

Recombinant Subunit Vaccines against *Neospora caninum*

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Abstract

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The intracellular protozoan parasite *Neospora caninum* is an important cause of bovine abortion and congenital infection in many countries. Currently there is no effective control method available, and development of a vaccine has been suggested as a possible strategy to prevent the disease neosporosis.

In this thesis an experimental recombinant subunit vaccine, consisting of the *N. caninum* antigen NcSRS2 and immunostimulating complexes (iscoms), was evaluated. The iscom is an adjuvant formulation into which antigens can be incorporated by hydrophobic interactions. Many recombinant proteins are hydrophilic, and special methods are needed for binding them to the iscoms and to achieve optimal adjuvant effects.

A novel method to combine recombinant proteins with iscoms utilizing the strong interaction between biotin and streptavidin was evaluated. Recombinant NcSRS2 was expressed in *Escherichia coli* as two different fusion proteins. One of them was biotinylated in *E. coli* and was bound to streptavidin-coated iscom matrix (iscom particles without any antigen). The other fusion protein contained recombinant streptavidin and was bound to biotinylated iscom matrix. These two strategies both resulted in efficient binding to the iscom matrix. Thus, this method might offer a convenient and effective alternative for association of other hydrophilic recombinant antigens with iscoms for evaluation in immunisation experiments.

Both the immunogenicity and the protective effect of the NcSRS2-iscoms were investigated by immunisation and challenge infection of mice. A real-time PCR assay was developed, and used for quantification of parasite DNA in the brain and blood of infected mice.

The NcSRS2-iscoms induced production of antibodies that recognised NcSRS2 of parasite origin, suggesting that the conformation of the recombinant protein was similar to that of the native antigen. Antigen-specific cellular responses were also induced as demonstrated by *in vitro* proliferation and cytokine production. After challenge infection, the immunised mice had significantly lower levels of *N. caninum* DNA in their brains and blood than did non-immunised control mice. These mice were also less affected by the disease, as judged by clinical symptoms and changes in body weight. These results, together with results from other studies, indicate that recombinant NcSRS2 might be considered as a potential candidate antigen for a future subunit vaccine against *N. caninum* infection in cattle.

Keywords: *Neospora caninum*, NcSRS2, iscom, subunit vaccine, recombinant immunogen, parasitaemia, real-time PCR

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Papers I–IV

This thesis is based on the following four papers:

- I. Wikman, M., Friedman, M., Pinitkiatisakul, S., Hemphill, A., Lövgren-Bengtsson, K., Lundén, A. and Ståhl, S. 2005. Applying biotin-streptavidin binding for iscom (immunostimulating complex) association of recombinant immunogens. *Biotechnology and Applied Biochemistry* 41, 163-74.
- II. Pinitkiatisakul, S., Mattsson, J.G., Wikman, M., Friedman, M., Lövgren-Bengtsson, K., Ståhl, S. & Lundén, A. 2005. Immunisation of mice against neosporosis with recombinant NcSRS2 iscoms. *Veterinary Parasitology* 129, 25-34.
- III. Pinitkiatisakul, S., Friedman, M., Wikman, M., Mattsson, J.G., Lövgren-Bengtsson, K., Ståhl, S. & Lundén, A. Immunogenicity and protective effect against murine cerebral neosporosis of recombinant NcSRS2 in different iscom formulations. *Vaccine*, in press, doi:10.1016/j.vaccine.2007.01.074.
- IV. Pinitkiatisakul, S., Mattsson, J.G. & Lundén, A. Quantitative analysis of parasite DNA in the blood of immunised and naïve mice after infection with *Neospora caninum*. Manuscript.

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Abbreviations

ABP	albumin binding protein
Bio	biotin / biotinylated
Bio-matrix	biotinylated iscom matrix
BioSRS2'-iscom(Ni)	Ni ²⁺ -matrix + His ₆ -SA + Bio-His ₆ -ABP-SRS2'
Con A	concanavalin A
cpm	counts per minute
Ct	cycle threshold
dBioSRS2'-iscom(Ni)	Ni ²⁺ -matrix + His ₆ -SA + Bio-Bio-His ₆ -ABP-SRS2'
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	enzyme-linked immunosorbent assay
His ₆	hexahistidyl
HRP	horse-radish peroxidase
IDA-matrix	matrix containing the chelating lipid IDA-Trig-DSGE
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
Iscom	immunostimulating complex
K _D	dissociation equilibrium constant
kDa	kilodalton
M5	peptide derived from the <i>Plasmodium falciparum</i> blood-stage antigen Pfl55/RESA
M5-iscom(Ni) matrix	Ni ²⁺ -matrix + His ₆ -SA + Bio-His ₆ -ABP-M5 iscom matrix <i>i.e.</i> iscom particles without any antigen
NCDG, NcGRA	dense granule antigen of <i>N. caninum</i>
NcMIC	microneme antigen of <i>N. caninum</i>
NcSAG1	surface antigen 1 of <i>N. caninum</i>
NcSRS2	SAG1 related sequences 2 of <i>N. caninum</i>
<i>N. caninum</i>	<i>Neospora caninum</i>
NK	natural killer cell
PCR	polymerase chain reaction
Quil A	saponin derived from <i>Quillaja saponaria</i> Molina
SA	streptavidin
SA-SRS2'-iscom(Bio)	Biotin-matrix + His ₆ -SA-SRS2'
SA-SRS2'-iscom(Ni)	Ni ²⁺ -matrix + His ₆ -SA-SRS2'
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
<i>T. gondii</i>	<i>Toxoplasma gondii</i>
TgSAG1	surface antigen 1 of <i>T. gondii</i>
TgSRS2	SAG1 related sequences 2 of <i>T. gondii</i>
Th1	type 1 T-helper
Th2	type 2 T-helper
TNF-α	tumor necrosis factor-alpha
ZZM5	recombinant protein containing two IgG-binding domains of the staphylococcal protein A as fusion partner to M5

Background

The protozoan parasite *Neospora caninum* is an important cause of abortion in cattle (the intermediate host), as well as causing paralysis in dogs (the definitive host). At present there is no efficient vaccine available against the disease neosporosis. This thesis investigates an experimental vaccine consisting of a recombinant *N. caninum* protein (NcSRS2) and immuno-stimulating complexes (iscoms) as adjuvant. A mouse model is used to evaluate a novel method of combining recombinant proteins with this kind of adjuvant.

Neospora caninum

Neospora caninum is an intracellular coccidian parasite closely related to *Toxoplasma gondii*, and placed in the family Sarcocystidae of the phylum Apicomplexa. *N. caninum* causes neosporosis, which affects both cattle and dogs. Today, it is recognised as a major cause of bovine abortion and neonatal mortality worldwide, and neosporosis is considered to be economically important to the farming industry (Dubey, 1999a; Dubey & Lindsay, 1996; Hemphill, 1999). The parasite was originally described in 1984 as an unidentified cyst-forming sporozoan causing encephalitis and myositis in dogs (Bjerkås, Mohn & Presthus, 1984). Later, Dubey and coworkers identified a similar parasite causing neuromuscular disease in dogs, and proposed the genus *Neospora* and species *N. caninum* (Dubey *et al.*, 1988a; Dubey *et al.*, 1988b). The complete lifecycle, including the dog as a definitive host, was described by McAllister and coworkers (McAllister *et al.*, 1998). Recently, the coyote has also been identified as a definitive host (Gondim *et al.*, 2004).

Parasite stages

N. caninum is an obligate intracellular parasite with a life cycle comprising three infectious stages: tachyzoites, bradyzoites within tissue cysts, and sporozoites within oocysts. Tachyzoites and bradyzoites are intracellular, asexual stages of the parasite found in both intermediate and definitive hosts. Oocysts are shed by the definitive host after sexual reproduction in its intestine (Dubey *et al.*, 2002).

The tachyzoites are ovoid, lunate or globular and 3–7 x 1–5 µm in size. They have been detected in various host tissues or cell types, including macrophages, fibroblasts, vascular endothelial cells, myocytes and hepatocytes, but mostly in the brain and spinal cord (Dubey & Lindsay, 1996). This ability to infect a wide range of cells implies that *N. caninum* has a low host-cell specificity (Hemphill *et al.*, 1999). The individual infected host cells may each contain many tachyzoites, located within a parasitophorous vacuole in the host-cell cytoplasm (Dubey *et al.*, 2002). They proliferate into two zoites by a process called endodyogeny (internal budding), producing hundreds of new tachyzoites within a few days after infection, resulting in cell lysis and necrosis. The new parasites that are released can invade nearby cells or be transported by blood or lymph to other organs (Dubey & Lindsay, 1996).

The bradyzoites are slender and elongate with a subterminal nucleus, approximately 8 x 2 µm in size and located in tissue cysts. Tissue cysts are often round to oval in shape, up to 100 µm long, with a smooth cyst wall 1–4 µm thick, depending on how long the infection has existed (Dubey *et al.*, 2002). Most tissue cysts are found in the central nervous system and other neural tissues, including retina and peripheral nerves (Daft *et al.*, 1997; Dubey, 1999a; Dubey *et al.*, 1988a; Dubey & Lindsay, 1996).

The oocysts, which are the result of the sexual phase of the life cycle, are spherical to subspherical in shape and approximately 11 µm in diameter (Dubey, 1999a; Lindsay, Upton & Dubey, 1999b). The oocyst wall is colourless and 0.6–0.8 µm thick. Sporulated oocysts contain two sporocysts with four sporozoites within. Oocysts of *N. caninum* are morphologically indistinguishable from those of *Hamondia heydorni*, found in canid faeces, and *T. gondii*, found in felid faeces (Dubey, 1999a).

Life cycle

N. caninum can infect a wide range of animal species that can serve as intermediate hosts. Natural infection has been recorded in cattle, sheep, goats, horses, deer and dogs. In addition, mice, rats, dogs, foxes, cats, coyotes, pigs, gerbils and rabbits have been experimentally infected (Dubey & Lindsay, 1996). Dogs and coyotes can act as both intermediate and definitive hosts; and after ingestion of tissue cysts in, for example, bovine placenta they can shed oocysts in their faeces (Dijkstra *et al.*, 2001; Gondim *et al.*, 2004; McAllister *et al.*, 1998). The oocysts sporulate and become infective within 24 hours (Lindsay, Dubey & Duncan, 1999a).

There are two main modes of transmission of *N. caninum* (Fig. 1): postnatal or horizontal transmission through ingestion of oocysts or tissue cysts, and transplacental or vertical transmission from an infected mother to her foetus. In cattle, vertical transmission is the dominant route of infection, and can occur repeatedly in consecutive pregnancies (Barr *et al.*, 1993; Barr *et al.*, 1994b; Thurmond & Hietala, 1997). Experimentally, transplacental transmission has been induced in several other species, e.g. dogs (Cole *et al.*, 1995a; Dubey & Lindsay, 1989a; Dubey & Lindsay, 1989b), cats (Dubey & Lindsay, 1989b), mice (Cole *et al.*, 1995b; Long & Baszler, 1996), sheep (Buxton *et al.*, 1997; McAllister *et al.*, 1996), goats (Lindsay *et al.*, 1995b), pigs (Jensen *et al.*, 1998) and non-human primates (Barr *et al.*, 1994a).

