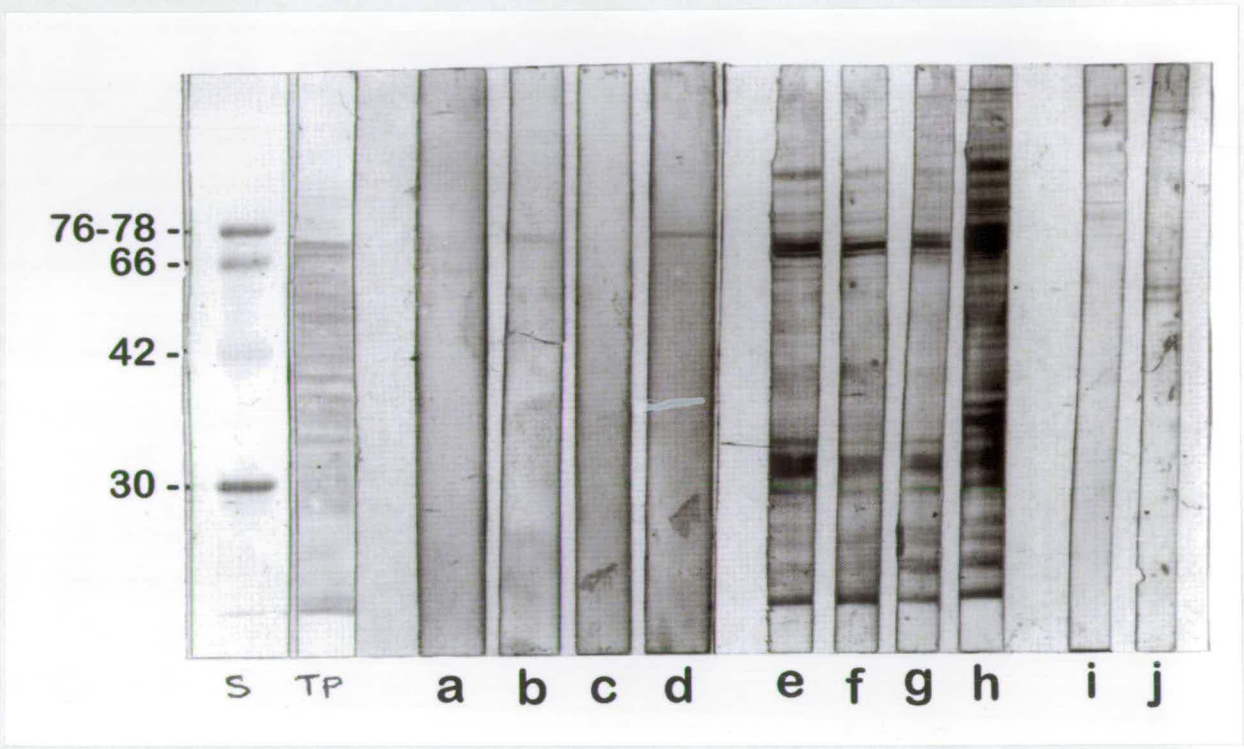


Figure 7.14 *Neospora caninum* NC1 antigen separated by SDS PAGE and probed with pre (day –1) (strips a-d) and post (day 28) (strips e-h) infection sera from calves 13 (a and g), 14 (b and f), 20 (c and g) and 21 (d and h) experimentally infected with 2.5×10^8 NC1 *N.caninum* tachyzoites on day 0. Antigen was also probed with sera from control animals 15 (i) and 25 (j). S, molecular weight standards, T, total protein stained with Ponceau dye.

7.4 Discussion

Results from the study of calves experimentally infected with *N.caninum* NC1 tachyzoites in the previous chapter showed that PBM cells from infected calves proliferated *in vitro* in response to whole crude *N.caninum* wsf antigen, whereas PBM cells from control cattle did not. A simple proliferation assay using crude lysate antigen prepared from NC1 tachyzoites was therefore able to distinguish infected from uninfected animals and showed that experimental infection of calves with *N.caninum* NC1 tachyzoites induces a cell mediated immune response. However PBM from infected calves which were sero-negative for *Toxoplasma* also cross reacted with *T.gondii* wsf antigen. In this study we attempted to identify antigen fractions of *N.caninum* which stimulated the cellular response to infection, and to investigate the response of the CD4+ T cell subset to both *N.caninum* and *T.gondii* antigen fractions as CD4+ cells have previously shown to be important in induction of antigen specific protective immunity to *T.gondii* in sheep.

Animals were inoculated with NC1 strain *Neospora caninum* tachyzoites which were first isolated from the neural tissues of a dog. A comparison of ribosomal DNA from the U1 region of the nss-rRNA gene of *N.caninum* NC1 strain and 4 bovine isolates, BPA 1-4, showed no variation between any of the isolates in this region, identified as being evolutionarily unstable (Marsh *et al*, 1995). A closer comparison of the 1.7kb of the nss-rRNA gene of BPA1 and NC1 also revealed no differences. To date no antigenic differences have been identified between these isolates. Therefore we believe it is appropriate to use the NC1 strain in experimental infection in cattle.

All inoculated animals in experiment A and animals 19 and 25 in experiment B responded to *N.caninum* and *T.gondii* wsf antigens and the non-specific mitogen Con A indicating a cell mediated response was occurring in the peripheral blood from these animals. The remaining two infected animals in experiment B, numbers 18 and 21, did not respond either to parasite wsf antigen or to Con A. This suggests that PBM cells from these animals were generally hyporesponsive, although why this occurred is not known. All infected animals did however sero-convert and were

antibody positive for *N.caninum* by week 4 post infection. Control animals remained sero-negative.

SDS PAGE separated nitrocellulose bound antigen was a combination of *N.caninum* SDS and wsf soluble antigens. As SDS is a detergent it is likely that the SDS fraction antigen included hydrophobic membrane antigens not present in the water soluble antigen preparation. Including both in the nitrocellulose bound preparation was an attempt to ensure a larger spectrum of antigens were incorporated. SDS PAGE soluble antigen in aqueous form could not be added directly to cells in culture as SDS is toxic to cells, therefore soluble antigen used in proliferation assays incorporated only the water soluble fraction of *N.caninum* tachyzoites. A cell mediated response to *N.caninum* antigen fractions separated by SDS PAGE could not be detected in pre or post infection PBM cells from infected calves. However *Neospora* specific CD4+ T cell lines cultured from PBM cells from infected calves consistently proliferated in response to SDS PAGE separated *Neospora* antigen fractions 14-20 of molecular weight ≤30kDa, and HPLC separated *Neospora* antigen fractions 10-21. Some pre-inoculation proliferation of PBM cells to S48 and NC1 fractions separated by HPLC was observed but it appeared to be non-specific and in each case the animals responded to different combinations of fractions from 1-21. Supernatants from the activated CD4+ T cells incubated with SDS PAGE fractions 14-20 contained high concentrations of IFNγ which as previously discussed is likely to be important in protective immunity (Innes *et al*, 1995a; Khan *et al*, 1997). These low molecular weight proteins may therefore be responsible for the induction of a potentially protective cell mediated immune response to neosporosis.

Peripheral blood mononuclear cells and CD4+ T cells from *N.caninum* infected cattle which were sero-negative for *T.gondii* proliferated in response to *T.gondii* wsf antigen indicating the parasites share some common antigens recognised by the cellular immune response. However CD4+ T cell lines generated post inoculation from 7 of the inoculated animals did not respond to S48 antigen fractions separated by either SDS PAGE or HPLC, with only one CD4+ T cell line from calf 19 proliferating in response to S48 antigen fractions separated by HPLC (10-16). Pre-inoculation PBM cells from calf 18 and calf 63 (control) did show some

response to S48 fractions separated by HPLC but again each of the animals responded to a different combination of fractions suggesting the response may have been non-specific. Lack of specific response to particular antigens suggests that the cross reactive cell mediated response is unlikely to be cross protective. These results therefore agree with the results of previous studies. PBM cells from mice experimentally infected with *N.caninum* have also been shown to proliferate in response to *T.gondii* antigen but production of significant levels of IFNγ did not protect against subsequent infection with *Toxoplasma* (Lindsay *et al*, 1990a). Lack of cross protection was also seen in a study by Innes (paper in preparation) where experimentally induced abortion in sheep inoculated with *Neospora* tachyzoites was not affected by pre-immunisation with the S48 Toxovax vaccine strain of *T.gondii.* By contrast experimental *N.caninum* infection in mice has been shown to stimulate a cell mediated response which is protective against challenge infection with *T.gondii* mediated by CD8+ T cells (Kasper and Khan, 1998). Protection was however dependant on 2 factors; (1) the size of the *N.caninum* inoculum, as mice infected with $5x10^4$ tachyzoites were not protected against toxoplasmosis, and (2) the size of the *T.gondii* challenge, as mice were not protected against a challenge dose of $2x10^3$ RH strain tachyzoites. Complete protection was only seen against the less virulent PLK strain of *T.gondii,* therefore protection is also dependant on virulence of the parasite. Some cross-protection may therefore exist but evidence suggests that it may only be partially protective.

The response of CD4+ T cells to *T.gondii* wsf antigen implies that proliferation may be induced by a *T.gondii* superantigen (Sag), previously identified by Denkers *et al* (1994), which is capable of activating T cells non-specifically and highly polarises the immune system to a Th1 response (Denkers, 1996). *Toxoplama gondii* Sag has previously been shown to activate CD4+ and CD8+ T cells (Herrmann *et al*, 1990; Herman *et al*, 1991) and stimulates production of high levels of IFNγ by binding to the outer face of the MHC II molecule rather than within the peptide binding groove and therefore does not require intracellular processing to non-specifically stimulate non-immune T cells and stimulate production of high concentrations of IFNγ. This response, however, may not be protective as

overstimulation of IFNγ can lead to increased disease pathogenesis and possibly result in deletion or anergy of responsive cells (Denkers, 1996).

Specific antibodies to the closely related parasite *T.gondii* have previously been shown to destroy tachyzoites in the presence of complement (Sabin & Feldman, 1948), overcome the parasite's ability to prevent its destruction within the host macrophage (Joiner *et* al, 1990) and may also inhibit host cell penetration by the tachyzoite (Schwartzman, 1986). Where protection against a pathogen is mediated by both humoral and cell mediated immunity an effective vaccine needs to incorporate antigens capable of activating both T and B cell epitopes (Lamb *et al*, 1988).The humoral immune response to *Neospora* may therefore play a role in the host's defence against clinical neosporosis. Several NC1 antigens of approximately 70, 32, 30 and 28kDa and a band at the base of the western blot were consistently recognised by sera from the infected animals. These antigens need to be further characterised to determine whether or not they may induce a protective immune response. Therefore the three lowest molecular weight antigens (≤30kDa) identified by SDS PAGE separation of NC1 antigen which were recognised by both the humoral and cell mediated responses may be important in the future development of a vaccine against neosporosis. Unfortunately I was not able to compare the molecular weight of HPLC separated NC1 antigens which stimulated a response from infected calves with the nitrocellulose bound SDS PAGE separated antigens, therefore further work is needed to clarify if these antigens are of the same molecular weight.

While the nature of protective immunity to *Neospora* has yet to be determined it is likely that both IFNγ and antibody production will play important roles. Therefore the specific parasite antigen fractions involved in induction of humoral and cell mediated responses identified in this study, which stimulate both production of IFNγ and a parasite specific antibody response, may be important in the development of a vaccine to induce a potentially protective immune response in naive animals. Further work is needed to characterise these important antigens and to identify the type of immune response that will protect against clinical neosporosis.

Publications arising from this work:

Marks J., Lundén A., Harkins D. and Innes E. (1997). Identification of *Neospora* antigens recognised by CD4+ve T cells and immune sera from experimentally infected cattle. *Parasite Immunology*, in press.

Marks J., Lunden A. and Innes E.A. (1997). Identification of specific *Neospora caninum* antigens which stimulate CD4+ T cells from immune cattle. Association of Veterinary Teachers and Research Workers, Scarborough, 25-27th March.

Marks J., Lunden A. and Innes E.A. (1997). Identification of Specific *Neospora caninum* antigens which stimulate CD4+ T cells from infected cattle. COST 820 Working Group 4 on Vaccines Against Animal Coccidiosis, Wusterhausen, Germany, 17-18th April.

Chapter 8

General Discussion and Concluding Remarks

8. General Discussion and Concluding Remarks

Neosporosis is emerging as an important disease for the dairy industry and beef production in the UK. Farmers sustain substantial financial losses through abortion of calves, production of weak calves and decreased milk production caused directly by abortions early in gestation. The effects on public health have not been adequately investigated but as experimental infection has been shown to cause congenital defects in primates similar to those caused by toxoplasmosis we must be aware that this disease may be of public health importance.

Neospora was first isolated into tissue culture only 10 years ago and very little is known about the disease it causes, pathological consequences of infection or the immune response to the parasite. Repeat abortion due to neosporosis does occur in some animals but clearly most develop an immune response to infection which prevents further loss. By understanding the immune mechanisms at work in the majority of animals it may be possible to develop a vaccine to prevent clinical neosporosis in cattle.

Development of effective disease control strategies depends on the ability to accurately diagnose the disease and the identification of modes of transmission and high risk groups most likely to be affected. Diagnostic tests currently available are not accurate enough to reliably identify individuals infected with *N.caninum*. Development of more sensitive and specific tests would complement development of treatment regimes, vaccines and control strategies by pinpointing those animals likely to abort or which provide a reservoir of infection in the herd. We must first understand the effects of disease on the host, persistence of the parasite and susceptibility of the host to reinfection/recrudesence of the disease before we can develop an accurate model of infection and develop reliable diagnostic tests. Very few longitudinal studies on the progression and effects of neosporosis on individual farms have previously been undertaken.

In this study we monitored serum antibody titres within a dairy herd which had suffered losses due to neosporosis over a three year period and in individual cattle that had previously aborted due to infection with *N.caninum.* A record was also kept of the incidence of *Neospora* linked abortions which occurred during this time. This study therefore monitored the long term effects of neosporosis on the dairy farmer. This project also reports on a study of the humoral immune response to infection with *N.caninum.* Previous studies have shown that some cross reaction may occur in response to conserved antigens shared by closely related parasites. Comparison of antigens recognised by sera from animals infected with a range of organisms closely related to *N.caninum* in this study has identified potentially *Neospora* specific antigens, which could be used to develop more accurate diagnostic tests. This study also reports marked variability in the intensity of antibody response to parasite antigens that could have far reaching consequences for the development of more sensitive and accurate diagnostic tests. And finally this project also investigates the cell mediated response to experimental infection with the parasite. *Neospora* is very closely related to *T.gondii* a parasite which causes abortion in sheep. An incomplete vaccine strain of *T.gondii,* S48, is already commercially available which protects against toxoplasmosis induced abortion. This study reports on the immune response in cattle, the natural host, to infection with *N.caninum* and therefore provides the first opportunity to compare the immune response to that observed in response to *T.gondii* in sheep. Previously no information on the cell mediated immune response to *Neospora* in cattle had been published.

8.1 Longitudinal sero-epidemiology survey of a dairy farm in the Ards Peninsula in Northern Ireland

Neosporosis is recognised as a major cause of bovine abortion worldwide, but the full implications of infection within the dairy herd and the disease epidemiology are

poorly understood. The main focus of this project has been to conduct a longitudinal sero-epidemiological study in seven cattle that aborted due to infection with *Neospora* and seven control cattle in the same herd, the abortion rate in the herd over the three-year period and also the annual herd sero prevalence. In addition, pre-colostral sera were taken from newborn calves to determine the presence of antibodies to *N.caninum* which would indicate that congenital infection had taken place.

Of a total of 29 reported abortions/resorptions 14 occurred at the beginning of the investigation during the abortion storm of August /September 1995. The sudden onset of a large number of abortions within a short time period suggests a point source of infection in the environment. The dog has only recently been identified as the definitive host of *Neospora* and the existence of oocysts has been confirmed making this route of infection a realistic possibility. Silage stored open to environmental contamination, communal feeders easily accessed by farm dogs, rodents and local wildlife and aborted foetal tissue may all have been sources of infection in this case. Improved feed storage in concrete or brick built bins, immediate disposal of aborted tissues and limited access of dogs and local wildlife to the herd could therefore be important for prevention of infection. In particular identification of possible wildlife reservoirs of infection, using highly sensitive methods of parasite detection such as PCR would be important in the development of control strategies.

Dams that aborted during the abortion storm of August/September 1995 became sero negative by ELISA/IFAT for *Neospora* antibodies 3-4 months post abortion and serum antibody levels in the herd were observed to fluctuate annually. It is likely however that animals were persistently infected rather than re-infected the following year when serum antibody levels increased during gestation in 1996 as no new cases of abortion were reported at that time. The fall in antibody titres may be due to low levels of parasite antigen persisting in tissues, as has been previously reported (ref), or to the parasite persisting only in the CNS of infected animals, an immunologically privileged site. As these animals can produce congenitally infected calves potentially creating a reservoir of infection in the herd, development of improved methods of diagnosis that

can accurately identify infected dams is important for control of this disease. Pre-colostral serum samples from calves infected *in utero* were strongly *Neospora* antibody positive at birth, therefore using this method one can accurately identify congenitally infected animals. One method of disease control discussed in the literature is the removal of infected calves by selective culling. However replacement of potentially immune animals with naïve calves onto pasture contaminated with *N.caninum* oocysts could result in an increase in abortions after primary infection, as is the case with the closely related coccidian *T.gondii*. Further work to assess levels of contamination in the environment and the abortion risk as a result of primary infection in cattle are needed before advice can be given to farmers on how to avoid losses caused by infection with *N.caninum*.

Stress induced by major changes in environment or diet may be a factor in reproductive loss. The farmer reported that abortions and resorptions occurred at the time the animals were taken off feed supplements and were put onto pasture around 3-4 months gestation. In several cases animals which showed signs abortion was imminent did not abort when they were removed from pasture, housed and given extra feed. Environmental stress during early pregnancy could therefore be a factor in onset of abortion in persistently infected animals, but clearly we have insufficient data to draw any firm conclusions from observations reported in this study.

The rate of repeat abortion due to neosporosis on the farm was estimated at between 10-21%, at least double the rate previously published (Dubey and Lindsay, 1996). This may be due to parasite strain variation relating to virulence or to the limited numbers of animals in this study. Other influences not previously taken into account such as regional changes in farm practices, climate and topography of the local environment may also affect abortion rates. Further work is needed to identify conditions that could increase susceptibility to infection and trigger abortion in a relatively small percentage of animals affected.

This study shows that neosporosis can cause considerable financial loss in a small dairy herd over an extended period of time. As the farmer in this study operates a

semi-closed herd, mainly replacing old stock with calves produced on the farm, future losses caused by congenitally infected but currently undiagnosed individuals, which may provide a reservoir of infection in the herd, are possible. The high calving index suggests that infection with *Neospora* may affect the fertility of the herd, resulting in further financial losses. Clearly identification of other possible sources and routes of infection is crucial for the development of effective strategies for the control of this economically important disease.

