Neospora caninum:

From genes to vaccines

Thèse

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KARIM DEBACHE

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Acceptée sur proposition du jury:

Directeur de thèse:	**Prof. Bruno Betschart**
Superviser:	**Prof. Andrew Hemphill**
Rapporteur:	**Prof. Luis Miguel Ortega-Mora**
	Prof. Michel Brossard

Université de Neuchâtel

FACULTE DES SCIENCES
Secrétariat-Décanat de la faculté
Rue Emile-Argand 11
CP 158
CH-2009 Neuchâtel

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Karim DEBACHE

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel,
sur le rapport des membres du jury

MM. Bruno Betschart (directeur de thèse, Neuchâtel),
Andrew Hemphill (co-directeur de thèse, Berne)
Michel Brossard (Neuchâtel) et Luis Miguel Ortega-Mora (Madrid, E)

autorise l'impression de la présente thèse.

Neuchâtel, le 13 avril 2010

Le doyen :
F. Kessler

UNIVERSITE DE NEUCHATEL
FACULTE DES SCIENCES
Secrétariat - décanat de la faculté
Rue Emile-Argand 11 - CP 158
CH-2009 Neuchâtel
Felix Kessler

Téléphone : +41 32 718 21 00 E-mail : secretariat.sciences@unine.ch www.unine.ch/sciences

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Résumé

Neospora caninum est un parasite intracellulaire obligatoire, découvert en 1984 et longtemps confondu avec *Toxoplasma gondii*. Cependant, au début des années 1990, il a été constaté que *Neospora* était responsable de nombreux avortements, d'une mortalité élevée, particulièrement chez les ruminants (principaux hôtes intermédiaires) et de troubles neuromusculaires chez le chien (à la fois hôte intermédiaire et hôte définitif). Chez les bovins, *N. caninum* est un facteur abortif majeur et la principale manifestation clinique de la néosporose est l'avortement, qui survient habituellement entre le quatrième et le septième mois de gestation. Chez la vache, il s'agit souvent du seul signe clinique observé. En général, une vache infectée a deux à trois fois plus de risque d'avorter qu'une vache non infectée. De plus, on a observé qu'une vache infectée pouvait avorter plus d'une fois d'un fœtus infecté. Le système immunitaire de la vache ne semble pas donc la protéger de manière adéquate, même après un premier avortement. On rapporte également qu'une vache infectée produit en moyenne moins de lait qu'une vache non infectée. Le mode principal de transmission du parasite est la transmission verticale (de mère en fille) et généralement, on considère qu'une vache infectée l'est pour la vie et qu'elle donnera naissance 9 fois sur 10 à un veau infecté. Accessoirement, *Neospora* se transmet suite à la contamination d'aliments par les excréments d'un autre animal (hôte définitif). Dans le mode de transmission dit horizontal, l'hôte définitif (chien ou coyote) se contamine en ingérant les tissus infectés de l'hôte intermédiaire. Par la suite, cet hôte définitif excréterait des oocytes dans ses fèces qui contamineraient les aliments de l'hôte intermédiaire. Les pathologies liées à *Neospora* sont diagnostiquées dans de nombreux pays à travers le globe, notamment en Suisse, *Neospora* est la cause la plus fréquemment diagnostiquée d'avortement chez les bovins, entraînant une perte économique considérable. En Suisse, cette perte est estimée à environ 9,7 millions d'euros par an. A ce jour, il n'existe pas de vaccin ou de traitement pouvant arrêter la transmission horizontale au fœtus et l'élimination total du parasite chez les hôtes intermédiaires et définitifs. En absence de traitement fiable, le seul moyen de contrôle est donc d'arrêter la transmission du pathogène par l'élimination des vaches infectées (séropositives). Cette solution n'est toutefois pas recommandée car elle s'avère trop coûteuse. De ce fait, il est impératif d'entamer une stratégie en vue de développer un vaccin sans effet négatif, ni sur l'animal à traiter, ni sur ses produits dérivés (lait et viande).

Plusieurs études ont permis de dévoiler un ensemble d'antigènes impliqués dans le processus d'infection des cellules hôtes. Ces antigènes sont produits essentiellement par les trois

principales organelles de sécrétion du parasite (rhoptries, micronèmes et granules denses). Dans ce travail, au moyen d'outils moléculaires, certaines de ces protéines ont été sélectionnées selon leur degré d'implication dans le processus d'invasion (interaction parasite-cellule hôte) et selon leur potentiel de stimulation du système immunitaire. Les protéines ont été produites sous forme recombinante grâce à un système d'expression procaryote. Les antigènes recombinants ont été évalués en tant que composants d'un vaccin d'après leur capacité à réduire la charge parasitaire, soit dans un modèle cellulaire *in vitro,* soit dans un modèle animal.

Les essais effectués durant ce travail de thèse sont résumés ci-dessous :

(i) NcROP2 recombinante. La protéine NcROP2 fait partie d'une large famille de protéines de rhoptries, impliquées dans l' invasion de la cellule hôte et la formation de la membrane de la vacuole parasitophore. *In vitro,* l'addition au milieu de culture cellulaire de la protéine NcROP2 recombinante ou de son antisérum spécifique affecte négativement la capacité d'invasion des cellules par le parasite. *In vivo,* une vaccination a été effectuée chez des souris par l'administration par voie intra-péritonéale de 3 doses de NcROP2 suivie d'un challenge infectieux. Plus d'un mois après l'infection expérimentale par *Neospora,* les souris ayant reçu NcROP2 montrent une diminution significative du taux d'infection cérébrale par rapport au groupe de contrôle ayant reçu l'adjuvant seul.

(ii) NcROP2 combinée avec les protéines de micronèmes. Des souris gestantes et préalablement vaccinées par voie intra-péritonéale avec un cocktail de NcROP2 et de protéines micronémiales NcMIC1 et NcMIC3 montrent un taux d'infection cérébrale faible. De plus, les nouveau-nés présentent également un taux très faible de parasites viables. Cette protection est obtenue dans un contexte de réponse immunitaire de type Th2.

(iii) NcPDI. Cette protéine (protéine disulfide isomérase) est principalement localisée au niveau du réticulum endoplasmique et à la surface du parasite. La vaccination de souris par voie intra-nasale des souris avec NcPDI adjuvantée de toxine cholérique, entraine une protection de 90% contre *Neospora caninum* et une importante sécrétion d'IgG1 et d'IgA. Par contre, aucun effet protecteur n'est obtenu si cette même protéine est injectée par voie intra-péritonéale.

(iv) NcPDI délivrée au moyen de nanogels de chitosan. NcPDI est administrée soit adjuvantée de saponine ou de toxine cholérique, soit en association avec un complexe alginate-chitosan ou alginate-chitosan recouvert de résidus mannose. Une réduction de la charge parasitaire est notée chez les souris ayant été vaccinées par l'alginate-chitosan seul et combiné avec NcPDI; cette protection est accompagnée d'une sécrétion élevée d'IgG1 et d'une réponse dominante

de type Th2. En revanche, aucun effet protecteur n'est observé suite à l'administration d'alginate-chitosan recouvert de mannose combiné à NcPDI.

Abstract

The coccidian parasite *Neospora caninum* has been identified as a veterinary health problem associated with important economic losses in the dairy industry. The corresponding disease, neosporosis, causes abortion, stillbirth and decreased milk yield in livestock, predominantly in cattle. Furthermore, it causes neonatal neuromuscular disorders in dogs. *Neospora caninum* is a cosmopolitan intracellular pathogen and has been found in a wide range of hosts including domestic and wild animals. The economic importance of neosporosis, especially in cattle, has led to intensive research on the development of strategies for prevention and treatment of *N. caninum* infections because at the present time no efficient vaccine capable of inducing complete protective immunity in cattle is available. Therefore, vaccination and chemotherapy strategies have been identified as economically promising control options. The present work will focus on the development of novel recombinant *Neospora* antigens either as mono or mixture vaccine candidates, and also involves testing of different formulations and delivery routes.

The *N. caninum* life cycle, governed by host cell invasion, intracellular development and long-term persistence presents a potential target for vaccination intervention. Several of the potential target antigens involved in these processes, are located either on the parasites surface or within secretory organelles such as micronemes, rhoptries and dense granules and so far, a limited number of recombinant proteins have been investigated as vaccine candidates. These include mostly immune-dominant antigens functionally involved in tachyzoite-host cell interaction, such as surface antigens and proteins localised in secretory organelles. The candidate antigens have been conveniently investigated in a murine model because of the ease in monitoring the disease and the biological similarities to cattle, in spite of the fact that murines and bovines exhibit somewhat different immunological characteristics.

Aiming to develop vaccines against neosporosis able to control the parasite transmission and infection, we have been focussing mainly on rhoptries and microneme proteins expressed in bacteria. We assessed the potential protectivity in *Neospora* infected cell-and mouse models. The trials are summarized as follow:

(i) **NcROP2 vaccine:** *Neospora caninum* rhoptrie protein 2, is one of ROPs family which are injected into the host cell cytoplasm at the invasion site. Of which, some ROPs remain associated with small vesicles that fuse with the developing parasitophorous vacuole membrane. This rhoptry protein

exhibits 46% identity with *Toxoplasma gondii* ROP2 and contains a serine/threonine protein kinase domain. Amino acids 257–546 were recombinantly expressed in *Escherichia coli*. Mice were vaccinated three times with purified recNcROP2, and challenged. Vaccinated mice remained clinically healthy, exhibited highly significantly reduced cerebral parasite burden and IgG-subclass ELISA indicated that NcROP2 induces a protective Th-1- or Th-2-biased immune response against experimental *N. caninum* infection, depending on the adjuvant type used for immunization. The protection achieved with recNcROP2 was not altered either by intraperitoneal or intranasal application.

(ii) **NcROP2 combined with microneme proteins:** Based on previous promising results obtained with recNcMIC1, recNcMIC3 and NcROP2, female mice were vaccinated with combined antigens (NcROP2-NcMIC1; NcROP2-NcMIC3; NcROP2-NcMIC1-NcMIC3). They were subsequently made pregnant, and challenged with *N. caninum* tachyzoites at day 7 of their pregnancy. Daily monitoring for occurrence of clinical signs and survival rate showed no morbidity or mortality in the dams receiving recNcROP2 and mixed recNcROP2-NcMIC1-NcMIC3 vaccines, and highest survival rates as well as lower cerebral parasite load were found among their newborns. No detectable surviving parasites were found in the group of offspring whose mothers had been vaccinated with recROP2/MIC1/MIC3. Based on serology tests also involving cytokine analysis, the obtained protective effects were achieved through a Th2-biased immune response.

(iii) **NcPDI vaccine:** protein disulfide isomerase, which is partially localized in the micronemes and on the surface of *N. caninum* tachyzoites, induced a strong humoral immune response but was completely ineffective in terms of reducing the *N. caninum* cerebral infection rate when inoculated intraperitoneally. However, when NcPDI suspended in cholera toxin adjuvant was administered by intranasal inoculation, there was a pronounced protective effect with 90% survivors, all with significantly

decreased cerebral parasite burden and high levels of IgG1 and IgA antibodies.

(iv) **NcPDI delivered by chitosan nanogels:** The recombinant protein was administrated either as mixture with saponin (i.p.) or cholera toxin adjuvant (i.n.) and associated with alginate-chitosan nanoparticles or alginate-mannose coated chitosan nanoparticles. Intraperitoneal application of recNcPDI antigen without nanoparticles led to protection against neosporosis in only 20% of the vaccinated mice. In contrast, 90% protection was achieved following intranasal administration with recNcPDI emulsified in cholera toxin. Mice vaccinated with alginate-coated particles alone or alginate-reNcPDI coated nanoparticles exhibited reduced parasite load, whereas alginate-mannose-associated recNcPDI showed no significant effect. Reduction of cerebral infection and viability in intranasal delivery was achieved through significantly higher IgG1 and dominant Th2-type cytokines.

1. Introduction

Neospora caninum is a cyst forming apicomplexan parasite originally described as a parasite of the domestic dog (Dubey et al. 1988) and was initially recognized in Norway as a cause of hind limb *Toxoplasma*-like illness (Bjerkas et al. 1984). The parasite (Apicomplexa: Eimeriina: Sarcocystidae) was identified and named by Dubey et al. (1988a, b) and soon researchers associated *Neospora* infection with bovine abortion (Dubey and Lindsay 1996). Later the intracellular pathogen was reported in various species of livestock, including cattle, sheep, goats, horses and deer (reviewed by Dubey and Lindsay 1996; Hemphill, 1999; Dubey et al., 2007). Today *N. caninum* is known as the most frequently diagnosed cause of abortion and stillbirth in cattle industry worldwide (Hemphill and Gottstein, 2000; Gondim et al., 2005; Dubey et al., 2007). Thus neosporosis represents an important veterinary health problem and leads to severe economic losses. In Switzerland 30% of all bovine abortions might be related to *Neospora* infection (Hasler et al., 2006a; b). The negative economical impact that the parasite has on the dairy industry including reduced milk yield (Hernandez et al., 2002), premature culling (Thurmond and Hietala, 1996) and reduced post-weaning weight gain in beef calves (Reichel, 2000; Pfeiffer et al., 2002; Hasler et al., 2006a, b) has given a great encouragement to research on the development of strategies for prevention and treatment of *N. caninum* infection.

1.2. Life cycle of *Neospora caninum*

N. caninum is an obligate intracellular parasite. Dogs and coyotes have been identified as definitive hosts for *N. caninum* (McAllister et al., 1998; Lindsay et al. 1999a; Gondim et al., 2004) and like *T. gondii*, this parasite is able to infect a broad range of intermediate hosts, including cattle, dogs, deer, horses, goats and chicken (Dubey and Lindsay, 1993, 1996; Costa et al., 2008). As coccidian parasite, the life cycle of the parasite (Fig. 1) consists of an asexual (sporogony and schizogony) and a sexual phase (gametogony). The asexual cycle occurs in intermediate (cattle, sheep, goats and horses) and definitive hosts (dogs and coyotes), whereas the sexual cycle occurs in definitive hosts only. Unsporulated oocysts are produced in the intestine of the definitive host and shed into faeces for one to several days after ingesting infected tissues. Sporulation (sporogony) of the oocyst begins outside the host within 72 hours. Each oocyst contains two sporocysts and each sporocyst contains four sporozoites

which are orally infective (McAllister *et al.*, 1998). The sporozoites transform into the rapidly dividing tachyzoites which are present during the acute phase of the infection. The tachyzoites are characterized by their ability to invade virtually any nucleated eukaryotic cell type. They locate inside host cells within a parasitophorous vacuole and replicated rapidly ("tachy" means fast) by a typical binary fission (endodyogeny). Tachyzoites reaching cerebral cells switch from tachyzoite to bradyzoites ("brady" means slow), which are surrounded by a protective cyst wall. This tissue cyst forming bradyzoites can persist in the infected host for several years without causing any clinical signs. When tissue cysts are taken up by the definitive host again (dog or coyote), bradyzoites are released and new schizonts are produced asexually by schizogony able to invade neighbouring cells. In the intestine of definitive host, oocysts are developed, shed into faeces and the life cycle is completed. Noting, that gametogony stages of *N. caninum* are unknown, no gamonts (macrogametocytes and microgametocytes) or zygotes were reported so far (Lindsay et al., 1999b, c; Schares et al., 2001).

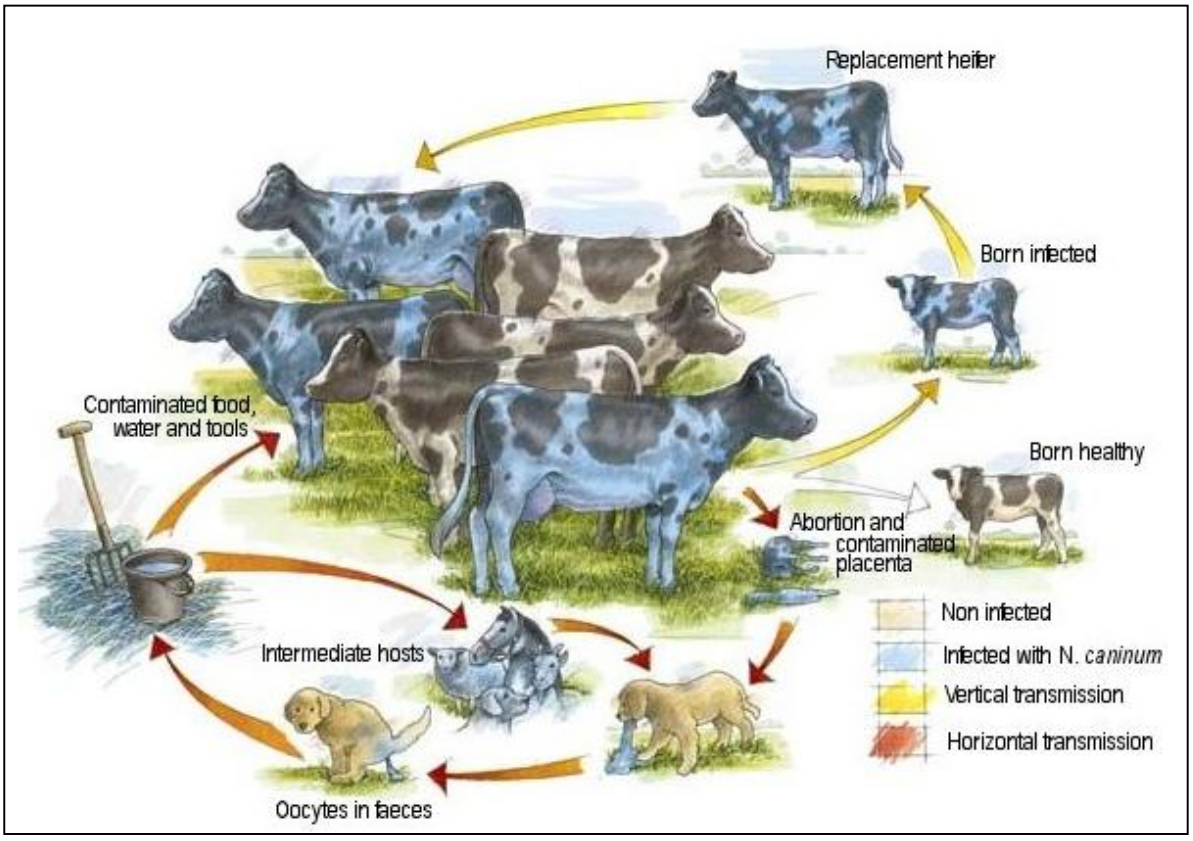

Figure 1: *Neospora caninum*, life cycles between definitive and intermediate hosts (Intervet, Schering-Plough Animal health).

Oocysts (end product of a sexual process): They are produced in the definitive host intestine, shed into feaces as unsporulated spherical bodies of 10-12 x 11,3 µm in diameter (Fig. 2a) and their sporulation (sporogony) begins outside the host and is completed within 72 hours (Fig. 2b). The colourless/smooth oocysts contain two sporocysts of 8,4 x 6,1 µm and each sporocyst contains four sporozoites of 6,5 x 2 µm and residuum (Dubey et al., 1996; McAllister et al., 1998).

Tachyzoites (asexual rapidly proliferating stage): Tachyzoites (Fig. 2c) have been detected in a variety of tissues and cell types (Dubey and Lindsay et al., 1993, 1996; Hemphill et al., 1999), suggesting a low host cell specificity. Depending on the stage of division, they presented an ovoid or globular shape with 3-7 x 1-5 µm in size and are enclosed within a parasitophorous vacuole (Lindsay and al., 1993; Speer et al., 1999).

Bradyzoites (asexual quiescent stage): They proliferate much more slowly and exhibit tissue cysts surrounded by a 1-4,5 µm thick cyst wall which is formed in reaction against the host immune system (Fig. 2d). Cysts are seen exclusively in the central nervous system and skeletal muscle tissues of both intermediate and definitive hosts (Thilsted and Dubey, 1989; Dubey et al., 1996; Peters et al., 2001). Each cyst can contain up to a hundred bradyzoites measuring approximately 4,8-8 x 1-1,9 µm and are elongated/curved with subterminal nucleus (Dubey et al., 2002). Morphologically, bradyzoites are similar to tachyzoites except that they contain fewer rhoptries organelles and also possess antigenic differences. This dormant form of the parasite can persist many years in a host without causing clinical manifestation and only in an immuno-compromised situation, such as pregnancy, bradyzoites can get reactivated and transform back into tachyzoites (Dubey et al., 1996; Rettigner et al., 2004).