It has been suggested that an additional mode of vertical transmission could be via the colostrum, since neonatal calves have been experimentally infected by feeding them colostrum containing tachyzoites (Uggla *et al.*, 1998). Recently, parasite DNA has been demonstrated in colostrum from seropositive cows, implicating the possibility of transmission through colostrum. However, it is uncertain if this occurs naturally, as high levels of specific antibodies in the colostrum might neutralise the infectivity of tachyzoites (Davison *et al.*, 2001; Moskwa *et al.*, 2007).

There are many aspects of the life cycle of *N. caninum* that are still unknown, including the survival of oocysts in the environment, frequency of oocyst shedding and whether canids other than the domestic dog and the coyote can act as definitive host. Moreover, the entero-epithelial stages of *N. caninum*, preceding oocyst formation in dogs, have not been described yet.

It was previously reported that *Neospora* infection might not be a zoonotic threat to healthy humans (Petersen *et al.*, 1999); however, it might possibly cause disease in immunocompromised patients, as specific antibody to *N. caninum* have been detected in sera from such individuals (Lobato *et al.*, 2006).

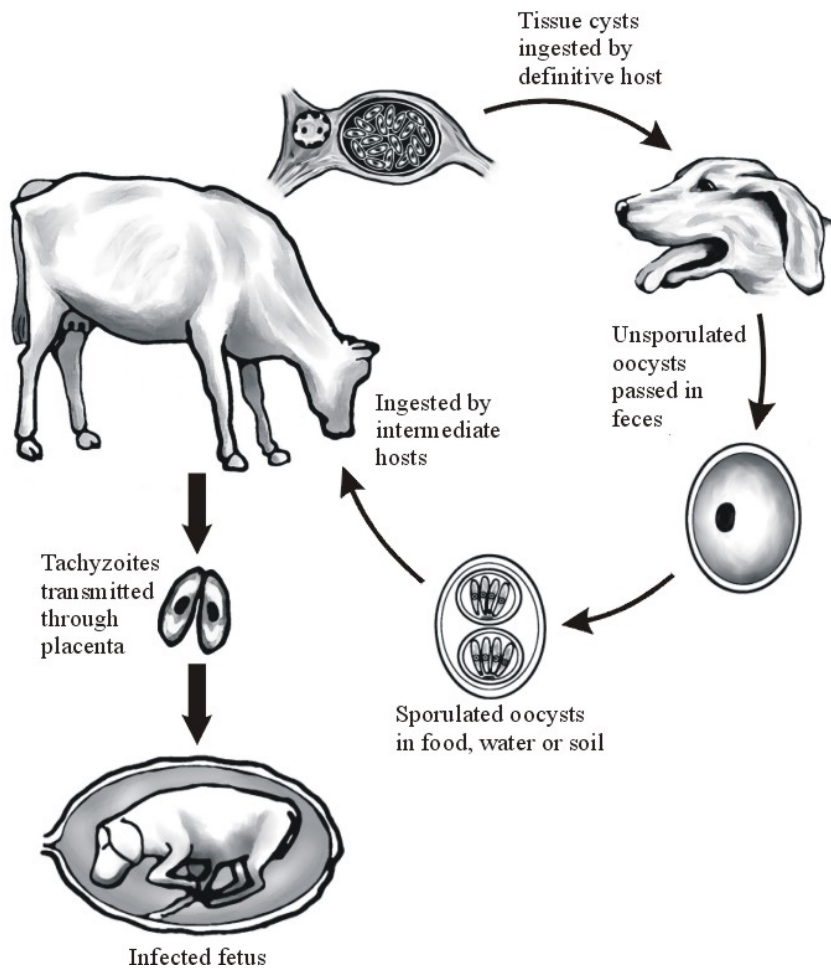


Fig. 1. The life cycle of *Neospora caninum*. The dog is a definitive host of the parasite, and can shed oocysts with its faeces. Oocysts can be ingested by intermediate hosts, such as the cow. Tachyzoites may be transmitted from the dam to her foetus. This is the main form of transmission for the parasite. The dog may ingest tissue cysts via uncooked tissue from an intermediate host, thus completing the life cycle of the parasite. Modified from a figure published in *Veterinary Parasitology* 84 (1999) with kind permission from J.P. Dubey.

Neosporosis in cattle

N. caninum is a major cause of abortion in both dairy (Dubey & Lindsay, 1996) and beef (Hoar *et al.*, 1996; Waldner, Janzen & Ribble, 1998) cattle, as reported from many countries over the world, including U.S.A., Canada, New Zealand, Australia, and several countries in Europe, Asia and Africa (Dubey, 1999a; Dubey, 1999b; Dubey & Lindsay, 1996; Hemphill & Gottstein, 2000).

Abortion is the only clinical sign observed in adult cows. It can occur from 3 months gestation to term, and most commonly occurs at 5–6 months gestation. Neosporosis-induced abortion happens throughout the year (Dubey, 2003). It may be epidemic, endemic or sporadic. It has been shown that *N. caninum*-seropositive cows are more likely to abort than are seronegative cows (Atkinson *et al.*, 2000; Jensen *et al.*, 1999; Moen *et al.*, 1998; Pfeiffer *et al.*, 2002; Thurmond, Hietala & Blanchard, 1997; Wouda, Moen & Schukken, 1998). The consequences of infection in a pregnant cow can be foetal death with resorption or mummification, autolysis or abortion of the foetus, a stillborn calf, birth of a clinically weak calf, or birth of a clinically healthy but congenitally chronically infected calf. Because vertical transmission can occur repeatedly in consecutive pregnancies (Barr *et al.*, 1993; Thurmond & Hietala, 1997), and congenitally infected heifers can transfer the parasite to their progeny, the parasite can persist in an infected herd for many years without involvement of a definitive host (Björkman *et al.*, 1996).

Clinical symptoms in calves congenitally infected with *N. caninum* include: neurological signs, an inability to rise, underweight, flexed or hyper-extended hindlimbs and/or forelimbs, ataxia, decreased patellar reflexes, and loss of conscious proprioception (Dubey, 1999a; Dubey, 2003).

Neosporosis is considered to be a costly disease for the cattle industry. In addition to the direct costs due to abortion, increased culling of affected cows and reduction of female breeding cattle, indirect costs should be considered, including diagnostic costs, milk yield loss, professional help expenses, and replacement costs for culled cows. In California the direct cost for *Neospora*-induced abortion was estimated to be \$US35 million per year (Dubey, 1999b). In a recent study the annual costs for the dairy industry due to neosporosis in Australia and New Zealand was estimated to \$AU 21 million and \$NZ 28, respectively (Reichel & Ellis, 2006). In Switzerland, losses attributed to *N. caninum* in the dairy industry was estimated to be approximately \$US12.8 million annually (Häsler *et al.*, 2006).

Presently, there are no effective methods to control *N. caninum* infections in cattle. Since the main route of infection is vertical transmission, from a dam to her foetus for several generations (Anderson *et al.*, 1997; Björkman *et al.*, 1996; Schares *et al.*, 1998), culling of infected animals may be an appropriate practice to control the disease in a herd (French *et al.*, 1999; Thurmond & Hietala, 1995). But this is not feasible if a large proportion of the animals is infected. Also, this will result in a non-immune herd, and if there is a risk of horizontal infection, for example by oocyst excretion from dogs, problems can arise.

To prevent horizontal transmission, dogs, coyotes or other potential definitive hosts should be prevented from contaminating cattle feed or water. Also, appropriate measures should be established to remove aborted bovine fetuses, foetal membranes, placentas and dead calves from infected cows (Innes *et al.*, 2000). However, there is no evidence of horizontal transmission between cows (Anderson *et al.*, 1997).

Embryo transfers from seropositive cows to seronegative recipients has been shown to result in the birth of uninfected calves, and can be used as a method of disease control (Baillargeon *et al.*, 2001; Landmann *et al.*, 2002).

Various chemotherapies have been tested *in vitro* (Dubey & Lindsay, 1996; Kim *et al.*, 2002; Lindsay, Butler & Blagburn, 1997) or *in vivo* in a mouse model (Gottstein *et al.*, 2001) and in cattle (Kritzner *et al.*, 2002). However, to date there are no known antimicrobial agents that can completely clear the parasites from chronically infected animals.

Neosporosis in dogs

Dogs can be both intermediate and definitive hosts (Dubey, 1999a). Antibodies to *N. caninum* have been demonstrated in dogs in several countries throughout the world (Dubey, 1999a; Dubey & Lindsay, 1996). Neosporosis is now recognised as an important cause of fatal disease in young as well as old dogs. However, the most severe cases of neosporosis occur in young, congenitally infected pups (Dubey, 1999a). Canine neosporosis has been reported from many countries (Dubey & Lindsay, 1996). Dogs with clinical neosporosis show hind-limb paresis that develops into a progressive paralysis and other neurological signs, myositis, pneumonia, and dermatitis (Barber & Trees, 1996; Dubey *et al.*, 1988b; Ruehlmann *et al.*, 1995). Other neurological signs include: forelimb ataxia, head tremors with tetraparesis, and rigid hyperextension of both hind and front limbs (although the hind limbs are more often affected than the forelimbs) (Barber & Trees, 1996; Pasquali *et al.*, 1998). Other dysfunctions which might be observed include: paralysis of the jaw, difficulty in swallowing, muscle atrophy, muscle flaccidity, and sudden collapse due to myocarditis and heart failure (Barber & Trees, 1996; Dubey & Lindsay, 1996).

Antimicrobial drugs, including clindamycin, sulfonamides and pyrimethamine, have been used in dogs with clinical neosporosis (Barber & Trees, 1996; Dubey *et al.*, 1998; Dubey *et al.*, 1995; Mayhew *et al.*, 1991; McGlennon, Jefferies & Casas, 1990). For example, ten of 16 dogs with demonstrated neosporosis partially recovered when treated with these drugs (Barber & Trees, 1996). Also, in a dog with neosporosis-associated dermatitis no viable *N. caninum* could be demonstrated after prolonged clindamycin therapy (Dubey *et al.*, 1995). However, the recovery rate of treated dogs may be less in cases with severe neosporosis (Barber & Trees, 1996; Dubey *et al.*, 1998).

Neosporosis in mice

The mouse is frequently used as a laboratory-animal model to investigate the biology of *N. caninum*, immune responses to the parasite, and the effect of antimicrobial drug therapy.