8.2 *N.caninum* specific Antigens Recognised by the Humoral Immune Response of Naturally Infected Cattle

Commercial tests currently available for diagnosis of neosporosis are not sufficiently accurate for identification of persistently infected individuals. As discussed in the previous section (8.1) development of a more specific and sensitive diagnostic test using a selection of *N.caninum* specific antigen fractions widely recognised by naturally infected cattle may improve diagnosis of infected animals. This approach to the development of a diagnostic test may also overcome possible cross reaction with antigens of closely related organisms. The aim of this study was to identify relevant parasite antigens that may be incorporated into a test of this kind.

Neospora caninum wsf antigen was probed with sera from calves experimentally infected with *N.caninum* NC1 tachyzoites, and with naturally infected cattle sera from both the study farm in Northern Ireland and a survey of neosporosis in Scotland. An antigen of approximately 65kDa present in both NC Liv and NC1 *N.caninum* isolates was commonly recognised, and in many cases strongly recognised, by serum antibody from the majority of these animals. This antigen, identified as a protein fraction, was also found to be parasite specific as it was not recognised using sera from cattle and sheep experimentally and naturally infected with the closely related coccidian parasites *Toxoplasma gondii, Sarcocystis tenella, Cryptosporidium parvum* and *Babesia sp.*

Baszler *et al*, (1996) previously reported the 65kDa antigen of the NC1 isolate as being widely recognised by sera from naturally infected cattle in Washington State, USA, but treatment of the antigen with an oxidising agent (periodate treatment) prevented it from being recognised. It was concluded that the epitope was not a protein but was in fact a carbohydrate molecule bound to the surface of the protein. In contrast this study showed that oxidation did not affect the binding of parasite specific antibody to the 65kDa antigen therefore suggesting that the epitope recognised is a protein. Culture of organisms *in vitro* over a long period of time can lead to antigens being 'lost' or no longer expressed which could explain the absence of recognition in the study by Baszler. But it is important to recognise that comparisons made between studies will not be accurate until isolates currently grown in tissue culture are typed and characterised. Results do however suggest that a combination of antigen fractions incorporated into a diagnostic test may be needed to overcome possible strain differences in different regions.

A *Neospora* antigen of approximately 18kDa cross reacted with both *Babesia bovis, Babesia bigemina* and *Sarcocystis tenella* positive sera, and other bands also bound *Babesia sp.* and *Toxoplasma gondii* positive sera, indicating that there are cross reactive antigens shared between the closely related coccidian parasites. This evidence supports the development of a diagnostic test which relies on a selection of parasite specific antigens. As the intensity of staining for individual protein bands varied between animals the sensitivity of a test of this kind may depend on the incorporation of several proteins which are widely recognised yet parasite specific.

Some success has already been achieved in the development of a diagnostic ELISA which uses recombinant *Neospora* antigens (Lally *et al*, 1996), but as only a few bovine serum samples were tested in the development of this kit it is not clear if the antigens used were parasite specific. However the results of tests using a limited number of serum samples showed that a combination of parasite antigens did provide more reliable results than incorporation of only one antigen into an ELISA of this kind. This study has identified a widely recognised *Neospora* tachyzoite antigen which could be

used to provide reliable and possibly more sensitive antigen specific diagnosis. It is important to recognise that the most readily available source of antigen for use in diagnostic tests is the tachyzoite which multiplies within cells in tissue culture and is a product of repeated selection of fast growing tachyzoites. Current opinion suggests that cattle may remain persistently infected following a primary challenge with *Neospora* when the parasite changes to the bradyzoite form and is maintained within a tissue cyst. Work on the closely related parasite *T.gondii* has shown that there are stage specific antigens characteristic of tachyzoites and bradyzoites. It is likely that the same will be true of *N.caninum* and it may be more pertinent to include bradyzoite antigens along with tachyzoite antigens in a diagnostic kit to enable the detection of persistently infected animals which are currently displaying fluctuating antibody titres to tachyzoite antigens when followed throughout gestation. Future work will characterise and investigate recognition of these immunodominant tachyzoite antigens and will identify *Neospora* bradyzoite antigens which could be of use in the development of more sensitive and specific diagnostic tests.

8.3 Cell Mediated Immune Response

Neospora caninum is very closely related to *T.gondii*, also an obligate intracellular coccidian parasite, which can cause abortion and congenital defects in sheep and humans. The immune response of the host to infection with *T.gondii* has been extensively researched and a live vaccine is currently commercially available for prevention of abortion in sheep. Using the technique of lymphatic cannulation it has been shown that the S48 vaccine works by stimulation of the cell mediated immune response resulting in protective immunity (Innes and Wastling, 1995). Previous studies have also shown that the cytokines IFN γ and IL12 are important in natural resistance to infection with *Neospora* tachyzoites in mice (Khan *et al*, 1997). At the time of conducting this study there were no references in the literature concerning cell mediated immunity to *N.caninum* in cattle.

In this study experimental infection of calves with *N.caninum* tachyzoites induced a measurable cell mediated immune response which could be detected in peripheral blood. Proliferation of PBM to a crude lysate *N.caninum* antigen was characterised by production of IFNγ. Therefore a simple cell proliferation assay can distinguish between infected and uninfected calves. However the role of the cell mediated response to infection with *N.caninum* in pregnant cattle is unknown. Studies of human gestation using *in vitro* models have identified factors released during gestation that can modulate the immune response to protect the foetus, which is essentially foreign tissue, from expulsion. Many of the factors produced only during pregnancy, including progesterone induced blocking factor (PIBF), trophoblast cell derived factor (TCDF) and placental suppressor factor (PSF), down regulate lymphocyte proliferation and activity *in vitro* and may act by shifting the immune response from a Th1 to a Th2 pathway (Ragupathy, 1997). It is therefore difficult to extrapolate results of this study to infection with *N.caninum* during gestation.

Although infection with *N.caninum* has been linked to high levels of bovine abortion, the cause of foetal death and expulsion is unknown. Infection during pregnancy with an intracellular organism may stimulate a strong Th1 response, possibly overriding the affects of Th2 inducing factors and indirectly inducing rejection of the foetus and abortion. Raised levels of IFNγ and tumor necrosis factor (TNF), both T cell cytokines, have been linked with significantly higher numbers of foetal resorptions in mice infected with *Leishmania major* compared to controls (Krishnan *et al*, 1996). Induction of cell mediated immunity during pregnancy has also been associated with increased rates of abortion caused by infection with *Plasmodium falciparum* in humans (Mendez, 1995). Another possible explanation for induction of abortion could be that the placenta, which produces some immuno-modulating factors, may become an immuno-privileged site incapable of mounting an effective local inflammatory response to intracellular infection. Uncontrolled parasite replication could cause extensive placental tissue damage, leading eventually to foetal death and expulsion. As only very few parasites persist in aborted foetal tissues and lesions associated with antigen are rare it is unlikely

that the foetus itself is a target for parasite invasion and replication. But placental tissue is often difficult to obtain and little is known about the effect of intracellular parasite infection on the placenta during pregnancy. The presence of immuno-modulatory factors may also affect recrudescence of neosporosis in persistently infected individuals. Further studies on the impact of infection with *N.caninum* in the pregnant cow and the effects on the foetus and placenta are needed if we are to understand how mummification and abortion of foetal tissue is induced by neosporosis.

This study also investigated the proliferative response of PBM from calves experimentally infected with *Neospora* to *Toxoplasma* antigen *in vitro*. PBM from calves, which were sero-negative for *T.gondii*, proliferated in response to both *T.gondii* and *N.caninum* lysate antigen after inoculation with *N.caninum* tachyzoites. This interesting observation led us to speculate whether this cross reactive T cell response may also imply cross protection between the two pathogens. However, as immunity to intracellular pathogens is highly dependant on cytokine production we may predict that cross protection to toxoplasmosis in *Neospora* infected animals is unlikely to occur as IFNγ levels in supernatants from PBM taken from animals infected with *N.caninum* and stimulated with *T.gondii* antigen were substantially lower than in those cultures stimulated with *N.caninum* antigen. Previous studies have also shown that experimental infection with *Neospora* in mice does not protect against toxoplasmosis (Lindsay *et al*, 1990) and that inoculation of sheep with *T.gondii* tachyzoites does not protect against challenge infection with *Neospora* tachyzoites (Innes *et al*, 1998). Further studies to investigate the specificity of this response and the cytokine profile of T cells induced by infection are needed before we can understand if this response has any applications in the immunological control of neosporosis or toxoplasmosis.

8.4 *N.caninum* specific Antigens Recognised by the Cell Mediated Immune Response of Experimentally Infected Cattle

Identification of specific *N.caninum* antigen fractions responsible for stimulation of the cell mediated immune response to infection with *Neospora* could be important for the future development of an effective vaccine against neosporosis. In addition, as discussed in the previous section (8.3), investigation of the specificity of the cross reactive immune response to *T.gondii* antigen fractions may also be beneficial for vaccine design.

Induction of the CD4+ T cell subset has previously been shown to be important in stimulation of antigen specific immunity to *T.gondii* in sheep (Innes *et al*, 1995b). In this study CD4+ T cell lines generated from *Neospora* infected calves consistently proliferated in response to a group of low molecular weight proteins of ≤30kDa separated by SDS PAGE and blotted onto nitrocellulose. Recognition of antigen fractions separated by HPLC was also examined. The proliferative response to the SDS PAGE separated fractions was characterised by production of IFNγ which as previously discussed is likely to be important in protective immunity to intracellular pathogens (Innes *et al,* 1995a) and maintenance of resistance to *N.caninum* infection in the mouse model (Khan *et al*, 1997). Several of these antigens of approximately 28 and 30kDa were also recognised by the humoral immune response in these calves. Specific antibodies to *T.gondii* have been shown to play an important role in preventing re-infection (Sabin and Feldman, 1948; Joiner *et al*, 1990; Schwartzman, 1986). Where protection against a pathogen is mediated by both humoral and cellular immune responses, an effective vaccine needs to incorporate antigens capable of activating both T and B cells responses (Lamb *et al*, 1988). As these *Neospora* derived low molecular weight antigens stimulate both the humoral and cell mediated response they may be of interest as potential vaccine candidates. However, we need to further investigate which are the critical immune responses important in resistance to the parasite and how these responses may be altered or compromised in the pregnant compared to the non-pregnant

host. Cell mediated immune responses have been shown to be important in development of immunity to the protozoan parasite *Theileria parva*, which can cause acute and often fatal disease in cattle. Adoptive transfer of CD8+ cytotoxic T lymphocytes specific for parasitised lymphoblasts and restricted by MHC class I can confer protection on naive animals subsequently challenged with the parasite (McKeever *et al*, 1994). Recipients rapidly controlled infection and did not develop clinical disease. Investigation of the role of MHC in determining variation of CTL response from the same animals to several different strains of *T.parva* used defined MHC phenotypes to quantify CTL pre-cursors of different MHC restriction specificity. The CTL response was frequently found to be strain specific and restricted by class I MHC haplotype. This restriction of CTL response in individual animals focused on a limited number of immunodominant antigenic determinants, selection of which is influenced by both host and parasite genotype (Taracha *et al,* 1995). This finding clearly has important implications for vaccine development indicating multiple proteins containing epitopes represented in all parasite strains need to be incorporated if protection against infection/disease is to be complete.

The observation that PBM from *Neospora* infected calves (which were naïve to *T.gondii)* will also react with *Toxoplasma* wsf antigen *in vitro* was reported in chapter 6 of this study. This pattern of response has also been reported in studies of cell proliferation responses in *N.caninum* infected mice (Khan *et al,* 1997). Interestingly this study showed that CD4+ T cell lines raised from PBM from *Neospora* infected calves did not proliferate in response to *Toxoplasma* antigen fractions separated by SDS PAGE and HPLC. This would suggest that the observed cross reactive response in PBM was not specific and therefore unlikely to be cross protective. The results of this study therefore agree with those of previous studies which showed that although the organisms are closely related experimental infection with *Neospora* or *Toxoplasma* did not induce cross protection in sheep (Innes *et al*, 1998) or mice (Lindsay *et al,* 1990a).

8.5 Future work and Concluding Remarks

Results of this study indicate that *Neospora caninum* is an economically important pathogen, causing substantial losses to the dairy farm investigated. A three year longitudinal serology study following infected animals within the herd showed that the antibody titre fluctuated substantially over time and throughout the gestation period. This fluctuation in antibody titre made it difficult to accurately identify individual infected animals using existing serology based assays. No strategies have yet been proposed for the control of *Neosporosis*. Development of better diagnostic tests to identify persistently infected animals which act as a reservoir of infection within the herd is clearly important. The recent finding that the dog is the definitive host of *N.caninum* indicates that contamination of pasture with oocysts may also be a source of infection to cattle and wildlife. Studies of toxoplasmosis have shown that management of wildlife reservoirs of disease are important for disease control. Future work should therefore investigate possible wildlife reservoirs of disease using the highly sensitive technique of PCR to identify parasite in tissues. If parasite DNA is detected in animal reservoirs further development of specific molecular probes to enable typing of *Neospora* strains would allow us to investigate the role of wildlife as a reservoir for infection in cattle.

A further study identified a *Neospora* specific antigen of approximately 65 kDa widely recognised by sera collected from both naturally and experimentally infected animals. It is hoped that this antigen may be developed further for use in an improved diagnostic test. Comparison of the work presented in this thesis with previously published data shows that antigenic differences exist between parasite isolates and also in preparations of parasite antigen made from tachyzoites of the same *Neospora* strain. The many differences between studies make comparisons difficult. Therefore future work should concentrate on a direct comparison of parasite isolates using standard sera to identify antigens which will be widely recognised. Further investigation into possible cross reaction with antigens of other protozoan parasites not investigated in this thesis such as *Eimeria* and *Hammondia* are also important in determining antigen specificity.

Identification of bradyzoite antigens which may be more pertinent in accurately identifying persistently infected animals could also be important for accurate diagnosis.

Finally, results of this study indicate that cell mediated immune responses, found to be important in immunity to other intracellular protozoans, may be important in the development of immunity to *Neospora*. As >95% of animals that abort due to infection with *N.caninum* do not abort for a second time it is likely that a protective immune response aimed against *N.caninum* induced abortion (similar to the S48 vaccine which prevents abortion due to *T.gondii* in sheep) may be a realistic aim. This study investigated the immune response in non-pregnant calves. Immunomodulation during pregnancy caused by release of soluble factors such as PIBF and PSF which cause a shift in immune response from cell mediated to humoral are likely to greatly affect the outcome of infection. Further studies should focus on the response to infection in the pregnant animal and therefore on the development of a model of disease in pregnant cattle for investigation of local immune responses to infection paying particular attention to cytokine profiles, and also for comparison of pregnant animals infected both early and late gestation. Also investigation into pathogenesis in the placenta caused by infection with *Neospora* may be important in determining the cause of foetal death.

There are still many questions to examine in this fascinating host parasite relationship. In particular the comparative biology of *N.caninum* and *T.gondii* is an extremely interesting area which I have just touched on in this thesis. The complete host range of *N.caninum* is not known, nor whether *N.caninum* can infect or cause disease in people. We still do not understand why *N.caninum* will cause disease in cattle but not in sheep and *T.gondii* causes disease in sheep but not in cattle. There is clearly a lot we can learn about *N.caninum* based on what we know of *T.gondii* but we should be cautious in extrapolating too freely. Results reported in this thesis indicate that management and control of neosporosis may be a realistic aim following further investigation of this complex and costly parasite.

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Appendix Chapter 4

Questionnaire: Farm study

Epidemiology of *Neospora caninum*

Farmer's Name

Farm Name

Address

Map Reference

Type of enterprise - dairy/sheep/mixed

upland/lowland/improved pasture

Number and type of animals

Calving/lambing dates

Does the farmer operate a closed/open herd

If animals are bought in, from where, how many, type, any health problems

Any special problems on the farm - flood, overrun with vermin, drought, etc

Management system;

Feed -

- Rotation of grazing on the farm, during which months
- What kind(s) of feed is used (type manufacturer, does it change at different times of the year)
- Is any winter feed grown on the farm (silage, barley, etc.)
- How is feed stored

- Do any other animals have access to it (dogs, cats, rodents, wild animals)
- How often are supplies delivered
- How are animals fed

Waste –

- How is it disposed of
- Area of application, topography, what is grown there (animal feed?)
- If applied as fertiliser, timing of application

Housing –

- How are animals housed
- What other animals have access to animal housing
- How are they cleaned out and how often
- Drainage systems in housing
- Bedding - where does it come from, how/where is it stored
- e.g. is it a deep litter system, and if so for which animals, time of year and for how long.

What borders the farm - Caravan parks, riding school, housing estate, etc.

Local climate - e.g. if near the sea less likely to develop frost, warmer, precipitation, temp., etc.

Any information on local wildlife, carnivores in particular, any hunting or shooting on the premises? If so when, any animals involved, how many people, etc.

People associated with the farm - how many, what are their occupations/ages

Water supply on the farm - do any streams or rivers pass through or along the edge of the property

Disease history of animals going back up to 5 years - is it possible to get access to records:

- fertility problems
- vaccination programmes
- unexplained reduction in milk yield

Milking parlour –

- Describe the routine of milking of cows
- What other animals have access to the milking parlour (farm dogs, rodents, etc.)
- How often is it cleaned and with what
- Where is it situated

Recent outbreak of Neosporosis –

- Date of outbreak
- How many heifers aborted
- Time scale
- Any tissues collected or stored
- Were any animals bought in before the outbreak

Did anything unusual occur in the previous few months before the outbreak or were any practices changed?

Documents

Try to obtain a plan/layout of the farm and medical records for heifers affected

Answers to epidemiology questionnaire

Study Farm
Ards Peninsula, Northern Ireland
Sept 1995

147 acres total (117 owned, 30 rented to the south of the farm).
Silage land is SW and S of WC on map.
28th Aug- Abortions occurred
In the next 14-21 days a total of 14 abortion took place
Foetal tissue of 6 cows went into VI Stormont
Cows that aborted were due to calve at christmas, therefore foetus' ~6.5 mths old
Half that aborted would be first calvers (7 cows)
Total of 127 heifers on the farm
Dairy farm, no other livestock, two sheep dogs (1 male, 1 female, no pups)
Farmer replaces his own stock mostly - semi closed
10 of the herd were bought in, two last year from Kinross, Fife, which were pedigree Ayrshires. Both healthy, have been no problems.
Cows went to grass on the 10th April and soon after two of the cows that had been PD'd +ve were PD'd -ve. Not known if they aborted in the field or reabsorbed -
*Could have been a source of infection to other cows
1st of these two cows, No. 408, was served again and will be calving in March.
7 of original aborting cows left. Were sold or slaughtered for reasons other than Neo., e.g. lameness, etc.
Are two farm dogs, 1 male, 1 female (has never had a litter of pups).

Tag numbers of those that did abort;
4, 7, 63,141, 42, 38, 114, 102, 9, 125, 101, 94, 45, 200, 408, 103, 17, 140.
Tag nos. of those that aborted and the farmer still has;
4, 7, 63, 141, 38, 114, 102, 103.

Some home reared beef cows on the farm which only stay on the farm until 15mths old. Then sold to a neighbouring farm.
There is a total of 127 cows, 17 of which will be culled next year ('96) before the rest are put to grass in the spring.