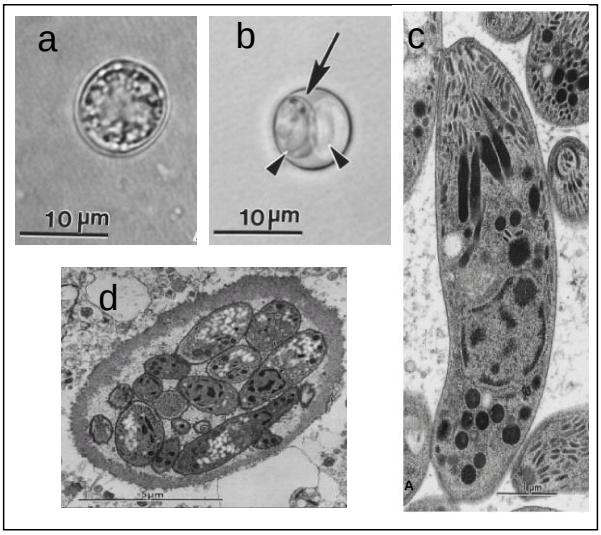

Figure 2: (a) unsporulated oocyst, (b) sporulated oocyst with two sporocysts and with sporozoites (arrowheads), (c) transmission electron microscopy *N. caninum* tachyzoite and (d) bradyzoite (McAllister et al., 1998; Speer et al., 1999).

1.3. Neosporosis

In general, dogs of all age suffer from neurological problems including an ascending paralysis of the hind limbs, encephalitis and myositis, leading to the disease named neosporosis. However, the most severe cases of disease where observed in congenitally infected neonates and dogs less than six month old. To date, anti-protozoal drugs, including clindamycin, sulfonamides and pyrimethamine, were used to treat canine neosporosis (Bjerkås et al., 1984; Dubey et al., 1988, 1998; Barber and Trees, 1996).

During the last years it became evident that neosporosis represents the most important cause of bovine foetal loss and abortion worldwide with the particularity that infected adult cattle do not exhibit any clear or visible clinical symptoms of disease (Quinn and Ellis, 2002). Wouda (1998) and Davison (2001) reported that not necessarily all infected cattle transmit the pathogen to all their live offspring and also Larson et al. (2004) published that not all infected foetuses are aborted. In Switzerland neosporosis has been registered as a notifiable disease since 2001. Approximately 30% of all abortions in Swiss dairy cow population are associated with *N. caninum* infection and a recent study estimated the median annual losses due to this veterinary health problem at 9.7 million Euros (Sager *et al.*, 2001; Hasler et al., 2006a). This high economic impact is also associated with reduced milk yield (Hernandez et al., 2002), premature culling (Thurmondand Hietala, 1996) and reduced post-weaning weight gain in

beef calves (Reichel, 2000; Pfeiffer et al., 2002; Hasler et al., 2006a,b). Abortion in *Neospora* infected cattle typically occurs in the first pregnancy at mostly midgestation phase (5-6 month) (Björkman et al., 1996; Anderson et al., 2000) and *Neospora* sero-positive cows were more likely to abort than sero-negative cows (Sager et al., 2001; Wouda et al., 1998). In infected pregnant cows, the foetuses may die in utero, may be born infected or born clinically normal but chronically sick (Wouda et al., 1998; Dubey et al., 1999a). In addition, infection with *N. caninum* can result from exogenous transplacental infection of the foetus by consumed oocysts during pregnancy where the dose of oocysts and the gestation time are the most influencing factors (McCann et al., 2007). The second main route results from endogenous transmission called also congenital transmission, which occurs by vertical transmission of the dam's parasite to the foetus (Rettigner et al., 2004). Another possible route of infection may be through bovine to bovine transmission via milk, calving fluids or foetal membranes. This type of transmission is documented in calves by oral challenge with experimentally infected colostrums or milk replacer even as late as one week post-calving. However, cattle were not infected when given milk or colostrum from cows naturally infected with *N. caninum* (Uggla et al., 1998; Davison et al., 2001).

1.4. Immune response to *N. caninum*

So far, the immunological response to *N. caninum* infection has not been well understood. There are two major immune response situations. On one hand, reducing parasite multiplication in sero-positive non pregnant dams known as a latent infection is controlled by Th1 immune response involving cell-mediated immunity marked with pronounced IgG2a immunoglobulin level and increased secretion of Th1 specific cytokines such as IFNγ, IL-12 and TNFα (Innes et al., 2000; Quinn et al., 2004). On the other hand, pregnancy-associated Th2-type immune responses favour foetus maintenance, but can also allow parasite reactivation (brady-tachyzoite switching), which results in foetus infection through vertical transmission. Evidence suggests that pathogen reactivation induced inflammatory responses such as those stimulated by IFNγ, are able to interface between the dam's placenta and foetus, which probably results in abortion (Ragupathy, 1997).

1.4.1. Bovine immune response to *N. caninum*

The most important points related to the immunity in *Neospora* infected cattle are: (i) not all infected cows transmit infection to their offspring (Wouda et al., 1998; Davison et al., 2001); (ii) abortion occurs in the first pregnancy and may occur in subsequent pregnancies also (Björkman et al., 1996); (iii) not all infected foetuses are aborted (Larson et al., 2004). In addition, the outcome of infection in cattle depends on the timing, quantity and duration of parasitaemia during gestation and the effectiveness of the cow's immune response plus the foetal ability to respond to the infection (Innes et al., 2000). Klevar et al. (2007) published that the first immune mechanism in infected cows is related to increased natural killer cell proliferation and IFNγ secretion leading to a Th1-type immune response, which is able to compromise the parasite multiplication through cytotoxic $CD8^+$ T cells, γδ T cells, but also may induce tachyzoite-bradyzoite switching leading to chronic infection. During gestation, this inflammatory response may be regulated with Th2 type cytokines, such as IL-4, IL-10 and TGFβ (transforming growth factor beta) aiming towards fetal maintenance, and this modulation of the immune response also favours bradyzoite reactivation (Innes et al., 2007) and infection of the foetus.

1.4.2. Murine immune response to *N. caninum*

The sensitivity to neosporosis in the murine model depends on the strains of both, parasite and mice, used in the studies as Ramamoorthy et al. (2005) demonstrated. According to this study, outbred mice are more resistant to neosporosis than some inbred mouse strains and *N. caninum* Liverpool strain causes a substantial inflammatory response in the mouse cerebral system, leading to disease, whereas *N. caninum* Nowra does not (Miller et al., 2002). Generally, infection of mice resulted in acute and chronic infection, which differ in clinical signs (hind limb paralysis / scruffy coat / inability to reach up for food and water) and production of tissue cysts (Collantes-Fernàndez et al., 2004; Rettigner et al., 2004). Protective immune responses against experimentally induced neosporosis in acute disease mouse models have been shown to be mainly associated with the development of a Th1-type immune response, whereas tests on B-cell deficient (μMT) mice revealed their increased susceptibility to *Neospora* infection, suggesting the importance of the humoral immune response in offering protective immunity against neosporosis in mice (Eperon et al., 1999). However, many

studies have shown that both humoral and cell-mediated immune responses are important components of protective immunity against *N. caninum* (reviewed in Hemphill et al., 2006; Innes and Vermeulen, 2006).

1.5. Epidemiology

The epidemiology of neosporosis is not very well understood because the data reported by different epidemiologist were not comparable due to several serologic methods and cut-off values used, as well as being based on convenience samples obtained for other purposes (Dubey et al., 2007). Abortion in an infected herd may manifest itself with either an endemic or epidemic pattern (Schares et al., 1999, 2002; Trees and Williams, 2005). However, the epidemic abortion due to primary infection of dams with food or water contaminated with *N. caninum* oocysts shed with the faeces of the definitive canine host (exogenous transplacental transmission) has been defined as epidemic if the abortion outbreak is temporary, and if 15% of the cows at risk abort within 4 weeks, 12.5% of the cows abort within 8 weeks, and 10% of the cows abort within 6 weeks. In contrast, an endemic situation has been defined if it persists in the herd for several months or years due to persistently infected dams that transmit the parasite to their progeny transplacentally (endogenous transplacental transmission) (Dubey et al., 2007; Trees and Williams, 2005).

The infection of cattle with *Neospora* either by horizontal (contaminated food/water) or vertical transmission (infected dam - the foetus) is mainly due to the following risk factors that cattle might be exposed to: (i) The age of cattle, the risk of being seropositive may increase with age or gestation number in beef and dairy cattle. (ii) The definitive hosts (dogs and coyotes) frequency in the cattle feeding area. It was demonstrated that dogs which have access to bovine placenta, uterine discharge and colostrums are at higher risk of being infected, by shed oocysts and thus promote the infection rate of cattle in the area. Dubey et al, (1999) published that prevalence in dogs shows a correlation with the prevalence in cattle, with a higher infection number in dogs that live in farm with abortions than in dogs from urban areas. In addition, in Switzerland 7.3% of pet dogs and 20% of the dairy farm dogs were shown to have antibodies against *N. caninum* (Sager et al., 2006). (iii) The presence of other intermediate hosts such as mice and cats. Cats failed to serve as definitive hosts for *N. caninum* and they are predators of mice which are putative intermediate hosts of the parasite. This could reduce the frequency by which dogs (definitive hosts) have access to the infected

tissues. (iv) The size and density of herd with an increased risk in larger herds due to more external replacement heifers and less control on food infection. (v) Seropositivity of individual cattle, the seropositive cows are more likely to abort than seronegative cows. (vi) Co-infection of cattle with *Neospora* and bovine herpes virus 1 (BHV1) and bovine viral diarrhoea virus (BVDV) has been reported to be associated with increased risk of abortion compared with an infection with a single agent. (vii) Some other putative factors such as climate, cattle breed, size of farmland, calving management or feeding colostrums or milk (Dubey et al., 2007).

Neosporosis is a worldwide expanded disease that induces important economical losses in cattle industry in many countries on all continents. For example, it has been estimated that in California approximately 40,000 abortions could be due to neosporosis, providing an estimated loss of $35 million per year. In Australia and New Zealand, losses are thought to be more than $100 million Australian per year. In Canada, the total annual loss was estimated to be $2,304 for a 50-cow dairy herd. In The Netherlands, 76% of seropositive herds with no episodes of abortion had no economic losses, whereas in the remaining 24% of herds, the economic losses increased notably, to a maximum of 2,000 euros per year (Barr et al., 1998; Reichel, 2000; Chi et al., 2002; Dubey et al., 2007).

In addition, to the possible horizontal transmission of the pathogen trough contaminated food/water, the major effective transmission is transplancentally (vertical) from infected dam to the foetus during pregnancy. In vertical transmission, one must distinguish between exogenous transmission where the foetus become infected by primary infected dam and endogenous transmission where the dam was persistently infected and the parasite virulence was reactivated by pregnancy. In this transmission case, the incidence of fœtal infection or abortion is positively related to seroprevalence, whereas where only exogenous transmission occurs, the incidence is negatively related to prevalence, due to infections of cattle at the same time, meaning at different gestation periods (Trees and Williams, 2005).

1.6. Diagnosis and treatment of neosporosis

Since the first isolation of *N. caninum*, several serological assays (indirect techniques) based on tachyzoite antigens have been developed for detecting infection in dogs, cattle and a variety of other potential species. The serological diagnosis include indirect fluorescence antibody test (IFAT), enzyme-linked immunosorbent assays (ELISAs), *Neospora*

agglutination test (NAT) and immunblotting (IB) (Dubey and Schares, 2006). The indirect methods measure specific *Neospora* antibody levels in serum samples from live animals where a possible discrimination between recent and chronic infection can be detected by IgG avidity ELISA (Björkman et al., 1999), but none of these assays can detect the parasite infection in aborted foetuses. Moreover, the serological assays are limited by the low specificity depending on the cut-off value for the assay or sensitivity due to cross-reactivity with other parasites. The most widely and highly specific assay used for detection of *N. caninum* DNA infection is polymerase chain reaction (PCR). This direct technique can also detect *Neospora* infection in body fluids such as amniotic and cerebrospinal fluid, blood, milk, and semen (Yao et al., 2009).

There are no safe and economical drugs on the market that would be able to control or to prevent the parasite proliferation and transmission. However, several studies have shown interesting results in experimental assays of chemical compounds against *N. caninum.* The chemotherapeutical potential of ponazuril and toltrazuril was underlined in *Neospora*-infected cattle. The results indicated a basic effectiveness of ponazuril against experimental *N. caninum* infection in calves and toltrazuril treated calves from *Neospora*-seropositive cows mounted a strong humoral immunity, while placebo-treated animals responded weakly to the persistent infection (Kritzner et al., 2002; Haerdi et al., 2006). Artemisinin and depudecin were also found to have an anti-parasitic activity against *Neospora* in vitro (Kim et al., 2002). In other studies, thiazolides derivatives such as nitazoxanide (NTZ) tested in vitro using *N. caninum* infected Human foreskin fibroblasts (HFF) cells showed an inhibition of intracellular tachyzoite proliferation and killed the parasite in a longer treatment by inhibiting the parasitophorous vacuole formation. Both NTZ and its deacetylated form tizoxanide (TIZ) were shown to have a high toxicity on mammalian cells (Esposito et al., 2007a,b; Müller et al., 2008b). Recently, Leepin et al. (2008) demonstrated the high parasiticidal activity of DB750, a diamidine compound, in inhibition of tachyzoite proliferation in vitro even during short treatment periods.

1.7. Vaccine strategy

Considering the economic and agricultural impact of neosporosis, there is an urgent need to develop control measures aimed at preventing its transmission and infection, as well as reducing the severity of the disease without compromising pregnancy (Hasler et al., 2006a, b;

Innes et al., 2002). Investigation at the molecular level of the host cell adhesion/invasion machinery of *N. caninum* should provide potential targets for vaccination. The processes that govern the physical interaction between the parasite and the host cell and representing eventually potential targets for vaccination may be summarized in the following two steps:

- Initial adhesion or the first physical contact with the host cell is mediated by the parasite surface antigens based on a low affinity contact and followed by perpendicular reorientation of parasite to bring its apical tip close to the host cell membrane which causes the secretion of micronemal proteins that interact with the glycoproteins on the host cell membrane leading to a tight attachment zone (Naguleswaran et al., 2002).
- Host cell entry is then achieved by the formation of a moving junction composed of microneme proteins and rhoptry proteins that propels the parasite into the host cell. Some rhoptry proteins injected into the cell target cytoplasm remain associated with small vesicles that fuse with the developing parasitophorous vacuole membrane in which the parasites develop (Martin et al., 2007). The parasitophorous vacuole membrane is formed and the dense granule proteins are secreted when it has completely established. The invasion process is powered by a parasite actin/myosin system (Buxton et al., 2002; Carruthers and Boothroyd, 2007).

The importance of microneme proteins (MICs) for the infection process has been extensively demonstrated in *Toxoplasma gondii* closely related to *N. caninum*. *T. gondii* mutants with genetically ablated TgMIC2 and TgMIC2-associated protein (M2AP) exhibited substantially reduced invasive capacities (Huynh et al., 2003; 2004). Cerede et al. (2005) showed that single deletion of the TgMIC1 gene decreased invasion in fibroblasts in vitro, whereas TgMIC3 deletion had no effect. Individual disruption of TgMIC1 or TgMIC3 genes slightly reduced virulence in mice, whereas doubly depleted parasites were severely impaired in virulence. The importance of microneme proteins as vaccine candidates against *T. gondii* infection was also documented by Ismael et al (2003), showing that intramuscular DNA vaccination of mice with TgMIC3 elicited a strong specific immune response and provided effective protection against *T. gondii* infection. In fact, the *T. gondii* MIC1-3 double knock-out mutant was a powerful vaccine and protected mice against chronic and congenital toxoplasmosis (Ismael et al., 2006). The use of this attenuated strain (*T. gondii* MIC1-3 knock-out) as heterologous vaccine has recently been shown to provide protection against *N. caninum* lethal infection in mice (Penarete-Vargas et al., 2010).

The importance of rhoptries proteins (ROP's) inserted into the vacuole membrane during invasion process has been demonstrated in *T. gondii* as well. Targeted depletion of ROP2 reduced invasive and replicative capacities of the parasite, and attenuation of virulence in mice (Nakaar et al., 2003). Antibodies directed against TgROP2 inhibit invasion of human fibroblasts by *T. gondii* in vitro (Mishima et al., 2002). *T. gondii* rhoptries proteins, injected into the host cell cytoplasm, are involved in crosstalk and manipulation of host cell functions, by co-opting gene expression through polymorphic kinase homologues (Saeij et al., 2007). El Hajj (2007) demonstrated that TgROP18 phosphorylated two parasite proteins, and TgROP18 overexpression in *T. gondii* resulted in increased proliferation. In contrast, TgROP16 is translocated to the host cell nucleus (Gilbert et al., 2007), subverts STAT3/6 signalling, and as a consequence IL12 production in infected host cells. Analysis of type I, II and III virulent *T. gondii* parasites showed that expression of TgROP16 correlated with high virulence strains.

Basically, an efficient vaccine against *N. caninum* infection should fulfill the following requirements:

(i) prevention of tachyzoite proliferation and dissemination in pregnant cattle (or other animals) to avoid transplacental transmission to the foetus;

(ii) prevention or reduction of oocyst shedding in dogs (or other possible final hosts);

(iii) prevention of tissue cyst formation in animals that have been infected with oocysts or tissue cysts (to avoid parasite transmission to carnivorous hosts) (Williams et al., 2007; Reichel et al., 2009).

Probably, this could be achieved by a live parasite immunization able to reflect the natural situation of infection and stimulates both humoral and cell-mediated immunity (Nishikawa et al., 2002). A number of vaccines against protozoa parasites closely related to *N. caninum* are commercially available. For example, a live attenuated *Besnoitia* vaccine for cattle based on a naturally occurring isolate from a wildebeest (Bigalke et al., 1974), a commercial *T. gondii* vaccine (Ovilis Toxovax®, Intervet) for sheep based on a strain of the parasite (S-48) that had lost its ability to form cysts after long-term passage in mice (Buxton, 1993), a live cattle vaccine for *Babesia bigemina* and *Babesia bovis* (Tick fever) has been employed successfully in Australia (Bock et al., 2004) and live attenuated vaccines for poultry coccidiosis (Shirley et al., 2005). However, in addition that these (live) vaccines may induce contamination with other animal pathogens (Bovine Viral Diarrhoea, Enzootic Bovine Leukosis) their production is mostly expensive (as they are usually prepared in cell culture or even in live animals) and present a limited shelf-life. Live vaccination may also present a problem with the timely

delivery of vaccine doses, as storage and distribution of such a vaccine will be dependent on the provision of an efficient cold chain (Reichel et al., 2009). In the case of *N. caninum* disease, the live vaccine may be dangerous for the vaccinated animal if the immune system did not stop the infection or stimulate the cyst formation. To avoid this intentionally induction of chronic infection it would be better to use attenuated tachyzoite strains which are not able to form cysts anymore (Nishikawa et al., 2002; Innes et al., 2006). Ramamoorthy et al. (2006) reported that vaccination with gamma-irradiated *N. caninum* tachyzoites protects mice against acute neosporosis. More recently, Bartley et al. (2008) published the effectiveness of immunisation with live attenuated tachyzoites against a lethal challenge of *N. caninum* in Balb/c mice model. Moreover, Williams et al. (2007) showed that vaccinated cows with live 10^7 *N. caninum*-Nowra tachyzoites prior to artificial insemination and challenged at day 70 post-gestation could prevent completely foetal death, whereas, in the same study a lysate of *N. caninum*-Nowra also failed to protect cattle from abortion. However, there are no reports yet of trialling these attenuated parasites in pregnant cattle. A persistent infection of the vaccinated cows was also rejected as no *N. caninum* DNA was detected in the cerebral tissue and other organs (Williams et al., 2007). Another study has shown a strong prevention of vertical transmission in outbred mice immunized with live tachyzoite and a potential inhibition with parasite lysate (Miller et al., 2005). They demonstrated also that the immune response against lysate prior to pregnancy and challenge was skewed towards IgG1, whereas both IgG1 and IgG2a were increased in response to live tachyzoite. Other isolates are now starting to emerge with biological properties like NC-Nowra. They are attenuated in their ability to cause disease in mice. Nine isolates were made from asymptomatic calves in Spain, and one of them (Nc-Spain-1H) failed to induce clinical signs in a BALB/c mouse, grew slowly in vitro and provided protection against foetal death in a pregnant mouse model (Rojo-Montejo et al., 2009).