Several inbred and outbred mouse strains have been used to study *N. caninum* infection. *N. caninum* is more or less non-pathogenic to outbred Swiss Webster mice, and the infection only occasionally establishes as tissue cysts in those mice (Dubey *et al.*, 1988b; Lindsay & Dubey, 1989a). However, in cortisonised outbred mice tissue cysts develop more often (Lindsay & Dubey, 1989a; McGuire, McAllister & Jolley, 1997). Inbred BALB/c mice are more susceptible to *N. caninum* infection than are outbred mice, and clinical neosporosis can be observed 2–3 weeks after parasite inoculation (Lindsay *et al.*, 1995a). Typical symptoms are head tilting, circling, hind limb paralysis, and progressive weakness. However, the severity of the clinical symptoms depends on the isolate, dose and number of passages in cell culture of *N. caninum* (Atkinson *et al.*, 1999; Bartley *et al.*, 2006; Lindsay *et al.*, 1995a). The main pathological lesion is meningoencephalitis (Lindsay & Dubey, 1990). Congenital transmission occurs in BALB/c mice after inoculation with tachyzoites during pregnancy (Long & Baszler, 1996).

In addition, severe clinical neosporosis can be induced by experimental infection of immunosuppressed and immunodeficient mice, such as: mice treated with methylprednisolone acetate (Lindsay & Dubey, 1989a; Lindsay & Dubey, 1990; Lindsay *et al.*, 1995a), athymic nude mice (Yamaga, Flechtner & Gottstein, 1996), mice treated with antibody against interferon gamma (IFN- γ) (Khan *et al.*, 1997), and IFN- γ knockout mice (McAllister *et al.*, 1998).

Diagnosis

Serology and immunoblotting

Demonstration of antibodies to *N. caninum* in the serum from an animal indicates that it is, or has previously been, exposed to the parasite. Antibodies can be demonstrated by the indirect fluorescent antibody test (IFAT) (Dubey *et al.*, 1988b), the direct agglutination test (*Neospora* agglutination test; NAT) (Packham *et al.*, 1998; Romand, Thulliez & Dubey, 1998), and enzyme-linked immunosorbent assay (ELISA) (Björkman, Holmdahl & Uggla, 1997; Pare, Hietala & Thurmond, 1995). To discriminate between recent and chronic *N. caninum* infection, an IgG avidity ELISA has been developed and applied for bovine samples (Björkman *et al.*, 1999). Since immunoblotting analysis has high specificity, it has been used as a confirmatory test in several studies of different animal species (Jakubek, Lundén & Uggla, 2006; Staubli *et al.*, 2006; Vardeleon *et al.*, 2001).

Histological examination and immunohistochemistry

N. caninum can cause histopathological changes in various tissues, but the most commonly affected tissues are the brain, heart and skeletal muscle (Barr *et al.*, 1991; Dubey *et al.*, 1988a; Lindsay *et al.*, 1995a). These tissues can be sectioned and stained using routine histological techniques with hematoxylin and eosin. The histopathological changes are degenerative inflammatory lesions found in those organs. However, the main histopathological lesions of neosporosis are found in infected brain tissue, and consist of focal, nonsuppurative encephalitis and a variable number of focal necroses (Dubey, 2003; Dubey & Lindsay, 1996). In neosporosis, there is no pathognomonic histological or gross lesion. The histopathological lesions could lead to a presumptive diagnosis (Dubey, 1999a), but other diagnostic methods, e.g. immunohistochemistry, serology and detection of *N. caninum* DNA, are necessary for a definitive diagnosis of neosporosis. By immunohistochemistry (Lindsay & Dubey, 1989b), specific antibodies are used to differentiate *N. caninum* from other apicomplexan parasites in infected tissue sections. However, false positive *N. caninum* results have been recently observed in *T. gondii*-infected tissue sections (van Maanen *et al.*, 2004).

Isolation of the parasite

To isolate the causative parasite, cell culture and inoculation of laboratory animals can also be used to recover *N. caninum* from infected tissues (Dubey *et al.*, 1988b). For example, BALB/c mice, IFN- γ gene knock out mice as well as gerbils have been used with success for *in vivo* parasite isolation (Cheah *et al.*, 2004; Rodrigues *et al.*, 2004; Vianna *et al.*, 2005). The success of the method depends on the number of parasites present and the state of the tissue sample.

Detection of parasite DNA

N. caninum DNA can be detected by polymerase chain reaction (PCR). The various PCR protocols to detect *N. caninum* DNA that have been developed have played an important role in the diagnosis *N. caninum* infections. Different types of repetitive DNA have most frequently been used as targets (Baszler *et al.*, 1999; Ellis, 1998; Gottstein *et al.*, 1998; Holmdahl & Mattsson, 1996; Kaufmann *et al.*, 1996; Liddell, Jenkins & Dubey, 1999; Yamane *et al.*, 1996). A competitive PCR assay has been developed to measure the level of *N. caninum* DNA in infected tissues (Liddell, Jenkins & Dubey, 1999). Presently, quantitative real-time PCR assays based on the use of SYBR-green detection systems or labelled probes have been successfully applied to detect and quantify the parasite DNA in infected tissue (Collantes-Fernandez *et al.*, 2006; Collantes-Fernandez *et al.*, 2002; Müller *et al.*, 2002). This type of tool can be used to study the pathogenesis, tissue distribution and parasite load, etc., in a more sophisticated manner. Most of the real-time assays target the Nc5 region, a repeated sequence specific to the *N. caninum* genome (Kaufmann *et al.*, 1996).

Immunity to *N. caninum*

To design an effective vaccine, it is important to understand how the immune system responds to *N. caninum*. Immune responses to intracellular parasites include both humoral and cellular responses. Specific antibodies may play a role in limiting the spread of the extracellular stages. Nevertheless, protective immunity is achieved mainly through cell-mediated responses characterised by type 1 T-helper (Th1) cells and interleukin (IL)-2, IL-12 and IFN- γ production (Innes *et al.*, 2000). Th2 responses, including IL-4 and IL-10 production, are generally considered to be unfavorable, and may lead to disease progression (Baszler, *et al.*, 1999; Khan, *et al.*, 1997). The protection is principally mediated by CD4⁺, CD4⁺ cytotoxic and CD8⁺ cytotoxic cells (Staska *et al.*, 2003; Tanaka *et al.*, 2000a). IL-12 and IFN- γ produced by CD4⁺ T cells in the spleens of mice infected with *N. caninum* are involved in disease resistance by inhibition of parasite proliferation and activation of macrophages (Baszler, *et al.*, 1999; Innes *et al.*, 1995; Khan, *et al.*, 1997; Nishikawa *et al.*, 2001c; Tanaka, *et al.*, 2000a; Yamane *et al.*, 2000). Suppression of IFN- γ and IL-12 production may allow greater parasite replication, resulting in more severe disease. Th1 and Th2 responses tend to down-regulate each other, and it has been shown that the balance of IFN- γ /IL-4 is important for the control of *N. caninum* infection (Long, Baszler & Mathison, 1998; Nishikawa *et al.*, 2003).

The acquired immunity is triggered by signals from the innate immunity during *Neospora* infection, and several components of the innate immunity have been shown to be activated. Proinflammatory cytokines such as IL-12, tumor necrosis factor (TNF)- α and IFN- γ are released from macrophages, natural killer (NK) cells and dendritic cells. These cytokines play roles in killing of parasites and parasite infected cells and increase T-cell response to the parasite (Innes *et al.*, 2000). For instance, activated macrophages induced by IFN- γ increase their nitric oxide production and killing activity against *N. caninum* (Tanaka *et al.*, 2000b). In addition, IFN- γ and TNF- α are able to inhibit *in vitro* intracellular multiplication of *N. caninum* tachyzoites (Innes *et al.*, 1995; Yamane *et al.*, 2000). Moreover, NK cells, triggered by heat-killed *N. caninum* tachyzoites, have been reported to increase their IFN- γ production and the killing of infected cells (Boysen *et al.*, 2006).

Vaccination against neosporosis

There are many different types of vaccines that could be considered. In general, live vaccines have the advantage of inducing long-lasting humoral and cell-mediated immune responses. Because of endogenous antigen processing in the infected cells, cell-mediated responses are effectively generated. However, the disadvantages of live vaccines are the risk of causing disease and the possibility of causing persistent infections. In addition, there is a risk of contamination with unwanted microorganisms.

An attenuated strain of *N. caninum* has been generated and found to induce protection in mice (Dreier *et al.*, 1999; Lindsay *et al.*, 1999c). However, further studies should be done in order to elucidate if it is safe for use in animals such as

cattle and dogs, before it could be introduced as a vaccine against *N. caninum* infection.

With killed vaccines there is no risk of causing persistent infection, and they are likely to be stable for storage and handling. Killed vaccines usually induce antibody production, although it might not be long-lasting. Also, they might not stimulate the cellular responses required for protection.

There is an inactivated *Neospora* vaccine, Neoguard™, commercially available in parts of the world. It is composed of a lysate of tachyzoites formulated with a havlogen adjuvant. The vaccine has been shown to be safe to use (Choromanski, 2002) and a reduction of the overall bovine abortion rate was demonstrated in a field study. However, the etiology of the abortions was not investigated (Romero et al., 2004). Another study showed that the proliferative response of peripheral blood mononuclear cells obtained from cattle immunised with a killed tachyzoite preparation associated with havlogen was significantly higher than of cells obtained from the negative control cattle, however, the production of IFN- γ was not different (Andrianarivo et al., 1999).

At present, several research groups have designed different experimental subunit vaccines (Cannas et al., 2003a; Cannas et al., 2003b; Liddell et al., 2003; Nishikawa et al., 2001a). This work includes selection of appropriate parasite antigens in combination with a suitable adjuvant, in order to stimulate an effective protective immune response against *N. caninum*.

***N. caninum* antigens**

A considerable amount of research has been done to define antigens of *N. caninum* using polyclonal antibodies, monoclonal antibodies and molecular biological methods (Dubey, 1999a). *Neospora* proteins and antigenic determinants are found in different locations of the parasite, and can be used for immunodiagnosis and vaccine development.

Surface antigens

Proteins located on the parasite surface are likely to be important for the survival of the parasites, and play an important role in the parasite-host interactions. Two major immunodominant surface antigens of *N. caninum* have been identified and the corresponding genes sequenced. Since their primary sequences are apparently homologous to the *T. gondii* surface antigen 1 (SAG1) (Burg et al., 1988) and SAG1-related sequences 2 (SRS2) (Manger, Hehl & Boothroyd, 1998), respectively, they have been designated NcSAG1 and NcSRS2 (Howe et al., 1998). NcSAG1 was previously named Nc-p29 (Howe et al., 1998) or Nc-p36 (Hemphill et al., 1997), and NcSRS2 was previously named Nc-p35 (Howe et al., 1998) or Nc-p43 (Hemphill & Gottstein, 1996). NcSAG1 is found only on the tachyzoite surface (Hemphill et al., 1997), while NcSRS2 is expressed on both tachyzoites and bradyzoites (Fuchs et al., 1998; Hemphill & Gottstein, 1996). Both

antigens are involved in the process of adhesion to and invasion of host cells (Hemphill & Gottstein, 1996). These proteins in their native or recombinant form are currently used in *Neospora*-vaccine experiments.