Buildings

Cows do not have access to drains

Herd Health

No particular signs of sickness before or a time of abortions
Last winter were some early foetal deaths, not a major problem, at 8-12 week stage of gestation (don't know how many).
Calving starts in Oct and ends in March, concentrated in Dec. and Jan.
All those that aborted were Friesians
Leptospirosis vaccination was lapsed last year, and the new calving heifers (high risk Neo. group) were not done. All cows were vaccinated for previous 5-6 years.
When the cows were served was different than usual - served in Jan-Feb by a mixture of servers (all AI).
Cows were dirty (discharge) after abortion, except for two that nearly went to term, e.g no. 102 was the latest gestation to abort and is the cleanest of all the Neo. pos. cows.
Also some drop in milk yield after abortion.

Feed and Water

All cows eat the same silage. Bioferm is added to the silage, a live lactobacillus strain which ferments and breaks down cellulose into sugars. The farm dogs (two collies) sometimes run along the passage that the cows eat the silage from, but do not soil there. Have access to this at any time. Silage is open plan, under a large open fronted barn and covered by black plastic. Cows diet supplemented with dairy meal - Bibbys fed from April, small amount fed all summer. Stored in a concrete built block bin, no access to

cats, dogs or rodents. Delivered every fortnight. Uses the mains water supply. The male dog sometimes swims in the water the cows drink.

Housing

Sawdust bought in last year for bedding and used during winter of '94-'95. 500 bags delivered in Nov. '94, bought from a local supplier, don't know where they came from originally. Sawdust was damp.

Housed in cubicles, 30 on rubber mats which are lymed, cleaned out twice a day, and 70 on sawdust, straw and lyme, bedded as necessary. All have drainage, automatic scrapers in the passages clear 6 times a day.

Milking Parlour

Herringbone system. 100 cows feed out of 10 troughs. Dogs can get access.

Rodents and Wildlife

Rats can be a problem from time to time, not that many. Dealt with within a week to 10 days. Can get into contact with feed in feeders (not in bins). Many hares in the area, and a few badgers and foxes

Slurry

Stored in underground tanks which can carry up to 350,000 gallons. Slurry passes directly through a slatted wooden floor in the housing into tanks. Put out onto the fields used to grow grass for silage during spring and all through summer. It is not usually put on grazing ground but last year as it was so hot and dry some was put on land where the cows were grazing.

Surrounding Area

The farm is 50 ft above sea level on flat land. It is therefore quite exposed, and is surrounded on three sides by the waters of Strangford lough. The sea acts as insulation

during winter, therefore there is little frost and practically no snow. This area gets approxomately 30 inches of rainfall each year.
There is a caravan park situated right on the border of the farm near the farmhouse. One man who uses the park during August walks his dog across the farm land.

Grazing

21 day grazing cycle. Some slurry put on grazing land last summer as it was so dry and hot. Not usually done. 2 heifers were grazed outside normal boundaries, both PD'd +ve, but later found to be -ve. Numberof only one of these cows known - 93 (PD'd 10wks +ve in March). Cows were put onto silage land to graze last summer which is not usually done.

Appendix Chapter 4 : Table 4.1, Bleed 1 Sera collected on 22/9/95. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by IFAT and ELISA. All animals in bleed 1 had aborted during August/September '95.

Cow No.	Reference No.	IFAT Titre	ELISA PP
4	1	1:2048	36
38	2	1:4096	27
63	5	1:4096	29
114	7	1:2048	21
141	8	1:4096	42

Appendix Chapter 4: Table 4.2, Bleed 2 Sera collected on 22/11/95. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by IFAT and ELISA.

Cow No.	Reference No.	IFAT Titre	ELISA PP
4	1	1:512	11
6	2	Neg	3
7	3	1:256	12
9	4	1:128	13
10	5	1:512	16
17	6	1:512	11
32	7	1:64	9
36	8	1:512	13
38	9	1:512	14
45	10	1:256	15
63	11	1:512	17
68	12	Neg	2
68	13	Neg	3
81	14	1:512	9
89	15	1:512	8
93	16	1:128	9
100	17	1:512	11
102	18	1:128	10
103	19	1:256	14
108	20	1:256	9
114	21	1:128	12
119	22	1:128	16
120	23	1:256	10
127	24	1:256	10
141	25	1:256	19
144	26	1:256	10
145	27	1:256	10
170	28	1:16,384	118

Appendix Chapter 4: Table 4.3, Bleed 3 Sera collected on 12/2/96. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by IFAT and ELISA.

Cow No.	Reference No.	IFAT Titre	ELISA PP
4	3	1:512	28
6	5	1:512	7
7	6	1:512	10
9	8	1:2048	9
32	20	1:128	9
36	22	1:128	8
38	24	1:1024	16
45	25	1:1024	11
63	39	1:1024	10
68	44	1:512	8
89	52	1:1024	7
100	57	1:1024	10
102	58	1:1024	7
103	59	1:2048	7
108	62	1:1024	8
114	66	1:2048	19
119	69	1:512	17
120	70	1:256	11
141	76	1:1024	22
144	79	1:512	9
145	80	1:512	12
170	81	1:512	10

Appendix Chapter 4: Table 4.4, Bleed 4 Sera collected on 5/4/96. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by IFAT and ELISA.

Cow No.	Reference No.	IFAT Titre	ELISA PP
4	1	1:1024	27
7	2	1:512	16
32	3	1:256	11
36	4	1:512	12
38	5	1:1024	29
45	6	1:1024	22
63	7	1:512	14
89	8	1:512	12
100	9	1:256	11
102	10	1:1024	9
103	11	1:1024	24
108	12	1:512	9
114	13	1:1024	22
119	14	1:256	18
120	15	1:1024	13
127	16	1:256	11
141	17	1:1024	28
144	18	1:1024	9
145	19	1:256	11
408	20	1:2048	66
Calf	21	1:8192	

Appendix Chapter 4: Table 4.5, Bleed 5 Sera collected on 25/6/96. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by IFAT and ELISA.

Cow No.	Reference No.	IFAT Titre	ELISA PP
4	1	1:512	69.8
7	2	1:64	46.2
32	3	1:256	38.5
36	4	Neg	32.4
38	5	1:256	66.8
45	6	1:256	59.5
63	7	1:256	39.3
89	8	1:32	24
102	9	Neg	34
103	10	1:128	56.9
108	11	1:512	35.1
114	12	1:256	59.5
119	13	1:512	54.2
120	14	Neg	42
140	15	Neg	34
141	16	1:128	51.5
144	17	Neg	27.9
145	18	1:64	25.6
200	19	1:128	55.7
408	20	1:512	67.9
2591	21	1:2048	145.8

Appendix Chapter 4: Table 4.6, Bleed 6 Sera collected on 26/9/96. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by ELISA.

Cow No.	Lab Ref No.	ELISA PP
4	1	170.2
32	3	12
38	5	44.7
103	10	51.3
108	11	4.9
114	12	89.7
119	13	82.2
120	14	14.3
141	16	25.6
144	17	24.6
145	18	19.4
153		138.7

Appendix Chapter 4: Table 4.7, Bleed 7 Sera collected on 1/11/96. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by ELISA.

Cow No.	Reference No.	ELISA PP
4	3	72.8
7	4	23.7
17	11	18.7
36	26	18.3
38	28	34.5
63	47	33.3
68	52	20.4
81	62	8.7
89	70	9.4
93	73	64
100	79	13.3
102	81	12.3
103	82	90.9
108	87	12.6
114	92	94.3
119	96	107.7
120	97	16.9
127	103	14.2
140	110	36
141	111	33.3
144	114	14.2
145	115	7.3
200	118	130.3

Appendix Chapter 4: Table 4.8, Bleed 8 Sera collected on 2/1/97. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by ELISA.

Cow No.	Reference No.	ELISA PP
4	7	71.2
7	8	139.3
32	1	28.8
38	9	77.5
89	2	20.5
100	3	22.7
102	10	23.7
103	11	79.5
108	4	16.8
114	12	37
119	13	66.8
120	14	26.6
127	5	12.6
140	15	34.5
141	16	33.8
144	18	37
145	6	20.1
200	17	66.9
7	19	141.7
104	20	116.9

Appendix Chapter 4: Table 4.9, Bleed 9 Sera collected on 8/5/97. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by ELISA.

Cow No.	Reference No.	ELISA PP
4	1	77
21	2	179.1
32	3	22.7
38	4	43.5
100	5	28.4
102	6	22.3
103	7	59.3
108	8	18.3
114	9	69.1
119	10	68.7
120	11	35.2
141	12	36.3
145	13	17.3
200	14	56.8

Appendix Chapter 4: Table 4.10, Bleed 10 Sera collected on 16/7/97. *Neospora* antibody titre in sera from cattle on the study farm in Northern Ireland, measured by ELISA.

Cow No.	Reference No.	ELISA PP
4	1	162
32	3	25.4
38	4	52.5
103	5	50.8
108	6	20.3
114	7	50
119	8	78.8
120	2	26.3
141	9	44.9
144	10	23.7
145	11	24.6
153	12	119.5

Appendix Chapter 4: Table 11, Herd Bleed 1 Sera collected on 12/2/96. *Neospora* antibody titre in sera from all cattle in the herd on the study farm in Northern Ireland, measured by IFAT and ELISA.

Cow No.	Reference No.	IFAT Titre	ELISA PP
1	1	1:512	7
3	2	1:512	7
4	3	1:1024	28
5	4	1:1024	13
6	5	1:512	7
7	6	1:512	10
8	7	1:1024	7
9	8	1:2048	9
11	9	1:512	11
12	10	1:512	7
15	11	1:1024	7
16	12	1:1024	10
22	13	1:512	7
23	14	1:512	8
24	15	1:512	9
26	16	1:1024	9
27	17	1:512	6
28	18	1:512	9
29	19	1:1024	9
32	20	1:128	9
35	21	1:256	7
36	22	1:128	8
37	23	1:512	9
38	24	1:1024	16
42	25	1:1024	21
44	26	1:1024	9

45	27	1:1024	11
48	28	1:256	12
49	29	1:512	5
50	30	1:512	10
51	31	1:1024	7
54	32	1:512	6
55	33	1:1024	10
56	34	1:1024	6
57	35	1:512	8
58	36	1:256	6
59	37	1:1024	10
62	38	1:512	9
63	39	1:1024	10
64	40	1:2048	14
65	41	1:1024	9
66	42	1:256	7
67	43	1:1024	5
68	44	1:512	8
70	45	1:1024	9
75	46	1:2048	8
78	47	1:1024	5
80	48	1:512	8
84	49	1:2048	7
85	50	1:1024	9
86	51	1:1024	11
89	52	1:1024	7
95	53	1:512	8
96	54	1:1024	7
97	55	1:512	8
99	56	1:1024	7
100	57	1:1024	10
102	58	1:1024	7
103	59	1:2048	19
104	60	1:1024	12

105	61	1:512	9
108	62	1:1024	8
109	63	1:512	16
111	64	1:512	6
113	65	1:256	9
114	66	1:2048	19
115	67	1:2048	27
117	68	1:512	12
119	69	1:512	17
120	70	1:256	11
124	71	1:1024	9
126	72	1:256	12
128	73	1;512	11
132	74	1:256	6
140	75	1:512	12
141	76	1:1024	22
142	77	1:512	7
143	78	1:512	9
144	79	1:512	9
145	80	1:512	12
170	81	1:512	10
171	82	1:512	8
200	83	1:256	14
202	84	1:512	14
203	85	1:256	10
204	86	1:256	8
205	87	1:512	15
206	88	1:512	8
207	89	1:256	10
208	90	1:512	10
411	91	1:512	7
1108	92	1:512	9
1171	93	1:256	9

Appendix Chapter 4: Table 12, Herd Bleed 2 Sera collected during Nov'96. *Neospora* antibody titre in sera from all cattle in the herd on the study farm in Northern Ireland, measured by ELISA.

Cow No.	Reference No.	ELISA PP
2	1	18.7
3	2	13.6
4	3	72.8
7	4	23.7
8	5	16
11	6	23.4
12	7	18.8
14	8	6.6
15	9	14.6
16	10	21.3
17	11	18.7
18	12	7.7
19	13	15.3
20	14	7.3
22	15	47.7
23	16	22.6
24	17	17.4
26	18	13.3
27	19	3.8
28	20	10.5
29	21	23.8
30	22	28.9
33	23	16.2
34	24	7.7
35	25	40.1
36	26	18.3
37	27	17.9

38	28	34.5
39	29	11.9
40	30	110.5
41	31	17
42	32	18.3
46	33	18.1
47	34	22.3
49	35	8.4
50	36	14.5
51	37	19.1
53	38	9.4
54	39	13.6
56	40	9.4
57	41	20
58	42	20
59	43	16.1
60	44	42.5
61	45	5.9
62	46	14.6
63	47	33.3
64	48	41.6
65	49	6.6
66	50	12
67	51	7.3
68	52	20.4
69	53	18.3
70	54	15.7
71	55	12.3
74	56	20.6
75	57	9.4
76	58	16.9
78	59	18.1
79	60	25.1
80	61	17.9

81	62	8.7
82	63	8.8
83	64	12.2
84	65	17.4
85	66	16.6
86	67	22.1
87	68	12.9
88	69	52.3
89	70	9.4
90	71	10.3
91	72	13.8
93	73	64
95	74	7.3
96	75	5.9
97	76	8.7
98	77	38.1
99	78	8.7
100	79	13.3
101	80	73.9
102	81	12.3
103	82	90.9
104	83	48.1
105	84	5.9
106	85	36
107	86	8.8
108	87	12.6
109	88	14.9
111	89	15.7
112	90	11.5
113	91	11
114	92	94.3
115	93	72.8
117	94	15
118	95	12.2

119	96	107.7
120	97	16.9
121	98	8
122	99	36.4
123	100	10.3
125	101	17.6
126	102	8.4
127	103	14.2
128	104	13.6
129	105	11.7
130	106	7
131	107	90.1
132	108	22
134	109	17.6
140	110	36
141	111	33.3
142	112	8.4
143	113	5.5
144	114	14.2
145	115	7.3
146	116	10.3
172	117	6.2
200	118	130.3
202	119	24.9
203	120	11.4
205	121	13.6
206	122	2.2
207	123	8.8
208	124	24.9
211	125	5.5
408	126	144.3
411	127	9.2
703	128	14.9
1012	129	8.8

1093	130	23
1108	131	9.2
1553	132	13.2
Bull ZWK	133	14.9
55	134	7
55	135	12.8
171	136	13.4
171	137	18.8

Appendix Chapter 4: Table 4.13, Herd Bleed 3 Sera collected during Nov'97. *Neospora* antibody titre in sera from all cattle in the herd on the study farm in Northern Ireland, measured by ELISA. Abs, absorbance.

Sample No	Abs	OD	PP
1	0.021	0.040514	4.05
	0.02		
2	0.001	0.025692	2.5
	0.025		
3	0.04	0.051383	5.13
	0.012		
4	0.631	1.219368	121.93
	0.603		
5	0.029	0.055336	5.53
	0.027		
6	0.107	0.205534	20.55
	0.101		
7	0.028	0.043478	4.347
	0.016		
8	0.054	0.073123	7.312
	0.02		
9	0.213	0.381423	38.14
	0.173		
10	0.027	0.062253	6.22
	0.036		
11	0.018	0.018775	1.87
	0.001		
12	0.024	0.04249	4.24
	0.019		
13	0.198	0.38834	38.83
	0.195		
14	0.029	0.063241	6.32

	0.035		
15	0.367	0.705534	70.55
	0.347		
16	0.029	0.050395	5.03
	0.022		
17	0.024	0.045455	4.54
	0.022		
18	0.014	0.038538	3.85
	0.025		
19	0.029	0.050395	5.03
	0.022		
20	0.233	0.492095	49.20
	0.265		
21	0.023	0.038538	3.85
	0.016		
22	0.282	0.570158	57.01
	0.295		
23	0.026	0.034585	3.45
	0.009		
24	0.025	0.047431	4.74
	0.023		
25	0.157	0.293478	29.34
	0.14		
26	0.388	0.781621	78.16
	0.403		
27	0.03	0.048419	4.84
	0.019		
28	0.368	0.706522	70.65
	0.347		
29	0.041	0.074111	7.41
	0.034		
30	0.006	0.029644	2.96
	0.024		
31	0.009	0.020751	2.07

	0.012		
32	0.333	0.672925	67.29
	0.348		
33	0.009	0.077075	7.70
	0.069		
34	0.055	0.08498	8.49
	0.031		
35	0.004	0.006917	0.69
	0.003		
36	0.01	0.01581	1.58
	0.006		
37	0.013	0.041502	4.15
	0.029		
38	0.044	0.086957	8.69
	0.044		
39	0.024	0.028656	2.86
	0.005		
40	0.356	0.710474	71.04
	0.363		
41	0.074	0.1917	19.16
	0.12		
42	0.485	1.023715	102.37
	0.551		
43	0.551	1.092885	109.28
	0.555		
44	0.018	0.032609	3.26
	0.015		
45	0.002	0.016798	1.67
	0.015		
46	0.014	0.029644	2.96
	0.016		
47	0.031	0.048419	4.84
	0.018		
48	0.01	0.02668	2.66

	0.017		
49	0.014	0.022727	2.27
	0.009		
50	0.034	0.079051	7.90
	0.046		
51	0.028	0.030632	3.06
	0.003		
52	0.01	0.047431	4.74
	0.038		
53	0.033	0.070158	7.01
	0.038		
54	0.034	0.04249	4.24
	0.009		
55	0.245	0.456522	45.65
	0.217		
56	0.002	0.021739	2.17
	0.02		
57	0.31	0.619565	61.95
	0.317		
58	0.002	0.01087	1.08
	0.009		
59	0.007	0.023715	2.37
	0.017		
60	0.027	0.033597	3.35
	0.007		
61	0.729	0.904732	90.47
	0.705		
62	0.098	0.143218	14.32
	0.129		
63	0.143	0.190536	19.05
	0.159		
64	0.228	0.276972	27.69
	0.211		
65	0.18	0.196845	19.68

	0.132		
66	0.625	0.771609	77.16
	0.598		
67	0.157	0.234069	23.40
	0.214		
68	0.17	0.205678	20.56
	0.156		
69	0.138	0.160252	16.02
	0.116		
70	0.103	0.135647	13.56
	0.112		
71	0.143	0.2347	23.47
	0.229		
72	0.164	0.202524	20.25
	0.157		
73	0.175	0.221451	22.14
	0.176		
74	0.112	0.18612	18.61
	0.183		
75	0.157	0.223344	22.33
	0.197		
76	0.177	0.201893	20.18
	0.143		
77	0.961	1.200631	120.0
	0.942		
78	0.141	0.186751	18.67
	0.155		
79	0.112	0.200631	20.06
	0.206		
80	0.172	0.204416	20.44
	0.152		
81	0.139	0.170347	17.0
	0.131		
82	0.104	0.156467	15.64