A protozoal vaccine based on killed tachyzoites has been licensed for use in the United States (Choromanski et al., 2001). This vaccine (Neoguard™, Intervet) contains 3 x 10^6 inactivated tachyzoites (and Havlogen as the adjuvant) and is applied prior to breeding or early in the first trimester of gestation twice (formulated in a 5 ml dose), 4 weeks apart, with one or two annual booster vaccinations. The induced immunological response appears to be mainly humoral in nature (Andrianarivo et al., 2000). Neoguard™ was tested by Romero et al. (2004) in Costa Rica dairy cows. In comparison to the placebo treated group, the abortion rate in the vaccinated group showed a limited efficacy, with 46% to 50% of protection. However, other studies gave other values of efficacy and the real protective effect of the vaccine is still

unknown (Innes et al., 2006). The failure of a killed tachyzoite lysate to induce protection against infection in cattle could be related to the presence of a multitude of different *Neospora* antigens that exhibit detrimental antigenic properties. More specific targets for vaccination are *N. caninum* surface proteins and/or components of secretory organelles (micronemes, rhoptries and dense granules), which are involved in adhesion/invasion mechanism of the host cell by the parasite (Hemphill et al., 1999). In contrast to live vaccines, vaccines composed of recombinant proteins have distinct advantages, such as ease of manufacture, longer shelf life, ease in handling and application.

The parasite surface antigen NcSRS2 and NcSAG1(see table 1), which are involved in tachyzoite-host cell interactions, were expressed in recombinant vaccinia virus and inoculated to the mice before mating and the prevention of vertical transmission was assessed. Mice vaccinated with NcSRS2 showed an effective protection against transplacental passage and were found to limit parasite dissemination, whereas NcSAG1 conferred only a partial protection (Nishikawa *et al.*, 2001a). Nishikawa et al. (2001b) also demonstrated the importance of IgG1 in the parasiticidal activity during the early stage of infection and of T-cell response against intracellular parasites in the late stage of infection. Reduced materno-fetal transmission was seen upon vaccination with NcGRA7 as well as NcHSP33 as a DNA vaccine (Liddell *et al.*, 2003). However, although DNA vaccines have the advantage to produce continuously the antigen and thus stimulating permanently the immune system, it could happen that anti-DNA antibodies are formed and induce an autoimmune response in the host (Kowalczyk et al., 1999). Further, the immune protective effect of NcGRA7 was enhanced upon use of it as a plasmid DNA together with CpG adjuvant, which improved the protective efficacy against congenital transfer (Jenkins *et al.*, 2004). Cho *et al.* (2005) reported vaccination efficacy in an acute disease model upon combining recombinant NcSRS2 and dense granule antigen NcDG1.

Expression of the *N. caninum* antigens NcMIC1, NcSRS2, NcGRA7 and NcGRA6, respectively, in the *Brucella abortus* vaccine strain RB51 and subsequent immunization of mice resulted in complete protection against experimentally induced acute disease (Ramamoorthy *et al.*, 2007a, 2007b), and NcGRA6 and NcMIC1 expressed in *Brucella abortus* protected against vertical transmission (Ramamoorthy et al., 2007b). Ellis et al. (2008) recently reported on very limited protection against transplacental transmission of *N. caninum* in mice following application of MIC10 and p24B recombinant antigens, but no protection at all using recombinant versions of NGRA1, NcGRA2, MIC10 and p24B alone.

Balb/c mice vaccinated with recombinant NcMIC3 protein were protected against cerebral infection with no *N. caninum* DNA detection (Cannas et al., 2003b). For another microneme protein NcMIC1, it was demonstrated that no significant protection was obtained by vaccination with a NcMIC1-DNA vaccine or with aNcMIC1-DNA combinated with recombinant NcMIC1 protein, but a significant reduction of cerebral parasite load was obtained with recombinant NcMIC1 antigen alone (Alaeddine et al., 2005). Generally, some microneme antigens, when inoculated into an animal, will modulate the immune response in favour of the host, thus confer protection, but others could also induce the opposite effect. This was demonstrated in a study by Srinivasan et al. (2007), where mice were vaccinated with different formulations of a vaccine that was based on NcMIC4.

TgROP2 has been found in all stages of *T. gondii* and it is therefore a potential candidate for vaccination also. The vaccination with recombinant NcROP2 was able to reduce neosporosis clinical symptoms and the cerebral infection rate (Debache et al., 2008). Moreover, recombinant NcROP2 was shown to reduce the vertical transmission in infected mice with a survival rate of the newborns of up to 50% when used as a single vaccine and up to 35% when used in combination with recombinant NCMIC1 and NCMIC3 but without affecting the parasite viability (Debache et al., 2009b).

A number of poly-epitope chimeric antigens incorporating multiple protective epitopes from multiple antigens and life cycle stages have been considered to be more effective than single stage vaccines. However, the desired outcome is not always achieved, and the success and efficiency is highly dependent on the configuration of the chimeric antigenic molecule (Cai et al., 2007; Shi et al., 2007). So far, chimeric vaccines have not been used against *N. caninum* infection either in murine or cattle *Neospora* infection model.

Thus, although a number of studies have provided evidence for limited efficacy, it is likely that an efficient vaccine should be able to keep the parasite in check, with immunity, at the same time, in favour of the host without compromising pregnancy.

Table 1: Selected recombinant vaccine candidates and the corresponding effects in experimental murine models.

Vaccine candidates	**Effects**	**References**
NcSRS2 antigen	protection against transplacental passage	Nishikawa et al., 2001a
NcSAG1 antigen	no protectivity	Cannas et al., 2003b
NcGRA7, HSP33 (DNA vaccine)	reduced materno-fetal transmission	Liddell *et al.*, 2003
NcSRS2 and NcDG1 (combination)	protectivity against acute disease	Cho et al., 2005
MIC1 antigen	no protectivity	Cannas et al., 2003a
MIC3 antigen	75% protectivity	Alaeddine et al., 2005
ROP2 antigen	protectivity against acute disease	Debache et al., 2008
NcSAG4 & NcGRA7 antigens	no protectivity	Aguado-Martìnez et al., 2009

1.8. Aim of the work

Hypothesis:

N.caninum antigens involved in cell adhesion/invasion represent useful targets for the development of a vaccine against *N. caninum*.

Objective:

The main objective of this work was to select such proteinic vaccine targets which are believed to be involved in the host cell adhesion/invasion process. These antigens were expressed as recombinant proteins in *E. coli*, and then their potential protective effect was assessed in vitro using cell culture invasion models based on Vero or HFF cells. Then, promising antigens were studied in murine models. Vaccination studies were done using different adjuvants and different delivery routes.

2. Results

2.1. Vaccination of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice infected with *Neospora caninum* tachyzoites.

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Vaccination of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice infected with *Neospora caninum* tachyzoites

Karim Debache [a,*], Christophe Guionaud [b], Ferial Alaeddine [b], Meike Mevissen [b], Andrew Hemphill [a,*]

[a] *Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland*
[b] *Institute of Veterinary Pharmacology & Toxicology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 124, CH-3012 Berne, Switzerland*

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Abstract

Rhoptry antigens are involved in a variety of cellular functions related to host cell invasion, formation of the parasitophorous vacuole and parasite–host cell interplay. The cDNA sequence of one of these antigens, NcROP2 was identified from *Neospora caninum* expressed sequence tags (ESTs), amplified by reverse transcription-PCR, expressed in *Escherichia coli* as a $(His)_6$-tagged recombinant protein (recNcROP2) and purified over Ni^{2+}-affinity chromatography. Both recNcROP2 and antibodies directed against recNcROP2 had a negative impact on *N. caninum* tachyzoite host cell invasion in vitro, indicating that this protein participates in the host cell entry process. Subsequently, the protective efficacy of NcROP2 as a potential vaccine candidate was evaluated in a C57BL/6 mouse cerebral disease model. Mice were vaccinated three times at 2-week intervals with recNcROP2 emulsified either in Freund's incomplete adjuvants (FIA) or saponin, and control groups were treated with adjuvants alone (adjuvants control) or PBS (infection control). Subsequently, mice were challenged with 2×10^6 *N. caninum* tachyzoites. Nine mice, all belonging to the infection control or adjuvants control groups, exhibited clinical signs of cerebral neosporosis and succumbed to infection, whilst no clinical signs were noted for recNcROP2-vaccinated mice. For all other animals, the experiment was terminated 35 days p.i. Cerebral parasite burdens were assessed by quantitative PCR in all mice, and were revealed to be significantly reduced in the recNcROP2-vaccinated mice. ELISA of sera revealed IgG1 to be elevated in recNcROP2–saponin vaccinated mice, whilst IgG2a was higher in recNcROP2–FIA vaccinated animals. This shows that, depending on the adjuvants used, vaccination with NcROP2 induces a protective Th-1- or Th-2-biased immune response against experimental *N. caninum* infection.
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Keywords: *Neospora caninum*; ROP2; Host cell invasion; Vaccination; Cerebral infection; Neosporosis

1. Introduction

Neospora caninum is an intracellular, apicomplexan parasite that causes abortion in cattle leading to significant financial losses (Dubey et al., 2007). *Neospora caninum* has been found in a wide range of species and tissues, and this parasite infects a wide range of host cells both in vivo and in vitro (Hemphill, 1999; Hemphill et al., 2006). Dogs and coyotes are the only known definitive hosts (McAllister et al., 1998; Gondim et al., 2004), and *N. caninum* also causes neuromuscular disease in dogs (Dubey et al., 1988). There are two major situations in which these parasites perpetrate the infection. Firstly, cattle acquire infection post-natally by exposure to and ingestion of oocysts excreted by the canine definitive host (De Marez et al., 1999; Trees et al., 2002). Second, during pregnancy, parasites cross the placenta and infect the foetus, causing

* Corresponding authors. Tel.: +41 31 631 2384; fax: +41 31 631 2477 (A. Hemphill).
E-mail addresses: karim.debache@ipa.unibe.ch (K. Debache), hemphill@ipa.unibe.ch (A. Hemphill).

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congenital infection and possibly abortion as a result from the altered balance of the immune system by foetus-derived regulatory cytokines (Williams et al., 2006). This mode of transmission accounts for the vast majority of infections, and vertical transmission occurs during repeated pregnancies (Anderson et al., 1997; Björkman et al., 1996) with up to 95% of the calves born to infected dams testing positive (Davison et al., 1999). Considering the economic and agricultural impact of neosporosis, there is an urgent need to develop biological control measures aimed at preventing its transmission and infection, as well as reducing severity of the disease. At the present time, there is no vaccine capable of inducing complete protective immunity in cattle. However, killed tachyzoite lysate prevents foetal infection in mice (Liddell et al., 1999) and a commercialised vaccine, containing tachyzoite lysate (Neoguard™), is currently marketed in the Unites States of America, with ambiguous efficacy data (Williams et al., 2006; Innes and Vermeulen, 2006).

Studies using murine models have led to a better understanding of the complex immune response that dictates *N. caninum* infection, and have contributed largely to the characterisation of novel antigens and innovative strategies that could lead to efficacious vaccination approaches (reviewed in Hemphill et al., 2006; Innes, 2007). Protective immune responses against experimentally induced neosporosis in acute disease mouse models have been shown to be mainly associated with the development of a Th1-type immune response, dominated by IgG2a antibody production, and natural killer (NK) cell proliferation with increased IFNγ production (Klevar et al., 2007). Tests on B-cell deficient (μMT) mice revealed their increased susceptibility to *N. caninum* infection, underlying the importance of the humoral immune response in offering protective immunity against neosporosis in mice (Eperon et al., 1999). However, many studies have shown that both humoral and cell-mediated immune responses are important components of protective immunity against *N. caninum* (reviewed in Hemphill et al., 2006; Innes and Vermeulen, 2006).

A limited number of recombinant proteins have been investigated as vaccine candidates. These include mostly immune-dominant antigens functionally involved in tachzyoite–host cell interactions, such as surface antigens and proteins localised in dense granules and micronemes. The surface antigen NcSRS2 expressed with recombinant vaccinia virus offered adequate protection against transplacental passage and was found to limit parasite dissemination (Nishikawa et al., 2001). On the other hand, Cannas et al. (2003a) reported reduced cerebral parasite load in mice using a combined recombinant protein and DNA vaccination procedure with NcSAG1 and NcSRS2 compared with vaccination with recombinant proteins alone. Reduced clinical signs and limitation of cerebral parasite load were also observed upon vaccination with recombinant microneme proteins NcMIC3 (Cannas et al., 2003b) and NcMIC1 (Alaeddine et al., 2005). In contrast, immunization with native, recombinant and DNA-vaccines based on NcMIC4 lead to higher cerebral parasite load and increased numbers of mice succumbing to infection (Srinivasan et al., 2007). Reduced materno-foetal transmission was seen after vaccination with NcGRA7 as well as NcHSP33 as a DNA vaccine (Liddell et al., 2003). Further, the immune protective effect of NcGRA7 was enhanced upon its use together with CpG adjuvant as a plasmid DNA that improved the protective efficacy against congenital transfer (Jenkins et al., 2004). Cho et al. (2005) reported vaccination efficacy upon combining recombinant NcSRS2 and dense granule antigen NcDG1. Expression of the two *N. caninum* antigens NcMIC1 and NcGRA6 in the *Brucella abortus* vaccine strain RB51 and subsequent immunization of mice resulted in complete protection against experimentally induced acute disease (Ramamoorthy et al., 2007).

Rhoptries are unique secretory organelles shared by all apicomplexan invasive stages. They are exocytosed upon host cell invasion and their contents are involved in creating the moving junction that propels the parasite into the cell (Dubremetz, 2007). In addition, rhoptry components are involved in the generation and modification of the parasitophorous vacuole in which the parasites develop (Martin et al., 2007), and some rhoptry proteins are targeted to the host cell nucleus and act as key virulence factors (Saeij et al., 2007). In *Plasmodium*, there is ample evidence that this class of proteins is appropriate for inclusion in a multivalent vaccine formulation (Kats et al., 2006).

The purpose of this study has been to investigate the involvement of the *N. caninum* protein NcROP2 in the tachyzoite host cell infection process, and to evaluate the protective efficacy of bacterially expressed recombinant $(His)_6$-tagged NcROP2 in the murine cerebral infection model. We found that vaccination of mice with recombinant NcROP2 conferred protection against challenge infection with *N. caninum* tachyzoites, associated with the presence of both anti-NcROP2 IgG1 and IgG2a antibodies depending on the adjuvants used. These findings show that NcROP2 induces protection against cerebral *N. caninum* infection in mice, and thus represents a promising vaccine candidate.

2. Materials and methods

Unless otherwise stated, all cell culture reagents were supplied by Gibco-BRL (Zurich, Switzerland) and chemicals were purchased from Sigma (St. Louis, MO, USA).

2.1. Vero cell and N. caninum tachyzoite culture and parasite purification

Vero cells and human foreskin fibroblasts (HFF) were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U of penicillin/ml and 50 μg of streptomycin/ml at 37 °C/5%

CO_2 in tissue culture flasks. Cultures were passaged at least once per week.

Neospora caninum tachyzoites of the Nc1 strain (Dubey et al., 1988) were maintained by serial passages in Vero cells or HFF during which time FCS was replaced with 5% immunoglobulin G (IgG)-free horse serum (HS). Parasites were harvested as described previously by (Hemphill et al., 1996). Infected cells were trypsinized, washed twice in cold RPMI 1640 medium, and the resulting pellet was resuspended in 2 ml cold RPMI 1640 medium. Cells were repeatedly passaged through a 25 G-needle and liberated tachyzoites were purified by passage through Sephadex-G25 columns (Amersham Biosciences, Otelfingen, Switzerland), previously equilibrated with cold RPMI 1640 medium. The eluted parasites were centrifuged at 600*g*/10 min/4 °C, resupended in cold RPMI 1640 medium, and counted in a Neubauer chamber.

2.2. Cloning of recNcROP2 and preparation of a recombinant N. caninum ROP2 (recNcROP2) antigen

The full-length cDNA sequence of *N. caninum* ROP2 (NcROP2) has recently been obtained (Alaeddine et al., unpublished data). NcROP2-specific primers were designed based on sequence information retrieved via ApiDots (http://www.cbil.upenn.edu/apidots/; DT.92484732 transcript (Li et al., 2003). All primers were purchased from MWG (Ebersberg, Germany). RecNcROP2 was expressed as a $(His)_6$-tagged fusion protein in *Escherichia coli*. A defined fragment of NcROP2, comprised of amino acids 238–594, was amplified by reverse transcription-PCR using the forward primer ROP-BamHI-F (5′-GGATCCTTGT GGCGTAATCAGAAGCAC-3′) and the reverse primer ROP-XhoI-R (5′-CTCGAGTTATAGCCTCGTGTCCTC CGT-3′) (BamHI and XhoI restriction sites underlined). The PCR product was cloned into pCR blunt II TOPO (Invitrogen, Carlsbad, CA), and verified by sequencing (Department of Clinical Research, University of Berne, Switzerland). The BamHI–XhoI insert was subcloned into BamHI- and SalI-digested pQE-30 (Qiagen, Hilden, Germany) using the XL1-Blue strain (Stratagene, La Jolla, CA) as a recipient. The resulting pQE-30-NcROP expression vector was used to transform *E. coli* BL21 (Novagen-EMD Biosciences, Madison, WI) harbouring the pREP4 repressor plasmid (Qiagen). To express the fusion protein, 1 L of 2× YT medium (per liter: 16 g bacto tryptone, 10 g bacto yeast extract and 5 g NaCl) prewarmed to 37 °C and supplemented with carbenicillin and kanamycin (100 and 25 μg/ml, respectively) was inoculated 1:100 with an overnight starter culture grown in the same medium. When the culture reached an OD_{600nm} of 0.5, isopropyl-ββ-D-1-thiogalactopyranoside was added to 1 mM, and expression of the recombinant protein was carried out for 3 h at 37 °C. Bacteria were harvested by centrifugation and disrupted by sonication. Insoluble bacterial components containing recNcROP2 were repeatedly washed with PBS containing 1 M urea and finally dissolved in PBS containing 20 mM DTT and 8 M urea. The solubilised recombinant protein was then purified by Ni^{2+}-chelate chromatography under denaturing conditions on a 1 ml HisTrap FF column (GE Healthcare, Piscataway, NJ), as recommended by the manufacturer. The eluate was precipitated as previously described (Wessel and Flügge, 1984) and the recNcROP2 was resuspended in PBS and sonicated to increase solubility.

2.3. Preparation of polyclonal anti-recNcROP2 antiserum

Antisera were obtained after immunization of two female white New Zealand rabbits, with 150 μg of recNcROP2 antigen in Gerbu adjuvant (Gerbu Biotechnik GmbH, Gaiberg, Germany) per injection, using a standard 10-week immunization protocol (Institut für Labortierkunde, Zurich, Switzerland). Antibodies were affinity-purified using recNcROP2 bound to nitrocellulose membranes, as previously described (Robinson et al., 1988).