Microneme antigens

Micronemes are secretory organelles found in the apical complex of *N. caninum* and related parasites. They are involved in the adhesion and invasion process, as the contents of these organelles are exocytosed at the onset of the parasite-host cell interaction (Carruthers & Sibley, 1997). In addition, microneme proteins have been shown to play a role in gliding motility of the tachyzoites (Sultan *et al.*, 1997). So far, more than 30 microneme proteins and genes of apicomplexan parasites have been identified (Tomley & Soldati, 2001), e.g. in *Cryptosporidium*, *Eimeria*, *Neospora*, *Plasmodium*, *Sarcocystis*, *Theileria* and *Toxoplasma*. A number of *Neospora* microneme proteins have been identified, including NcMIC1, NcMIC2, NcMIC3 and NcMIC4. Recombinant microneme proteins such as recombinant NcMIC1 and NcMIC3 have been used in vaccine experiments (Alaeddine *et al.*, 2005; Cannas *et al.*, 2003b).

NcMIC1 is a 460 amino acid microneme protein of *N. caninum* expressed in the tachyzoite stage. Keller *et al.* (2002) showed that NcMIC1 is secreted and involved at the onset of the attachment between the parasite and the host cell, and also that NcMIC1 can bind to host cell-surface glycosaminoglycans.

NcMIC2 is a member of the thrombospondin-related adhesive protein (TRAP) family found in apicomplexan parasites (Naitza *et al.*, 1998). It is 61% identical to *T. gondii* MIC2 as regards its amino acid sequence, and it is localised to the micronemes (Hemphill *et al.*, 1999).

NcMIC3 is a 38 kDa antigenic protein expressed in both tachyzoites and bradyzoites, and could be involved in adhesion and invasion of the host cell (Sonda *et al.*, 2000). NcMIC3 is secreted and bound to the tachyzoite surface, and could be detected on the surface membrane within 2–3 h after host-cell invasion (Naguleswaran *et al.*, 2001). It has been shown that NcMIC3 interacts with host cell-surface proteoglycan (Naguleswaran *et al.*, 2002).

NcMIC4 is a 55 kDa lectin-like microneme protein exhibiting lactose-binding properties. It has been shown that NcMIC4 is also secreted by *N. caninum* tachyzoites at the onset of adhesion to the host cell. It can bind to the host cell-surface glycosaminoglycans (Keller *et al.*, 2004).

Dense granule antigens

The dense granules are secretory organelles found in all coccidian parasites. The parasites secrete dense granule contents into the lumen of the parasitophorous vacuole after host-cell invasion. To date, five dense granule antigens of *N. caninum* have been identified, including NcGRA1 (Atkinson *et al.*, 2001), NcGRA2 (Ellis *et al.*, 2000), NcGRA6 or NCDG2 (Liddell *et al.*, 1998), NcGRA7

or NCDG1 or Nc-p33 (Hemphill *et al.*, 1998; Lally *et al.*, 1997), and the nucleoside triphosphate hydrolase (NcNTP or NTPase) (Asai *et al.*, 1998).

Immunostimulating complexes (iscoms)

One of the adjuvants that has been used in experimental vaccines against *N. caninum* with promising results (Lundén *et al.*, 2002) is the immunostimulating complexes (iscoms), invented by Morein and coworkers in 1984 (Morein *et al.*, 1984). Iscoms are spherical 40 nm cage-like structures, composed of the saponin adjuvant Quil A from the bark of the tree *Quillaja saponaria*, cholesterol, phospholipids, and multiple copies of one or several proteins or antigens. Similar particles without any antigen can also be produced, and are called iscom matrix. They can be used for coupling to different antigen molecules.

Iscoms have been shown to increase the immunogenicity of the incorporated antigens, and to induce humoral and cell-mediated immunity in animals (Morein *et al.*, 1995; Sjolander, Cox & Barr, 1998) and humans (Sjolander *et al.*, 2001). The adjuvant and carrier properties of iscoms include:

1. Physical presentation of several copies of proteins or antigens incorporated into the iscoms (Morein *et al.*, 1995).
2. Enhancement of uptake and internalisation of antigens by antigen presenting cells (Villacres *et al.*, 1998).
3. Stimulation of proinflammatory cytokine production by antigen presenting cells. Both iscoms and the iscom matrix stimulate IL-1, IL-6 and IL-12 production (Behboudi, Morein & Villacres-Eriksson, 1996; Behboudi, Morein & Villacres-Eriksson, 1997; Villacres-Eriksson *et al.*, 1997), and therefore T-helper cells and cytotoxic T cells are activated, and antibody responses are generated.
4. Increased expression of major histocompatibility complex class II (Bergström-Mollaoglu *et al.*, 1992; Watson *et al.*, 1992).

In addition to vaccine development, the iscom technology can be used to prepare antigens for immunoassays. Serological assays using iscom antigen preparations have been successfully employed for the diagnosis of *T. gondii* and *N. caninum* using ELISA (Björkman & Lundén, 1998).

Iscoms have been constructed with a wide range of viral, bacterial or parasite antigens. Several methods have been developed to construct iscoms containing different proteins and peptides, including recombinant antigens. The ultracentrifugation and dialysis methods could be used for hydrophobic or amphipathic antigens, which are incorporated into iscoms through hydrophobic interaction (Morein & Lövgren Bengtsson, 1999).

Electrostatic interaction can also be utilised for preparation of iscoms, since matrix particles generally are negatively charged and positively charged molecules such as nucleoprotein can attach to them (Morein, Hu & Abusugra, 2004).

For incorporation of hydrophilic antigens, several techniques could be applied, for instance: low pH treatment of the antigen to expose hidden hydrophobic parts, or by conjugating lipids to the antigen. Chemical covalent binding could be used for coupling hydrophilic antigens and synthetic oligopeptides to a pre-formed iscom matrix containing a phospholipid suitable for conjugation, e.g. phosphatidylethanolamine (Morein & Lövgren Bengtsson, 1999).

Different affinity interactions have recently been tried to couple recombinant proteins to iscom matrix. For example, recombinant proteins with a hexahistidyl peptide (His₆) tag can, via divalent anions, bind to iscom matrix containing a chelating lipid (Andersson *et al.*, 2001).

Aims

The aims of the present study were:

- To evaluate methods for incorporation of recombinant proteins into iscoms using the affinity between biotin and streptavidin (**Paper I**).
- To investigate if the recombinant *N. caninum* antigen NcSRS2, expressed in *Escherichia coli* with fusion partners selected for affinity purification and association to iscoms, could induce antibodies that recognised the native parasite protein (**I, II**).
- To investigate whether the NcSRS2 iscoms could generate protective immunity against cerebral neosporosis in mice (**II**).
- To compare different NcSRS2-iscoms prepared by the methods described in **Paper I** as regards immunogenicity, including both humoral and cellular responses, and protective capacity against challenge infection (**III**).
- To explore the possibility of using real-time PCR for the detection and quantification of parasitaemia in mice, and to use this parameter for further evaluation of the protective effect of the NcSRS2-iscoms (**IV**).

Comments on materials and methods

The materials and methods are described in detail in **Papers I–IV**.

Recombinant proteins and iscoms

The well-known bio-specific interaction between biotin and streptavidin, as well as the chemical affinity between histidine and metal ions, were utilised for binding recombinant antigens to iscom matrix. Two different concepts for binding via biotin and streptavidin were followed: (i) indirect binding of a biotinylated antigen via recombinant streptavidin with a His₆-tag that was bound to iscom matrix loaded with Ni²⁺ ions (Fig. 2A), and (ii) direct binding of streptavidin fusion proteins to biotinylated matrix (Fig 2B). In addition, since the streptavidin fusion proteins contained a His₆-tag, one of them was also bound to Ni²⁺-loaded matrix for comparative studies (Fig. 2C).

The model antigens selected for evaluation of these concepts were SRS2', a 232 amino acid segment derived from the central region of the *N. caninum* major surface antigen NcSRS2, and the malaria peptide M5, a 53 amino acid peptide derived from the central region of the *Plasmodium falciparum* blood-stage antigen Pf155/RESA. The recombinant antigens and their fusion partners were expressed in *E. coli* strain BL21 (DE3).

Three kinds of iscom matrix were used: regular, metal-chelating and biotinylated iscom matrix (denoted matrix, IDA-matrix and Bio-matrix, respectively). All three types of matrix were prepared from cholesterol, phosphatidylcholine and Quil A. The IDA-matrix also contained the chelating lipid IDA-Trig-DSGE. For activation, the IDA-matrix was incubated with NiCl₂ and thereafter dialysed to remove unbound Ni²⁺ ions. The resulting preparation was denoted Ni²⁺-matrix. To prepare Bio-matrix, matrix containing phosphatidylethanolamine was biotinylated with N-hydroxysuccinimide-biotin.

Recombinant fusion proteins

For the first concept (indirect binding), two fusion proteins consisting of a tag (Bio) for *in vivo* biotinylation by *E. coli*, a His₆-tag, an albumin binding protein (ABP) and SRS2' or M5 were designed, and denoted Bio-His₆-ABP-NcSRS2' and Bio-His₆-ABP-M5. The ABP and His₆-tag were included for affinity purification. To investigate whether increased biotinylation would increase binding to the iscoms, Bio-His₆-ABP-SRS2' was biotinylated *in vitro*, and the double biotinylated fusion protein was denoted Bio-Bio-His₆-ABP-SRS2'. For linking the biotinylated antigens to Ni²⁺-matrix, a His₆-tagged streptavidin fusion protein (His₆-SA) was produced.

For the second concept (direct binding), recombinant NcSRS2 and M5 were expressed as His₆-tagged streptavidin fusion proteins (denoted His₆-SA-SRS2' and

His₆-SA-M5, respectively). The His₆-tag was originally included for affinity purification, but was also used for binding to Ni²⁺-matrix, to enable comparison between iscoms prepared from Bio-matrix and Ni²⁺-matrix (III).

After sonication of *E. coli* cells, the His₆-SA, His₆-SA-SRS2' and His₆-SA-M5 fusion proteins were purified by His₆-tag-mediated immobilized metalloaffinity chromatography (IMAC) on Co²⁺-charged affinity resin columns under denaturing conditions. The Bio-His₆-ABP-M5' and Bio-His₆-ABP-NcSRS2' fusion proteins were purified by ABP-mediated affinity chromatography on HSA (human serum albumin)-Sepharose. The presence and size of the fusion proteins were determined by SDS-PAGE and Western blotting, using M5-specific polyclonal antibodies and a monoclonal antibody to NcSRS2 (I).