	0.144		
83	0.165	0.215773	21.57
	0.177		
84	0.161	0.191798	19.17
	0.143		
85	0.171	0.215142	21.5
	0.17		
86	0.886	1.125552	112.5
	0.898		
87	0.144	0.22082	22.08
	0.206		
88	0.152	0.174132	17.41
	0.124		
89	0.873	1.056782	105.67
	0.802		
90	0.1	0.13817	13.81
	0.119		
91	0.141	0.200549	20.05
	0.151		
92	0.109	0.154533	15.45
	0.116		
93	0.139	0.206731	20.67
	0.162		
94	0.165	0.221841	22.18
	0.158		
95	0.168	0.203297	20.32
	0.128		
96	0.383	0.504808	50.48
	0.352		
97	0.182	0.272665	27.26
	0.215		
98	0.165	0.216346	21.63
	0.15		
99	0.182	0.237637	23.76

	0.164		
100	0.091	0.14217	14.21
	0.116		
101	0.15	0.238324	23.83
	0.197		
102	0.277	0.37706	37.70
	0.272		
103	0.152	0.197802	19.78
	0.136		
104	0.114	0.158654	15.86
	0.117		
105	0.179	0.263049	26.30
	0.204		
106	0.581	0.789148	78.91
	0.568		
107	0.136	0.178571	17.85
	0.124		
108	0.677	0.943681	94.36
	0.697		
109	0.136	0.214973	21.49
	0.177		
110	0.873	1.18544	118.54
	0.853		
111	0.11	0.157967	15.79
	0.12		
112	0.14	0.199863	19.98
	0.151		
113	0.181	0.26717	26.71
	0.208		
114	0.176	0.245879	24.58
	0.182		
115	0.112	0.161401	16.14
	0.123		
116	0.092	0.149038	14.90

	0.125		
117	0.144	0.223901	22.39
	0.182		
118	0.175	0.224588	22.45
	0.152		
119	0.174	0.197115	19.71
	0.113		
120	0.098	0.143544	14.35
	0.111		
121	0.155	0.175659	17.56
	0.138		
122	0.134	0.159472	15.94
	0.132		
123	0.322	0.470024	47.00
	0.462		
124	0.638	0.72482	72.48
	0.571		
125	0.271	0.320144	32.01
	0.263		
126	0.599	0.666067	66.60
	0.512		
127	0.181	0.228417	22.84
	0.2		
128	0.697	0.802758	80.27
	0.642		
129	0.173	0.264388	26.43
	0.268		
130	0.369	0.395683	39.56
	0.291		
131	0.144	0.197242	19.72
	0.185		
132	0.306	0.264388	26.43
	0.135		
133	0.137	0.243405	24.34

	0.269		
134	0.121	0.134892	13.48
	0.104		
135	0.297	0.368705	36.87
	0.318		
136	0.301	0.282374	28.23
	0.17		
137	0.173	0.193046	19.30
	0.149		
138	0.114	0.140887	14.08
	0.121		
151	0.154	0.207729	20.77
	0.147		
152	0.626	0.882678	88.26
	0.653		
153	0.9	1.198068	119.80
	0.836		
154	0.307	0.449965	44.99
	0.345		
155	0.166	0.224293	22.42
	0.159		
156	0.125	0.181504	18.15
	0.138		
157	0.174	0.256039	25.60
	0.197		
158	0.165	0.222222	22.22
	0.157		
159	0.168	0.214631	21.46
	0.143		
160	0.122	0.188406	18.84
	0.151		
161	0.175	0.276743	27.67
	0.226		
162	0.193	0.244997	24.49

	0.162		
163	0.177	0.222912	22.29
	0.146		
164	0.136	0.193927	19.39
	0.145		
165	0.15	0.242926	24.29
	0.202		
166	0.166	0.242236	24.22
	0.185		
167	0.9	1.203589	120.35
	0.844		
168	0.145	0.199448	19.94
	0.144		
169	0.194	0.311249	31.12
	0.257		
170	0.193	0.251898	25.18
	0.172		
171	0.711	0.995859	99.58
	0.732		
172	0.14	0.206349	20.63
	0.159		
173	0.176	0.275362	27.53
	0.223		
174	0.209	0.276052	27.60
	0.191		
175	0.192	0.254658	25.46
	0.177		
176	0.103	0.135266	13.52
	0.093		
177	0.158	0.31815	31.81
	0.303		
178	1.134	1.529331	152.93
	1.082		
179	0.306	0.344375	34.43

	0.193		
180	0.13	0.152519	15.25
	0.091		
181	0.041	0.079051	7.90
	0.039		
182	0.022	0.041502	4.15
	0.02		
183	0.044	0.074111	7.41
	0.031		
184	0.021	0.045455	4.54
	0.025		
185	0.033	0.038538	3.85
	0.006		
186	0.036	0.060277	6.02
	0.025		
187	0.042	0.045455	4.54
	0.004		
188	0.113	0.176877	17.68
	0.066		
189	0.026	0.027668	2.76
	0.002		
190	0.032	0.032609	3.26
	0.001		
191	0.021	0.021739	2.17
	0.001		
192	0.042	0.081028	8.10
	0.04		
193	0.49	0.914032	91.40
	0.435		
194	0	0.009881	0.98
	0.01		
195	0.038	0.037549	3.75
	0		
196	0.019	0.044466	4.44

	0.026		
197	0.049	0.062253	6.22
	0.014		
198	0.02	0.025692	2.56
	0.006		
199	0.16	0.29249	29.24
	0.136		
200	0.704	1.420949	142.09
	0.734		
201	0.064	0.08004	8.00
	0.017		
202	0.007	0.014822	1.48
	0.008		

Appendix Chapter 4: Table 4.14, Herd Bleed 3 Sera collected during Nov'97. *Neospora* antibody titre in sera from cattle in the herd on the study farm in Northern Ireland which can be matched to cow numbers, measured by ELISA.

Cow No.	PP
2	19.3
3	20
7	17.4
10	26.7
12	4.2
14	22.4
15	24.6
17	2.7
18	45
20	119
21	91.4
22	90.5
23	2.6
24	1
25	80.3
29	20.6
30	94.4
34	26.3
39	21.5
40	77.2
41	4.4
42	18
43	4.5
44	1.6
46	34.4
49	27.3
51	7

52	14.2
53	19.7
54	26.4
55	20.3
56	20.7
65	31.8
67	22.39
71	16
74	23.8
78	14.3
80	18.6
81	1.5
82	7.9
83	8.7
85	13.5
86	8
87	24.3
88	119.8
90	1.7
91	3.4
93	66.6
95	19.7
96	2.2
99	19.7
101	99.6
102	3
105	23.5
106	62
109	13.5
110	8
111	2.1
114	16.1
116	19.4
118	15.9

122	15.9
123	28.2
125	25.2
126	8.1
127	0.7
128	18.1
129	8.5
131	112.6
134	2.3
135	27.5
136	26.4
138	14.1
139	105.7
140	20.8
142	17.9
143	24.2
144	32
145	2.8
146	14.4
147	27.7
148	22.3
151	5.5
152	18.8
154	4.7
157	24.5
172	22.8
206	24.3
1108	19

Appendix chapter 5

Appendix chapter 5, Table 5.1 Antibody titres of sera used to probe SDS PAGE separated NC Liv *N.caninum* antigen, shown in figure 5.1.

Strip No.'s	**Serum**
1→3	*Neospora* Natural Infection (Cattle)
4	Control for 1-3
5→7	*Neospora* Experimental Infection (Cattle)
8	Control for 5-7(pre-infection)
9→11	*Toxoplasma* Experimental Infection (Cattle)
12	Control for 9-11 (pre-infection)
13→15	*Toxoplasma* Natural Infection (Sheep)
16	Control for 13-15
17	*Sarcocystis cruzi* Infection (Cattle) (titre not known)

Strip No.	**Animal No.**	**Infection Status/IFAT**	
1	38 (Batch 1)	+ve	1:4096
2	63 (Batch 1)	+ve	1:4096
3	170 (Batch 2)	+ve	1:16,384
4	6 (Batch 2)	-ve	0
5	525	+ve	1:4096
6	3679	+ve	1:2048
7	619	+ve	1:4096
8	525	-ve	Pre-Infection
9	2716	+ve	1:1024
10	2713	+ve	1:1024
11	2401	+ve	1:1024
12	2401	-ve	1:128
13	5227/8	+ve	1:1024
14	580/8	+ve	1:1024
15	5227/5	+ve	1:1024
16	5226	-ve	negative
17	NA	+ve	

Appendix chapter 5, Table 5.2 Antibody titres of sera used to probe SDS PAGE separated NC1 *N.caninum* antigen, shown in figure 5.2.

Strip No.'s	**Serum**
1→3	*Neospora* Natural Infection (Cattle)
4	Control for 1-3
5→7	*Neospora* Experimental Infection (Cattle)
8	Control for 5-7
9→11	*Toxoplasma* Experimental Infection (Cattle)
12	Control for 9-11
13→15	*Toxoplasma* Natural Infection (Sheep)
16	Control for 13-15
17	*Sarcocystis cruzi* Infection (Cattle)

Strip No.	**Animal No.**	**Infection Status/IFAT**	
1	38 (Batch 1)	+ve	1:4096
2	63 (Batch 1)	+ve	1:4096
3	170 (Batch 2)	+ve	1:16,384
4	6 (Batch 2)	-ve	0
5	525	+ve	1:4096
6	3679	+ve	1:2048
7	619	+ve	1:4096
8	525	-ve	Pre-Infection
9	2716	+ve	1:1024
10	2713	+ve	1:1024
11	2401	+ve	1:1024
12	2401	-ve	1:128
13	5227/8	+ve	1:1024
14	580/8	+ve	1:1024
15	5227/5	+ve	1:1024
16	5226	-ve	negative
17	NA	+ve	

Appendix chapter 5, Table 5.3 Antibody titres of sera collected for the Scottish survey of neosporosis. Sera from aborted foetuses and cows sero-positive for *N.caninum* was used to probe SDS PAGE separated NC Liv *N.caninum* antigen, shown in figure 5.3.

Strip No.	Cow No.	Status	IFAT	VI Centre
1	64	M	1:16,384	Auch
2	122a	F	1:4,096	Dumfries
3	149	M	1:8,192	Dumfries
4	156	MM	1:8,192	St.Bos
5	156	F	1:16,384	St.Bos
6	189	F	1:1,024	Bush
7	1	M	1:2,048	Inverness
8	6	F	1:8,192	Perth
9	16	M	1:4,096	Perth
10	27	M	1:4,096	Perth
11	36	M	1:8,192	Aberdeen
12	36	F	1:2,048	Aberdeen
13	70	M	1:8,192	Thurso
14	70	F	1:8,192	Thurso
15	81	F	1:512	Aberdeen
16	86	M	1:8,192	Perth
17	124	M	1:8,192	Dumfries
18	129/31	F	1:2,048	St.Bos
19	233	M	1:4,096	Thurso
20	238	M	1:4,096	Thurso
21	238	F	1:512	Thurso
22	251	M	1:8,192	Perth
23	251	F	1:4,096	Perth
24	264	F	1:16,384	Bush
25	274a	F	1:512	Aberdeen
26	348	M	1:16,384	St.Bos
27	385	M	1:8,192	Dumfries
28	6	M	1:4,096	Auch

29	10	M	1:16,384	Auch
30	11	M	1:4,096	Auch
31	42	M	1:16,384	Bush
32	48	M	1:16,384	Inverness
33	80	M	1:2,048	Thurso
34	80	F	1:4,096	Thurso
35	100	M	1:4,096	Dumfries
36	103	M	1:4,096	St.Bos
37	107	F	1:1,024	St.Bos
38	128	M	1:4,096	Bush
39	141	M	1:2,048	Aberdeen
40	141	F	1:16,384	Aberdeen
41	147	F	1:2,048	Dumfries
42	150	M	1:8,192	Bush
43	168	M	1:4,096	Bush
44	177	M	1:16,384	Thurso
45	189	M	1:16,384	St.Bos
46	189	F	1:16,384	St.Bos
47	192	F	1:512	Perth
48	196	F	1:512	Bush
49	203	M	1:8,192	Inverness
50	203	F	1:16,384	Inverness
51	215	F	1:16,384	Bush

Appendix chapter 5, Table 5.4 Antibody titres of sera collected from cows on the study farm (chapter 4) in Northern Ireland. All animals were sero-positive for *N.caninum*. Sera was used to probe SDS PAGE separated NCLiv *N.caninum* antigen, shown in figure 5.4.

Strip No.	Ref No.	IFAT
1	4*11	1:1024
2	4*12	512
3	4*13	1:1024
4	4*15	1:1024
5	4*17	1:1024
6	4*18	1:1024
7	4*20	1:2048
8	0*1	1:2048
9	0*2	1:4096
10	0*5	1:4096
11	0*7	1:2048
12	0*8	1:4096
13	8*1	1:512
14	8*5	1:512
15	8*6	1:512
26	8*8	1:512
27	8*9	1:512
28	8*11	1:512
29	8*14	1:512
30	8*15	1:512

Appendix chapter 5, Table 5.5 Antibody titres of sera collected from calves congenitally infected with *N.caninum* and used to probe SDS PAGE separated NC1 *N.caninum* antigen, shown in figure 5.5.

Calves	Animal Nos.	Status
D	1657	Congenitally Infected *Neospora* Pos (from No.119)
E	1658	Congenitally Infected *Neospora* Pos (from No.114)
H	2652	Control
U	1671	Control

Strip No.'s	Serum	*Neospora* ELISA (%OD)
1	Calf D, Bleed 2	101
2	Calf D, Bleed 4	97
3	Calf E, Bleed 2	146
4	Calf E, Bleed 4	135
5	Calf H, Bleed 2	6
6	Calf H, Bleed 4	5
7	Calf U, Bleed 2	3
8	Calf U, Bleed 4	3

Bleed 2: 15th Nov '96

Bleed 4: 22nd Nov '96

Appendix chapter 5, Table 5.6 IFAT antibody titres of *Toxoplasma* and *Neospora* positive sera used to probe SDS PAGE separated and oxidised (periodate treated) NC Liv *N.caninum* antigen, shown in figure 5.6.

Strip No.'s	Serum
1	*Neospora* Natural Infection (Cattle)
2	Control
3	*Neospora* Experimental Infection (Cattle)
4	Control
5	*Toxoplasma* Experimental Infection (Cattle)
6	Control
7	*Toxoplasma* Natural Infection (Sheep)
8	Control
9	*Sarcocystis cruzi* Infection (Cattle) (titre not known)

Strip No.	Animal No.	Infection Status/IFAT	
1	38 (Batch 1)	+ve	1:4096
2	6 (Batch 2)	-ve	0
3	525	+ve	1:4096
4	525	-ve	Pre-Infection
5	2401	+ve	1:1024
6	2401	-ve	Pre-Infection
7	5227/8	+ve	1:1024
8	5226	-ve	1:128
9	NA	+ve	

Appendix chapter 5, Table 5.7 *Babesia* positive sera used to probe SDS PAGE separated NC1 *N.caninum* antigen, shown in figure 5.7.

	Strip No	Serum	Animal No./Serum Ab
	1	*Neospora* pos - bovine Scottish survey no.156F	
	2	*Neospora* neg - bovine pre infection no.525	
Natural Infection	3	*B.divergens*	A/F 17 Positive
	4.	*B.divergens*	A/F 20 Positive
Experimental Infec.	5.	*B.divergens*	4 Post infection
	6	*B.divergens*	4 Pre infection
	7	*B.divergens*	5 Post infection
	8	*B.divergens*	5 Pre infection
	9	*B.divergens*	125 Post infection
	10	*B.divergens*	125 Pre infection
	11	*B.divergens*	122 Post infection
	12	*B.divergens*	122 Pre infection
	13	*B.bovis*	A 13 Post infection
	14	*B.bovis*	A 13 Pre infection
	15	*B.bovis*	C 47 Post infection
	16	*B.bovis*	C 47 Pre infection
	17	*B.bovis/bigemina*	A3 Post infection
	18	*B.bovis/bigemina*	A3 Pre infection
	19	*B.bovis/bigemina*	A5 Post infection
	20	*B.bovis/bigemina*	A5 Pre infection
	21	*B.bigemina*	GR.A.9 Post infection
	22	*B.bigemina*	GR.A.10 Post infection

Appendix chapter 5, Table 5.8 IgM antibody titre in calves experimentally infected with *Cryptosporidium parvum.* Sera was used to probe SDS PAGE separated NC1 *N.caninum* antigen, shown in figure 5.8.

Strip No.'s	Serum
1	Bovine experimental infection NC1 no.525, wk 4 PI
2	Bovine pre infection serum, no.525
3	Calf 2, Sample 1
4	Calf 2, Sample 2
5	Calf 4, Sample 1
6	Calf 4, Sample 2
7	Calf 6, Sample 1
8	Calf 6, Sample 2

Samples 3-8 are all *Cryptosporidium* positive sera, collected from infected calves 3 weeks PI.

Appendix chapter 5, Table 5.9 IFAT Antibody titres of *Sarcocystis* positive sera used to probe SDS PAGE separated *N.caninum* antigen, shown in figure 5.9.