2.4. Studies on effects of recNcROP2 and anti-recNcROP2 antibodies on cellular tachyzoite invasion in vitro

Invasion of HFF by *N. caninum* was investigated by employing HFF monolayers grown in six well flat bottomed tissue culture plates. All assays were carried out in quadruplicate and each experiment was repeated twice with virtually identical results. Freshly purified tachyzoites (1.2×10^6) were resupended in 200 μl RPMI 1640 medium and were incubated for 30 min at room temperature in the presence of one of the following additives: (i) rabbit pre-immune serum (1:100), (ii) anti-recNcROP2 (1:100), (iii) anti-*Giardia lamblia*-PDI4 (Müller et al., 2007; 1:100), (iv) anti-*N. caninum* hyperimmune serum (Hemphill et al., 1996; 1:100), (v) 10 μg purified His-tagged recNcROP2, (vi) 10 μg purified His-tagged recombinant *Giardia* nucleohydrolase (rec-NH (kindly provided by Dr. Joachim Müller; Sterk et al., 2007) and (vii) RPMI 1640 alone. Subsequently, each pre-treated parasite suspension was added to one well to invade for 30 min at 37 °C/5% CO_2. Infected monolayers were washed three times with cold RPMI 1640 and incubated with RPMI 1640 medium containing 100 μM pyrrolidine dithiocarbamate (PDTC), 0.2 μM $CuSO_2$ and anti-*N. caninum* hyperimmune serum (1:200) for 2 h at 37 °C/5% CO_2 to kill and permeabilise the adherent extracellular tachyzoites (Naguleswaran et al., 2003). Wells were then washed three times with cold RPMI 1640 and incubated for 1 h at 37 °C/5% CO_2 with RPMI 1640 containing 1 mg/ml DNaseI. All wells were washed twice with cold RPMI 1640, and a third time with RPMI 1640 containing 1 mM EDTA. Finally, monolayers were trypsinized, and the DNA was purified using an AllPrep DNA/RNA Mini Kit (Quiagen). Numbers of invaded parasites were determined by quantitative PCR according to Müller et al. (2002). The extracted DNA samples were analysed by UV spectrophotometry and then adjusted to 20 ng DNA/μl with DNAse-free water. The parasite count for a given sample was assessed by using

DNA standards extracted from *N. caninum* tachyzoites (Naguleswaran et al., 2003).

2.5. Studies on recNcROP2 vaccination in C57BL/6 mice and experimental challenge of mice with N. caninum tachyzoites

Two independent experiments, one after the other, were carried out under the same conditions, with virtually identical outcomes. 10-week-old female C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained under conventional day/night-cycle housing conditions as required by the animal welfare legislation of the Swiss Veterinary Office. Mice were divided into five groups of nine or 10 animals each (Table 1). They were used for experimentation on reaching 10 weeks of age, having been checked serologically for the absence of anti-*N. caninum* immunoglobulins (pre-immune sera) according to Eperon et al. (1999). Prior to infection with *N. caninum* tachyzoites mouse groups were treated by i.p. injection as described below:

Group 1 was treated with 100 µl PBS (positive control). Group 2 was treated with 100 µl saponin adjuvant (SAP) at 100 µg/ml. Group 3 was immunised with 100 µl recNcROP2 (100 µg/ml) in SAP. Group 4 was injected with 100 µl Freund's incomplete adjuvant (FIA), and group 5 was immunised with 100 µl recNcROP2 (100 µg/ml) emulsified in FIA. Injections were carried out on days 1, 15 and 28.

On day 53, animals were challenged by i.p. inoculation of a suspension (200 µl) containing 1×10^6 freshly purified *N. caninum* tachyzoites. On day 83, the experiment was terminated, and mice were sacrificed by CO_2-euthanasia. Animals exhibiting clinical signs of neosporosis (ruffled coat, apathy, hind limb paralysis) were euthanized at the onset of these clinical signs. Blood was drawn by cardiac puncture for serum antibody and brains were collected using individual sterile instruments and stored at −20 °C for subsequent quantitative PCR analysis.

2.6. Serology

To evaluate the humoral immune response, individual sera (pre-immune sera, post-immune sera prior to infection (BI), and post-challenge sera (PI)) were analysed for *N. caninum* and rec NcROP2 specific immunoglobulin G (IgG), IgG1, and IgG2a by ELISA. Somatic antigen extract from *N. caninum* tachyzoites (Nc-extract) was obtained by subjecting tachyzoites to three freeze–thaw cycles and sonication (Alaeddine et al., 2005). Cellular debris were removed by centrifugation and the protein concentration in the supernatant was measured employing the Bio-Rad protein assay using acetylated bBSA as a standard.

Table 1
Clinical signs of neosporosis in mice following challenge with 1×10^6 *N. caninum* tachyzoites

Mouse groups (*n*)	No. of symptomatic mice	Time of death (day p.i.)
PBS ($n = 9$)	4	6/7/20/20
SAP ($n = 9$)	2	8/9
recNcROP2–SAP ($n = 10$)	0	–
FIA ($n = 9$)	3	7/8/20
recNcROP2–FIA ($n = 10$)	0	–

PBS = infection control (positive control); SAP and FIA = saponin and Freund's adjuvant controls, respectively; recNcROP2–SAP = vaccinated with recNcROP2 emulsified in saponin; recNcROP2–FIA = vaccinated with NcROP2 emulsified in Freund's incomplete adjuvant.

For ELISA, 96 wells plates (Maxisorp, Nunc, Wiesbaden, Germany) were coated overnight at 4 °C with 100 µl of either Nc-extract (0.7 µg/ml) or purified recNcROP2 (0.2 µg/ml), diluted in 0.1 M $NaHCO_3$–Na_2CO_3, pH 9. To block non-specific binding of antibodies, the plates were incubated 2 h with 200 µl of PBS containing 3% BSA and 0.2% Tween 20. Serum samples were diluted 1:50 in PBS containing 0.3% BSA/0.2% Tween 20 (BSAT) and incubated for 2 h at room temperature. After washing three times with BSAT, anti-mouse IgG alkaline phosphatase-conjugated (Promega, Madison, WI, USA), and goat anti-mouse alkaline phosphatase-conjugated IgG1 and IgG2a, respectively (SouthernBiotech, Birmingham, USA) were added at dilution of 1:1000 in BSAT and incubated for 2 h at room temperature. Following three washes in BSAT, the wells were incubated with 100 µl of 1 mg/ml *p*-nitrophenyl-phosphate-disodium in 10% diethanolamine containing 0.5 mM $MgCl_2$, pH 9.8. After allowing the colour to develop for 30 min at room temperature, the absorbance values (405 nm) were read in a tunable microplate reader (Dynatech, Embrach, Switzerland).

2.7. Quantitative real-time PCR

Cerebral parasite burden in mice was determined by *Neospora*-specific quantitative real-time PCR as previously described (Cannas et al., 2003a,b; Alaeddine et al., 2005; Srinivasan et al., 2007). Brain DNA extraction was performed using the DNeasy® Blood & Tissue Kit (Quiagen) as previously described by Müller et al. (2002). The DNA concentrations in all brain samples were determined by UV spectrophotometry, were adjusted to 100 ng/µl with sterile DNAse free water, and the assessments of *N. caninum* tachyzoite loads were performed using a LightCycler™ Instrument (Roche Diagnostic, Basel, Switzerland). The parasite counts were calculated by interpolation from a standard curve with DNA equivalents from 1000, 100 and 10 parasites included in each run.

2.8. Statistical analysis

In vitro invasion assays were statistically evaluated by Student's *t*-test using the Microsoft Excel program and a value of $P < 0.05$ was considered statistically significant. For serological assays, the differences amongst end-point values of the controls (PBS/adjuvants) and experimental data were also analysed by Student's *t*-test. Cerebral para-

site loads in different treatment and control groups were analysed by Kruskal–Wallis multiple-comparison Z-value tests ($P < 0.05$).

3. Results

3.1. Expression and purification of recombinant NcROP2 (recNcROP2)

Recombinant NcROP2 (recNcROP2) was expressed in *E. coli* and purified by Ni^{2+}-affinity chromatography, yielding a single protein band that, by SDS–PAGE, migrated at approximately 43 kDa (Fig. 1). Immunization of rabbits with the recombinant protein resulted in the production of a polyclonal antiserum, which by immunoblotting was shown to react with bacterially expressed recNcROP2 and with a protein of approximately 54 kDa in *N. caninum* extracts (Fig. 1, lane 1). The *Mr* of this band corresponded to the calculated *Mr* of NcROP2, and to the M_r reported for ROP2 from the closely related *Toxoplasma gondii* (Herion et al., 1993; Saavedra et al., 1996).

3.2. Anti-NcROP2 antiserum and purified recNcROP2 both inhibit N. caninum host cell invasion in vitro

Incubation of *N. caninum* tachyzoites with polyclonal anti-NcROP2 antiserum and subsequent assessment of the numbers of parasite invading HFF monolayers showed that the addition of anti-NcROP2 antibodies caused a significant ($P < 0.05$) inhibition of tachyzoite host cell invasion, compared with control incubations carried out with the corresponding rabbit pre-immune serum and a polyclonal antiserum directed against an unrelated His-tagged protein, respectively, (Fig. 2A). Inhibition of host cell entry by a polyclonal anti-*N. caninum* antiserum served as a positive control.

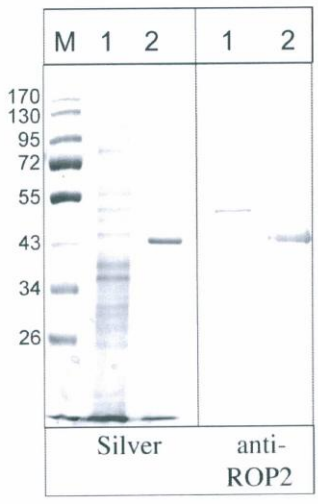

Fig. 1. NcROP2 antigen and reactivity of anti-NcROP2 antibodies. Silver stained SDS–PAGE and corresponding immunoblot labelled with anti-recNcROP2 antibodies of *N. caninum* extract (lane 1) and purified recNcROP2 (lane 2). Note that anti-ROP2 antibodies react with the 43 kDa recNcROP2, and also specifically label a 54 kDa protein in *N. caninum* extract. *M* = molecular weight markers.

The addition of purified recNcROP2 to *N. caninum* tachyzoites during invasion of HFF cell monolayers had a similar effect, in that the host cell entry process was significantly ($P < 0.05$) inhibited (Fig. 2B). Addition of another unrelated His-tagged recombinant protein (*G. lamblia* nucleoside hydrolase) also reduced the number of invaded tachyzoites, although to a much lesser degree. Crude tachyzoite extract was added to invading tachyzoites as a positive control. These results indicated that antibodies against NcROP2, as well as recNcROP2 itself, interfered in processes associated with invasion of host cells in vitro. These findings provided the rationale for subsequent vaccination studies.

3.3. Clinical signs caused by parasite challenge following immunization of mice with recNcROP2 emulsified in saponin (SAP) or Freund's incomplete adjuvants (FIA)

This experiment was carried out twice with virtually identical outcomes. The results of one trial are shown. Following challenge infection of vaccinated and unvaccinated mice, clinical signs (hind limb paralysis/scruffy coat/inability to reach up for food and water) were observed from day 6 p.i. onwards. Overall nine mice had to be sacrificed prior to termination of the experiment, including four of nine mice in the PBS-treated infection control group, two of nine mice in the SAP control group, and three of nine mice in the FIA control group (Table 1). No clinical signs could be detected in those mice vaccinated with recNcROP2 emulsified in SAP or FIA.

3.4. Cerebral parasite burden in experimental groups

Previous studies had demonstrated that experimental infection of C57BL/6 mice with *N. caninum* tachyzoites resulted predominantly in CNS infection (Cannas et al., 2003a,b; Alaeddine et al., 2005; Srinivasan et al., 2007). We compared the cerebral parasite load in these animals by quantitative PCR (Fig. 3). Vaccination with recNcROP2 emulsified in SAP or FIA resulted in a significant ($P < 0.05$) reduction in the numbers of tachyzoites compared with the mice treated with adjuvant alone, with up to 93% reduction for vaccination with recNcROP2 emulsified in SAP and 75% reduction for vaccination with recNcROP2 emulsified in FIA. Thus, the protective efficacy was slightly higher for recNcROP2 in SAP.

3.5. Humoral immune responses to vaccination and infection

The sera of all mice were analysed for the presence of total IgG and for IgG1 and IgG2a isotypes, directed

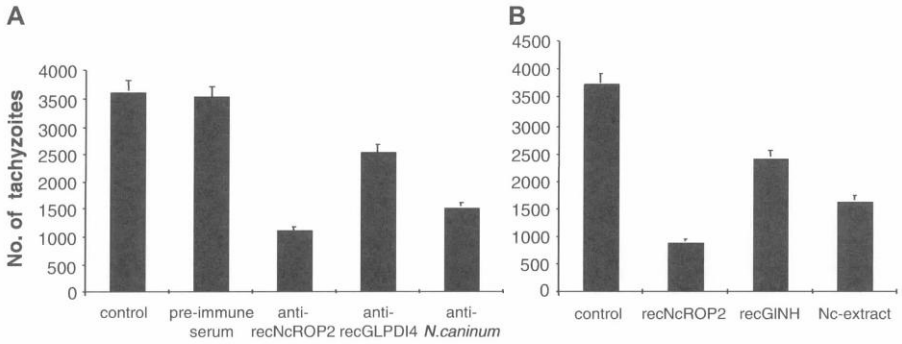

Fig. 2. Treatment of invading *N. caninum* tachyzoites with anti-recNcROP2 antibodies or recNcROP2 leads to strongly decreased tachyzoite invasion of host cells. The parasite numbers per 20 ng of DNA were obtained using real-time PCR. (A) Addition of anti-recNcROP2 antibodies resulted in an invasion reduction of 70% compared with the pre-immune serum control (PI), and 57% compared with a control with added polyclonal anti-GlPDI4 antiserum as an irrelevant control antibody. Addition of polyclonal anti-*N. caninum* antiserum resulted in a 50% invasion inhibition. (B) Addition of recNcROP2 resulted in a 75% reduction in invaded tachyzoites compared with an untreated control, and a reduction of 63% compared with added recGLNH. Addition of crude *N. caninum* (Nc) extract resulted in an inhibiton of tachyzoite invasion by 50%. Error bars indicate SD.

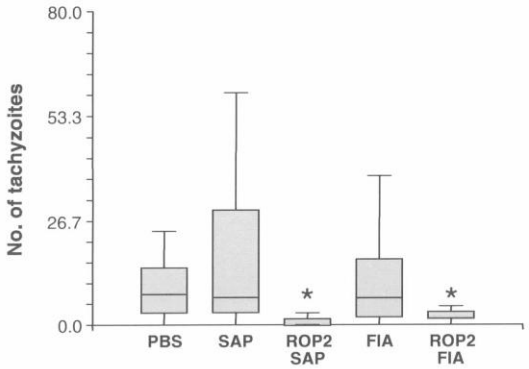

Fig. 3. Box plot of cerebral parasite burden in all immunized animals assessed by real-time PCR. The numbers on the *y*-axis correspond to the parasite number/100 ng of DNA. Note, marked by ⋆, the significantly reduced cerebral parasite burden in those mice receiving recNcROP2 vaccination. See Fig. 4 for abbreviations.

against native NcROP2 (in Nc-crude extract; Fig. 4A) and purified recNcROP2 (Fig. 4B) by ELISA. Pre-immune sera of all mice were negative for Nc-extract and recNcROP2 (data not shown). Sera were analysed post-immunization (BI) and following infection (PI).

As shown in Fig. 4A, the specific IgG, IgG1 and IgG2a antibody levels directed against Nc1 crude extract in BI-sera of the infection control and adjuvants controls were similarly low, whilst vaccination with recNcROP2 with both FIA and SAP resulted in increased total IgG. Following *Neospora* challenge infection, mice vaccinated with recNcROP2–SAP elicited an IgG response with a more pronounced increase of IgG1 levels after challenge compared with the increase of IgG2a ($P < 0.01$). In contrast, in mice vaccinated with recNcROP2 emulsified in FIA and challenged with *N. caninum* tachyzoites, the IgG2a increase was the more pronounced ($P < 0.01$; Fig. 4A).

Fig. 4B shows that the specific IgG, IgG1 and IgG2a antibody levels directed against purified recNcROP2 in BI sera of the infection control and adjuvants controls were similarly low, whilst vaccination with recNcROP2 with both FIA and SAP resulted in increased IgG1 antibody titres. Following challenge, total IgG levels increased in all groups, with a more dominant increase of IgG2a isotype levels following challenge ($P < 0.01$) in mice vaccinated with recNcROP2–FIA. Surprisingly, IgG1 and IgG2a antibody levels decreased following infection in the recNcROP2–SAP vaccinated mice, as well as the IgG1 levels in the recNcROP2–FIA-group (Fig. 4B).

4. Discussion

In analogy to other apicomplexan parasites including *Toxoplasma* and *Plasmodium*, *N. caninum* rhoptry proteins are most likely to play a crucial role in the host cell invasion process, modification of the parasitophorous vacuole membrane, host–parasite communication, and maintenance of the intracellular niche in which these parasites reside (Dubremetz, 2007; Martin et al., 2007; Saeij et al., 2007). Whilst a number of bacterially expressed surface antigens, dense granule and microneme proteins have been evaluated as potential vaccine candidates (reviewed in Hemphill et al., 2006; Innes and Vermeulen, 2006; Innes et al., 2007), to our knowledge no studies have yet been conducted on the potential protective efficacy of recombinant *N. caninum* rhoptry proteins. NcROP2 is a rhoptry-associated protein that is homologous to the members of the ROP2 family of *T. gondii*. The ROP2 family is a dominant group of rhoptry proteins comprised of protein kinase homologues, of which only some possess all critical residues needed for kinase activity (El Hajj et al., 2006).

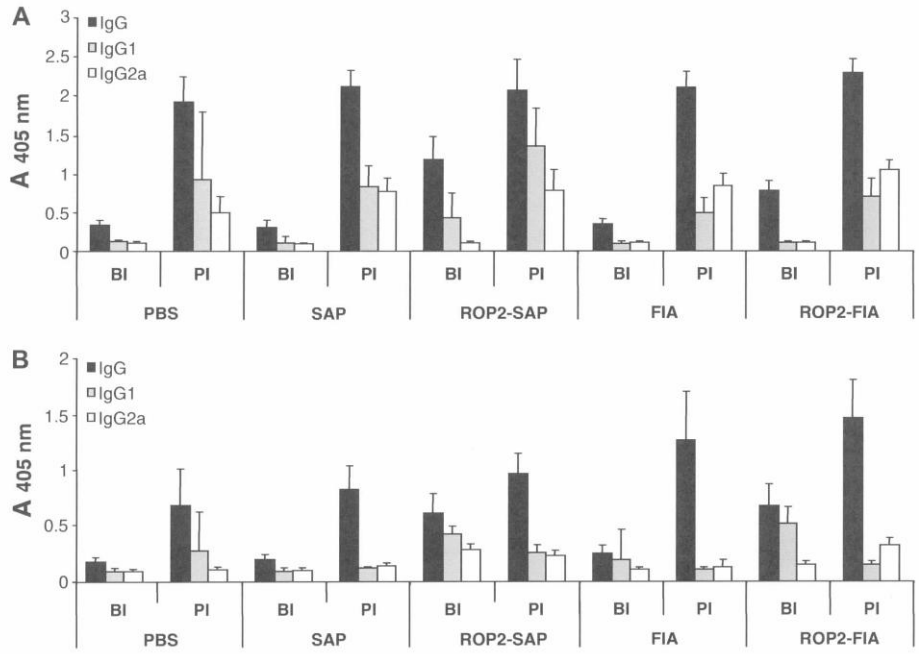

Fig. 4. Serological analysis (total IgG, IgG1, IgG2a) of all mice in all experimental groups. Serum samples were taken either following vaccination but prior to infection (BI) or following infection prior to euthanasia (PI). In (A), ELISA wells are coated with *N. caninum* (Nc) antigen, in (B) wells are coated with purified recNcROP2. PBS = infection control treated with PBS only, SAP = saponin control, ROP2–SAP = vaccinated with recNcROP2 in SAP, FIA = vaccinated with Freund's incomplete adjuvant, ROP2–FIA = vaccinated with recNcROP2 in FIA. Values are indicated as A405 nm ± SD.

TgROP2 and other rhoptry proteins share antigenic determinants, and mice receiving DNA vaccination with a plasmid encoding TgROP2 showed partial protection against a lethal challenge with *T. gondii* cysts, with significantly lower numbers of brain cysts compared with controls (Vercammen and D'Hooghe, 2000; Leyva et al., 2001).