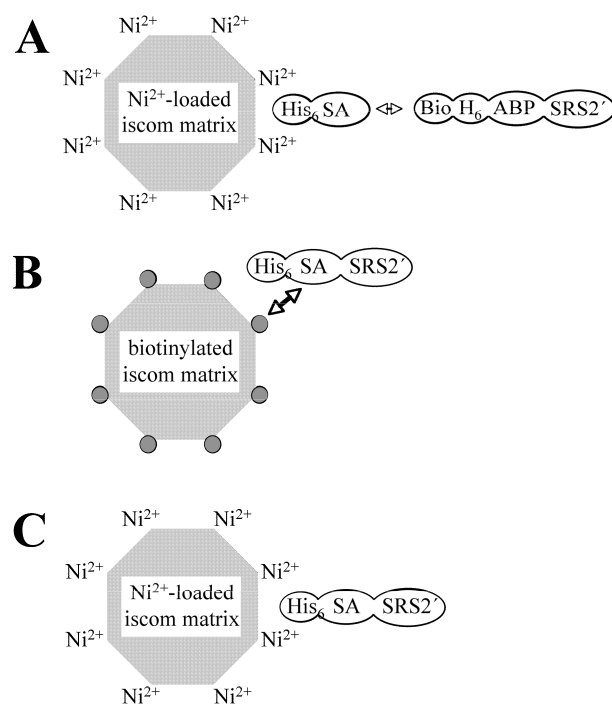


Fig. 2. Schematic presentation of the two concepts (A & B) used for association of recombinant antigens by biotin-streptavidin interaction. (A): indirect binding of a biotinylated protein via His₆-SA to Ni²⁺-matrix; (B): direct binding of a streptavidin fusion protein to biotinylated matrix; (C): binding of a His₆-tagged protein to Ni²⁺-matrix.

Iscom preparation

According to the first concept, pre-formed Ni²⁺-matrix was incubated with the His₆-SA protein. Thereafter, the Bio-His₆-ABP-M5', Bio-His₆-ABP-NcSRS2' or Bio-Bio-His₆-ABP-SRS2' fusion proteins were added, and the mixture incubated again before dialysis overnight.

For the second concept, the His₆-SA-SRS2' or His₆-SA-M5 fusion proteins were directly mixed with Bio-matrix and incubated, in order to establish the direct association through the biotin-streptavidin interaction, and dialysed overnight. A similar procedure was used to bind His₆-SA-SRS2' to Ni²⁺-matrix.

To analyse the association of the different proteins to the matrix and to each other, the iscom preparations were centrifuged through a sucrose gradient and fractionated. The fractions were analysed for the presence of Quil A and proteins by measuring the absorbance at 214 and 280 nm, respectively. The protein content was also determined by Bradford analysis. To trace the different fusion proteins, aliquots of each fraction were coated into separate wells of ELISA plates, followed by incubation with specific antibodies direct against NcSRS2, M5 or streptavidin.

Table 1. *Iscom preparations used for immunisation*

Full name used in Paper I	Abbreviated name used in Papers II–IV*		
	II	III	IV
Ni ²⁺ -matrix + His ₆ -SA + Bio-His ₆ -ABP-SRS2'	NcSRS2 iscom	BioSRS2'-iscom(Ni)	
Ni ²⁺ -matrix + His ₆ -SA + Bio-bio-His ₆ -ABP-SRS2'		dBioSRS2'-iscom(Ni)	
Biotin-matrix + His ₆ -SA-SRS2'		SA-SRS2'-iscom(Bio)	as in III
Ni ²⁺ -matrix + His ₆ -SA-SRS2'		SA-SRS2'-iscom(Ni)	as in III
Ni ²⁺ -matrix + His ₆ -SA + Bio-His ₆ -ABP-M5	M5 iscom	M5-iscom(Ni)	

* The abbreviations from Paper III are used in the text of the thesis.

Immunisations and challenge infections

Inbred BALB/c mice were used, since they are susceptible to infection with *N. caninum* and develop clinical neosporosis (Lindsay *et al.*, 1995a; Long, Baszler & Mathison, 1998). All immunisations and inoculations with parasites were done subcutaneously at the base of the tail. The different SRS2'-iscom preparations used are listed in Table 1, and are referred to with the abbreviated names used in **Paper III**.

In **Paper I**, two iscom preparations, prepared according to the first concept with the *in vivo* biotinylated fusion proteins Bio-His₆-ABP-M5 and Bio-His₆-ABP-SRS2' (Table 1) were selected to investigate whether the recombinant antigens could induce specific antibodies recognizing the target immunogens M5 and NcSRS2. In addition, a third group of mice was immunised with a mixture of regular matrix, His₆-SA and Bio-His₆-ABP-SRS2', to evaluate the immunogenicity of Bio-His₆-ABP-SRS2' when not specifically bound the matrix. The antibody responses were analysed by ELISA with ZZM5, an unrelated M5 fusion protein, or iscoms containing NcSRS2 of parasite origin as coating antigens.

In **Paper II**, the protective effect of the SRS2'-iscom preparation used in **Paper I** (BioSRS2'-iscom(Ni)) was evaluated by challenge infection. Control mice were immunised with the corresponding M5-iscom preparation (BioM5-iscom(Ni)). The antibody responses against native NcSRS2 were analysed by ELISA and Western blotting. After inoculation with 10^7 live *N. caninum* tachyzoites, the mice were weighed daily and clinical signs of neosporosis were recorded. After 31 days the mice were killed, and their brains analysed by competitive PCR for quantification of parasite DNA.

Paper III further evaluated and compared the immunogenicity of the different NcSRS2 iscom formulations prepared according to the two concepts of iscom association via biotin-streptavidin binding. In a first experiment, groups of mice were immunised with Bio-His₆-ABP-SRS2', Bio-Bio-His₆-ABP-SRS2' or His-SA-SRS2' alone or bound to streptavidin-coated or biotinylated matrix as described in **Paper I**. After analysis of the antibody and cellular immune responses, two of the iscom formulations (BioSRS2'-iscom(Ni) and SA-SRS2-iscom (Bio)) were selected for a second experiment, along with a new preparation of His₆-SA-SRS2' bound to Ni²⁺-matrix (SA-SRS2'-iscom(Ni)). After two immunisations, as well as at the end of the challenge experiment, sera from the individual mice were analysed for antibodies to native NcSRS2 (experiments 1 and 2) and recombinant SRS2' (experiment 2), including IgG subclass analysis. From some of the mice, the spleens were removed after the booster injection, and splenocyte suspensions were prepared for analysis of antigen specific proliferation and cytokine production. In the second experiment, the remaining mice were inoculated with live tachyzoites and monitored as in **Paper II** for 23 days. At the end of the experiment their brains were collected and analysed by quantitative real-time PCR.

The aims of the work described in **Paper IV** were to evaluate whether the real-time PCR assay that was set up and described in **Paper III** was sensitive enough to detect parasitaemia after experimental infection, and to investigate whether the immunity induced by the NcSRS2 iscoms could reduce the level of parasitaemia. The relationship between the level of parasitaemia during the acute stage and the amount of parasites in the brain in the chronic stage was also investigated. In an initial experiment, mice were inoculated with different numbers of tachyzoites, blood samples were collected every second day for 12 days, and thereafter with longer intervals until the end of the experiment at 25 days post inoculation. For the immunisation experiment the two iscom preparations SA-SRS2'-iscom(Bio) and SA-SRS2'-iscom(Ni) were selected, and blood was collected daily for nine days after challenge infection.

Parasites

The NC-1 isolate of *N. caninum* (Dubey *et al.*, 1988b) was used to prepare antigen for ELISA (**I–IV**), immunoblotting (**II, III**) and lymphocyte stimulation test (**III**), as well as for challenge infection (**II–IV**). The parasites were propagated *in vitro* in Vero cells. For challenge infection, parasites with a low passage number were used, since it has been shown that *N. caninum* can lose its virulence after many

passages in cell culture (Bartley et al; 2006). Furthermore, care was taken to harvest the parasites at an optimal time point, to minimise loss of infectivity after the rupture of the host cells (Hemphill, 1999).

Antibody and IgG subclass analysis

Blood was collected from the tail vein after each immunisation (**I–III**) and after challenge infection (**III**), and the antibody response was analysed by ELISA. The antigens used to measure the response against recombinant M5 and NcSRS2 were the recombinant protein ZZM5 (**I**) and His₆-SA-SRS2 (**III**), respectively. To detect antibodies that reacted with native NcSRS2, iscoms containing surface antigens extracted from *N. caninum* tachyzoites were used as coating antigen (**I–IV**). The sera were titrated in serial three-fold dilutions from 1:30, and the results were expressed as reciprocals of the dilution giving an absorbance of 1.0. Horse-radish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (Ig) was used for total Ig analysis, and HRP-conjugated caprine antibodies to murine IgG1 and IgG2a were used for analysis of these two subclasses. Since IgG2a production is stimulated by Th1 cytokines, and Th2 cytokines promote IgG1 production, these subclasses are often used as a measure of the Th1/Th2 balance.

The antibody response of the immunised mice was also analysed by immunoblotting (**II**). *N. caninum*-iscoms and BioSRS2'-iscoms(Ni) were separated by SDS-PAGE under non-reducing conditions, and transferred to nitrocellulose membranes. The reactivity of the sera from the immunised mice was compared to that of sera from *N. caninum*-infected mice, a monoclonal antibody against NcSRS2, and polyclonal sera against streptavidin. HRP-conjugated rabbit anti-mouse Ig was used as secondary antibody.

Analysis of cellular responses

Cellular immune responses after immunisation were evaluated by a lymphocyte stimulation test (**III**). The test was used to measure antigen-specific proliferative responses and cytokine production. Since resistance to neosporosis is associated with Th1 cytokines such as IFN- γ , and susceptibility with Th2-cytokines such as IL-4, these two cytokines are often measured to evaluate the cellular immune responses against *N. caninum*.

Spleen cells were isolated from mice after booster immunisation. The cells were plated into 96-well culture plates, and stimulated with *N. caninum* lysate or the mitogen Concanavalin A (Con A). The cells were incubated for 96 h, and tritium-labelled thymidine was added before another 18 h incubation. Thymidine incorporation was measured as count per minute (cpm) using a β -scintillation counter. The proliferative responses were expressed as net cpm according to the formula:

$$\text{Net cpm} = \text{mean cpm of stimulated cultures} - \text{mean cpm of control cultures.}$$

The cytokine-producing ability was studied in cultures of splenocytes incubated with *N. caninum* lysate as specific stimulator or Con A as positive control. The culture supernatants were analysed by commercial ELISA kits, each containing two monoclonal antibodies (capture and detection antibody) to murine IL-4 or IFN- γ , and recombinant IL-4 or IFN- γ for the standard curves used for quantification.

Detection and quantitative analysis of *N. caninum* DNA

Two different PCR assays, including semiquantitative competitive PCR (II) and real-time PCR (III, IV), were used to demonstrate and quantify *N. caninum* DNA after challenge infection. The competitive PCR is based on amplification of the Nc5 repeated sequences, with the primers Np6+ and Np21+ (Liddell, Jenkins & Dubey, 1999). This PCR assay is performed with a conventional thermocycler. The basis of the assay is to transform a qualitative assay to a quantitative assay by adding a known amount of a mimic competitor molecule. This competitor is specifically amplified by the same primers as the target DNA, although its size has to be different. Both target sequence and competitor are amplified in the same tube; and by setting up the reaction with serial dilutions of the competitor, the quantity of the target amplicon can be estimated after visualisation of the bands on agarose gels. The concentration of the competitor that gives equally strong bands for the target and mimic is assumed to correspond to the concentration of the target. The competitor molecule also functions as a control for false-negative reactions. Although this assay is considered to be a sensitive method, with a detection level of 0.09 pg *N. caninum* tachyzoite DNA (Liddell, Jenkins & Dubey, 1999), it has several disadvantages compared to quantitative real-time PCR. For example, the serial-diluted tube format and post-PCR gel electrophoresis are laborious and time-consuming.