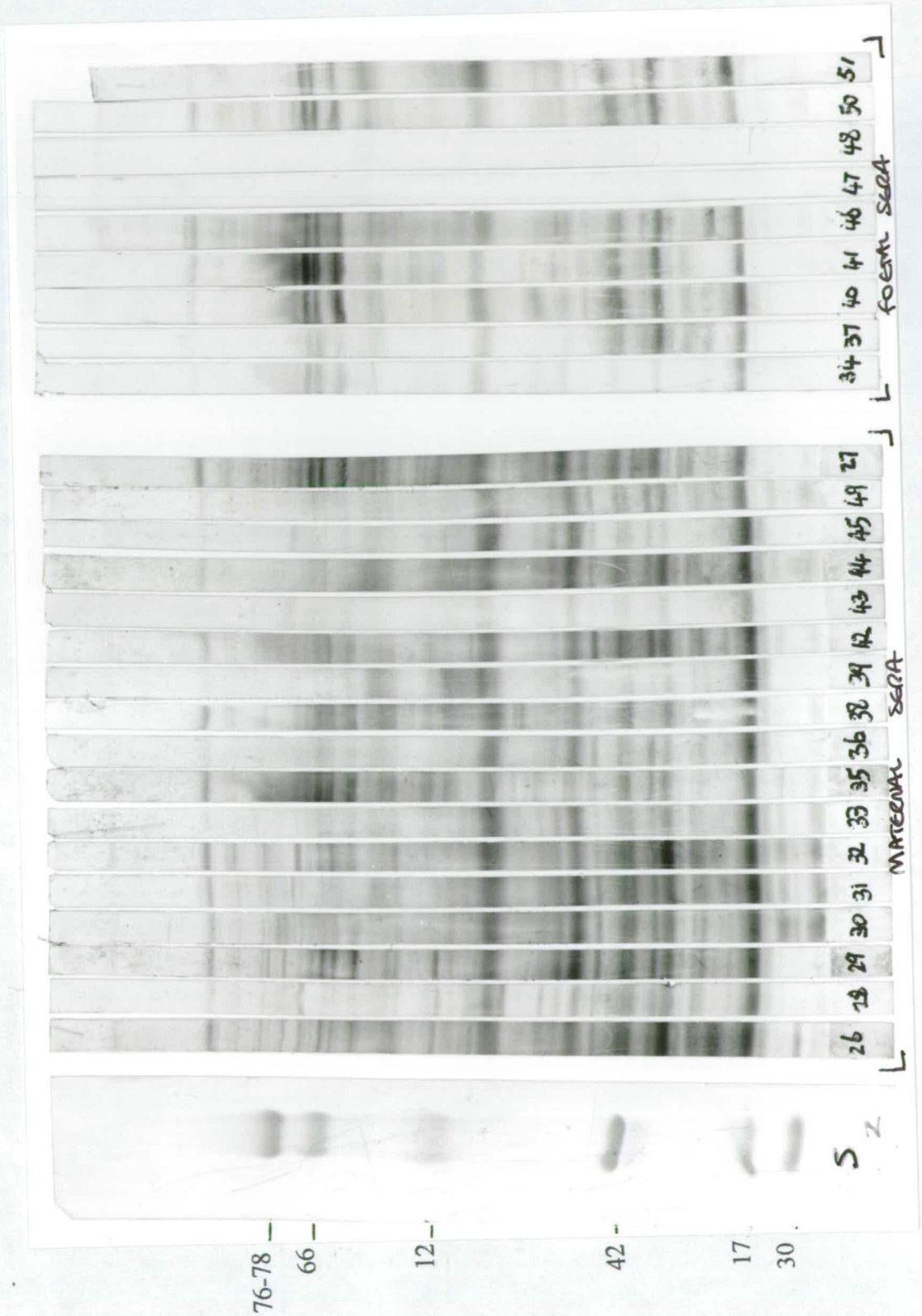
	Strip No.	Serum	
Controls	1	Ovine M3 Experimental Infection, Week4 PI +ve	
	2	Ovine M3 Experimental Infection, Pre-Infec. Serum -ve	
Experimental	3	Sheep 1 - 107 DPI, 1:81920	+ve
Sera	4	Sheep 1 - 0 DPI,	-ve
	5	Sheep 2 - 107 DPI, 1:81920	+ve
	6	Sheep 2 - 0 DPI,	-ve
	7	Sheep 3 - 107 DPI, 1:81920	+ve
	8	Sheep 3 - 0 DPI,	-ve
	9	Sheep 4 - 107 DPI, 1:81920	+ve
	10	Sheep 4 - 0 DPI,	-ve
	11	Sheep 5 - 107 DPI, 1:81920	+ve
	12	Sheep 5 - 0 DPI,	-ve

DPI – Days post infection

+ve – positive anti-parasite antibody titre

-ve – negative anti-parasite antibody titre

Appendix chapter 5, Figure 5.1 *N.caninum* NC Liv antigen separated by SDS PAGE probed with Scottish survey maternal and foetal sera which tested strongly antibody positive by *Neospora* IFAT. The number on each strip correlates to an animal number, details of which are given in appendix chapter 5, table 5.3. S = standards.



Appendix Chapter 6

Appendix Chapter 6, Table 6.1 Temperature values (°C) for control and infected calves from 1 day pre to 14 days post infection.

	Infected				Controls	
Day PI	**Calf 13**	**Calf 14**	**Calf 20**	**Calf 21**	**Calf 15**	**Calf 25**
-1	39.31	38.5	38.99	39	39.21	39.46
0	38.91	38.81	39.33	39.13	38.85	39.27
1	39.09	39.02	39.35	40.05	38.89	38.93
2	38.89	39.01	39.12	40.04	39.1	39.13
3	39.29	39.08	39.22	39.69	38.93	39.13
4	38.89	38.87	39.15	39.06	38.78	39.07
5	39.45	39.37	39.07	39.06	39.12	39.13
6	39.82	39.87	39.53	39.41	39.18	39.07
7	39.28	39.85	39.42	39.45	38.93	39.01
8	39.4	39.73	39.73	39.66	39.14	38.97
9	39.08	39.06	39.29	39.22	38.97	38.9
10	39.11	39.09	39.23	39.17	38.98	38.92
11	39.01	39.15	38.71	39.37	39.33	39.25
12	39.01	39.17	38.99	39.18	39.08	39.11
13	38.69	39.54	38.7	38.49	38.74	39.75
14	38.61	39.37	38.72	39.25	39.47	39.35

Appendix Chapter 6, Table 6.2 Mean temperature values for control and infected calves from 1 day pre to 14 days post infection. Significance testing (P value) of the mean temperature value for each day is compared with the base line value (average of pre-infection temperatures) of 39.1°.

Day	Calves	Mean Temp	P VALUE
-1	4	38.950	0.78
0	4	39.045	0.67
+1	4	39.377	0.16
+2	4	39.265	0.29
+3	4	39.320	0.096
+4	4	38.993	0.89
+5	4	39.237	0.13
+6	4	39.658	0.0077
+7	4	39.500	0.023
+8	4	39.630	0.0033
+9	4	39.163	0.17
+10	4	39.150	0.11
+11	4	39.060	0.60
+12	4	39.088	0.59
+13	4	38.855	0.81

Appendix Chapter 6, Table 6.3 *N.caninum* serum antibody concentration, measured by ELISA, of infected calves in experiment A from week 1 pre to week 4 post infection. Values are given in %OD.

	Infected				Controls	
Week	**Calf 13**	**Calf 14**	**Calf 20**	**Calf 21**	**Calf 15**	**Calf 25**
0	21.8	6.9	3.4	3.4	11.5	5.7
1	39	22	18	22.3	10.6	18
2	45	25	19	38.9	17.9	13.7
3	56	33.8	16	60	15	15
4	76	38.8	38.8	70	12.5	16.2

Appendix Chapter 6, Table 6.4 Mean *N.caninum* serum antibody concentration, measured by ELISA, of infected calves in experiment A from week 1 pre to week 4 post infection. Significance testing (P value) of the mean %OD value for each week is compared with the base line value (average of pre-inoculation antibody values) of 8.8%.

Week PI	Calves	Mean	P VALUE
-1	6	7.04	0.50
+1	6	9.49	0.011
+2	6	12.59	0.0090
+3	6	20.95	0.019
+4	6	26.44	0.014

Appendix Chapter 6, Table 6.5 Mean proliferation values of PBM from calves infected with $2.5x10^8$ *N.caninum* NC1 tachyzoites (13, 14, 20 and 21) incubated with *N.caninum* NC1 and *T.gondii* S48 wsf antigen *in vitro* at a final concentration of 10μg per ml from day 3 pre-infection to day 20 post infection. Proliferation was measured in CPM (n=3) of ^{3}H thymidine selectively incorporated into proliferating cells. Positive control - cells were incubated with Con A at a final concentration of 5μg. per ml. Med, medium control.

Calf 13								
Day PI	**Med**	**SE**	**NC1**	**SE**	**S48**	**SE**	**Con A**	**SE**
-3	150.75	16.5	150.75	38.3	1423.75	415.1	11796	137.3
-2	31.25	5.1	26.7	9.1	174.75	52.5	9785.75	346.5
-1	361.25	61.6	220.75	56.4	418	38.7	24904	182.5
0								
2	244.75	54	290.25	126.4	478.5	160.4	19358.75	632.3
4	325.25	114.8	585.25	150.5	609	45.6	25915.5	153.6
6	182.25	31.1	480.75	31.8	593.5	71.3	20490	377.2
8	480.5	58.1	4513	105.1	5626	361.9	14151	202.5
10	362.5	36.7	5650.75	588.3	6254.25	260.6	15789	515.6
12	584.5	55.6	4270.75	363.6	4559.5	646.2	19494.5	180.9
14	270.75	40.3	3068.25	293.8	2417.75	337.9	13721.75	326.1
16	232.25	26.2	1338	149.4	972.5	370.4	10048.75	2114.7
18	141.75	5.8	2970.75	215.3	2326	434.8	19602	1056.5
20	386.75	32	5758.25	283.7	5078	735	18847	1140.1

Calf 14								
Day PI	**Med**	**SE**	**NC1**	**SE**	**S48**	**SE**	**Con A**	**SE**
-3	115	98.5	328	112.9	680.75	253	11733.75	257.1
-2	2851.5	319.7	745.5	45.8	5479.5	1157.9	7783	269.6
-1	271	69.7	204.5	64	520	245.3	19765.25	793.2
0								
2	125.85	19.8	269.25	31.5	304.25	20.4	16251.75	792.1
4	455.25	84.7	419.5	32.9	470.5	25.2	23054	289.8
6	163.75	63.4	882.25	227.6	1038	248.4	21200	332.3
8	552.25	31.9	8213.5	852.5	6599.75	826.3	7016.75	255.5
10	334.25	21.2	12574.75	1141.4	13501.75	1036.9	8484.5	553.3
12	68.425	18	12654.25	480.4	7595.75	867.4	14676	140
14	186.25	26.8	8893.75	821.2	3122.25	360.2	10705.25	550.7
16	625.75	242	15640.25	1255.4	8382.75	690.9	7377.75	153.8
18	94.8	18	9641	561.8	4126.75	212.7	14817	294.2
20	2032.5	231.1	14887.75	888.3	8038.5	273.4	19034	658

Calf 20								
Day PI	**Med**	**SE**	**NC1**	**SE**	**S48**	**SE**	**Con A**	**SE**
-3	22	3.3	151	46.7	1782.75	439	8863.25	522.9
-2	1111.25	496.8	1769.75	730.3	5103.25	336.8	6923	29.5
-1	906.25	213.2	5890.75	864.9	8775.25	554.4	21350	311
0								
2	224.75	93.8	257.75	38.5	266.5	43.1	15806	1755.5
4	347.75	95.9	1228.25	205.2	1235.5	165.6	23100.75	489.2
6	55.825	10.8	2155.75	445.6	3417.75	435.3	22463.25	411.1
8	524.5	27.5	4254.5	200.2	5356.5	430	9764.25	138.3
10	335.25	25	13148.75	508.7	10897.5	582.9	14425.25	372
12	487.75	17.1	7792.5	186.2	6592	125.7	18984.25	281.2
14	1282.5	77.8	6332.75	580.9	4493.75	216	10997	310.4
16	44.4	2	10618.75	491.7	7639.25	169	20479	734.5
18	21.68	2.3	3466.5	287.3	2410	401.6	22495	1049.1
20	204	100.4	8840	1167.1	6486	534.7	17755.25	779.3

Calf 21								
Day PI	Med	SE	NC1	SE	S48	SE	Con A	SE
-3	9602.5	470.8	3787.75	581	6234.75	526.6	12351.5	886.6
-2	6856.5	446.5	2494.25	567.3	7049.5	360	4974	54.9
-1	4011	1096.6	2690.5	419.7	12667	787.7	19677	278.5
0								
2	159.75	15.9	226.25	24.4	288.5	70.3	13588	439.9
4	486.5	133.8	717.75	68.2	878	189.8	17054.5	678
6	217	11	3954.75	287.5	1851.25	211.1	17700.75	105.6
8	321.5	42.2	1666.75	165.6	2531.75	342.4	4533.75	310.9
10	446	60.8	5575.25	836.8	6642.5	404.6	3253.5	148.6
12	567.75	184	7399.75	264	6316	92.2	12854.25	379.4
14	313.75	49.6	5289.5	478.4	4589.25	405.7	7839	260.7
16	407	157.1	6538.25	417.7	7156.75	812.2	6464.75	141.4
18	313	85.7	6487.75	484.5	4475.75	390.9	10190.75	301.5
20	2320	525.5	9376.5	576	5920.75	686.1	8921.75	228.1

Appendix Chapter 6, Table 6.6 Concentration of IFNγ produced by PBM from *Neospora* infected calves in response to *Neospora* and *Toxoplasma* wsf antigen in vitro. The concentration of active IFNγ (units per ml) was measured using a bioassay. DPI, day post infection. Significance testing (P value) of the concentration if IFNγ for each day is compared with the base line value (average of pre-inoculation IFNγ concentrations) for each calf.

Calf 13

	Medium			***N.caninum* wsf Antigen**			***T.gondii* wsf Antigen**		
dpi	**IFN**	**Non IFNγ**	**IFNγ**	**IFN**	**Non IFNγ**	**IFNγ**	**IFN**	**Non IFNγ**	**IFNγ**
-3	0	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0	0
-1	4	4	0	64	64	0	0	0	0
0									
2	4	4	0	16	8	8	0	0	0
4	4	4	0	4	4	0	8	4	4
6	4	4	0	16	4	12	16	4	12
8	4	4	0	128	16	112	128	4	124
10	4	4	0	256	32	224	64	4	60
12	4	4	0	128	32	96	64	4	60
14	4	4	0	256	64	192	32	4	28
16	0	0	0	0	0	0	0	0	0
18	4	4	0	128	64	64	64	32	32
20	4	4	0	256	32	224	128	32	96

Calf 14

	Medium			*N.caninum* wsf Antigen			*T.gondii* wsf Antigen		
dpi	IFN	Non IFNγ	IFNγ	IFN	Non IFNγ	IFNγ	IFN	Non IFNγ	IFNγ
-3	4	0	4	4	0	4	0	0	0
-2	4	4	0	8	4	4	16	4	12
-1	4	4	0	8	4	4	16	4	12
0	4								
2	4	0	4	4	4	0	0	0	0
4	4	4	0	4	4	0	8	4	4
6	4	0	4	4	4	0	4	4	0
8	4	4	0	64	4	60	64	4	60
10	4	0	4	256	4	252	128	4	124
12	4	0	4	64	8	56	64	4	60
14	4	0	4	128	4	124	64	4	60
16	4	0	4	64	4	60	16	4	12
18	4	0	4	128	8	120	16	4	12
20	8	4	4	128	8	120	32	4	28

Calf 20

	Medium			*N.caninum* wsf Antigen			*T.gondii* wsf Antigen		
dpi	IFN	Non IFNγ	IFNγ	IFN	Non IFNγ	IFNγ	IFN	Non IFNγ	IFNγ
-3	8	4	4	64	4	60	0	0	0
-2	4	4	0	32	4	28	64	4	60
-1	4	4	0	16	4	12	64	4	60
0									
2	4	0	4	8	4	4	8	4	4
4	4	0	4	4	4	0	8	4	4
6	4	0	4	16	4	12	32	4	28
8	4	0	4	32	4	28	128	4	124
10	4	0	4	256	8	248	64	4	60
12	8	4	4	64	4	60	64	4	60
14	4	4	0	128	8	120	64	4	60
16	4	0	4	128	8	120	32	4	28
18	4	0	4	32	4	28	16	4	12
20	4	0	4	32	4	28	64	4	60

Calf 21

	Medium			*N.caninum* wsf Antigen			*T.gondii* wsf Antigen		
dpi	IFN	Non IFNγ	IFNγ	IFN	Non IFNγ	IFNγ	IFN	Non IFNγ	IFNγ
-3	16	4	12	125	4	121	128	4	124
-2	32	4	28	128	4	124	32	4	28
-1	4	4	0	256	8	248	32	4	28
0									
2	4	4	0	8	4	4	4	4	0
4	4	4	0	8	4	4	8	4	4
6	4	4	0	128	8	120	256	32	224
8	4	4	0	64	4	60	16	4	12
10	4	4	0	128	4	124	32	4	28
12	4	4	0	256	4	252	32	4	28
14	4	4	0	256	4	252	32	4	28
16	4	4	0	128	4	124	32	4	28
18	4	4	0	128	4	124	64	4	60
20	16	4	12	256	8	248	64	4	60

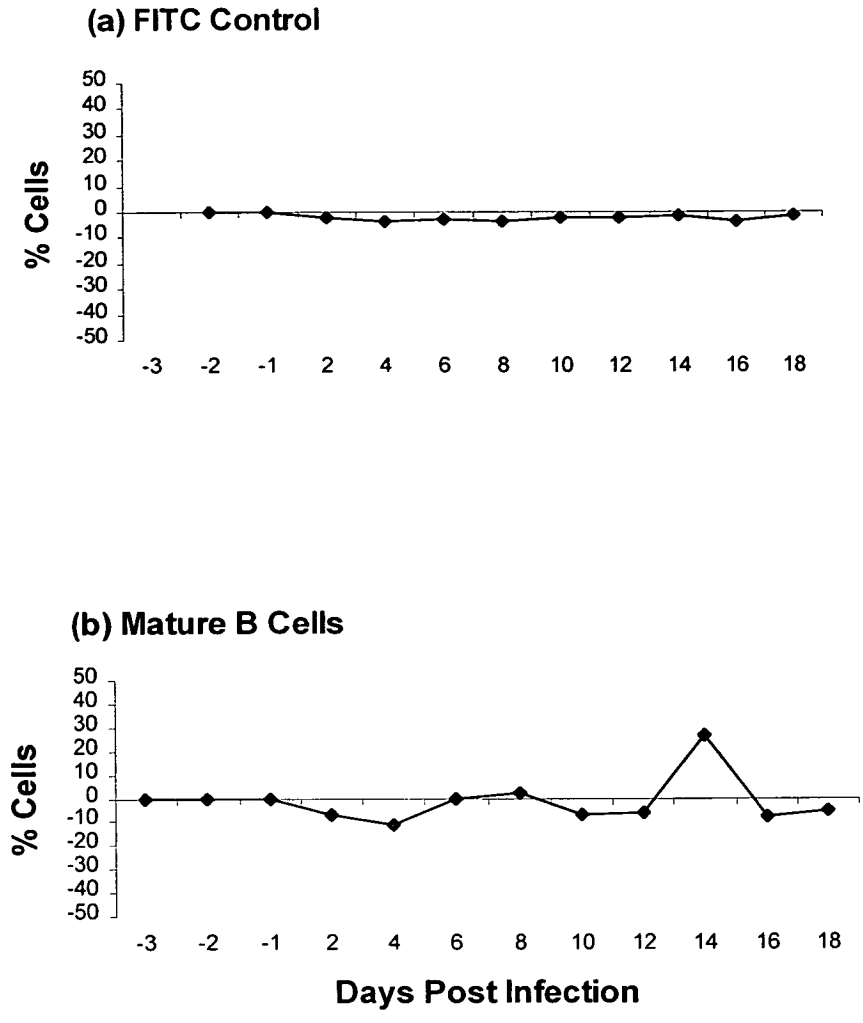
Appendix chapter 6, Table 6.7a Mean IFNγ concentration (u/ml) in supernatants of PBM cultures from 3 calves (13, 14 and 20) incubated with *N.caninum* NC1 wsf antigen. All calves were experimentally infected with *N.caninum* tachyzoites. The concentration of biologically active IFNγ was measured by *in vitro* inhibition of Semliki Forest Virus. Samples were collected from week 1 pre to week 4 post infection. Significance testing (P value) of the mean IFNγ concentration is compared with the base line value (average of pre-inoculation IFNγ concentration in PBM cultures incubated with *N.caninum* NC1 wsf antigen) of 12.4u/ml.