Bacterially expressed recNcROP2 was purified and we showed that this protein, as well as a corresponding antiserum directed against recNcROP2, significantly impaired host cell invasion by *N. caninum* tachyzoites in vitro. How this impairment is achieved is presently unclear. However, it is possible that anti-NcROP2 antibodies associate with secreted NcROP2 at the onset of host cell invasion and thus exert a functional inhibitory effect, whilst recNcROP2 is likely to interact with potential host cell receptors and could interfere with the establishment of necessary physical contact with the host cell. However, such NcROP2 binding host cell receptors have not been identified so far, and further work is needed to study the exact mechanisms of host cell invasion and the involvement of NcROP2 in these processes.

Nevertheless, our results suggested that NcROP2 could be regarded as a potential vaccine candidate. Similarly, Haldorson et al. (2006) recently demonstrated that both polyclonal and monoclonal antibodies directed against affinity-purified native NcSRS2 significantly inhibited the invasion of Vero cells and bovine trophoblasts by *N. caninum* tachyzoites in vitro. Thus, we hypothesised that any interference in parasite–host interaction may be exploited to control the spread of the parasite within the host. Therefore, we comparatively assessed the protective efficacy of recNcROP2 emulsified in two different adjuvants, SAP and FIA.

The cerebral infection model for *N. caninum* has been widely used for primary assessments of vaccine candidates against neosporosis (reviewed in Hemphill et al., 2006). Several lines of evidence suggest that recNcROP2 should be investigated further. Firstly, animals exhibiting symptomatic infection were only found in the infection control and adjuvants control groups. Second, assessment of cerebral parasite burdens by quantitative PCR showed that cerebral infection was clearly reduced in recNcROP2-vaccinated mice, with protective effects achieved with both SAP and FIA adjuvants. The high protectivity against cerebral infection in the recNcROP2–SAP-vaccinated group was associated with the presence of total IgG and IgG1 antibody titres prior to infection, and an increase of IgG1 levels following infection that was higher than the increase in IgG2a levels. In the recNcROP2–FIA vaccinated group, total IgG levels were not elevated upon vaccination, but following infection these mice exhibited significant total IgG levels, and a higher increase in IgG2a- than IgG1-titer.

Thus, recNcROP2-mediated protection against experimental *N. caninum* infection and cerebral disease in mice is associated with both IgG1 and IgG2a dominated immune responses, and indicated that both Th1- and Th2-like pathways could confer protection.

Earlier studies carried out by Long et al. (1998) using Balb/c, C57Bl/6 and B10.D2 mice have shown that mouse strains more resistant to cerebral disease produce higher levels of IgG2a and thus are protected through a Th1-type immune response, whilst the immune response in more susceptible mice was associated with high IgG1 antibodies. On the other hand, as in our study, Baszler et al. (2000) showed that antigen lysates mixed with Freund's incomplete adjuvant induced Th2-type responses and exacerbated the disease in Balb/c mice. Balb/c mice have an inherent Th2-bias, in contrast to C57Bl/6 mice, which will preferentially mount a Th1-biased immune response (Charles et al., 1999). Thus the induction of high levels of IgG1 upon recNcROP2 vaccination and cerebral *N. caninum* infection is rather surprising, and in this case clearly successful in terms of preventing disease. A recent study investigating the *N. caninum* microneme protein NcMIC4 also reported on a IgG1-dominated humoral response against recombinant NcMIC4, but the effects were not protective (Srinivasan et al., 2007). However, investigations on another recombinant microneme protein, recNcMIC3 (Cannas et al., 2003b), have shown that immunization of mice with this antigen induced significant levels of protection against cerebral infection, which were also associated with an IgG1 antibody response against crude *N. caninum* extract. This would most likely be more compatible with a successful outcome of pregnancy (Innes and Vermeulen, 2006).

For protection against foetal infection and abortion, a Th2-type immune response capable of inhibiting parasite proliferation and dissemination during pregnancy would be preferable. In this respect, Haldorson et al. (2005) had shown that vaccination of mice with native, purified NcSRS2 resulted in mice producing antigen-specific antibody, primarily of the IgG 1 subtype. Following challenge during gestation with 10^7 tachyzoites, immunized mice had a statistically significant decreased frequency of congenital transmission compared with non-immunized mice or mice inoculated with adjuvant alone. Decreased congenital transmission amongst immunized mice correlated with a predominantly Th2 immune response compared with non-immunized mice as indicated by an increased ratio of IL-4 to IFN-γ secretion from antigen-stimulated splenocytes (Haldorson et al., 2005). Thus, further studies will focus on the effects of recNcROP2-based vaccination procedures for the prevention of foetal *N. caninum* infection and *N. caninum*-induced abortion.

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Vaccination with recombinant NcROP2 combined with recombinant NcMIC1 and NcMIC3 reduces cerebral infection and vertical transmission in mice experimentally infected with *Neospora caninum* tachyzoites

Karim Debache [a,*], Ferial Alaeddine [b], Christophe Guionaud [b], Thierry Monney [a], Joachim Müller [a], Maria Strohbusch [a], Stephen L. Leib [c], Denis Grandgirard [c], Andrew Hemphill [a,*]

[a] Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland
[b] Institute of Veterinary Pharmacology & Toxicology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 124, CH-3012 Berne, Switzerland
[c] Institute for Infectious Diseases, University of Berne, Friedbühlstrasse 51, CH-3010 Berne, Switzerland

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ABSTRACT

We investigated the protective potential of recombinant his-tagged antigens recNcMIC1, recNcMIC3 and recNcROP2, applied either as single vaccines or as vaccine combinations, in BALB/c mouse models for cerebral and fetal infection. Subsequently, mice were mated and challenged by i.p. inoculation of 2×10^6 *Neospora caninum* tachyzoites at day 7 of pregnancy. The mortality and morbidity of adult mice (non-pregnant and dams) and of the newborn pups was studied for a period of 40 days following birth. Vaccination of non-pregnant mice with recNcROP2 or combinations of recNcROP2 with recNcMIC antigens significantly reduced the numbers of mice suffering from clinical signs, and morbidity was completely prevented with the combination of all three antigens. Of the dams, the groups receiving either recNcROP2 alone or the combination of all three antigens did not exhibit any morbidity, the groups receiving ROP2 mixed with either MIC1 or MIC3 exhibited reduced numbers of deaths, and in the infection control group and the adjuvant group 50% and 43% of mice, respectively, succumbed to disease. For pups, the highest survival rates were noted for the groups receiving recNcROP2 (50%) and recNcROP2/NcMIC1/NcMIC3 (35%), while in the infection- and adjuvant- control groups all pups died, the latest at days 25 and 30, respectively. Quantification of parasite DNA by *N. caninum*-specific real-time PCR revealed consistently lower parasite burdens in brain tissue of pups from vaccinated groups compared with the controls. However, dense granule antigen 2 (GRA2) real-time reverse transcriptase-PCR on brain tissue of surviving pups (applied here to detect viable parasites) demonstrated that only the pups from the group vaccinated with all three antigens in combination appeared free of viable tachyzoites, while in all other groups viable parasites were still present. Serological analysis of humoral (total IgG, IgG1 and IgG2a) and serum cytokine (IL-4 and IFN-γ) responses showed that this effect was associated with a Th-2-biased immune response, with a clearly elevated IL-4/IFN-γ ratio in the mice receiving all three antigens in combination. In conclusion, a mixture of recombinant antigens representing important secretory micronemal and rhoptry proteins leads to a significant protection against vertical transmission of *N. caninum* in mice.

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1. Introduction

Neospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) was first reported as an unidentified protozoan in dogs with encephalomyelitis and myositis (Bjerkas et al., 1984). The parasite was described and named by Dubey et al. (1988), and was later also reported in various species of livestock including cattle, sheep, goats, horses and deer (reviewed in Dubey and Lindsay, 1996; Hemphill, 1999; Dubey et al., 2007). In the USA, the European Union (EU) and in Switzerland, neosporosis is reported as the leading cause of abortions in cattle (Hasler et al., 2006a,b; Hemphill and Gottstein, 2000; Dubey et al., 2007), thus the disease represents an important veterinary health problem and is of high economic significance, as it is also associated with reduced milk yield (Hernandez et al., 2002), premature culling (Thurmond and Hietala, 1996) and reduced post-weaning weight gain in beef calves (Reichel, 2000; Pfeiffer et al., 2002; Hasler et al., 2006a,b).

Dogs and coyotes have been identified as definitive hosts for *N. caninum* (McAllister et al., 1998; Lindsay and Dubey, 1999; Gondim et al., 2004, 2005). One possible route of transmission in

* Corresponding authors. Tel.: +41 31 6312384; fax: +41 31 6312477.
E-mail addresses: karim.debache@ipa.unibe.ch (K. Debache), hemphill@ipa.unibe.ch (A. Hemphill).

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cattle is through the oral uptake of sporozoite-containing oocysts. This will not cause clinical disease in an immuno-competent host, but liberated sporozoites are believed to cross the intestinal epithelium, reach blood and lymphatic vessels, and infect other cells including macrophages and lymphocytes. Following or during dissemination, sporozoites transform to the rapidly proliferating tachyzoite stage and immunological and physiological factors will trigger stage conversion to the slowly proliferating and rather quiescent bradyzoites, which form intracellular tissue cysts almost exclusively within the CNS and possibly in muscle tissue (Peters et al., 2001). Bradyzoites can survive within a latently infected but immuno-competent animal for many years without causing any clinical signs. However, in special situations such as pregnancy, bradyzoites get reactivated and the loss of an efficient Th-1 response in a pregnant dam can lead to limited suppression of the cell-mediated immunity which normally keeps tachyzoite proliferation in check (Innes et al., 2002; Innes, 2007), most likely contributing to the reactivation of bradyzoites and transplacental infection of the foetus. In utero transmission of *N. caninum* is highly efficient, rendering neosporosis an important veterinary medical problem (Dubey et al., 2007).

Thus, the economic importance of neosporosis, especially in cattle, has lead to research on the development of strategies for prevention of *N. caninum* infection. Vaccination and chemotherapy have been identified as economically promising options (Hasler et al., 2006a,b). *Neospora caninum* can only survive, proliferate and proceed during most stages of its life cycle as an intracellular parasite, thus the processes which govern host cell invasion and intracellular development are of crucial importance, and they represent potential targets for vaccination and/or chemotherapeutic intervention (Hemphill et al., 2006a,b). Many of these potential targets are located either on the parasite surface or within secretory organelles such as micronemes, rhoptries and dense granules. A limited number of recombinant proteins have been investigated as vaccine candidates against neosporosis. These include mostly immune-dominant proteins such as the major surface antigens and proteins localised in secretory organelles. For instance, the surface antigen NcSRS2 expressed in recombinant vaccinia virus offered adequate protection against transplacental passage and was found to limit parasite dissemination (Nishikawa et al., 2001a,b). Immunization of mice against neosporosis with different recombinant NcSRS2 iscom formulations was reported to induce specific antibody responses to native NcSRS2 and a significant reduction of cerebral parasite load in immunized mice was observed (Pinitkiatisakul et al., 2005, 2007). In fact, NcSRS2 in its native form has been shown to protect against *N. caninum* congenital transmission in mice (Haldorson et al., 2005). Reduced materno-fetal transmission was seen upon vaccination with the dense granule antigen NcGRA7 as well as with heat-shock protein 33 (NcHSP33) as a DNA vaccine (Liddell et al., 2003). Further, the immune protective effect of NcGRA7 was enhanced when used as a DNA vaccine together with the synthetic oligodeoxynucleotide CpG adjuvant, which improved the protective efficacy against congenital transfer (Jenkins et al., 2004). Cho et al. (2005) reported vaccination efficacy in an acute disease model upon combining recombinant NcSRS2 and dense granule antigen NcDG1. Expression of the *N. caninum* antigens NcMIC1, NcSRS2, NcGRA7 and NcGRA6, respectively, in the *Brucella abortus* vaccine strain RB51 and subsequent immunization of mice resulted in complete protection against experimentally induced acute disease (Ramamoorthy et al., 2007a), and NcGRA6 and NcMIC1 expressed in *Brucella abortus* protected against vertical transmission (Ramamoorthy et al., 2007b). Ellis et al. (2008) recently reported on limited protection against transplacental transmission of *N. caninum* in mice following application of MIC10 and p24B recombinant antigens.

Two recombinant microneme proteins have been demonstrated to confer partial protection against *N. caninum* challenge infection in C57BL/6 mice, namely his-tagged recNcMIC3 (Cannas et al., 2003b) and GST-tagged recNcMIC1 (Alaeddine et al., 2005). Recently Debache et al. (2008) showed that vaccination with a recombinant his-tagged rhoptry antigen, recNcROP2, induced a protective immune response against challenge infection in the C57BL/6 cerebral infection mouse model, leading to significantly reduced parasite burden and abrogation of disease in the vaccinated mice. In this study, we report on the protective potential of bacterially expressed recNcROP2, and combinations of recNcROP2 with recNcMIC1 and recNcMIC3, in a BALB/c mouse model, by assessing cerebral infection and fetal infection in non-pregnant and pregnant mice, respectively.

2. Materials and methods

Unless otherwise stated, all cell culture reagents were supplied by Gibco-BRL (Zurich, Switzerland) and chemicals were purchased from Sigma (St. Louis, MO, USA).

2.1. Cell culture

Vero cells and human foreskin fibroblasts (HFF) were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U of penicillin/ml and 50 μg of streptomycin/ml at 37 °C/5% CO_2 in tissue culture flasks. Cultures were passaged at least once per week.

Neospora caninum tachyzoites of the Nc1 strain (Dubey et al., 1988) were maintained by serial passages in Vero cells or HFF during which time FCS was replaced with 5% IgG-free horse serum (HS). Parasites were harvested as described previously by Hemphill et al. (1996). Infected cells were trypsinized, washed twice in cold RPMI 1640 medium and the resulting pellet was resuspended in 2 ml cold RPMI 1640 medium. Cells were repeatedly passaged through a 25 G-needle and liberated tachyzoites were purified on Sephadex G25 columns (PD-10; Amersham Biosciences, Otelfingen, Switzerland), previously equilibrated with cold RPMI 1640 medium. The eluted parasites were centrifuged at 600*g* for 10 min at 4 °C, resuspended in cold RPMI 1640 medium and counted in a Neubauer chamber.

2.2. Preparation and purification of recombinant *N. caninum* antigens recNcROP2, recNcMIC1 and recNcMIC3

The gene-specific forward and reverse primers (see Table 1) were designed according to the published cDNA sequences from

Table 1
Primers used in this study to amplify the cDNA coding for the rhoptry antigen2 (NcROP2), and the microneme antigens, NcMIC1 and NcMIC3, from *Neospora caninum* cDNA.

Primer name	Primer sequence 5′–3′	Annealing temperature (°C)	Product size (bp)
ROP2 forward	CACCTTGTGGCGTAATC AGAAGCAC	58	1600
ROP2 reverse	TATAGCCTCGTGTC CTCCGT	58	
MIC1 forward	CACCGAAGCGTCGCAAC CATCGG	65	1314
MIC1 reverse	TTACAATTCAGATTCACC CGGAGA	65	
MIC3 forward	CACCGTTCAGAAATCG AGTCATC	60	1081
MIC3 reverse	TTATCGAGCCGTTCCGCA	60	

NcMIC3 (Sonda et al., 2000), NcMIC1 (Keller et al., 2002) and NcROP2 (Debache et al., 2008). Primers were purchased from MWG (Ebersberg, Germany) and corresponding fragments were obtained by PCR using *N. caninum* cDNA. The PCR products were cloned into a pET151 vector (Champion™ Expression Kit, Invitrogen) and verified by a commercial sequencing service (Microsynth AG, Balgach, Switzerland). The resulting plasmids were used to transform *Escherichia coli* strain BL21(DE3) (Invitrogen) and bacteria were grown overnight at 37 °C in LB (10 g NaCl, 5 g yeast extract, 10 g bacto tryptone) medium supplemented with ampicillin (100 μg/ml). One ml of each culture was transferred into 500 ml of LB-ampicillin medium, and after 3 h at 37 °C, induction of recombinant protein synthesis was done by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 4 h, bacteria were pelleted by centrifugation and disrupted by sonication. Inclusion bodies were repeatedly washed with PBS containing 1 M urea and finally dissolved in PBS containing 20 mM β-mercaptoethanol and 8 M urea. The solubilised recombinant proteins were then purified by Ni^{2+}-chelate chromatography under denaturing conditions on a 1 ml HisTrap FF column (GE Healthcare, Piscataway, NJ, USA), as recommended by the manufacturer. The eluates were precipitated as previously described (Wessel and Flügge, 1984) and recNcMIC1, recNcMIC3 and recNcROP2 were resuspended in PBS. Purified proteins were checked by SDS–PAGE and immunoblotting as previously described (Debache et al., 2008).

2.3. Production of polyclonal antisera

Antisera against recNcMIC1 and recNcMIC3 were produced in rats, with three injections at 2 week intervals of 20 μg of each recombinant antigen emulsified in Gerbu™ adjuvant (Gerbu Biotechnik GmbH, Gaiberg, Germany). Antisera against recNcROP2 have been described previously (Debache et al., 2008).

2.4. Antibody inhibition assays

HFF monolayers were grown to confluency in 6-well, flat bottom tissue culture plates. Freshly purified tachyzoites (1.2×10^6) were suspended in 200 μl RPMI 1640 medium and were incubated at room temperature for 30 min in the presence of the following additives: (i) RPMI 1640, (ii) anti-recNcROP2 (1:100), (iii) anti-recNcROP2/anti-recNcMIC1 (1:100 each), (iv) anti-recNcROP2/anti-recMIC3 (1:100 each), (v) anti-recNcROP2/anti-recNcMIC1/anti-recNcMIC3 (1:100 each). Subsequently, 200 μl of these parasite suspensions were added to the monolayers (in 5 ml medium) and tachyzoites were allowed to invade for 30 min at 37 °C/5% CO_2. Infected monolayers were washed three times with cold RPMI 1640 and incubated with RPMI 1640 medium containing 100 μM pyrrolidine dithiocarbamate (PDTC), 0.2 μM $CuSO_4$ and anti-*N. caninum* hyperimmune serum (1:200) for 2 h at 37 °C/5% CO_2 to kill and permeabilize the adherent, extracellular tachyzoites. Subsequently, wells were washed three times with cold RPMI 1640 and were incubated for 1 h at 37 °C/5% CO_2 with RPMI 1640 containing 1 mg/ml DNaseI, in order to remove all extracellular DNA. All wells were washed twice with cold RPMI 1640 and a third time with RPMI 1640 containing 1 mM EDTA. Finally, monolayers were trypsinized and the DNA was purified by using an AllPrep DNA/RNA Mini Kit (Qiagen, Basel, Switzerland). Numbers of invaded parasites were determined by real-time PCR according to Müller et al. (2002). All samples were run in triplicate, and the parasite count for a given sample was assessed per 20 ng of DNA. This experiment was done twice, with virtually identical results.

2.5. Studies on vaccination with recNcROP2 and recNcROP2/NcMIC1/NcMIC3 in pregnant and non-pregnant BALB/c mice and experimental challenge of mice with N. caninum tachyzoites

We have employed the BALB/c mouse model for congenital *N. caninum* infection as described by López-Pérez et al. (2006, 2008). Eight-week-old female BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained under conventional day/night-cycle housing conditions as required by the animal welfare legislation of the Swiss Veterinary Office. They were used for experimentation on reaching 10 weeks of age, having been checked serologically for the absence of anti-*N. caninum* immunoglobulins (preimmune sera) according to Eperon et al. (1999). Mice were randomly allocated into six groups of 16 animals each. Each group was vaccinated three times by i. p. injection at 2 week intervals as follows: Group 1 (PBS) was treated with 100 μl sterile PBS (infection control). Group 2 (SAP) was injected with saponin adjuvant (100 μg/ml in PBS = adjuvant control). Group 3 (ROP2) was immunized with 20 μg recNcROP2. Group 4 (ROP2/MIC1) was injected with 20 μg recNcROP2 and 20 μg recNcMIC1. Group 5 (ROP2/MIC3) was vaccinated with 20 μg recNcROP2 and 20 μg recNcMIC3, and group 6 (ROP2/MIC1/MIC3) received a combination of 20 μg recNcROP2, 20 μg recNcMIC1 and 20 μg recNcMIC3. Just prior to use, all recombinant antigens were suspended at the appropriate concentrations in saponin adjuvants, sonicated for 2×30 s at 57 W in a Bransonic sonifier B-12 (Bransonic Power Company, Cleveland, OH, USA), and were extensively vortexed. Ten days after the third immunization, females were stimulated with a nine-week-old male for 4 days (Whitten effect). Then, groups of two females were housed with one male for three nights. Females were then housed separately to rear their pups, and were challenged at day 7 post-mating by i.p. inoculation of 2×10^6 freshly purified *N. caninum* tachyzoites. At day 60 of the experiment (day 19 of pregnancy), pregnant and non-pregnant mice were separated.