Real-time PCR is a high-throughput method that does not involve any post-amplification manipulations, which decreases the risk of cross-contamination and saves time. The assay uses fluorescent labels for continuous monitoring and detection of the amplicon (PCR product) formation. Depending on the amount of starting target DNA, it will take a certain number of cycles before the fluorescent signal crosses a threshold level. This cycle number is named the cycle threshold (Ct) value, which is inversely correlated to the initial amount of the template. A standard curve with Ct values of known standards can be generated, and the starting amounts of the template in unknown samples are calculated from the curve according to their Ct values.

The real-time PCR assay used in the present work was based on detection of the Nc5 sequence, using the primer pairs Np6+ and Np21+ and SYBR Green as detection system. SYBR Green binds to double-stranded DNA, and emits strong fluorescence measured at a particular point during each amplification cycle.

The main limitation of using SYBR Green is non-specific binding to any double-stranded DNA. Therefore, SYBR Green would bind to primer-dimers, and any

products that have been amplified un-specifically, as well as to the target amplicon. To improve the specificity of the assay, highly specific primers with low risk of primer-dimer formation, and the optimisation of the PCR protocol, should be considered. In the present study, samples from **Paper III** were initially analysed with the real-time PCR assay described by Collantes-Fernandez et al. (2002). However, the product melting curve analysis suggested that some degree of unspecific amplification occurred. This observation was verified after agarose-gel electrophoresis. The performance of the real-time PCR was significantly improved after switching primers to those used in **Paper II** and optimisation of the cycling conditions. Consequently, all samples were analysed with this new PCR system. Nevertheless, end-point agarose gel electrophoresis with ethidium bromide staining was performed on samples that had Ct-values close to those of the negative control. In the present studies, a product-melting curve analysis was performed to reveal the presence of primer-dimer formation or unspecific amplification. It was found that some unspecific amplification occurred, and sometimes the melting point of the unspecific product was close to that of the target. Consequently, agarose gel electrophoresis with ethidium bromide staining was also performed, before final judgement of samples that had Ct values close to those of the negative control.

Results and discussion

Binding of recombinant proteins to iscom matrix (Papers I, III)

The association of the different proteins to iscom matrix was evaluated by analytical ultracentrifugation. Typically, iscoms are located in the mid fractions of the gradients, while most of the unassociated proteins are found in the upper fractions. When iscoms were prepared according to the first concept (Fig. 2A), a large proportion of the fusion proteins were detected in the Quil A-rich mid fractions (Fig. 3A). In contrast, when the proteins were analysed alone they were found in the upper fractions of the gradient. This indicates that the two *in vivo* biotinylated fusion proteins (Bio-His₆-ABP-M5 and Bio-His₆-ABP-SRS2') were successfully bound to streptavidin-coated iscom matrix.

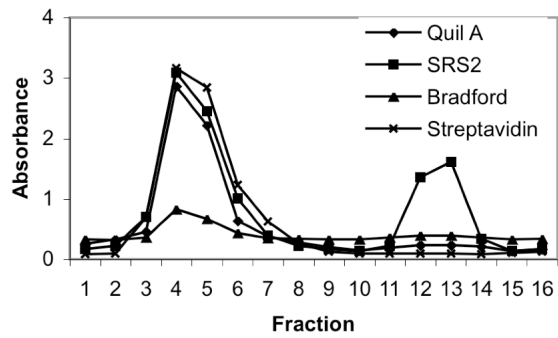
Interestingly, control experiments showed that regular matrix to some extent associated with His₆-SA and the biotinylated immunogens (Bio-His₆-ABP-M5 or Bio-His₆-ABP-SRS2'). However, the vast majority of the proteins remained unassociated, indicating that this unspecific adherence was not as strong as the binding to Ni²⁺-matrix via His₆-SA. The immunogenicity of Bio-His₆-ABP-SRS2' mixed with regular matrix was investigated (I), and is discussed in a later section.

Since the biotinylated immunogens (Bio-His₆-ABP-SRS2' and Bio-His₆-ABP-M5) contained a His₆-tag, it could be argued that they might bind directly to the Ni²⁺-matrix instead of indirectly via the His₆-SA. To minimize this unintended binding, the His₆-SA protein was pre-incubated with Ni²⁺-matrix before the biotinylated immunogens were added. Moreover, since the biotin-streptavidin interaction is at least 100 times stronger than the His₆-Ni²⁺ binding (Wilchek & Bayer, 1990; INDIATM HRP-method, Pierce), this former interaction is likely to dominate over the latter.

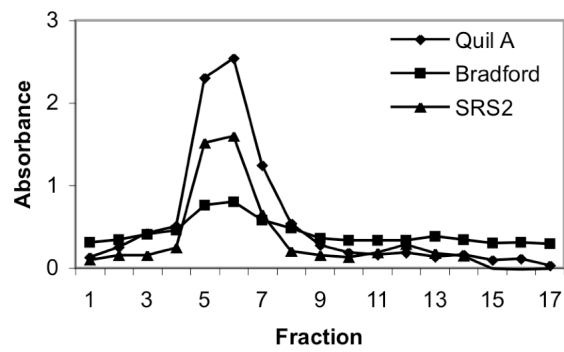
To explore the effect of increased biotinylation, Bio-His₆-ABP-SRS2' was biotinylated *in vitro*, and this double-biotinylated protein was used for preparation of iscoms according to the first concept. Comparison of iscoms prepared with the *in vivo* biotinylated protein and with the double-biotinylated protein indicated that the increased bitinylation seemed to give a more efficient binding (Fig 3A and B). However, the interpretation of the analytical centrifugations was complicated by the fact that when the double-biotinylated protein was analysed without matrix a substantial proportion of it was located in the mid fractions of the gradient, probably due to aggregation of the protein (Fig. 3C). All three preparations shown in Fig. 3 were used in an immunisation experiment (III), presented in a later section.

Evaluation of iscoms prepared according to the second concept revealed that both His₆-SA-M5 and His₆-SA-SRS2' were found in the Quil A-rich middle fractions, indicating successful association to the biotinylated matrix. In control experiments, His₆-SA-M5 was found in the upper fractions when analysed alone or with regular matrix, while His₆-SA-SRS2' was localized in the mid fractions,

A



B



C

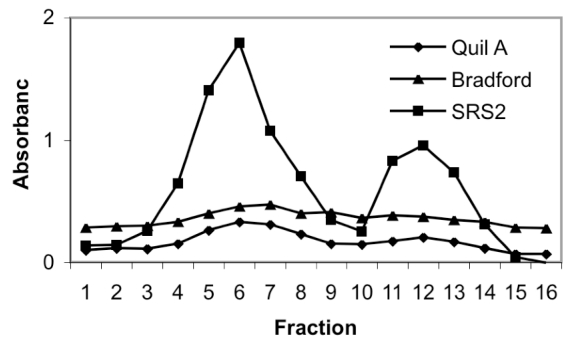


Fig. 3. Analysis of Quil A and protein content in fractions collected from sucrose-density-gradient centrifugation of iscom preparations. (A): BioSRS2'-iscom(Ni); (B): dBioSRS2'-iscom(Ni); (C): Bio-Bio-His₆-ABP-SRS2'.

whether analysed alone or together with regular matrix. Thus, it seems likely that both proteins were successfully bound to the Bio-matrix, although it could not be shown as clearly for the SRS2' protein as for the M5 protein (**I**).

The iscoms prepared by binding His₆-SA-SRS2' to Ni²⁺-matrix (**III**) were also analysed by centrifugation through a sucrose gradient, and the protein was only detected in the Quil A-rich fractions.

Thus, both concepts for binding (via biotin and streptavidin) were demonstrated to give successful iscom association of immunogens. However, the expression levels of the *in vivo* biotinylated protein used according in the first concept seemed to be unimpressive (2–4 mg/l of cell culture), whereas the streptavidin fusion proteins used in the second concept were expressed at higher level (62–84 mg/l of cell culture). Furthermore, the second concept is obviously simpler; involving only one fusion protein binding to the biotinylated matrix. Also, the biotin-streptavidin interaction is much stronger than the histidine-Ni²⁺ binding. Thus, a direct association through biotin-SA interaction is probably preferable to the indirect approach.

Analysis of B-cell epitopes of the recombinant NcSRS2 (I, II, III)

The amino acid sequence of NcSRS2 shows homology to the SAG and SRS antigens of *T. gondii* (Howe *et al.*, 1998). Although antigenically distinct from these proteins (Fuchs *et al.*, 1998), NcSRS2 can be assumed to have a similar secondary and tertiary structure (Howe *et al.*, 1998). For TgSAG1, it has been shown that many B-cell epitopes are conformational and dependent on the proper formation of disulphide bridges (Velge-Roussel *et al.*, 1994). Production of correctly folded TgSAG1 in *E. coli* has met considerable problems, while expression in eukaryotic systems has generally been successful (Biemans *et al.*, 1998; Kim *et al.*, 1994). Since NcSRS2 contains 12 cysteine residues that are conserved in the *T. gondii* SAG and SRS antigens (Howe *et al.*, 1998), similar problems with expression in prokaryotic cells can be expected.

Therefore, to investigate if the recombinant SRS2' could induce antibodies that recognised native NcSRS2, mice were immunised with one of the iscom preparations (BioSRS2'-iscom(Ni)). After two immunisations, *N. caninum*-specific antibodies were detected with an average titre of 1:10784, which was only about two dilution steps lower than for pooled sera collected from mice inoculated with live *N. caninum* tachyzoites (**I**). This strong reactivity to the native NcSRS2 antigens, together with the recognition of recombinant SRS2' by the monoclonal antibody mab 5.2.15 raised to native *N. caninum* antigens, as well as by sera from *N. caninum* infected mice in immunoblotting (**I**, **II**), suggested that the recombinant SRS2' protein was properly configured.

Similarly, mice were immunised with one of the M5-iscom preparations (M5-iscom(Ni)), in order to evaluate their capability to induce antibodies that

recognised the target antigens M5. These mice produced antibodies reacting with an unrelated recombinant M5 fusion protein with a mean titre of 1:452 (I).

To further investigate the antigenic structure of the recombinant SRS2', the four different kinds of SRS2' iscom preparations were analysed with immunoblotting using the two monoclonal antibodies mab 5.2.15 and NcMab-10 to NcSRS2 (Björkman & Hemphill, 1998; Schares *et al.* 1999; A. Hemphill, personal communication), and serum from infected mice. The two monoclonal antibodies were first tested in a competitive ELISA, which showed that they recognise different epitopes (data not shown). The recombinant SRS2', in all four iscom formulations, was recognised by the polyclonal serum and mab 5.2.15, but not by NcMab-10, while native NcSRS2 antigen was stained by both monoclonal antibodies (III). This indicated that the recombinant SRS2' possess some B-cell epitopes present in the native NcSRS2, but lack at least one of them. It is possible that the epitope recognised by NcMab-10 is located outside the segment of NcSRS2 that constitutes SRS2'.