Day PI	Calves	Mean	P VALUE
-3	3	21.3	0.34
-2	3	10.7	0.57
-1	3	5.3	0.91
+2	3	4.0	0.97
+6	3	4.7	0.91
+8	3	66.7	0.078
+10	3	241.3	0.0007
+12	3	70.7	0.022
+14	3	145.3	0.015
+16	3	60.0	0.15
+18	3	70.7	0.081
+20	3	124.0	0.094

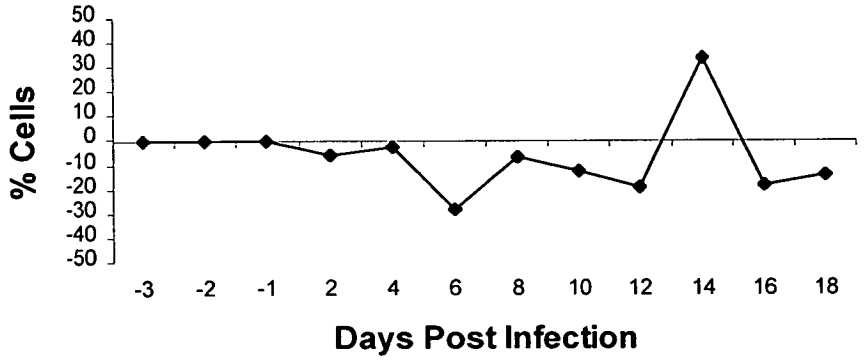
Appendix chapter 6, Table 6.7b Mean IFNγ concentration (u/ml) in supernatants of PBM cultures from 3 calves (13, 14 and 20) incubated with *T.gondii* S48 wsf antigen. All calves were experimentally infected with *N.caninum* tachyzoites. The concentration of biologically active IFNγ was measured by *in vitro* inhibition of Semliki Forest Virus. Samples were collected from week 1 pre to week 4 post infection. Significance testing (P value) of the mean IFNγ concentration is compared with the base line value (average of pre-inoculation IFNγ concentration in PBM cultures incubated with *T.gondii* S48 wsf antigen) of 12.4u/ml.

Day PI	Calves	Mean	P VALUE
-2	3	24.0	0.35
-1	3	24.0	0.35
+2	3	1.3	1.00
+6	3	13.3	0.61
+8	3	102.7	0.028
+10	3	81.3	0.046
+14	3	49.3	0.044
+16	3	13.3	0.61
+18	3	18.7	0.36
+20	3	61.3	0.074

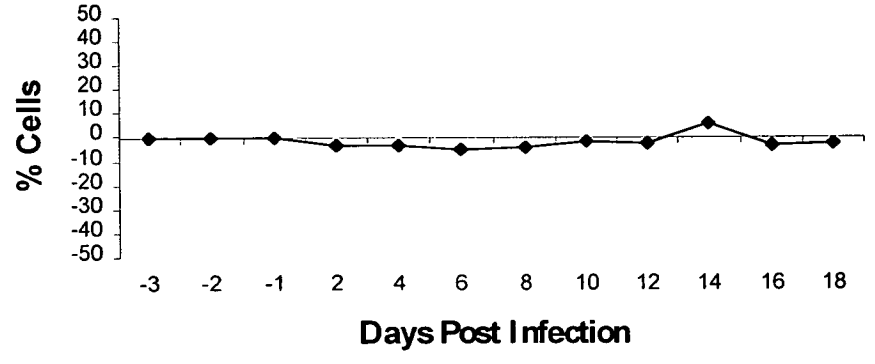
Appendix chapter 6, Figure 6.1 Mean percentage change from base line values of bovine peripheral blood lymphocyte subsets following experimental infection with $2.5x10^8$ NC1 N.caninum tachyzoites on day 0. Cell populations were identified by FACS analysis.



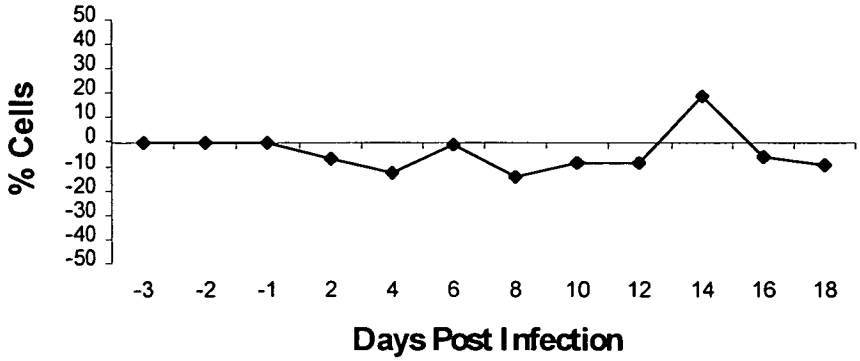
(c) Monocytes/Macrophages



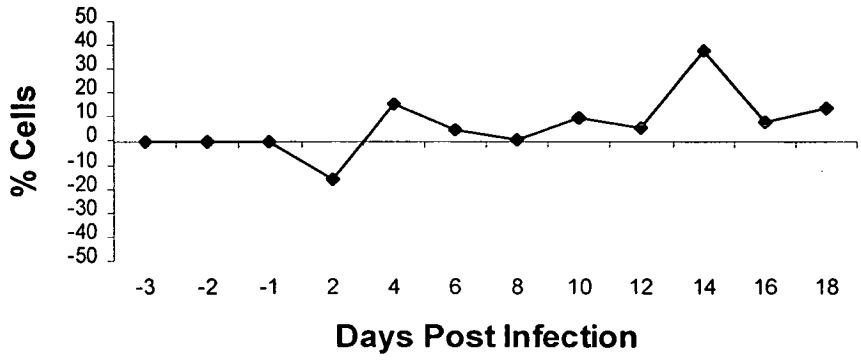
(d) Gamma/Delta T Cells



(e) B Cells



(f) B and Activated T Cells

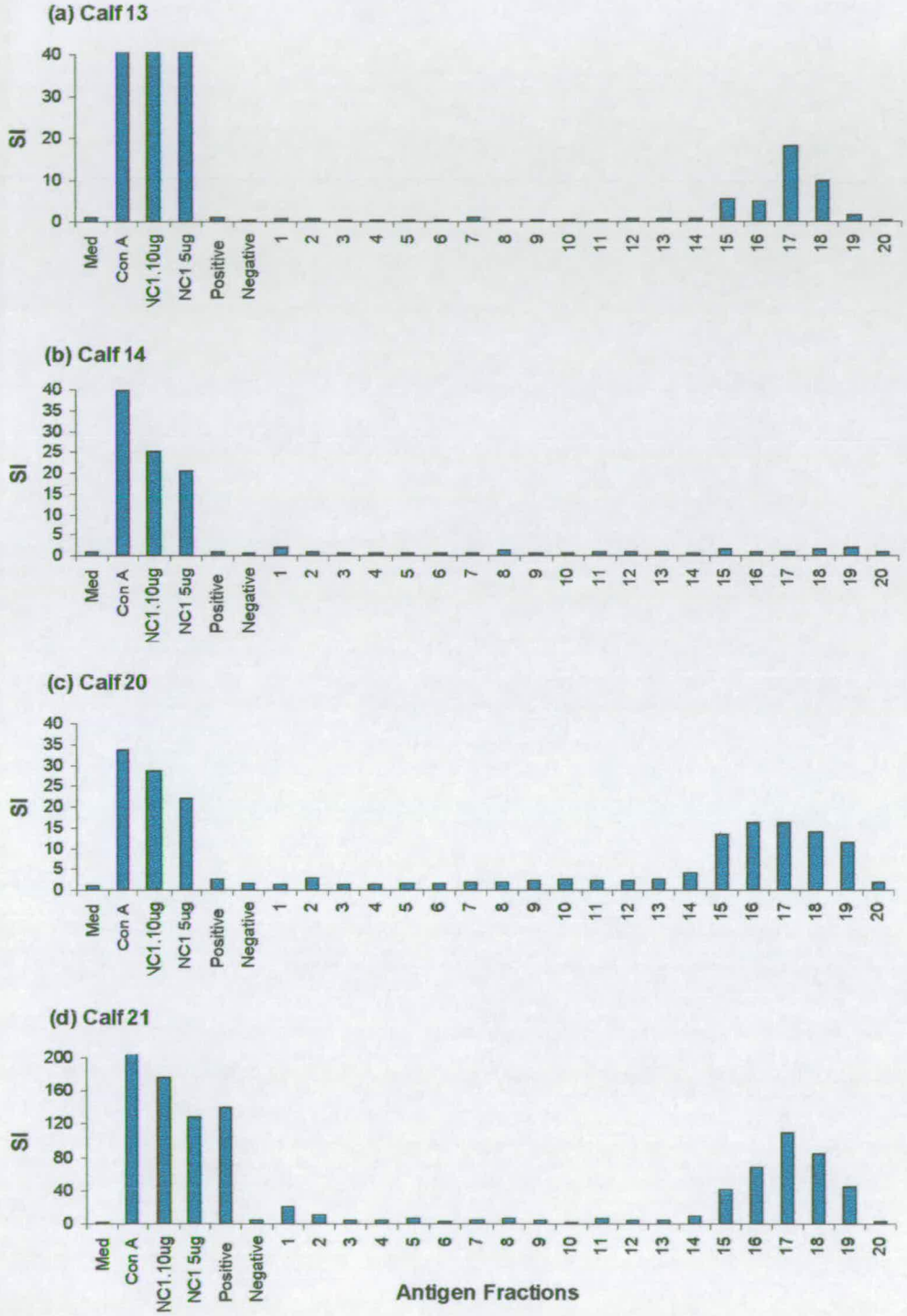


Appendix Chapter 6, Table 6.8 FACS data showing baseline percentage values of cell populations present in PBMC from calves. Cells were stained with monoclonal antibodies pre infection with *N.caninum* tachyzoites. This data was used to calculate changes in PBMC populations from baseline after infection with $2.5x10^8$ NC1 tachyzoites.

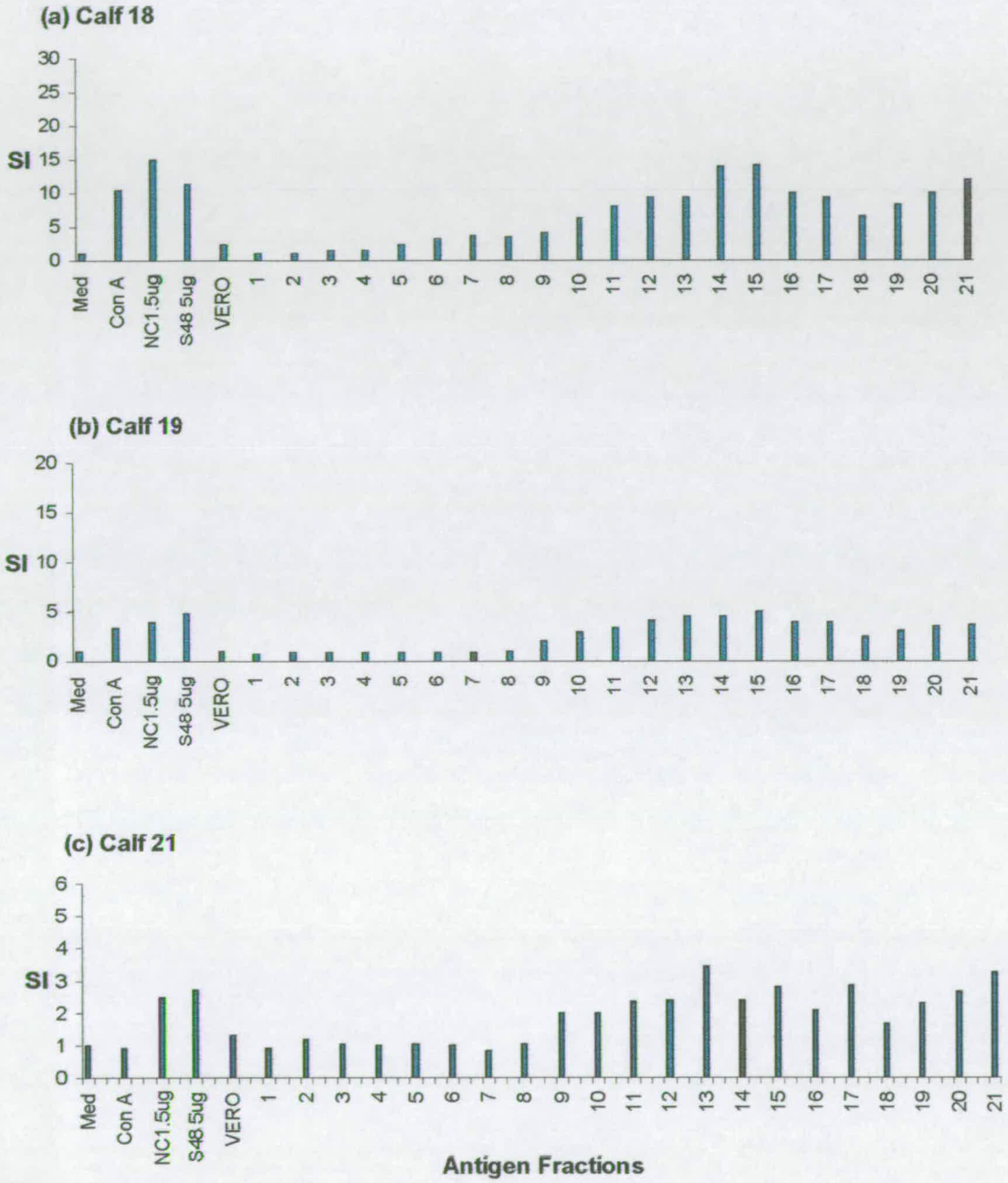
Antibody	Day Pre Infection	Percentage of Cells Stained				Average % of Cells Stained	Average % of Cells Stained Pre Infection
		Calf 13	Calf 14	Calf 20	Calf 21		
Anti CD4 (CC8)	3	16.01	23.98	31.48	24.61	24.0	27
	2	28.31	20.7	29.77	41.27	30.0	
	1	14.14	16.42	50.4	35.95	29.2	
Anti CD8 (CC63)	3	11.88	7.66	16.31	17.55	13.3	10.5
	2	4.15	5.89	7.12	21.79	9.7	
	1	12.45	2.87	4.74	10.6	7.6	
CD2 (CC42)	3	49.57	42.38	57.54	44.15	48.4	49.7
	2	70.2	34.23	49.75	68.99	55.8	
	1	41.42	29.26	47.71	61.64	45.0	
IL2 R (ILA111)	3	40	45.01	19.17	24.97	32.3	30.7
	2	26.39	39.62	20.88	21.47	27.1	
	1	46.68	49.11	22.24	12.85	32.7	

Appendix Chapter 7

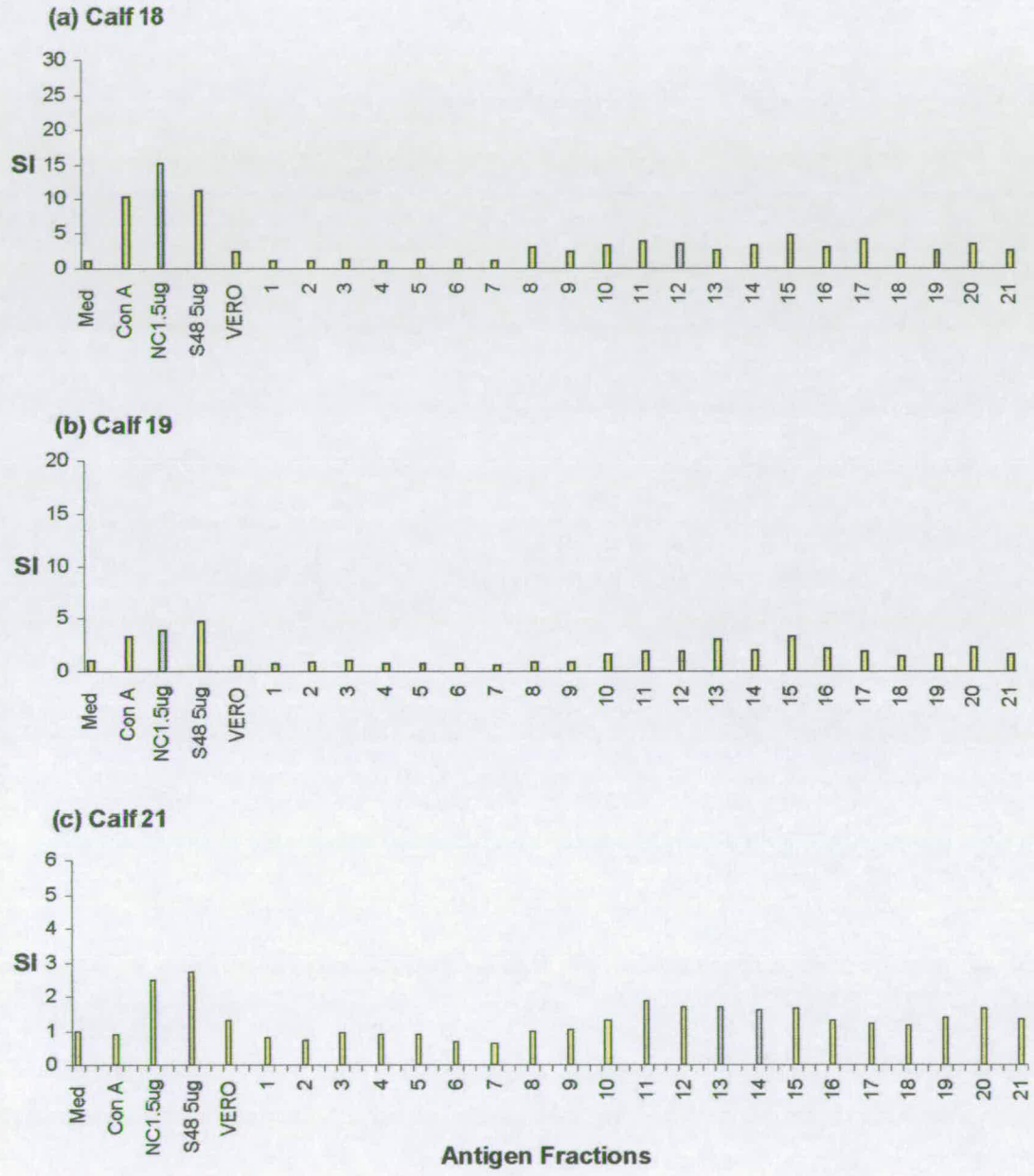
Appendix Chapter 7, Figure 7.1 Proliferative response of CD4+ T cell lines from calves infected with NC1 *N.caninum* tachyzoites to *N.caninum* antigen fractions 1-20 Separated by SDS PAGE.



Appendix Chapter 7, Figure 7.2 Proliferative response of CD4+ T cell lines from calves infected with NC1 *N.caninum* tachyzoites to *N.caninum* antigen fractions 1-20 separated by HPLC.



Appendix Chapter 7, Figure 7.3 Proliferative response of CD4+ T cell lines from calves infected with NC1 *N.caninum* tachyzoites to *T.gondii* antigen fractions 1-20 separated by HPLC.



Appendix Chapter 7, Table 7.1 Temperature values for control and infected calves in experiment A from 1 day pre to 14 days post infection. Significance testing (P value) of the mean temperature value for each day is compared with the base line value (average of pre-infection temperatures) of 39.1°.