All the dams allocated to the pregnant mouse model delivered 21 ± 1 days after mating, and they were allowed to nurture their offspring normally. The further development of dams and offspring was recorded for 7 weeks until day 112 of the experiment. Neonates were weighed from 14 days post partum until 49 days after birth. On day 112, the experiment was terminated and all animals were killed by CO_2-euthanasia. The non-pregnant females were allocated to the cerebral infection model. They were followed-up and checked for clinical signs on a daily basis for up to 64 days p.i. Animals exhibiting clinical signs of neosporosis (ruffled coat, apathy, hind limb paralysis) were killed at the onset of these clinical signs.

Blood was drawn by cardiac puncture for serum antibody and cytokine analysis. Brains were collected using individual sterile instruments and were vertically divided into two hemispheres. The one destined for subsequent quantitative PCR analysis from genomic DNA was immediately frozen at −20 °C until further use. The other hemisphere was minced into small pieces with a mortar and the tissue placed into 500 μl QIAzol™ Lysis Reagent (Qiagen) prior to freezing at −80 °C.

2.6. Measurement of serum antibody (total IgG, IgG1 and IgG2a) levels

To evaluate the humoral immune response, individual sera (pre-immune sera, post-immunization sera prior to infection (BI), and post-challenge sera (PI)) were analysed for the presence of antigen-specific IgG, IgG1 and IgG2a by ELISA. Sera from surviving pups were taken at the end of the experiments only (49 days post partum). Somatic antigen extract from *N. caninum* tachyzoites (Nc-extract) was obtained by subjecting tachyzoites to three freeze–

thaw cycles as described earlier (Alaeddine et al., 2005; Debache et al., 2008).

Coating of 96-wells plates (Maxisorp, Wiesbaden, Germany) was performed overnight at 4 °C with 100 µl of *N. caninum* extract (0.7 µg/ml) or 100 µl of individual recombinant antigens (0.4 µg/ml) diluted in 0.1 M $NaHCO_3$–Na_2CO_3, pH 9. To block non-specific binding of antibodies, the plates were incubated for 2 h with 200 µl of PBS containing 3% BSA and 0.2% Tween 20. Serum samples were diluted 1:50 in PBS containing 0.3% BSA/0.2% Tween 20 (BSAT) and incubated for 2 h at room temperature. After washing three times with BSAT, anti-mouse IgG alkaline phosphatase-conjugate (Promega, Madison, WI, USA), and goat anti-mouse IgG1 and IgG2a alkaline phosphatase-conjugate, respectively (Southern-Biotech, Birmingham, USA) were added at dilution of 1:1,000 in BSAT and incubated for 2 h at room temperature. Following three washes in BSAT, the wells were subsequently incubated with 100 µl of 1 mg/ml *p*-nitrophenyl-phosphate-disodium in 10% diethanolamine containing 0.5 mM $MgCl_2$, pH 9.8. After allowing the colour to develop for 30 min at room temperature, the absorbance values (405 nm) were read in a tunable microplate reader (Dynatech, Embrach, Switzerland).

2.7. Measurement of serum IL-4 and IFNγ levels

The serum levels of IFN-γ and IL-4 were measured by means of multiplex bead immunoassay using the mouse cytokine/chemokine LINCO*plex* kit (Billerica, MA, USA). Undiluted post-immunization sera (BI) and post-infection sera (PI) were analyzed according to the manufacturer's protocol. Microtiter filter plates were run on Luminex instrument (Bio-Plex™ 200 system). Calibration curves from the provided recombinant standards were calculated with Bio-Plex Manager software version 4.1.1 using a five parametric logistic curve fitting. When cytokine concentration was below the detection limit an arbitrary value corresponding to the detection limit of undiluted samples (provided by the kit manufacturer) was used for statistical analysis.

2.8. Quantitative real-time PCR using genomic brain DNA

Classical *Neospora*-specific quantitative real-time PCR to determine the number of tachyzoites that have reached the cerebral tissue was performed as previously described (Alaeddine et al., 2005; Cannas et al., 2003a,b; Srinivasan et al., 2007; Debache et al., 2008). DNA extraction from brain tissue was performed using the DNeasy® Blood & Tissue Kit (Qiagen) as previously described by Müller et al. (2002). The DNA concentration in each sample was determined by UV spectrophotometry (NanoDrop™, Thermo Scientific, Delaware, US), was adjusted to 100 ng/µl with sterile DNase-free water, and real-time PCR was performed using the LightCycler™ Instrument (Roche Diagnostic, Basel, Switzerland). The parasite counts were calculated by interpolation from a standard curve with DNA equivalents from 1,000, 100 and 10 parasites included in each run (Müller et al., 2002).

2.9. Quantitative real-time PCR using cerebral cDNA

Chloroform (200 µl) was added to all brain samples stored in QIAzol™ and the upper phase, obtained after vigorously vortexing and centrifuging the sample for 2 min at 10,000g, was transferred to a new tube. Ethanol (70% v/v) was added, the sample mixed by pipetting and loaded onto RNeasy® mini kit (Qiagen) spin columns, and total RNA isolation was performed according to the standard protocol. RNA was eluted in RNase-free water and boiled at 95 °C for 3 min. Using the Omniscript® Reverse Transcription kit (Qiagen) and 0.5 µg random primers (Promega, Wallisellen, Switzerland), cDNA synthesis was performed according to the standard protocol for first-strand cDNA synthesis from 1,000 ng of RNA (measured and adjusted by NanoDrop™). Finally, cDNA was boiled at 95 °C for 3 min and frozen at −80 °C prior to use. In order to determine the number of viable tachyzoites in brain tissues of infected mice, we performed NcGRA2-specific real-time PCR according to the method recently described by Strohbusch et al. (2008a,b).

2.10. Statistical analysis

In vitro invasion assays and differences in antibody binding assessed by ELISA were statistically evaluated by Student's *t*-test using the Microsoft Excel program and a value of $P < 0.05$ was considered statistically significant. For evaluation of the newborn survival percentage at each day-point after parturition, the Kaplan–Meier survival method was applied following by log-rank statistical test. Cerebral parasite loads in different treatment and control groups were analyzed by Kruskal–Wallis multiple-comparison. The χ^2-test was performed by an appropriate tool programmed in Excel for comparing the ratio between live and stillborn pups.

3. Results

3.1. Expression and purification of recNcROP2, recNcMIC1 and recNcMIC3

The three recombinant *N. caninum* proteins used in this study were expressed in *E. coli* strain BL21 and purified by Ni^{2+}-affinity chromatography under denaturing conditions. Polyclonal antibodies were raised against each antigen, and SDS–PAGE and immunoblots stained with the corresponding antisera are shown in Fig. 1A. Immunoblots using a polyclonal anti-*N. caninum* antiserum showed the same results.

3.2. Inhibition of in vitro host cell invasion by N. caninum tachyzoites with a combination of antisera directed against recombinant NcROP2, NcMIC1 and NcMIC3

Treatment of *N. caninum* tachyzoites with antibodies directed against recNcROP2 resulted in a significant reduction of host cell invasion by 50% ($P < 0.05$) compared with untreated controls (Fig. 1B). Combining anti-recNcROP2 antiserum with anti-recNcMIC1 or anti-NcMIC3 antibodies, respectively, reduced the invasive capacities of tachyzoites even further, and combining all three antisera yielded a 75% significant inhibition of tachyzoite invasion ($P < 0.05$) compared with controls without antiserum. This confirms earlier studies which had demonstrated that these three proteins are contributing to the host cell invasion process (Keller et al., 2002; Naguleswaran et al., 2001, 2002; Debache et al., 2008), and they thus represent potential vaccine candidates.

3.3. Effects of vaccination in non-pregnant mice

In all treatment groups, approximately 50% of all females became pregnant (see Table 2), indicating that the vaccination by itself did not affect pregnancy. In the non-pregnant mice, neosporosis symptoms occurred in the PBS-treated infection control group and in the adjuvant-control group in three mice at an early time point p.i., and in another two and three mice, respectively, at a later time point p.i. (Table 3). A clearly lower number of animals succumbed to infection in the vaccinated groups and most notably, no clinical signs occurred for a period of 64 days p.i. in those mice vaccinated with the recNcROP2/NcMIC1/NcMIC3 combination (Table 3). Assessment of the parasite burden by real-time PCR, detecting a specific *N. caninum* DNA fragment routinely used for

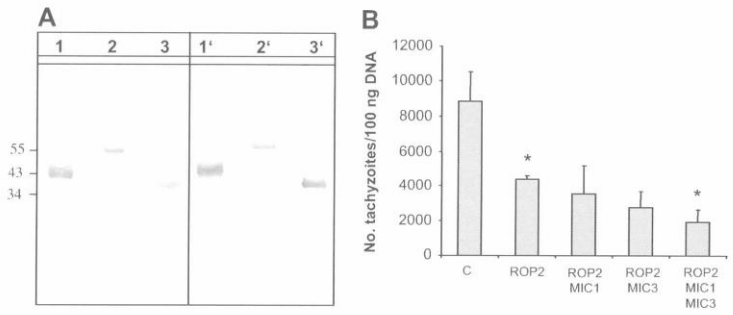

Fig. 1. Purified recombinant antigens inhibit host cell invasion. (A) RecNcROP2 (lane 1), recNcMIC1 (lane 2) and recNcMIC3 (lane 3) were separated by SDS–PAGE and stained with Coomassie blue. These antigens were used to raise polyclonal antisera. The corresponding Western blots are shown in lanes 1′–3′, with recNcROP2 stained with anti-NcROP2, recNcMIC1 stained with anti-NcMIC1, and recNcMIC3 labelled with anti-NcMIC3. (B) Tachyzoites were allowed to invade human foreskin fibroblast (HFF) monolayers in vitro and the addition of anti-NcROP2 antiserum inhibited host cell invasion. The combination of anti-NcROP2 with anti-NcMIC1 and anti-NcMIC3 antisera lead to a significant infection-block (*).

diagnostic purposes (Müller et al., 2002), showed that a significantly ($P < 0.05$) lower number of tachyzoites had reached the brain in the vaccinated (recNcROP2, recNcROP2/recNcMIC1, and recNcROP2/NcMIC1/NcMIC3) groups, compared with the control groups (Fig. 2A). As this assessment merely indicates the numbers of tachyzoites that have reached the brain tissue, but does not discriminate between viable and non-viable tachyzoites, we applied a recently established NcGRA2-specific reverse transcriptase (RT)-PCR, which was designed to quantify NcGRA2-transcripts, and thus only the number of actual live parasites, in cerebral tissues of mice (Strohbusch et al., 2008a,b). Fig. 2B demonstrates that (i) no viable tachyzoites could be detected in those tissue samples originating from mice vaccinated with the recNcROP2/NcMIC3 combination, (ii) the numbers of detectable viable parasites were significantly reduced in the recNcROP2/NcMIC1 vaccinated group and in the group vaccinated with all three antigens, and (iii) surprisingly, the number of viable parasites was similar in the control groups and in the group vaccinated with recNcROP2 alone (Fig. 2B). This suggests that the major portion of tachyzoites that were detected in the control group tissues no longer harboured intact NcGRA2-mRNA and were thus probably non-viable.

3.4. Effects of vaccination in pregnant mice

In the pregnant mice, vaccination had no adverse effects in terms of the numbers of offspring (see Table 2). Neosporosis symptoms occurred in the PBS-treated infection control group and in the adjuvant-control group in three mice at early time points p.i. (days 7 and 10 and days 9 and 11, respectively), and in another two and one mice, respectively, at later time points (days 19 and 16, respectively) (see Table 4). The slight, but not significant, reduction in deaths in the saponin group could reflect a marginal protective effect obtained due to non-specific stimulation of the immune system by treatment with the adjuvant alone. One mouse in the recNcROP2/NcMIC1-vaccinated group and two mice in the recNcROP2/NcMIC3 vaccinated group also showed severe signs of disease between days 10 and 18 p.i., indicating that these two vaccine formulations did not exhibit pronounced protection compared with the saponin control group. No clinical signs occurred in mice vaccinated either with recNcROP2 alone, or with the recNcROP2/NcMIC1/NcMIC3 combination (Table 4). Assessment of the overall cerebral parasite burden by real-time PCR showed that a significantly ($P < 0.05$) lower number of tachyzoites had infected the brains in the vaccinated groups, compared with the control groups, with the exception of the recNcROP2/NcMIC3-vaccinated group (Fig. 3A). The values for the overall cerebral parasite burden in dams were generally half of the values obtained in the non-pregnant groups (see Fig. 2A). In addition, largely similar numbers of viable parasites were detected in every group (Fig. 3B).

Table 3
Clinical signs of neosporosis in non-pregnant mice following challenge with *Neospora caninum* tachyzoites. PBS indicates the infection control, SAP indicates the adjuvants control, ROP2 was vaccinated with recNcROP2, ROP2/MIC1 was vaccinated with a combination of recNcROP2 and recNcMIC1, ROP2/MIC3 was vaccinated with a combination of recNcROP2 and recNcMIC3, ROP2(MIC1/MIC3 was vaccinated with a combination of recNcROP2, recNcMIC1 and recNcMIC3.

Experimental groups	No. of symptomatic mice	Time of death (day p.i.)
PBS ($n = 8$)	5	7/7/10/19/19
SAP ($n = 9$)	6	6/8/8/14/16/20
ROP2 ($n = 8$)	1	21
ROP2/MIC1 ($n = 7$)	2	13/17
ROP2/MIC3 ($n = 9$)	1	10
ROP2/MIC1/MIC3 ($n = 7$)	0	–

Table 2
Number of mice per treatment and number of offspring (live and stillborn) in each experimental group of the present study. The significance of the reduction in numbers of stillborn pups relative to saponin (SAP) as a control was assessed using a χ^2-test.

Experimental group	Non-pregnant (n°)	Dams (n°)	Offspring (n°)		χ^2 (P)
			Total	Live/stillborn	
PBS	8	8	41	29/12	0.45
SAP	9	7	43	30/10	**Control**
ROP2	8	8	42	35/7	0.30
ROP2/MIC1	7	9	51	36/15	0.51
ROP2/MIC3	9	7	52	38/14	0.49
ROP2/MIC1/MIC3	7	9	52	47/5	**<0.05**

The statistical difference between control and the combination of all three antigens is indicated in bold.

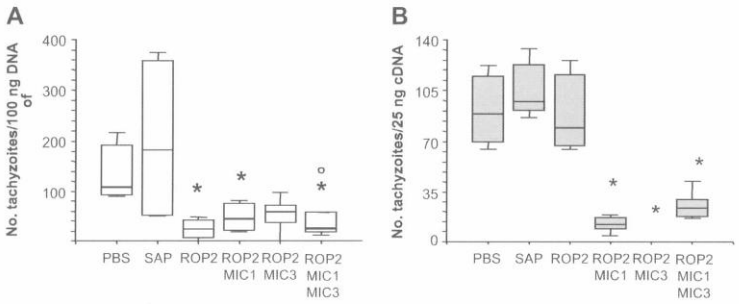

Fig. 2. Effects of vaccination on cerebral infection in non-pregnant mice. (A) Box plot of cerebral *Neospora caninum* infection in all control (PBS, saponin (SAP)) and immunized animals assessed by real-time PCR. The numbers on the *y*-axis correspond to the parasite number/100 ng DNA. Marked by ⁎, the significantly reduced cerebral parasite burden in those mice receiving recNcROP2, a combination of recNcROP2 and recNcMIC1 (recNcROP2/NcMIC1) and a combination of recNcROP2, NcMIC1 and recNcMIC3 (recNcROP1/NcMIC1/NcMIC3). (B) Assessment of parasite viability in control and immunized groups. Note the significantly reduced viability observed in the recROP2/NcMIC1 vaccinated group (⁎), and the absence of viable parasites detected in the cerebral tissue of the group vaccinated with recNcROP2/NcMIC3.

Table 4
Clinical signs of neosporosis in pregnant mice following challenge with *Neospora caninum* tachyzoites. PBS indicates the infection control, SAP indicates the adjuvants control, ROP2 was vaccinated with recNcROP2, ROP2/MIC1 was vaccinated with a combination of recNcROP2 and recNcMIC1, ROP2/MIC3 was vaccinated with a combination of recNcROP2 and recNcMIC3, ROP2(MIC1/MIC3 was vaccinated with a combination of recNcROP2, recNcMIC1 and recNcMIC3.

Experimental groups	No. of symptomatic mice	Time of death (day p.i.)
PBS ($n = 8$)	5	7/7/10/19/19
SAP ($n = 7$)	3	9/11/16
ROP2 ($n = 8$)	0	–
ROP2/MIC1 ($n = 9$)	1	13
ROP2/MIC3 ($n = 7$)	2	10/18
ROP2/MIC1/MIC3 ($n = 9$)	0	–

3.5. Effects of vaccination in pups

Comparison of the ratios between live and stillborn pups in the control and the different treatment groups showed that the number of stillborn pups was significantly lower in the group vaccinated with recNcROP2/MIC1/NcMIC3 (Table 2). All stillborn pups were found to be PCR-positive (data not shown). Survival curves of pups following birth for each group are shown in Fig. 4A. None of the pups in the PBS infection control group and in the adjuvant control group survived this period of 7 weeks; they succumbed to infection, the latest at days 25 and 30, respectively.

The cerebral parasite burden was determined by real-time PCR from those pups that survived until 49 days post partum (Fig. 4B). *Neospora caninum* DNA could be detected in all groups with mean values of 10–40 tachyzoites/100 ng DNA. Thus, cerebral infection had taken place in all pups of all experimental groups. However, when assessing the number of viable parasites using NcGRA2-real-time RT-PCR, the mice whose dams were vaccinated with a combination of all three recombinant antigens were shown to be essentially free of viable tachyzoites, since no GRA2 transcripts could be detected in these samples (Fig. 4C). The pups originating from dams vaccinated with recNcROP2/MIC1 also exhibited a significantly reduced number of viable tachyzoites compared with the other two vaccinated groups.

3.6. Serological responses and IgG subclasses

The antibody responses in non-pregnant mice, in dams and in surviving pups, were analyzed by ELISA employing the purified recombinant antigens used for vaccination, and strong IgG responses against respective antigens were noted in all immunized animals (data not shown). With respect to the serological reactivity of *N. caninum* tachyzoite extract antigens (Fig. 5), total IgG, and IgG1 and IgG2a subclasses, were measured using sera obtained from the two control groups and the groups vaccinated with recNcROP2 and the combination of all three antigens. Non-pregnant mice (Fig. 5A) vaccinated with recNcROP2 only exhibited a minor

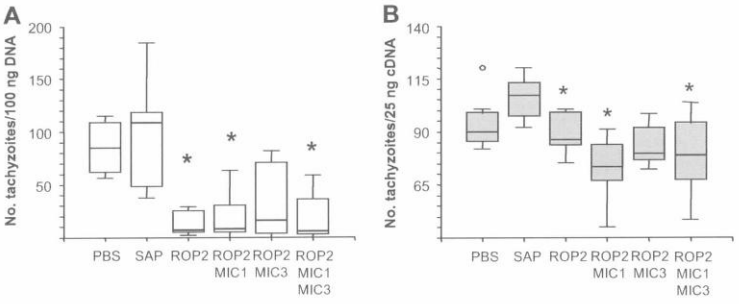

Fig. 3. Effects of vaccination on cerebral infection in dams. (A) Box plot of cerebral *Neospora caninum* infection in all control (PBS, saponin (SAP)) and immunized animals assessed by real-time PCR. The numbers on the *y*-axis correspond to the parasite number/100 ng DNA. Note, marked by ⁎, the significantly reduced cerebral parasite burden in those mice receiving recNcROP2, recNcROP2 and recNcMIC1 (recNcROP2/NcMIC1) and the combination of all three antigens (recNcROP1/NcMIC1/NcMIC3). (B) Assessment of parasite viability in control and immunized groups. Note that there are relatively high values in all groups.