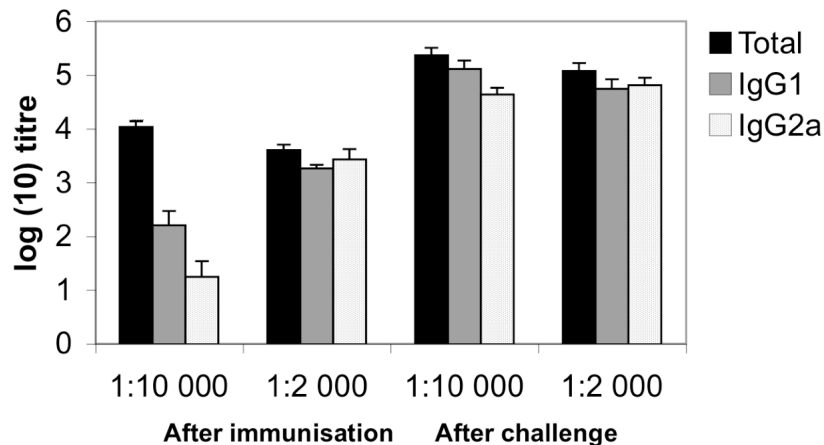


Fig. 4. Antibody titres against native *N. caninum* antigens for mice measured both after immunisation with SRS2'-iscoms and after challenge infection with live parasites. The subclass-specific conjugate was used at two different dilutions, 1: 10 000 and 1:2 000.

Futhermore, the IgG subclass analysis of sera from immunised and infected mice revealed possible differences in the antigenicity of the recombinant antigens and the native NcSRS2. The subclass titres against the native antigens, IgG1 as well as IgG2a, induced by immunisation were low in relation to the total Ig titres. In contrast, the subclass titres against the recombinant protein were much closer to the total Ig titres, a pattern that was also seen in the *N. caninum*-specific antibody response after infection (III). Theoretically, the discrepancy between the total Ig and subclass titres could be due to the dominance of isotypes other than IgG, or subclasses other than IgG1 and IgG2a. Alternatively, and more likely, it could be caused by differences in the ELISAs used for detection of total Ig and the subclasses.

To test this hypothesis, the concentration of the subclass-specific secondary antibodies was increased, to test some sera collected from immunised mice before and after challenge infection. This resulted in increased subclass titres for sera collected before infection, but did not affect the titres of sera taken after challenge infection (Fig. 4). This can be interpreted as a low specificity and/or affinity of the antibodies induced by the recombinant proteins, as compared to those induced by infection. Thus, it is likely that, although the recombinant antigens obviously contain some of the B-cell epitopes of the native antigen, others are missing or not properly exposed.

Nevertheless, the finding that the SRS2'-iscoms induced antibodies that reacted with the native antigen was considered to be promising, since previous studies using other recombinant NcSRS2 proteins indicate that protective immunity is associated with production of antibodies that react with native NcSRS2. Cannas *et al.* (2003a) reported that mice immunised with a recombinant SRS2 protein produced antibodies reacting to the recombinant protein but not to native parasite antigen, whereas immunisation with NcSRS2-DNA followed by a single injection of recombinant NcSRS2 induced antibodies recognising the native antigen. After challenge infection, a significant reduction in the number of mice with detectable parasite DNA in their brains was observed in the group given the DNA vaccine, but not in mice immunised with recombinant protein without priming with DNA-vaccination. Therefore, it was worthwhile continuing to evaluate the immunogenicity and protective capacity of the SRS2'-iscoms.

Comparison of immune responses induced by the different NcSRS2 iscom formulations (I, II, III)

In **Paper III**, the immunogenicity of three different SRS2'-iscom preparations was compared with that of the corresponding recombinant SRS2' without any adjuvant. The preparations used were:

BioSRS2'-iscom(Ni) and Bio-His₆-ABP-SRS2'
dBioSRS2'-iscom(Ni) and Bio-Bio-His₆-ABP-SRS2'
SA-SRS2'-iscom(Bio) and His₆-SA-SRS2'.

It was found that two of the iscom preparations (BioSRS2'-iscom(Ni) and SA-SRS2'-iscom(Bio)) induced high levels of *N. caninum*-specific antibodies, while only very low titres were detected in mice given the corresponding recombinant proteins Bio-His₆-ABP-SRS2' or His₆-SA-SRS2' without adjuvant. Analysis of antigen-specific cellular responses revealed that only cells obtained from mice immunised with BioSRS2'-iscom(Ni) or SA-SRS2'-iscom(Bio) produced significantly higher level of IFN- γ and IL-4 than did cells from unimmunised mice ($P < 0.05$ and $P < 0.01$ for IFN- γ ; $P < 0.05$ and $P < 0.05$ for IL-4). These results clearly demonstrate the adjuvant effect of the iscom, and the capacity of these two SRS2'-iscoms to induce *N. caninum*-specific cellular response.

In contrast, the double biotinylated SRS2' without adjuvant induced high antibody titres that were comparable to those generated by the corresponding

iscom preparation. Furthermore, no *N. caninum*-specific IFN- γ or IL-4 production was detected in cultures from mice immunised with this protein, either alone or in association with iscoms. These results suggest that the high antibody titres observed in mice injected with double-biotinylated SRS2'-iscoms might not be attributable to the adjuvant effect of the iscom. Since there was no proliferative response of cells obtained from mice injected with double-biotinylated SRS2' iscoms, it is possible that the protein was not properly associated with the matrix, and that aggregation of the protein was the reason for its localisation in the Quil A-rich fractions of the gradient. This is also a likely explanation for its capacity to stimulate an antibody response even without any adjuvant.

In **Paper I**, mice immunised with SRS2'-iscoms (BioSRS2'-iscom(Ni)) produced specific antibodies reacting to *N. caninum* antigens with a mean titre 1:10784. However, the same level of *N. caninum*-specific antibodies (with a mean titre of 1:14,355) were also observed in mice immunised with regular iscom matrix mixed with His₆-SA and Bio-His₆-ABP-SRS2'. As mentioned above, analysis of sucrose-gradient ultracentrifugation of the former iscom preparation showed successful iscom association, whereas the latter mixture showed minimal iscom association of both fusion proteins. The reason for this unspecific binding is not clear. Nevertheless, the addition of regular matrix to the protein was sufficient for inducing an antibody response to *N. caninum* SRS2 antigen. To further examine the immunogenicity of this preparation, it would be of interest to investigate whether cellular responses could be generated.

In **Paper II**, immunoblotting analysis confirmed that the antibody responses to *N. caninum* antigens measured by ELISA were directed against NcSRS2.

In a further experiment (**III**), the immunogenicity and protective effect of the two iscom preparations BioSRS2'-iscom(Ni) and SA-SRS2'-iscom(Bio), was compared to another preparation containing SA-SRS2'-iscom(Ni), which was included to test the influence of biotinylated matrix (Bio-matrix) and Ni²⁺-loaded matrix on the immunising effect. Control groups were injected with M5-iscom(Ni) or with IDA matrix.

The results of antibody and subclass analyses after the second immunisation showed that high levels of *N. caninum*-specific antibodies were detected in sera from all mice in all three SRS2'-iscom immunised groups, without any significant differences between the groups. Only very low antibody titres were detected in sera from mice immunised with the M5-iscoms, and no antibodies were detected in sera from mice given IDA-matrix. Also, *N. caninum*-specific IgG subclasses (IgG1 and IgG2a), of which IgG1 dominated, were detected in all three SRS2'-iscom immunised groups, although at relatively low levels compared with the titres for total Ig to *N. caninum*.

Significant antigen-specific proliferative responses were detected in cell cultures obtained from mice immunised with all three different SRS2' iscoms [BioSRS2'-iscom(Ni) (Group 1), SA-SRS2'-iscom(Bio) (Group 2), and SA-SRS2'-iscom(Ni) (Group 3)], but not in cell cultures obtained from mice immunised with either the

M5-iscoms or IDA-matrix. In addition, high levels of both IFN- γ and IL-4 were detected in antigen-stimulated cultures from all three groups given different SRS2' iscom preparations. The cytokine analysis revealed that cells from mice immunised with SA-SRS2'-iscom(Bio) (Group 2) produce significantly higher IFN- γ than did the other immunised mice (Groups 1 and 3), and the IL-4 levels in Group 2 were significantly higher than in cultures from mice in Group 3.

In conclusion, the three SRS2' iscom preparations, BioSRS2'-iscom(Ni), SA-SRS2'-iscom(Bio) and SA-SRS2'-iscom(Ni), were all capable of inducing *N. caninum*-specific humoral and cellular responses. However, the highest levels of IFN- γ production were detected in cell cultures from mice given Bio-matrix+His₆-SA-SRS2', indicating that this preparation might be superior to the other two.

Detection and quantification of parasitaemia (II, III, IV)

To further evaluate the different iscom formulations, a series of challenge experiment was performed. The parameters used to assess resistance against infection were clinical symptoms, including changes in body weight, and the amount of parasite DNA detected in the brain three weeks after infection (II, III). For this purpose a real-time PCR was developed to detect *N. caninum* DNA (III). Study IV assessed if this assay was sensitive enough to detect and quantify parasitaemia in mice after infection, for evaluation of protective immunity.

In a first experiment, three groups of 12 mice were inoculated with 5×10^6 (Group 1), 10^6 (Group 2) or 10^5 (Group 3) live tachyzoites. As expected, clinical symptoms were observed in all of the inoculated mice, and the mice inoculated with the lowest dose were the least affected. Six mice in Group 1, three in Group 2 and two in Group 3 showed severe symptoms, and were killed before the end of experiment. Analysis of blood samples collected every second day for 12 days, and thereafter with longer intervals, showed that *N. caninum* DNA could be detected most frequently from days 2 to 8 post inoculation, and thereafter only sporadically. This was consistent with a similar study, in which parasitaemia was detected in most inoculated mice on days 1 to 7 post inoculation and thereafter only intermittently (Collantes-Fernandez *et al.*, 2006).

In the present study, *N. caninum* DNA was detected in the brain from all inoculated mice, except three mice given the lowest number of tachyzoites. The levels of parasite DNA in the brain were significantly different between groups ($P < 0.01$), with the lowest levels detected in the group inoculated with the lowest dose. Thus, the level of parasitaemia seemed to be correlated to the amount of parasite DNA in the brain and to disease severity. Results from other studies support a correlation between disease severity and parasite load in the brain (Collantes-Fernandez *et al.*, 2006; Long, Baszler & Mathison, 1998).

The results showed that the real-time PCR was sensitive, and could be used for detection and quantification the parasite DNA in the relative small blood volume

that can be collected from mice on multiple occasions. The real-time PCR had a detection limit at 0.008 pg *N. caninum* DNA, which is approximately equivalent to 0.1 tachyzoite genome. Despite this, only a few of the samples from the groups inoculated with the lowest dose were positive. Thus, analysis of larger volumes of blood, or more frequent sampling, would be desirable to increase the probability of parasite DNA detection, but this is obviously difficult in the murine model. The results from this experiment were used to design the study of parasitaemia in immunised mice described below. A high infection dose was chosen, and blood was collected every day for nine days after infection.