Day	Calves	Mean	P VALUE
-1	4	38.950	0.78
0	4	39.045	0.67
+1	4	39.377	0.16
+2	4	39.265	0.29
+3	4	39.320	0.096
+4	4	38.993	0.89
+5	4	39.237	0.13
+6	4	39.658	0.0077
+7	4	39.500	0.023
+8	4	39.630	0.0033
+9	4	39.163	0.17
+10	4	39.150	0.11
+11	4	39.060	0.60
+12	4	39.088	0.59
+13	4	38.855	0.81

Appendix Chapter 7, Table 7.2 Temperature values for control and infected calves in experiment B from 1 day pre to 14 days post infection. Significance testing (P value) of the mean temperature value for each day is compared with the base line value (average of pre-infection temperatures) of 39.1°.

Day PI	Calves	MEAN	P VALUE
-3	4	38.825	0.30
-2	4	38.425	0.98
-1	4	38.400	0.95
0	4	39.025	0.030
+1	4	39.350	0.10
+2	4	40.150	0.0022
+3	4	40.250	0.012
+4	4	39.450	0.028
+5	4	39.050	0.077
+6	4	39.950	0.016
+7	4	39.600	0.0086
+8	4	39.225	0.014
+9	4	39.000	0.095
+10	4	38.200	0.95
+11	4	38.725	0.46
+12	4	38.950	0.032
+13	4	38.750	0.25
+14	4	38.925	0.059

Appendix Chapter 7, Table 7.3 *N.caninum* serum antibody concentration values measured by ELISA infected calves in experiment A from 1 week before to 4 weeks after infection. Significance testing (P value) of the mean %OD value for each week is compared with the base line value (average of pre-inoculation antibody values) of 8.8%.

Week PI	Calves	Mean	P VALUE
-1	6	8.78	0.50
+1	6	21.65	0.011
+2	6	26.58	0.0090
+3	6	32.63	0.019
+4	6	42.05	0.014

Appendix Chapter 7, Table 7.4 *N.caninum* serum antibody concentration values measured by ELISA infected calves in experiment B from 1 week before to 4 weeks after infection. Significance testing (P value) of the mean %OD value for each week is compared with the base line value (average of pre pre-inoculation antibody values) of 11.6%.

Week PI	Calves	Mean	P VALUE
-1	4	13.49	0.20
+1	4	11.51	0.52
+2	4	33.55	0.016
+3	4	56.25	0.0065
+4	4	71.71	0.0033

Appendix Chapter 7, Table 7.5 Proliferation results of PBM from experiment B calves incubated with *N.caninum* NC1 and *T.gondii* S48 wsf antigen at 5ug/ml. Values are given in counts per minute of ^{3}H thymidine selectively incorporated into proliferating cells. SE, standard error; ND, not done.

Calf No.	Day PI	Med	SE	NC1	SE	S48	SE	Con A	SE
18	-3	114	55.3	2275.5	463.3	3219.8	630.3	6499.8	891.2
	-2	131.7	85.6	5150.7	372.7	3837.5	902.3	12127	122.1
	-1	105.4	53.4	1856	390.9	2860.5	310.1	13247	127.6
	7	112.6	95	355.5	132.3	243.8	52.6	12793	219.5
	14	1016	92.7	10727	543.9	6863.5	684.4	11354	531.2
	21	382.6	135.3	10215	347.8	4374.8	427.8	15438	441.8
19	-3	209.85	173.8	471	242.9	4066	814.7	8089.5	1757.6
	-2	1052.2	944.4	805	340	3715	280.3	9907.8	214.3
	-1	372.2	347.6	1316	383.9	4132.2	606.3	18436	341.4
	7	117.8	72.9	159.8	17.6	402.8	75.9	17269	343.7
	14	39.8	13	4484.8	299.8	3712.5	251.1	8334.2	88.2
	21	495.3	169	4421	61.7	2510.2	158.8	17482	213
21	-3	8379.5	459.9	9738.5	680.2	8402.8	565.3	50508	4209.8
	-2	2572.8	484.2	8234.2	637.8	7555.8	896.8	18381	1136.6
	-1	7456.5	2187.4	6435.5	800	6805.2	1312.4	18233	160.2
	7	1123.7	394.6	1161.5	100.3	888.75	61.9	16371	710
	14	2598.2	199.7	3483.7	241.6	2517.2	244.4	16878	243.1
	21	2311.8	145	4488.8	162.6	2676.5	217	15486	272

Calf No.	Day PI	Med	SE	NC1	SE	S48	SE	Con A	SE
25	-3	ND		ND		ND		ND	
	-2	100.4	45.7	5906.8	502.3	3908.8	858.2	16978	860.6
	-1	1343	555.4	1874.5	376.8	2112.5	339.1	18110	1341
	7	127.6	69.8	206.8	43.3	266.2	42.7	18388	563.6
	14	128.9	30.9	8960.2	222.4	4680.5	374.5	15521	488.5
	21	355.6	195	8417	239.5	3101	227	19904	396
63	-3	1818.2	1591.9	4360	849.2	11863	857.8	47119	1866.9
	-2	69.4	42	1420.5	847.4	8578.5	930.8	18963	943.6
	-1	551.3	303.7	2711	153.7	5325.5	341.4	17531	294.8
	7	520.5	188.4	402.8	128.1	672.2	71	19491	294.8
	14	41.7	19.2	1024	70.1	1715.5	103.9	20364	212.7
	21	60.25	33.3	1249	81.3	1140.5	74.4	17647	217.4
64	-3	466	411.3	2437.5	599.4	7130.5	740.3	18185	3153.9
	-2	159	114.8	8999.7	2070.2	8330.8	960.9	16977	115.2
	-1	3702.2	1636	2515.5	613.9	7749.2	1024.5	14212	313.5
	7	794.9	347.3	615.8	198.9	1109	173	15974	89.6
	14	1277.5	320.9	1194.5	147.4	1334	78.5	19920	150.9
	21	487.9	344.6	1362.5	167.2	1780	286.4	15321	203.7

Appendix

Publications and Proceedings of Meetings

Refereed Publications

Innes E.A., Panton W.R.M., Marks J., Trees A.J., Holmdahl J. and Buxton D. (1995). Interferon gamma inhibits intracellular multiplication of *Neospora caninum,* quantified by incorporation of ^{3}H uracil. *Journal of Comparative Pathology*, **113**, 95-100

Buxton D., Caldow G.L., Maley S.W., Marks J. and Innes E.A. (1997). Neosporosis and bovine abortion in Scotland. *The Veterinary Record,* **141**, 649-651.

Marks J., Lundén A., Harkins D. and Innes E. (1998). Identification of *Neospora* antigens recognised by CD4+ve T cells and immune sera from experimentally infected cattle. *Parasite Immunology*, **20**, 303-309.

Lundén A., Marks J., Maley, S.W. and Innes E. (1998). Cellular Immune Responses in cattle experimentally infected with *Neospora caninum*. *Parasite Immunology*, in press.

Harkins, D., Clements, D.N., Maley, S., Marks, J., Wright, S., Esteban, I. And Innes, E.A. (1998). Western blot analysis of the IgG responses of ruminants infected with *Neospora caninum* and *Toxoplasma gondii*. *Journal of Comparative Pathology*, **119**, 45-55.

Proceedings of Meetings

Marks, J., Esteban, I., Panton, W.R.M. and Innes, E.A. (1995). Inhibition of *Neospora caninum* multiplication within ovine and bovine fibroblast cells following treatment with recombinant IFNγ. Meeting of the Scottish Universities Molecular Parasitology Group, Kindrogan, 5-6th May.

Innes E.A., Buxton D., Caldow G.L., Maley S.W. and Marks J. (1996) A serological survey of bovine neosporosis in Scotland: interim results. Association of Veterinary Teachers and Research Workers, Scarborough, 2-4th April.

Marks J., Esteban I., Panton W. and Innes E.A. (1996). Inhibition of *Neospora caninum* multiplication within ovine and bovine fibroblast cells following treatment with recombinant IFNγ. Association of Veterinary Teachers and Research Workers, Scarborough, 2-4th April.

Innes E.A., Marks J., Bartley P., Maley S., Wright S., Harkins D. and Buxton D. (1996). Cell mediated immunity to *Neospora caninum*. COST 820 Working Group 4 on Vaccines Against Animal Coccidiosis, Copenhagan, 10-12th October.

Marks J., Lunden A. and Innes E.A. (1997). Identification of specific *Neospora caninum* antigens which stimulate CD4+ T cells from immune cattle. Association of Veterinary Teachers and Research Workers, Scarborough, 25-27th March.

Lunden A., Marks J. and Innes E.A. (1997). Proliferation of peripheral blood mononuclear cells and production of IFNγ in *Neospora* infected calves. COST 820 Working Group 4 on Vaccines Against Animal Coccidiosis, Wusterhausen, Germany, 17-18th April.

Marks J., Lunden A. and Innes E.A. (1997). Identification of Specific *Neospora caninum* antigens which stimulate CD4+ T cells from infected cattle. COST 820 Working Group 4 on Vaccines Against Animal Coccidiosis, Wusterhausen, Germany, 17-18th April.

Buxton D., Caldow G.L., Maley S.W., Marks J. and Innes E.A. (1997) Neosporosis and bovine abortion in Scotland. Proceedings of the XIX World Buiatrics Congress, Edinburgh, 8-12th July.

Innes E.A., Marks J., Lunden A., McLean-Tooke A., Maley S.W., Wright S. and Buxton D. (1997). Cell mediated immune responses to *Neospora caninum*. VII International Coccidiosis Conference and European Union COST820 Workshop, Keble College, Oxford, 1-5th September.

Marks J., Graham D., McNamee P., Lunden A. and Innes E.A. (1997). Sero-epidemiological studies of a dairy herd infected with *Neospora caninum* in Northern Ireland. VII International Coccidiosis Conference and European Union COST820 Workshop, Keble College, Oxford, 1-5th September.

Marks J., Graham D., McNamee P. and Innes E.A. (1997). Immune response in calves congenitally infected with *Neospora caninum.* PhD second year assessment, Faculty of Science and Engineering, Edinburgh University.

Innes, E.A., Marks, J., Esteban, I., Lunden, A., Maley, S., Wright, S., Harkins, D., Rae, A., Buxton, D. and Vermeulen, A. (1998). Immunity to *Neospora caninum*. 52nd Meeting of the Association of Veterinary Teachers and Research Workers, Scarborough, 7th-9th April.

Marks, J., Esteban-Redondo, I., Lunden, A., Maley, S., McLean-Tooke, A., Duggan, T. and Innes, E.A. (1998). Immunity to *Neospora caninum*. Vaccines Against Animal Coccidiosis: Proceedings of the EU COST 820 Annual Workshop, Toledo, Spain, 22nd-25th October.

Parasite Immunology, 1998: **20**: 303–309

Identification of *Neospora* antigens recognized by CD4$^+$ T cells and immune sera from experimentally infected cattle

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SUMMARY

Neospora caninum *is recognized as a major cause of infectious abortion in cattle. Very little is known about immunity to* Neospora. *Cell mediated responses have previously been shown to be important in the development of protective immunity to the closely related parasite* Toxoplasma gondii, *and may therefore be an important component in the immune response to* Neospora. *In this paper we report that a group of low molecular weight NC1 strain tachyzoite antigens (≤ 30 kDa) separated by SDS PAGE and bound to nitrocellulose membrane stimulated proliferation in vitro of CD4$^+$ T cells from calves experimentally infected with* N. caninum. *Proliferation was accompanied by production of high concentrations of IFNγ. Several of these antigens were also recognized by antibody produced in these animals. As the most effective vaccines require the stimulation of both humoral and cell mediated immune responses, these antigens may be important in the development of a vaccine against neosporosis.*

Keywords Neospora caninum, *CD4$^+$T cells*, *antigens*, *IFNγ*

INTRODUCTION

Neospora caninum is a recently recognized protozoan parasite which is closely related to, and previously misdiagnosed as, *Toxoplasma gondii* (Dubey *et al.* 1988). The parasite is recognized as being a major cause of infectious abortion in cattle world-wide (Dubey *et al.* 1989, Thilsted & Dubey 1989, Anderson *et al.* 1991). Unlike toxoplasmosis in sheep, which stimulates protective immunity following primary infection, neosporosis can cause repeat abortion in cattle (Anderson *et al.* 1995, Moen *et al.* 1995). But the rate of repeat abortion is estimated to be less than 5% (Anderson *et al.* 1995, Moen *et al.* 1995), therefore it is probable that many animals do develop some form of protective immunity. *Neospora caninum*, like *T.gondii*, is an obligate intracellular parasite, therefore it is likely that cell mediated immune mechanisms are an important component of the immune response. We have previously shown that treatment of fibroblast cells with recombinant interferon gamma (rIFNγ) caused significant inhibition of intracellular multiplication of *N. caninum* compared to untreated control cells (Innes *et al.* 1995a). The aim of this study was to identify *Neospora* antigens that are important in the humoral and cell mediated host immune response to the parasite. These antigens could be of use as potential vaccine candidates. Antigens recognized by the humoral immune response in particular may also be important in the development of serological diagnostic assays. In this study CD4$^+$ T cells and sera from calves inoculated with *N. caninum* tachyzoites were tested for their ability to recognize *Neospora* antigen fractions separated by SDS PAGE and transferred to nitrocellulose membrane (Young & Lamb 1986).

MATERIALS AND METHODS

Animals and inoculum

Six calves ≈ 6 months old were selected which were seronegative for *N. caninum* and *T. gondii* by an indirect fluorescent antibody test (IFAT). Four of the calves were

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injected subcutaneously on day 0 with $2{\cdot}5 \times 10^8$ NC1 strain *N. caninum* tachyzoites (Dubey *et al.* 1988). *Neospora caninum* was cultured within vero cell monolayers as described by Innes *et al.* (1995a). Two control calves were inoculated subcutaneously with a dose of vero cells estimated to be similar to that received by the *N.caninum* infected calves. Inoculated and control calves were penned separately throughout the experiment.

Preparation of NC1 antigen

N. caninum antigen was prepared by 3 × freeze/thaw cycle of *N. caninum* tachyzoites suspended in distilled water followed by 7 × 15 s cycles of sonication on ice and centrifuged at 10 000 *g* for 30 min. The water soluble fraction (wsf) antigen was stored in aliquots of 100 μl at − 20°C and used as antigen in proliferation assays (Innes *et al.* 1995b). To prepare sodium dodecyl sulphate (SDS) (Sigma, UK) soluble antigen 200 μl of 10% SDS solution was added to the crude sonicated antigen prior to centrifugation. Protein concentration was assayed using BCA reagent (Pierce, USA) and read using a Monarch 2000 spectrophotometer (Instrumentation Laboratories, USA).

SDS PAGE and transfer to nitrocellulose

(a) Preparation of antigen for T cell assays

NC1 *N.caninum* proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions as described by Laemmli (1970). 50 μl of SDS soluble antigen and 50 μl wsf antigen were mixed with an equal volume of reducing buffer and boiled for ≈ 5 min in a water bath. The mini Protean system (Biorad, UK) was used to separate antigen on a 10% polyacrylamide gel, and a combination of molecular weight markers in the range of 12 000–78 000 kDa was used to determine molecular mass (BDH, UK). Proteins were transferred from the gel to a nitrocellulose membrane (Schleicher & Schnell, Germany) by electroblotting with a trans-blot electrophoretic transfer cell (Biorad, UK) at a constant current of 100 v for 45 min to one h (not exceeding 0·7 A). The blot was dried and cut into 20 strips 2–3 mm wide, each strip containing antigen of the same molecular weight. Fraction number 1 had the highest molecular weight, and number 20 was the lowest. Strips were then cut into pieces of ≈ 2 mm^2 and placed for one h in Hanks balanced salt solution supplemented with 2% foetal calf serum (Sigma, UK), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, UK) and 0·01% sodium azide (Sigma, UK) (FACS medium) followed by four washes with sterile PBS. Nitrocellulose bound antigen fractions were stored at 4°C in PBS until used in the proliferation assay.

(b) Western blot analysis

Neospora specific protein fractions were separated from a combination of equal volumes of SDS and wsf *Neospora* antigen under reducing conditions by SDS PAGE and proteins transferred to nitrocellulose membrane as previously described. The membrane was then cut into 0·5 cm wide strips and whole protein bound to the membrane shown by staining one strip with Ponceau dye (Sigma, UK). Strips were treated with 5% Marvel (dried skimmed milk) in tris buffered saline tween (TBST) for one h to block nonspecific background staining before incubation for 90 min with sera from the infected calves taken at four weeks post inoculation and on day − 1 preinfection. Sera from the control animals was also tested. After several washes and a further 90 min incubation with secondary antibody (alkaline phosphatase conjugated rabbit antibovine IgG, whole molecule) (Sigma Immuno-Chemicals, UK) the strips were developed using a freshly prepared mixture of nitro blue tetrazolium (NBT) (Sigma, UK) and bromochloroindolyl phosphate (BCIP) (Sigma, UK) in alkaline phosphatase buffer (Sigma, UK).

Preparation of peripheral blood mononuclear cells

Blood was collected in heparinized vacutainers containing 10 U/ml heparin, diluted 1:2 with sterile PBS and centrifuged for 20 min at 450 *g* at 12°C. The buffy coat was removed and diluted 1:2 in Hanks balanced salt solution (HBSS) supplemented with 2%FCS, 100 U/ml heparin (Sigma, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, UK) (wash medium). The cell suspension was layered over lymphoprep (Nycomed, UK) and centrifuged at 550 *g* for 30 min. Peripheral blood mononuclear cells (PBM) were collected from the interface, washed three times in wash medium and resuspended in Iscove's modified Dulbecco's medium supplemented with 10%FCS (Sigma, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, UK) (T cell medium) at a concentration of 2×10^6/ml.

Generation of $CD4^+$ T cell lines

Infected and control animals were bled 4–6 weeks post infection and PBM were separated from whole blood as previously described. Equal volumes of PBM at 2×10^5 cells/well and NC1 wsf antigen at 5 μg/ml were cultured in 96 well round bottom tissue culture plates (Nunc, Denmark) at 37°C in a humidified 5% CO_2 incubator. After seven days the cells were harvested and diluted 1:3 in T cell medium supplemented with 10 U/ml of human recombinant IL2 (Proleukin, Eurocetus). Cells were cultured for a further seven days prior to harvesting when they were used in proliferation assays.

Proliferation assays

Peripheral blood mononuclear cells were separated from whole blood as previously described and resuspended at

2×10^6/ml. Equal volumes of PBM and NC1 wsf antigen at 5 and 10 μg/ml or 2 mm^2 sterile nitrocellulose bound *Neospora* antigen fractions were cultured in 96 well round bottom tissue culture plates (Nunc, Denmark) at 37°C in a humidified 5% CO_2 incubator for 5 days. Cells were pulsed for the final 18 h with 18·5 kBq [^{3}H]-thymidine (Amersham, UK) per well before harvesting onto fibreglass filters (Packard Canberra, USA). Cell-associated radioactivity was quantified in a gas proportional counter (Packard Canberra, USA). Proliferation assays with the $CD4^+$ T cells were cultured in the presence of autologous PBM irradiated with 3000 rad from a ^{37}Cs source as antigen presenting cells (APC). The ratio of APC:CD4 was 10:1. Cells cultured in 5 μg/ml Concanavalin-A (Con-A) (Sigma, UK) and in medium alone were used as positive and negative controls, respectively. Unfractionated NC1 wsf antigen was dot blotted directly onto nitrocellulose and used as a positive control, and cells were also tested for nonspecific stimulation by nitrocellulose membrane alone. In proliferation assays cells from the control animals were set up in quadruplicate, and assays measuring responses of cells from the infected animals to the nitrocellulose bound antigens were set up in triplicate.