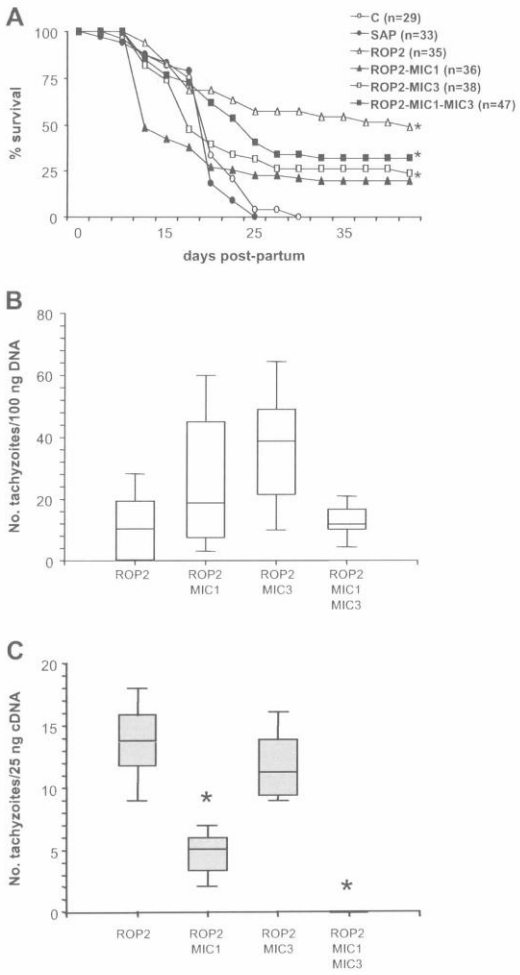

Fig. 4. Effects of vaccination in pups. (A) Survival curves of pups in the different experimental groups. Note that all newborn mice have died in the PBS- and saponin (SAP)-control groups on days 25 and 30, respectively, while several mice (25–50%) survived in the vaccinated groups. (B) Box plot of cerebral *Neospora caninum* infection as assessed by real-time PCR in those newborn pups that survived 7 weeks post partum. The numbers on the *y*-axis correspond to the parasite number/100 ng DNA. Note that the most consistent results were achieved in the group vaccinated with all three antigens in combination (recNcROP2/NcMIC1/NcMIC3). (C) Assessment of parasite viability in newborn pups that had survived until 7 weeks post partum. Note the significantly reduced (*) numbers of viable parasites in the recNcROP2/NcMIC1 vaccinated group, and viable parasites could not be detected in those pups born to recNcROP2/NcMIC1/NcMIC3-vaccinated dams.

humoral response to *Neospora* antigen after vaccination, with IgG1 and IgG2a levels comparable to these in the saponin adjuvant group, and both IgG1 and IgG2a levels increased following infection, but to levels that were also observed for the saponin control. The situation after vaccination with NcROP2 was similar in the dams (Fig. 5B). However, in post-infection sera, IgG1 levels in the NcROP2-group increased significantly, while IgG2a contents remained largely at the pre-infection level (Fig. 5B). In contrast, the saponin control mice responded to infection with an increase in both IgG1 and IgG2a. Both, non-pregnant mice and dams, exhibited a very similar antibody response following vaccination with all three antigens, with almost identical IgG1 and IgG2a levels. Following infection, non-pregnant mice vaccinated with all three antigens responded with a pronounced IgG1 response and IgG2a levels diminished almost entirely (Fig. 5A), while the dams responded with a significant increase in IgG1, and IgG2a levels remained the same as prior to infection. Surviving pups (Fig. 5C), of which the offspring of recNcROP2/NcMIC1/NcMIC3-vaccinated mice apparently no longer harboured any viable parasites in their brains (see Fig. 4C), clearly exhibited an IgG1-response that was significantly higher than in the offspring originating from the dams immunized with recNcROP2 only.

3.7. Cytokines

IFN-γ and IL-4 serum cytokine levels were determined from non-pregnant mice and dams of the control groups, recNcROP2- and recNcROP2/NcMIC1/NcMIC3-vaccinated mice following vaccination (prior to infection) and at the end of the experiment day 118, and from surviving neonates at day 45 post partum (Table 5). In all groups, IFN-γ levels of both dams and non-pregnant mice increased several fold after infection. However, vaccination of non-pregnant mice and dams with all three antigens resulted in IL-4 levels which were already 15–20 times higher than the other groups prior to infection, and they increased dramatically after infection to levels that were much higher compared with the other groups. Similar increased IL-4 levels were detected in p.i. sera in neonates from vaccinated mice. The corresponding IL-4/IFN-γ ratios are shown in Fig. 6. Thus, vaccination with recNcROP2/NcMIC1/NcMIC3 leads to a strong Th2-biased immune response.

4. Discussion

The development of a vaccine to prevent congenital transmission of *N. caninum* would be an important step in reducing the economic losses associated with neosporosis in cattle. It has been demonstrated earlier that immunity to experimental neosporosis in mice is associated with a Th1-immune response dominated by the production of IFN-γ and IgG2a antibodies (Khan et al., 1997; Long et al., 1998; Baszler et al., 1999). It was also shown that immunization of mice with tachyzoite lysates resulted in exacerbation of disease in both cerebral and fetal infection models (Liddell et al., 1999; Baszler et al., 2000). Thus, the identification of antigens inducing protective immunity is of prime importance and those antigens involved in parasite–host cell interactions represent important candidates for incorporation into an effective vaccine.

Neospora caninum rhoptry and microneme proteins play a crucial role in the initial host cell interaction and in the host cell invasion process, and during the subsequent phases of infection (Dubremetz et al., 1998; Hemphill et al., 2006a,b; Dubremetz, 2007; Martin et al., 2007; Saeij et al., 2007). Of these, NcROP2, NcMIC1 and NcMIC3 have earlier been shown to significantly reduce mortality and cerebral infection in an acute disease model comprised of C57BL/6 mice challenged by i.p. infection with *N. caninum* tachyzoites (Alaeddine et al., 2005; Cannas et al., 2003b; Debache et al., 2008). All three antigens were bacterially expressed as $(His)_6$-fusion proteins and purified (see Fig. 1A). In vitro invasion assays using antibodies directed against these recombinant antigens resulted in consistently decreased numbers of invading parasites (Fig. 1B) confirming the involvement of these antigens in host cell interaction processes. All three antigens are secreted by tachyzoites at the onset of host cell interaction/invasion (Naguleswaran et al., 2001; Keller et al., 2002). NcMIC1 binds to sulphated glycosaminoglycans (Keller et al., 2002), and NcMIC3 bind to heparansul-

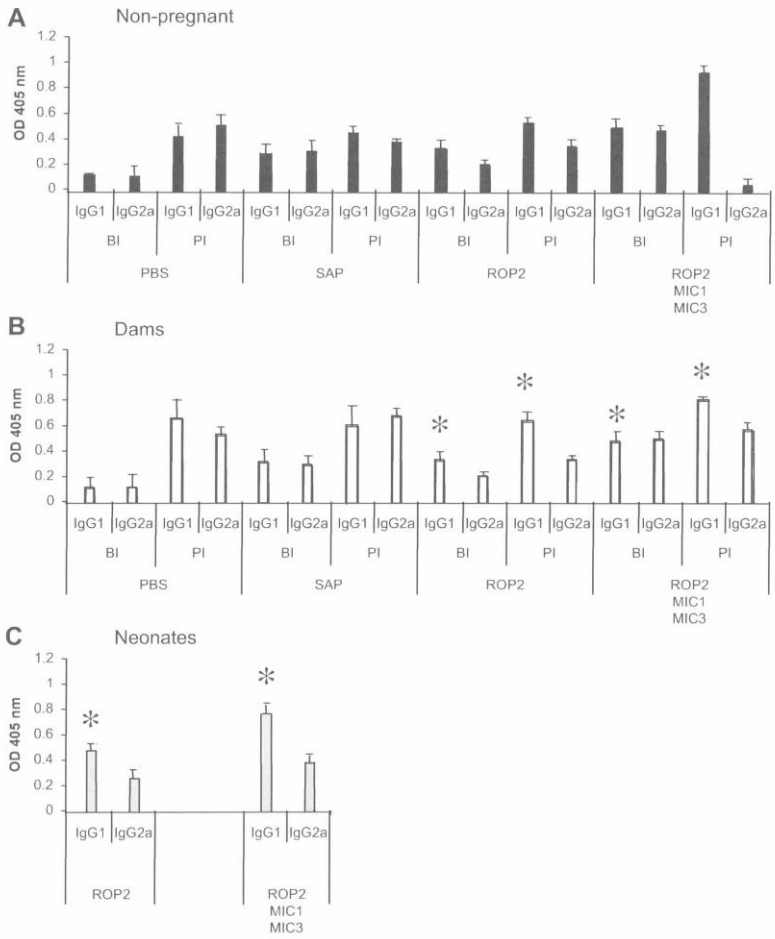

Fig. 5. Antibody responses (IgG, IgG1 and IgG2a) in non-pregnant mice (A), dams (B) and surviving neonates (C) vaccinated with recNcROP2 and all three antigens in combination (recNcROP2/NcMIC1/NcMIC3). Serum samples were taken either following vaccination but prior to infection (BI) or following infection prior to euthanasia (PI). ELISA wells were coated with *Neospora caninum* crude antigen. Only results from the recNcROP2 and recNcROP2/NcMIC1/NcMIC3-vaccinated groups are shown. Values are indicated as A405 nm ±SD. PBS = infection control treated with PBS only, SAP = saponin control.

fate residues (Naguleswaran et al., 2002). NcROP2-binding host cell receptors have not been identified so far. However, it is likely that corresponding antibodies block the adhesion functionality of these proteins or other cellular functions and thus interfere in the establishment of physical contact of the parasite with the host cell. Similarly, Haldorson et al. (2005) demonstrated earlier that both polyclonal and monoclonal antibodies directed against affinity purified native NcSRS2 significantly inhibited the invasion of Vero cells and bovine trophoblasts by *N. caninum* tachyzoites in vitro. Thus, interference in parasite–host cell interaction may be exploited to control the spread of the parasite within the host.

Results obtained in the non-pregnant BALB/c mice basically confirmed earlier findings obtained in C57BL/6 mice by Debache et al. (2008) with respect to the protection against cerebral infection achieved by vaccination with recNcROP2. Combining recNcROP2 with MIC antigens did not result in any further reduction in the numbers of tachyzoites infecting the CNS (see Fig. 2A). To quantify the numbers of viable tachyzoites in the different treatment groups, we employed NcGRA2- real-time RT-PCR as recently described by Strohbusch et al. (2008a,b). This assay is based on the rationale that intact RNA is only detectable when viable organisms are present in a given tissue, since mRNA is rapidly degraded upon cell death. In contrast, genomic DNA is inherently more stable, thus in the case where non-viable organisms are present in the brain tissue, these might be still be detected using conventional real-time PCR, but not by the GRA2-real-time RT-PCR assay (Strohbusch et al., 2008a,b). Surprisingly, mice vaccinated with recNcROP2 did not exhibit a reduced number of viable parasites in the CNS compared with the control groups, but the numbers of detectable viable parasites were significantly reduced when recNcROP2 was applied in combination with MIC antigens (Fig. 2B). These relative differences between overall parasite numbers and numbers of viable parasites in the different treatment groups can be explained by the extensive proliferation of tachyzoites in the CNS of unvaccinated control group mice, (resulting in death of 11 out of 17 animals), thus leading to an increased proportion of non-viable parasite organisms. In contrast, vaccination with recNcROP2 and respective combinations with MIC proteins kept the overall cere-

Table 5
Serum IFN-γ and IL-4 levels from mice obtained from the two control groups (PBS = infection control and SAP = adjuvant control), and from the recNcROP2-vaccinated (ROP2) and recNcROP2/NcMIC1/NcMIC3 vaccinated (ROP2/MIC1/MIC3) groups. Sera were obtained following vaccination, thus before infection (BI) and post-infection (PI).

Groups		IFN-γ ± SD (pg/ml)		IL-4 ± SD (pg/ml)	
		BI	PI	BI	PI
PBS	Dams	1.1 ± 0.2[a]	5.5 ± 3.5	0.2 ± 0.01[b]	10.2 ± 0.1
	Neonates	–	–	–	–
	N. Preg.	0.9 ± 0.6	4.6 ± 1.6	0.5 ± 0.3	4.8 ± 1.5
SAP	Dams	0.91 ± 0.5	2.4 ± 0.2	0.3 ± 0.9	3.3 ± 0.9
	Neonates	–	–	–	–
	N. Preg.	0.2 ± 0.04	5.6 ± 2.4	1.8 ± 0.8	5.7 ± 4.1
ROP2	Dams	0.5 ± 0.1	2.1 ± 0.5	0.5 ± 0.2	3.9 ± 1.3[b]
	Neonates	–	1.3 ± 0.7	–	3.1 ± 0.7
	N. Preg.	0.3 ± 0.2	4.4 ± 1.9	0.5 ± 0.4	6.4 ± 2.3
ROP2/	Dams	0.4 ± 0.1	5.6 ± 2.3[b]	15.9 ± 8.9[a]	171.8 ± 9.2[b]
MIC1/	Neonates	–	6.5 ± 2.2	–	187.4 ± 7.6
MIC3	N. Preg.	0.2 ± 0.2[a]	5.4 ± 0.5	7.6 ± 4.4[a]	30.6 ± 2.4[b]

N. Preg. indicates "non-pregnant".
[a] $P < 0.05$ compared values of BI sera with SAP, as determined by Student's *t*-test.
[b] $P < 0.01$ compared values of PI sera with SAP, as determined by Student's *t*-test.

bral parasite burden at a lower level, with the recNcROP2/NcMIC3 combination being the most efficient. The fact that no viable tachyzoites could be detected in the brain tissues of mice vaccinated with recNcROP2/NcMIC3 indicates that parasites, once they have crossed the blood–brain barrier, could still be eliminated. Recently Ramamoorthy et al. (2007a) demonstrated that infection of mice with *Brucella abortus* vaccine strain RB51 expressing NcMIC1 and NcMIC3 also resulted in protection rates of 100% and 50%, respectively (Ramamoorthy et al., 2007a). However, this study had performed parasite detection by only quantifying tachyzoite DNA, and did not attempt to assess the viability of the tachyzoites located within the cerebral tissue.

For all vaccinated and non-vaccinated treatment groups the ratio between pregnant/non-pregnant mice ranged from 7/9 to 9/7, indicating that the vaccination regime did not negatively affect the initiation of pregnancy (see Table 2). However, it is possible that the final outcome of pregnancy could have been affected by the infection of mice with *N. caninum* tachyzoites at day 7 post-mating. This could lead to mummification and resorption of fetuses, a process which is difficult to monitor, and we will not further elaborate on that in this study. Vaccinated dams gave birth to a higher number of pups compared with mice in the PBS-treated and saponin-treated control groups, and especially in the group vaccinated with all three antigens, the number of live offspring was highest (see Table 2). This suggests that vaccination, most notably recNcROP2/NcMIC1/NcMIC3-vaccination, could possibly prevent fetal resorption during pregnancy. Pregnant mice were completely protected against disease in the groups vaccinated with recNcROP2 and recNcROP2/NcMIC1/NcMIC3 (Table 4), and they exhibited the lowest cerebral infection rates (Fig. 3A). The facts that (i) the mean values for parasite numbers obtained in the pregnant mice were consistently lower than in non-pregnant mice, and (ii) in contrast to non-pregnant mice, dams in all groups failed in eliminating viable tachyzoites, indicates important differences in the immune responses directed against experimental *N. caninum* infection in pregnant versus non-pregnant mice.

During pregnancy in cattle, parasites cross the placenta and infect the foetus, causing congenital infection and possibly abortion as a result from the altered balance of the immune system by foetus-derived regulatory cytokines (Williams and Trees, 2006). This mode of transmission accounts for the vast majority of infections and vertical transmission occurs during repeated pregnancies (Bjorkman et al., 1996; Anderson et al., 1997) with up to 95% of the calves born to infected dams testing positive (Davison et al., 1999). Prevention of vertical transmission and thus of abortion and postnatal disease, is crucial for limiting the economic losses caused by the parasite in cattle (Dubey et al., 2007). Although there are notable differences in the immunological parameters that lead to the control of neosporosis between cattle and different mouse models (Williams and Trees, 2006; Williams et al., 2007; Innes and Mattsson, 2007), studies in mice are very useful to provide a proof of concept in terms of evaluating protective properties of vaccine candidates (Hemphill, 2007). Thus in our study the analysis of the newborn pups and their fate during postnatal development has been of primary interest.

In this BALB/c model, challenge of pregnant mice at day 7 resulted in a transmission rate of 100% (data not shown), confirming the findings published earlier by Lòpez-Pérez et al. (2006, 2008). Interestingly, there were significantly less stillborn mice in the group vaccinated with recNcROP2/NcMIC1/NcMIC3 compared with the control. While pups from both control groups did not survive the postnatal phase, surviving pups were found in all vaccinated groups, with 50% survivors in the recNcROP2-vaccinated group and 35% survivors in the group vaccinated with all three antigens in combination. These survival rates are lower than in other studies employing crude *N. caninum* antigen, NcSRS2-expressing vaccinia virus or antigens expressed in *B. abortus* vaccine strain RB51 (Liddell et al., 1999; Nishikawa et al., 2001a; Ramamoorthy et al., 2007b). However, in our study a relatively high challenge dose was used (2×10^6 as compared with 10^5–10^6 in others). Also, we have investigated pup mortality during a timeframe of 7 weeks post partum, while in other studies the corresponding data has been obtained from a few days to 30 days after birth. In fact, the timing of analysis of pups during postnatal development is crucial and should be maintained at least for several weeks, since congenital *N. caninum* infection can have a considerable impact on the postnatal development of newborns

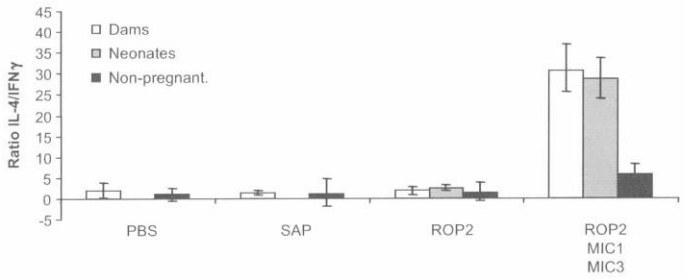

Fig. 6. IL-4 and IFN-γ in p.i. sera of control groups (PBS and saponin (SAP)) and groups vaccinated with recNcROP2 and all three antigens in combination (recNcROP2/NcMIC1/NcMIC3). Sera were taken at the time of euthanasia and values are given as IL-4/IFN-γ-ratio ±SD.

(Lòpez-Pérez et al., 2008; this study). Other factors such as age and strain of mice, and the *N. caninum* isolate used, are also crucial determinants and could explain the differences among the present and former studies. The cerebral infection intensities of the pups that survived until 7 weeks post partum were variable for all three groups, but the assessment by NcGRA2-specific real-time RT-PCR of brain tissues clearly showed that brain tissues from those pups coming from dams vaccinated with recNcROP2/NcMIC1/NcMIC3 were essentially free of viable tachyzoites. Thus, vaccination of mice by the combination of all three antigens did not effectively prevent disease in newborns, but those that survived had eliminated viable tachyzoites beyond the detection level. Clearly, future studies will focus on the consecutive generation and analysis of the parasite burden of the offspring of those congenitally infected mice, to clearly confirm that no congenital transmission to the next generation can take place.