Protective effect of NcSRS2 iscoms (II, III, IV)

In the first challenge experiment (II), BioSRS2'-iscom(Ni) was used to investigate whether the recombinant SRS2' could induce protective immunity. After challenge infection, mice immunised with these SRS2'-iscoms exhibited only mild and transient symptoms. In contrast, the control mice immunised with M5-iscom(Ni) became progressively more affected, and some of them developed neurological symptoms. These mice started to lose weight from day 15 after infection, while the weights of mice given SRS2'-iscoms and of the uninfected controls remained at the same level until the end of the experiment. The differences in average weight change between the three groups were close to significant ($P=0.051$). However, the level of parasite DNA in the brains was significantly lower in immunised mice compared to the control mice ($P<0.01$). Moreover, while parasite DNA was detected in the brain of all mice in the infected control group, 3 of 10 mice immunised with this NcSRS2'-iscom formulation were negative. Taken together, these results clearly show that BioSRS2'-iscom(Ni) can induce immunity sufficient to reduce infection of the brain and disease severity.

Thereafter, the protective effects of the three different SRS2'-iscom preparations [BioSRS2'-iscom(Ni) (Group 1), SA-SRS2'-iscom(Bio) (Group 2) and SA-SRS2'-iscom(Ni) (Group 3)] were compared (III). Control mice were immunised with M5-iscoms (Group 4). After challenge infection, all immunised groups (Groups 1–3) showed mild clinical signs for 7 days, and thereafter they appeared normal until the end of the experiment, except 4 out of the 10 mice in Groups 1 and 3 still remained mildly affected. The infected control mice (Group 4) developed obvious clinical symptoms, and became progressively more affected.

In this experiment, the overall differences in average weight change between the groups were clearly statistically significant ($P<0.001$). Pairwise comparisons showed that the infected controls lost significantly more weight than did the mice in all of the immunised groups. In addition, the weight changes in Group 2 and in the uninfected control mice were not significantly different, whereas the mice in Groups 1 and 3 lost significantly more weight than did the uninfected controls. Moreover, the parasite DNA load in the brain was significantly different between groups ($P<0.001$). Parasite DNA was detected in all infected control mice, whereas no parasite DNA could be detected in 1, 6 and 1 of the 10 immunised mice in Groups 1, 2 and 3, respectively.

Thus, immunisation with all three SRS2'-iscom formulations induced immunity sufficient to reduce cerebral infection and clinical symptoms. Of the three formulations compared, SA-SRS2'-iscom(Bio) afforded the best protection. This was also the formulation giving the strongest antigen-specific cellular responses with the highest levels of IFN- γ production. Since the balance between Th1 and Th2 responses is considered to be important for resistance against *N. caninum*, the relationship between IFN- γ and IL-4 levels, and between IgG1 and IgG2a titres, induced by the different iscoms were compared, but no differences were found. Thus, the degree of protection seemed to be correlated to the strength of the cellular responses, rather than to differences in Th1/Th2 balance.

In **Paper IV**, the protective effect of two of the SRS2'-iscoms was further evaluated, as regards reduction of parasitaemia after challenge infection. In this experiment, two groups of mice were immunised with either SA-SRS2'-iscom(Bio) or SA-SRS2'-iscom(Ni). Analysis of blood samples collected daily during 9 days after challenge infection revealed significant differences in the concentration of *N. caninum* DNA between groups and over time ($P < 0.001$ and $P < 0.01$, respectively). Also, the duration of parasitaemia seemed to be shorter in immunised mice than in non-immunised infected mice, since parasite DNA was detected in at least three of the non-immunised mice on days 1 to 7 post inoculation, but in the immunised groups this number of mice were found positive only during the first 3 days.

As in the previous experiments, the immunised mice showed mild clinical signs for some days after infection, and thereafter most of them recovered. However, some of them still showed mild signs of illness until the end of experiment, and two mice given SA-SRS2'-iscom(Bio) and one given SA-SRS2'-iscom(Ni) became worse and were killed on day 20 after infection. The non-immunised infected mice developed severe symptoms and 5 of the 18 mice had to be killed. They also lost significantly more weight ($P < 0.001$) and had significantly more *N. caninum* DNA in their brains ($P < 0.001$) than the immunised mice, but there was no significant difference between the two immunised groups. There appeared to be a good correlation between the degree of parasitaemia and the amount of parasite DNA in the brain 3 weeks after infection.

The results from this study indicate that the immunised mice were able to control the infection during the early stage, which resulted in reduced parasitaemia. Similar results have been reported from a study of *T. gondii* infection, in which mice immunised with an attenuated strain had reduced parasitaemia after challenge infection (Kirisits *et al.* 2000). It is likely that the lower brain parasite load in immunised mice was a result of reduced parasitaemia and fewer parasites reaching the brain.

The difference in protective effect of the two formulations SA-SRS2'-iscom(Bio) and SA-SRS2'-iscom(Ni) that was recorded in the previous experiment (**III**) was not evident this time (**IV**). The reasons for this discrepancy could be that different batches of iscoms were used, variations in the virulence of the parasites, or individual mouse responses.

In conclusion, the present studies show that the bacterially expressed NcSRS2 recombinant immunogen was successfully associated with iscom matrices by different approaches. It was found that the three different NcSRS2' iscom preparations were capable of inducing antibody and cellular immune responses. Hence, immunised mice showed sufficient resistance to reduce the level and duration of parasitaemia, and the intensity of infection in the brain. In addition, both approaches of indirect and direct iscom association via biotin-streptavidin interaction are likely to constitute valuable means to achieve efficient iscom association of recombinant immunogens.

The potential of NcSRS2 as a vaccine candidate

Vaccination against neosporosis is a possible strategy for disease control. However, to design an effective vaccine against *N. caninum* infection is a challenge for vaccinologists. Several research groups have evaluated different immunodominant proteins associated with different adjuvant systems in experimental vaccines, and partial protective immunity has been achieved in mouse experiments. However, comparison of the degree of protection obtained in different experiments is difficult because of dissimilarities of certain circumstances; e.g. antigen/immunogen used, (laboratory) animal species and strains, immunisation dose, administration route, the parasite isolate and number of parasites used for challenge infection, and finally the parameters and methods used for evaluation of the protective immunity.

A number of *N. caninum* antigens have been considered as vaccine candidates. Many of them are immunodominant and/or have been shown to be functionally involved in different phases of the infection process (i.e. play a crucial role in infection). Some of them have been evaluated in vaccination trials, either as recombinant subunit, as DNA vaccines or expressed by live vectors. For example, live recombinant vectors expressing NcSAG1 or NcSRS2 (Nishikawa *et al.*, 2001b), recombinant NcSAG1 or NcSRS2 alone or after priming with cDNA (Cannas *et al.*, 2003a), NcMIC1 (Alaeddine *et al.*, 2005), NcMIC3 (Cannas *et al.*, 2003b), NcGRA7 (Jenkins *et al.*, 2004; Liddell *et al.*, 2003), and NcDG1 and NcDG2 (Cho *et al.*, 2005) have been studied. In most of these experiments partial protection was observed.

Among these antigens, NcSRS2, the major immunodominant surface antigen found in both tachyzoites and bradyzoites, has been considered as a potential antigen in future vaccines (Fuchs *et al.*, 1998; Hemphill & Gottstein, 1996; Howe *et al.*, 1998). In the present studies, it was shown that the *E. coli*-expressed recombinant NcSRS2 in different iscom formulations gave partial protection against clinical disease and significant reduction in brain parasite load (**II**, **III**, **IV**). Similar protective effects have been demonstrated after priming with cDNA coding for NcSRS2 and boosting with the recombinant protein (Cannas, *et al.*, 2003a). Immunisation with NcSRS2 constructed in a live vector has been shown both to reduce infection in non-pregnant mice (Nishikawa *et al.*, 2001a) and to prevent transplacental transmission (Nishikawa *et al.*, 2001b). Affinity-purified

native NcSRS2 has also been shown to reduced transplacental transmission (Haldorson *et al.*, 2005). Furthermore, the selection of antigens recognised by specific antibodies and immune T cells might be useful to identify candidate antigens. Recently, it has been reported that NcSRS2, rather than NcSAG1, could induced activation of memory CD4⁺ and CD8⁺ T lymphocytes from experimentally infected cattle (Staska *et al.*, 2005).

In conclusion, NcSRS2 seems to be a promising candidate antigen for a vaccine against neosporosis (i.e. that can elicit elements of potentially protective immune responses against the infection). However, the partial protection induced so far by the various experimental NcSRS2 vaccines needs to be improved. This might be achieved by improvement of recombinant protein conformation, optimisation of the adjuvants used, or selection of other delivery adjuvant systems. Another strategy that should be considered is to use a combination of different recombinant antigens, to enable the induction of complete protection.

Conclusions

Efficient association of recombinant immunogens to iscoms was achieved by a novel method taking advantage of the strong biospecific interaction between biotin and streptavidin. This was achieved either by indirect binding of biotinylated proteins to Ni²⁺-loaded matrix via His₆-SA or by direct binding of streptavidin-fusion proteins to biotinylated iscom matrix.

Since many recombinant proteins are hydrophilic, they need an appropriate modification in order to become efficiently associated to the adjuvant. The approaches described might offer convenient and effective methods to achieve iscom association of various recombinant antigens for development of the new generation of adjuvant-subunit vaccines.

Mice immunised with bacterially expressed NcSRS2 and associated with iscoms according to the two described concepts induced promising humoral and cellular immune responses. The immunisation resulted in production of specific antibodies that reacted with the native *N. caninum* antigen, and the recombinant antigens were recognised by polyclonal and monoclonal antibodies raised to *N. caninum*. Therefore, it was concluded that the conformation of the recombinant proteins were similar to that of the native protein. Furthermore, immunisation with the NcSRS2-iscoms generated an antigen-specific cellular response including production of IFN- γ and IL-4.

The real-time PCR assay based on the detection of the Nc-5 repeated sequence of *N. caninum* was found to be a sensitive means for rapid quantitative detection of *N. caninum* DNA in blood and brain samples from infected mice.

It was demonstrated that in murine neosporosis the level of parasitaemia during the acute stage of infection appeared to be correlated to disease severity and the amount of parasites in the brain during the chronic stage.

The recombinant NcSRS2-iscoms induced partial protection against challenge infection, which was demonstrated as reduced clinical symptoms, brain parasite load and parasitaemia. Although comparison of the protective effect of the different iscom formulations was not conclusive as regards which of them was best, it showed that protection seemed to be associated with efficient induction of cellular responses.

The recombinant NcSRS2 is a potential candidate antigen for a future vaccine against neosporosis. However, such a vaccine should probably contain a combination of different protective antigens to be able to induce complete protection.

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