Phenotypic analysis of $CD4^+$ T cell lines

The cellular composition of the $CD4^+$ T cell lines was analysed using a panel of monoclonal antibodies (MoAbs) recognizing specific bovine leucocyte populations (Table 1). The method used to stain the cells is largely described by Innes *et al.* (1995b). In brief 2×10^6 washed PBM were resuspended in 50 μl of FACS medium and mixed with 50 μl of the appropriate MoAb at a predetermined optimal dilution. Cells and MoAb were incubated at 4°C for 30 min, washed three times by repeated resuspension and centrifugation at 300*g* for 5 min. Cells were then resuspended in 50 μl of a 1:50 dilution of fluorescein isothiocyanate (FITC) conjugated antimouse immunoglobulin (Dakopatts, Denmark) and incubated in the dark for a further 30 min. Cells were then washed three times with FACS medium and finally fixed in FACS medium containing 1% paraformaldehyde. The percentage of cells stained with FITC was determined using flow cytometry (FACScan, Becton Dickinson, UK).

Assay for interferon gamma (IFNγ)

Supernatant was collected from $CD4^+$ T cells stimulated with *Neospora* antigen fractions 14–20 for four days. The concentration of bovine IFNγ in these samples was quantified using an ELISA kit produced by CSL Veterinary, Melbourne, Australia. Recombinant bovine IFNγ (rBovIFNγ) (Ciba Geigy, Switzerland) was used as a control to calibrate results. Samples were analysed in duplicate.

Table 1 Phenotypic analysis of cells taken from infected calves (nos 13, 14, 20 and 21) after stimulation with NC1 *Neospora caninum* wsf antigen for one week followed by expansion for a further seven days with human recombinant IL2.

		Animal No.			
Antibody	Specificity	13	14	20	21
CC8	CD4	99	98	99	98
CC63	CD8	8	11	9	22
CC42	CD2	100	99	100	99
ILA111	IL2R	82	79	100	99

The table illustrates the percentage of cells staining with each of the monoclonal antibodies. Figures are rounded up to the nearest percent. MoAbs CC8, CC63 and CC42 were obtained from IAH, Compton and ILA111 from ILRI, Nairobi, Kenya.

RESULTS

Proliferation of PBM to unfractionated NC1 antigen

Peripheral blood mononuclear cells from the four infected cattle proliferated in response to whole *Neospora* wsf antigen at final concentrations of 5 and 10 μg/ml, but did not respond to any of the 20 distinct nitrocellulose bound antigen fractions of NC1. Figure 1 illustrates the proliferative activity of a representative infected animal.

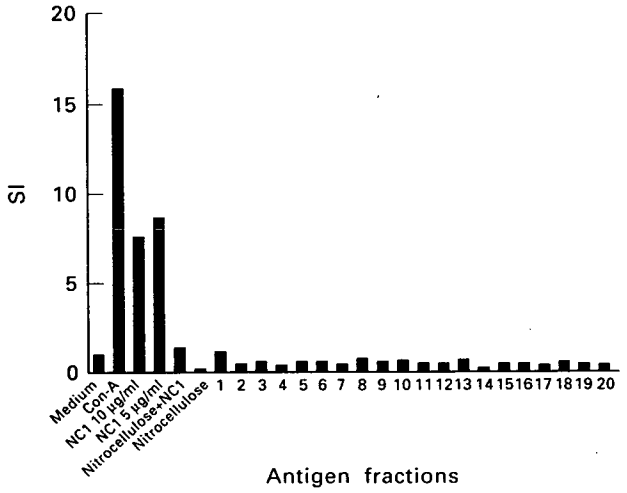

Figure 1 Proliferation of PBM taken on day 36 post infection from a representative infected calf (no. 14) to *Neospora* antigen fractions. Cells were stimulated by Con-A and whole *N. caninum* water soluble antigen at final concentrations of 10 and 5 μg/ml, and *N. caninum* specific antigen fractions (numbered 1–20). Cell growth was measured in counts per min ^{3}H thymidine incorporated into proliferating cells and represented above as a stimulation inded (SI units = mean CPM cells stimulated by antigen/mean CPM medium control, $n = 3$). Mean CPM of medium control 1390, standard error ± 253.

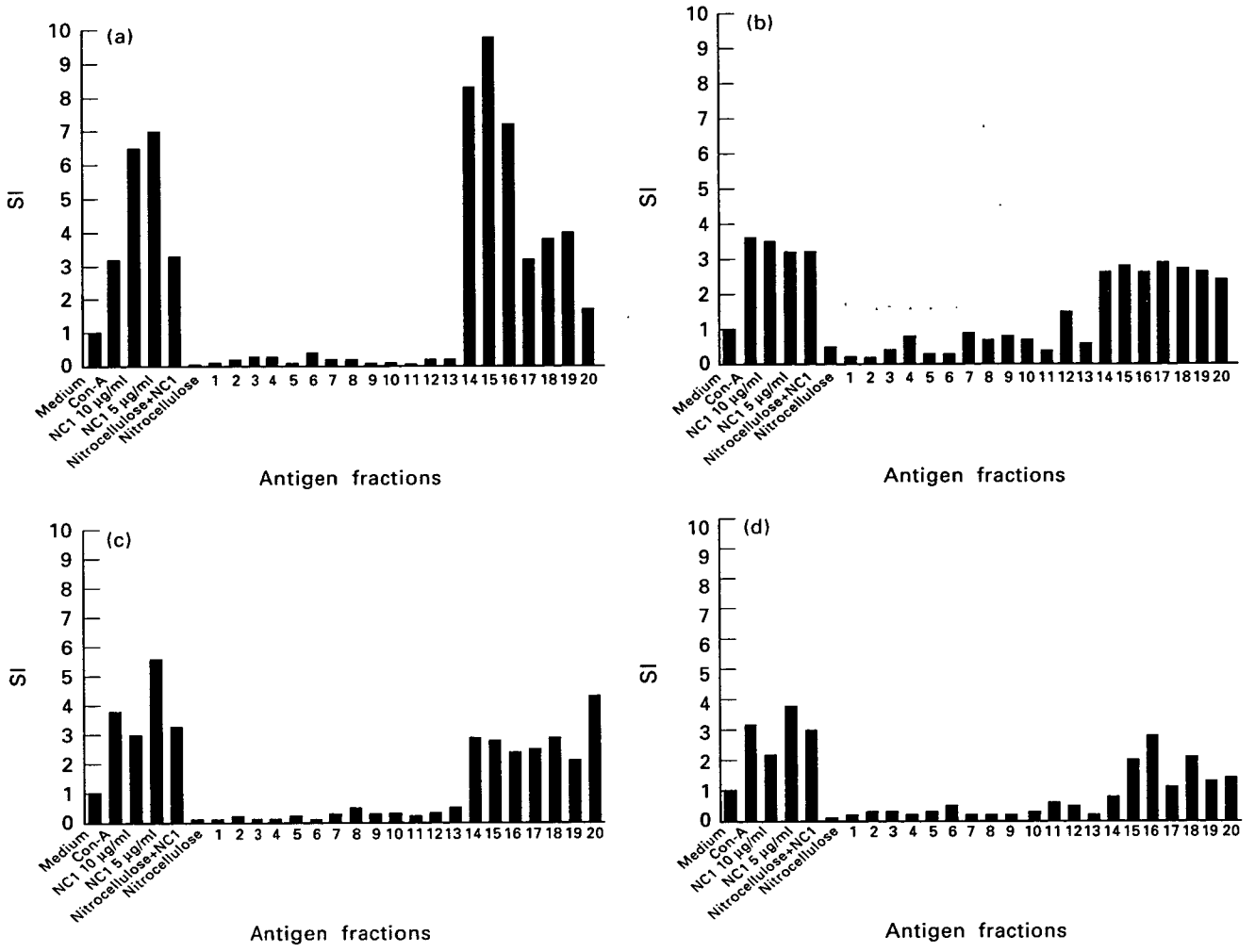

Figure 2 Proliferative response of T cell lines generated from PBM taken on day 36 post infection from a) calf 13, b) calf 14, c) calf 20 and d) calf 21, to nitrocellulose bound *N. caninum* specific antigen fractions numbered 1–20 is shown above. Cell proliferation was measured in CPM as before (Figure 1) and is represented as SI values ($n = 3$). Mean CPM and standard error of medium controls; a) 1595, ± 629, b) 5943, ± 1913, c) 4306, ± 856, d) 4576, ± 479.

The proliferative response to the antigen fractions was not greater than the response to the medium control (Figure 1). Peripheral blood mononuclear cells from control animals did not show any proliferative activity to the whole NC1 wsf antigen (results not shown) and therefore were not tested with the nitrocellulose antigen fractions. Strong proliferative responses were observed in cells from control and infected animals stimulated by 5 µg/ml Con-A.

Phenotypic analysis of T cell lines

$CD4^+$ T cell lines were cultured from PBM taken from infected cattle. Phenotypic analysis of the cell lines at the time they were used in proliferation assays with antigen fractions is shown in Table 1. T cell lines from infected cattle had > 97% $CD4^+$ cells.

Response of $CD4^+$ T cell lines to specific antigen fractions

The results are illustrated in Figure 2. $CD4^+$ T cell lines generated from the four infected cattle show consistent proliferative activity in response to unfractionated wsf NC1 antigen. When tested with the *Neospora* antigen fractions proliferation of $CD4^+$ cells was seen in response to a particular group of lower molecular weight antigen fractions (≤ 30 kDa) numbered 14–20. Cells did not proliferate in response to nitrocellulose membrane alone but did

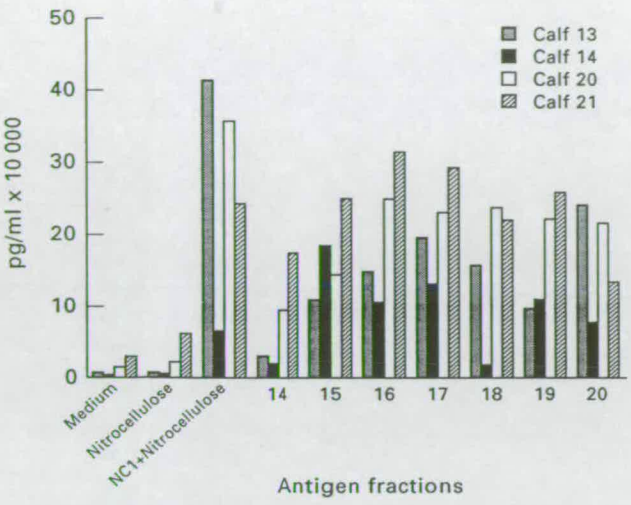

Figure 3 IFNγ concentration (picograms/ml) in supernatant from $CD4^+$T cells generated from the four *Neospora* infected calves and cultured with nitrocellulose bound antigen fractions 14–20. Cells were incubated with antigen for four days and IFNγ concentration in supernatant determined by specific ELISA. Cells cultured with medium and nitrocellulose membrane alone were used as background controls, and with whole NC1 wsf dot blotted onto nitrocellulose as a positive control ($n = 2$).

proliferate in the presence of whole wsf NC1 antigen dot blotted onto nitrocellulose. We were not able to obtain $CD4^+$ T cell lines from naive animals and cells cultured from these animals did not respond to *Neospora* antigen fractions. All cells proliferated strongly in the presence Con-A.

IFNγ analysis

Nitrocellulose bound whole wsf *Neospora* antigen and antigen fractions 14–20 consistently stimulated production of high concentrations of IFNγ from $CD4^+$ T cells generated from the infected animals (Figure 3). Comparatively low levels of this cytokine were detected in cells cultured in medium alone and in cultures of $CD4^+$ cells incubated with nitrocellulose membrane alone.

Humoral immune response

Antigens recognized by sera from the infected cattle were identified by Western blot (Figure 4). No *Neospora* specific antibodies were present in sera from the control or preinfection sera, whereas all four infected cattle showed a strong antibody response at four weeks post infection. *Neospora* specific sera from the infected cattle recognized proteins with molecular weights of ≈ 70, 32, 30, 28 kDa and also a band at the base of the blot.

DISCUSSION

Neosporosis is recognized as a major cause of bovine abortion (Anderson *et al.* 1991, Buxton *et al.* 1997). Very little is known about the host immune response to *N. caninum* and the precise cause of abortion is unknown. Repeat abortion has been observed, but the rate seems to be very low, at less than 5% (Anderson *et al.* 1995, Moen *et al.* 1995). Therefore current evidence suggests the majority of animals do develop some form of protective immunity. Cell mediated responses have previously been shown to be important in the development of protective immunity to the closely related parasite *T. gondii* (Suzuki & Remington 1988, Parker *et al.* 1991, Innes *et al.* 1995b). IFNγ, an important T cell cytokine produced mainly by $CD4^+$T cells, is a major mediator of resistance to *Toxoplasma* in

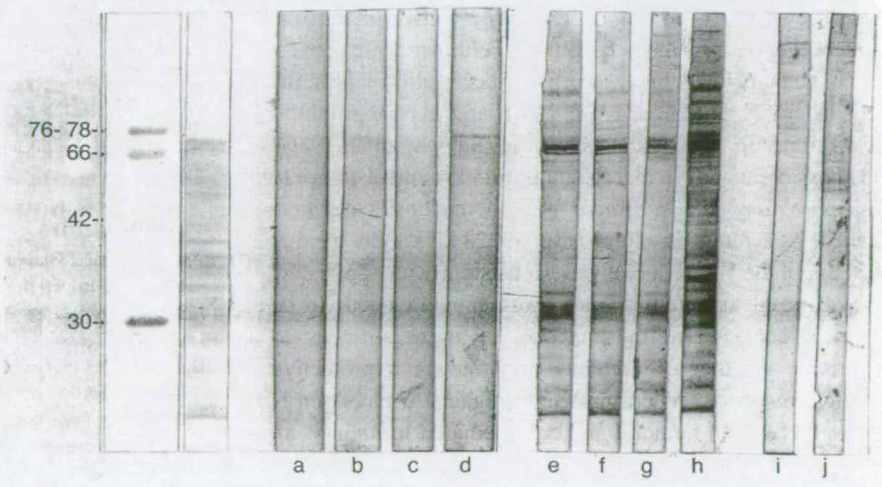

Figure 4 A combination of SDS and wsf soluble *Neospora* antigens on a Western blot probed with pre- (day −1) (strips a–d) and post- (day 28) (e–h) infection sera from calves 13 (a & e), 14 (b & f), 20 (c & g) and 21 (d & h). Calves were subcutaneously inoculated with $2{\cdot}5 \times 10^8$NC1 tachyzoites on day 0. Nitrocellulose bound antigen was also probed with sera from control animals 15 (i) and 25 (j).

sheep (Innes *et al.* 1995b). We have previously shown that recombinant IFNγ suppresses the growth of NC1 strain *N. caninum* tachyzoites cultured in fibroblasts *in vitro* (Innes *et al.* 1995a). Also recent data by Khan *et al.* (1997), demonstrated a protective cell mediated immune response in three inbred strains of mice (C57BL/6, A/J, Balb/c) experimentally infected with NC1 strain *N. caninum* tachyzoites (Khan *et al.* 1997). This immune response was characterized by the induction of IL-12 and upregulation of IFNγ mRNA *in vivo*. Introduction of antibody to IL12 and IFNγ into these mice ablates protection and results in death. IFN γ plays an important role in the activation of other cell types, in particular macrophages, to destroy tachyzoites and promotes maturation of $CD8^+$ T cells into effector cytotoxic cells (Zanovello *et al.* 1988). Therefore $CD4^+$ T cells which produce IFNγ may be a critical component of the cell mediated response to *N. caninum*. Identification of the *Neospora* antigens that induce $CD4^+$ T cells to produce IFNγ may therefore be important in the development of a vaccine to prevent *Neospora* associated abortion in cattle.

PBM from the experimentally infected cattle proliferated *in vitro* in response to whole crude *N. caninum* wsf antigen, whereas PBM from control cattle did not. Therefore a simple proliferation assay using crude lysate antigen prepared from NC1 tachyzoites was able to distinguish infected from uninfected animals. *Neospora* specific $CD4^+$ T cell lines from the 4 infected calves consistently responded to *Neospora* antigen fractions 14–20 of molecular weight ≤ 30 kDa. Supernatants from the activated $CD4^+$ T cells contained high concentrations of IFNγ which as previously discussed is likely to be important in protective immunity (Innes *et al.* 1995a, Khan *et al.* 1997). These low molecular weight proteins may therefore be responsible for the induction of a potentially protective cell mediated immune response to neosporosis.

Specific antibodies to the closely related parasite *T. gondii* have previously been shown to destroy tachyzoites in the presence of complement (Sabin & Feldman 1948), overcome the parasite's ability to prevent destruction within the host macrophage (Joiner *et al.* 1990) and may also inhibit host cell penetration by the tachyzoite (Schwartzman 1986). It is possible therefore that the humoral immune response to *Neospora* may play a similar role in the host's defence. Several NC1 antigens of ≈ 70, 32, 30, 28 kDa and a band at the base of the Western blot were consistently recognized by sera from the infected animals. Further work needs to be done to characterize the function of these antigens to determine whether or not they may induce a protective immune response. Where protection against a pathogen is mediated by both humoral and cell mediated immunity, an effective vaccine will need to incorporate antigens capable of activating both T and B cell epitopes (Lamb *et al.* 1988). Therefore the three lowest molecular weight antigens (≤ 30 kDa) which were recognized by both the humoral and cell mediated responses may be important in the future development of a vaccine against neosporosis.

Animals were inoculated with NC1 strain *N. caninum* tachyzoites which were first isolated from the neural tissues of a dog. Comparison of ribosomal DNA from the U1 region of the nss-rRNA gene of *N. caninum* NC1 strain tachyzoites, an area which has been identified as evolutionarily unstable, with 4 bovine *Neospora* isolates, BPA 1–4 showed that there is no variation between NC1 and the 4 bovine isolates in this region (Marsh *et al.* 1995). A closer comparison of the 1·7kb of the nss-rRNA gene of BPA1 and NC1 also revealed no differences. To date there have been no antigenic differences identified between these isolates. Therefore we believe it is appropriate to use the NC1 strain in experimental infection in cattle.

While the nature of protective immunity to *Neospora* has yet to be determined it is likely that both IFNγ and antibody production will play important roles. Therefore identification of the specific parasite antigens involved in induction of humoral and cell mediated responses, and in particular the production of IFNγ, will have a considerable impact on our understanding of how to induce a potentially protective immune response in naive animals.

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