Serological analysis prior to and post-infection of non-pregnant mice, dams and newborn pups of the two control groups and the groups receiving recNcROP2 and recNcROP2/NcMIC1/NcMIC3 showed that vaccinated animals responded to the challenge by eliciting an IgG1-biased humoral response, suggesting the involvement of Th2-like immunity (Fig. 5). Analysis of the cytokine levels of IL-4 and IFN-γ in the same sera confirmed these findings, and most notably the IL-4/IFN-γ ratio was dramatically elevated in the mice and pups originating from recNcROP2/NcMIC1/NcMIC3-vaccinated groups (Table 5). Thus the protective effect of the combined vaccine is associated with a strong Th2-biased immunity.

Conflicting reports exist from other studies with respect to the serological responses and cytokines associated with protection against *N. caninum* infection in mice. Long et al. (1998) using BALB/c, C57Bl/6 and B10.D2 mice showed that mouse strains more resistant to cerebral disease produce higher levels of IgG2a and thus are protected through a Th1-type immune response, while the immune response in more susceptible mice was associated with high IgG1 antibodies. Others have demonstrated that mice treated with anti-IL-12 or anti-IFN-γ antibodies succumb to lethal infection, similar to IFN-γ knock-out mice (Khan et al., 1997; Baszler et al., 1999). Moreover, Baszler et al. (2000) showed in BALB/c mice that antigen lysates mixed with Freund's incomplete adjuvant induced Th2-type responses and exacerbated the disease. A study investigating the *N. caninum* microneme protein NcMIC4 reported on an IgG1-dominated humoral response against recombinant NcMIC4, but the effects were not protective, most likely because these antibodies did not recognise the native antigen (Srinivasan et al., 2007). Vaccination with recNcMIC3 (Cannas et al., 2003b) and recNcMIC1 (Alaeddine et al., 2005) induced high levels of protection against cerebral infection in non-pregnant C57BL/6 mice, which were also associated with an IgG1-dominated antibody response against crude *N. caninum* extract. Live vaccines have also been studied: BALB/c mice vaccinated with recombinant vaccinia virus expressing the major *N. caninum* surface protein NcSRS2 resisted parasite dissemination through out the body, and compared with unvaccinated animals, low levels of IFN-γ production and high levels of IL-4 production (similar to our work) were observed in vaccinated animals (Nishikawa et al., 2003). On the other hand, other live vaccines such as the *B. abortus* vaccine strain RB51 expressing protective *N. caninum* antigens or irradiated tachyzoites elicit an immune response in C57BL/6 mice that is characterised by high IFN-γ and IL-10 production by isolated splenocytes (Ramamoorthy et al., 2007a,b). Vaccination of Qs-mice with the naturally attenuated isolate NC-Nowra that confers high levels of protection against infection in pregnant Qs-mice against the *N. caninum* Liverpool isolate, elicited an IgG2a-dominated response against *N. caninum* crude antigen, which is indicative for a Th1-biased immunity (Ellis et al., 2008).

Not many non-live subunit vaccines have been assessed as vaccines for the prevention of congenital transmission of *N. caninum* and fetal death to date. Vaccination of BALB/c mice with affinity-purified native NcSRS2 resulted in reduced congenital transmission that was associated with a high IL-4/IFN-γ ratio in splenocytes stimulated with native NcSRS2 (Haldorson et al., 2005). In contrast, vaccination trials assessing systemic disease in C57BL/6 mice with bacterially expressed recombinantNcSRS2 have been rather unsuccessful, although protection could be enhanced by a combined application of recNcSRS2 followed by the corresponding DNA vaccine pcDNA–NcSRS2 (Cannas et al., 2003a). Thus bacterially expressed recombinant NcSRS2 has not yet been assessed in the fetal infection model. More recently, Ellis et al. (2008) reported on the evaluation of recombinant proteins NcGRA1, NcGRA2, NcMIC10, p24B and combinations thereof as vaccines in pregnant Qs-mice challenged with *N. caninum* Liverpool isolate, and received very marginal protection rates despite using a lower (10^6) infection dose. In contrast, the very good protection rates achieved by live vaccination with the naturally attenuated NC-Nowra isolate lead to the conclusion that most likely live vaccines will be the way forward, similar to those vaccines currently available for besnoitiosis in cattle (Bigalke et al., 1974), *Toxoplasma*-induced abortion in sheep (Buxton and Innes, 1995) and coccidiosis in poultry (Shirley and Bedrník, 1997). However, there could be significant risks associated with using live parasite populations as vaccines in cattle, and there is always the potential of reversion to a disease-causing phenotype. Thus, we propose that the search for promising antigens must continue, and the ideal recombinant vaccine against neosporosis should be composed of different subunits conferring efficient protective immunity. The combined application of recNcROP2/NcMIC1/NcMIC3 antigens could serve as a basis for the development of such a multivalent recombinant vaccine.

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2.3.Intraperitoneal and intra-nasal vaccination of mice with three distinct recombinant *Neospora caninum* antigens results in differential effects with regard to protection against experimental challenge with *Neospora caninum* tachyzoites. Debache K, Guionaud C, Alaeddine F, Hemphill A. (2009).

Intraperitoneal and intra-nasal vaccination of mice with three distinct recombinant *Neospora caninum* antigens results in differential effects with regard to protection against experimental challenge with *Neospora caninum* tachyzoites

K. DEBACHE[1], C. GUIONAUD[2], F. ALAEDDINE[2] *and* A. HEMPHILL[1]*

[1] *Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland*
[2] *Institute of Veterinary Pharmacology & Toxicology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 124, CH-3012 Berne, Switzerland*

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SUMMARY

Recombinant NcPDI(recNcPDI), NcROP2(recNcROP2), and NcMAG1(recNcMAG1) were expressed in *Escherichia coli* and purified, and evaluated as potential vaccine candidates by employing the C57Bl/6 mouse cerebral infection model. Intraperitoneal application of these proteins suspended in saponin adjuvants lead to protection against disease in 50% and 70% of mice vaccinated with recNcMAG1 and recNcROP2, respectively, while only 20% of mice vaccinated with recNcPDI remained without clinical signs. In contrast, a 90% protection rate was achieved following intra-nasal vaccination with recNcPDI emulsified in cholera toxin. Only 1 mouse vaccinated intra-nasally with recNcMAG1 survived the challenge infection, and protection achieved with intra-nasally applied recNcROP2 was at 60%. Determination of cerebral parasite burdens by real-time PCR showed that these were significantly reduced only in recNcROP2-vaccinated animals (following intraperitoneal and intra-nasal application) and in recNcPDI-vaccinated mice (intra-nasal application only). Quantification of viable tachyzoites in brain tissue of intra-nasally vaccinated mice showed that immunization with recNcPDI resulted in significantly decreased numbers of live parasites. These data show that, besides the nature of the antigen, the protective effect of vaccination also depends largely on the route of antigen delivery. In the case of recNcPDI, the intra-nasal route provides a platform to generate a highly protective immune response.

Key words: Recombinant *Neospora caninum* antigen, tachyzoites, vaccination, mice, intraperitoneal, intra-nasal.

INTRODUCTION

Neospora caninum is an apicomplexan parasite that causes abortion and stillbirth in cattle and neuromuscular disease in dogs. Many other species can also be affected. The parasite has emerged as a significant veterinary public health problem, since it has been shown to represent the most important abortion-causing pathogen in bovines, responsible for economic losses to the cattle industry worldwide (McAllister *et al.* 1998; Gondim, 2004; Dubey *et al.* 2007). Protection of fetal death in cattle has been achieved experimentally by immunization of dams with live *N. caninum* tachyzoites (Williams *et al.* 2007). However, at the present time, there is no non-live vaccine capable of inducing complete protective immunity against neosporosis in cattle.

* Corresponding author: Andrew Hemphill, Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland. Tel. +41 31 6312384. Fax +41 31 6312477. E-mail: hemphill@ipa.unibe.ch

Mice have been widely used as laboratory models for proof-of-concept studies involving vaccine candidates, in spite of the fact that murines and bovines exhibit somewhat different immunological characteristics (reviewed by Hemphill *et al.* 2006; Innes, 2007; Innes and Mattsson, 2007). Infection with killed tachyzoite lysate prevents foetal infection in mice (Liddell *et al.* 1999), but not in cattle. A commercialized vaccine, consisting of tachyzoite lysate (Neoguard^{TM}), is currently marketed in the US, but efficacy data are difficult to interpret (Williams and Trees, 2006; Innes and Vermeulen, 2006).

To date, the major portion of antigens that have been investigated as subunit vaccine candidates are involved in tachyzoite-host cell interactions, and are localized either on the surface of tachyzoites, or within secretory organelles such micronemes, rhoptries and dense granules (reviewed by Hemphill *et al.* 2006). Various strategies have been used to enhance the post-vaccination immune response, such as application of multiple recombinant antigens in one shot (Cho *et al.* 2005; Ellis *et al.* 2008), different adjuvants and dosages, various formulations and

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different routes of administration (Innes and Mattsson, 2007).

For non-live vaccines such as recombinant antigens, the classical application routes are either intraperitoneal (i.p.) or subcutaneous (s.c.) injection. In this study, we have investigated whether changing the route of application will alter the efficacy of 3 putative *N. caninum* vaccine candidates in terms of cerebral neosporosis in mice. First, NcROP2 is a *N. caninum* rhoptry protein, and pre-incubation of parasites with antibodies directed against NcROP2 and of host cells with recombinant recNcROP2, inhibited host cell invasion by *N. caninum* tachyzoites *in vitro* (Debache *et al.* 2008). Secondly, NcPDI, a protein disulfide isomerase, is partially found within micronemes and exposed on the surface of *N. caninum* tachyzoites, and anti-NcPDI antibodies also impair adhesion of parasites to the host cell surface *in vitro* (Naguleswaran *et al.* 2005). In addition, inhibition of NcPDI activity with a number of sulfhydryl blockers and the PDI inhibitor bacitracin also have a negative influence on the interaction of *N. caninum* tachyzoites with their host cells (Müller *et al.* 2008). Third, NcMAG1 is located within dense granules in tachyzoite-stage parasites, which incorporates into the cyst wall during tachyzoite-to-bradyzoite stage conversion, similar to what has been described for NcGRA2 and NcGRA7 (Vonlaufen *et al.* 2004).

We here show that intra-nasal application of a vaccine based on recNcPDI produced fundamentally improved outcomes in terms of occurrence of clinical signs, parasite burden and serological responses upon challenge infection. In contrast, intra-nasal vaccination with recNcMAG1 had a negative impact on protection and, in the case of recNcROP2, no substantial differences were noted.

MATERIALS AND METHODS

Unless otherwise stated, all cell culture reagents were supplied by Gibco-BRL (Zurich, Switzerland) and chemicals were purchased from Sigma (St Louis, MO, USA).

Neospora caninum *tachyzoite culture and purification*

Vero cells and human foreskin fibroblasts (HFF) were routinely cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U of penicillin/ml and 50 μg of streptomycin/ml at 37 °C/5% CO_2 in tissue culture flasks. Cultures were passaged at least once per week. *N. caninum* tachyzoites of the Nc1 strain (Dubey *et al.* 1988) were maintained by serial passages in Vero cells or HFF during which time FCS was replaced with 5% immunoglobulin G (IgG)-free horse serum (HS). Parasites were harvested as described previously by Hemphill *et al.* (1996). Infected cells were trypsinized, washed twice in cold RPMI-1640 medium, and the resulting pellet was resuspended in 2 ml of cold RPMI-1640 medium. Cells were repeatedly passed through a 25 G-needle and liberated tachyzoites were purified by passage through Sephadex-G25 columns (Amersham Biosciences, Otelfingen, Switzerland), previously equilibrated with cold RPMI-1640 medium. The eluted parasites were centrifuged at 600 ***g***/10 min/4 °C, resupended in cold RPMI-1640 medium, and counted in a Neubauer chamber. Viability assessment was done by Trypan blue exclusion, and in all experiments, over 95% of the tachyzoites were viable.

Expression and purification of recombinant NcPDI (recNcPDI), NcROP2 (recNcROP2), and NcMAG1 (recNcMAG1)

RecNcPDI was cloned into the His-tag expression vector pET151 and expressed in *Escherichia coli* BL21 Star (Invitrogen, Carlsbad, Canada) as previously described (Müller *et al.* 2008). RecNcROP2 was cloned into pQE-30 (Qiagen, Hilden, Germany) as described by Debache *et al.* 2008, 2009), and expressed in *E. coli* BL21 (Novagen-EMD Biosciences, Madison, WI, USA) harbouring the pREP4 repressor plasmid (Qiagen).

The cDNA sequence coding for the NcMAG1 antigen was retrieved in GenBank under the Accession number EF580924. The deduced protein sequence is comprised of 456 amino acids, and shares 54% identity to the homologous TgMAG1 protein (Parmley *et al.* 1994). RecNcMAG1 cloning was performed by isolation of total RNA from 3×10^6 purified *N. caninum* tachyzoites using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First strand cDNA synthesis reactions were performed for 1 h at 42 °C in a 20 μl reaction mixture containing 2 μg of total RNA, 1× first-strand buffer (Invitrogen), 0·5 mM of each dNTP (Endotell, Allschwil, Switzerland), 10 mM dithiothreitol, 20 U of RNasin RNase inhibitor (Promega, Madison, WI), 200 U of SuperScript II reverse transcriptase (Invitrogen). A portion of NcMAG1 (aa 31–394, numbering according to the precursor protein) was amplified by RT-PCR using MAG1-BamHI-F (5′-GGATCCCAAAGGGTGCCTCGCTACCC-3′) and MAG1-SmaI-R (5′-CCCGGGTTATTCCTCCACTATTTCGTCCGC-3′) primers (*Bam*HI and *Sma*I restriction sites underlined). The PCR product was cloned into pCR blunt II TOPO (Invitrogen), verified by sequencing and the BamHI-SmaI MAG1 insert subcloned into pQE-30 (Qiagen, Hilden, Germany) using the XL1-Blue strain (Stratagene, La Jolla, CA, USA) as a recipient. The resulting pQE-30-MAG1 expression vector was used to transform *E. coli* BL21

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(Novagen-EMD Biosciences, Madison, WI) harbouring the pREP4 repressor plasmid (Qiagen). To express the fusion protein, 1 l of 2× YT medium (per litre: 16 g bacto-tryptone, 10 g bacto-yeast extract, and 5 g NaCl), pre-warmed to 37 °C and supplemented with carbenicillin and kanamycin (Sigma; 100 and 25 μg/ml, respectively), was inoculated with 10 ml of an overnight starter culture grown in the same medium. When the culture reached an OD of 0·5 at $\lambda = 600$ nm, isopropyl-β-D-1-thiogalactopyranoside (Sigma) was added to 1 mM and expression was carried on for 3 h at 37 °C. Bacteria were harvested by centrifugation and disrupted by sonication.

Purification of recombinant His-tagged proteins was performed under non-denaturing conditions for recNcPDI and recNcMAG1 (Müller *et al.* 2008) and denaturing conditions for recNcROP2 (Debache *et al.* 2008), using Protino Ni-IDA columns (Macherey-Nagel, Düren, Germany), as recommended by the manufacturer. The purified recombinant proteins were analysed by SDS–PAGE and Western blotting, and the protein concentrations were measured employing the Bio-Rad protein assay using acetylated BSA as a standard. Following dialysis into PBS, the recombinant proteins were stored at −80 °C prior to use.

Immunization and challenge infection

Female C57Bl/6 mice (6 weeks of age) were purchased from Charles River Laboratories (Sulzheim, Germany) and were housed under conventional day/night conditions according to the standards set up by the animal welfare legislation of the Swiss Veterinary Office. Two independent experiments, one after the other, were carried out under the same conditions, with virtually identical outcomes, and the results of the second experimental trial are shown.

At the age of 8–9 weeks, mice were randomly distributed into 10 experimental groups of 10 mice each, and the serological status (*Neospora*-negative) was checked by enzyme-linked immunosorbent assay (ELISA). Mice in groups 1–5 were treated by i.p. injection; mice in group 1 received 100 μl of PBS each (i.p. infection control), group 2 received 100 μl saponin adjuvant (SAP) at 100 μg/ml, group 3 received 10 μg of recNcPDI in SAP, group 4 received 10 μg recNcROP2 in SAP, group 5 received 10 μg recNcMAG1 in SAP. Mice in groups 6–10 were treated by i.n. application through the nares, which was performed under mild isoflurane anaesthesia. Mice in group 6 received 100 μl of PBS/mouse (i.n. infection control), group 7 received 20 μl of cholera toxin adjuvant (CT) at 250 μg/ml, group 8 received 10 μg recNcPDI/mouse in CT, group 9 received 10 μg recROP2 in CT, group 10 received 10 μg recNcMAG1 in CT. These procedures were carried out on days 1, 15 and 30. We have not included a non-vaccinated and non-treated group in these trials, since in several similar vaccine trials carried out to date no spontaneous deaths of mice occurred under the conditions used (Cannas *et al.* 2003*a*, *b*; Alaeddine *et al.* 2005; Srinivasan *et al.* 2007; Debache *et al.* 2008).

On day 46 all animals were challenged by i.p. inoculation of 1×10^6 freshly purified *N. caninum* tachyzoites. On day 74, the experiment was terminated and mice were euthanized by CO_2 asphyxiation. Those animals exhibiting clinical signs of neosporosis (ruffled coat, apathy, hind limb paralysis) prior to day 74 were euthanized at the onset of these signs. From each animal, blood was drawn by cardiac puncture for serological assays, brains were dissected under aseptic conditions and the tissue immediately frozen at −20 °C.

Brains from the mice that were vaccinated intranasally were divided into the 2 hemispheres. One hemisphere, destined for the isolation of genomic DNA and subsequent quantitative assessment of parasite burden by real-time PCR (Müller *et al.* 2002) was immediately frozen at −20 °C. The other hemisphere, destined for RNA isolation, cDNA synthesis and quantitative assessment of viable parasites by real-time PCR on cDNA was hashed in 500 μl of QIAzol™ Lysis Reagent (Qiagen) with a small mortar in dry-ice before being frozen at −20 °C.

Serology

Individual blood samples including pre-immune sera and post-immunization sera (taken prior to infection = BI) were obtained from the tail vein, and the final blood samples (post-infection sera = PI) were obtained by cardiac puncture post-euthanasia. To evaluate the humoral immune response, samples were analysed for *N. caninum*-specific immunoglobulin G (IgG), IgG1, IgG2a and immunoglobulin A (IgA) by ELISA, employing somatic antigen extracts from *N. caninum* tachyzoites obtained from cell culture (Nc-extract). Nc-extract was prepared by subjecting tachyzoites to 3 freeze-thaw cycles followed by sonication as described by Alaeddine *et al.* (2005). Cellular debris was removed by centrifugation, and the protein concentration in the supernatant was measured employing the Bio-Rad protein assay using acetylated bovine serum albumin (BSA) as a standard (Debache *et al.* 2008). To carry out the ELISAs, 96-well plates (Maxisorp, Wiesbaden, Germany) were coated overnight at 4 °C with 100 μl of Nc-extract (7 μg/ml), diluted in 0·1 M $NaHCO_3$-Na_2CO_3, pH 9. To block non-specific binding of antibodies, plates were incubated for 2 h with 200 μl of PBS containing 3% BSA and 0·2% Tween 20. Serum samples were diluted 1 : 50 in PBS containing 0·3% BSA/0·2% Tween 20 (BSAT) and incubated for 2 h at 20 °C. After 3 washes with BSAT, anti-mouse IgG conjugated to alkaline

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phosphatase (Promega, Madison, WI, USA), or goat anti-mouse alkaline phosphatase-IgG1 or IgG2a-conjugates (SouthernBiotech, Birmingham, USA) and goat anti-mouse IgA-alkaline phosphatase conjugates (Sigma, St Louis, MO, USA) were added, respectively, at dilution of 1 : 1000 in BSAT. Conjugates were incubated for 2 h at 20 °C. Following 3 washes in BSAT, the wells were subsequently incubated with 100 µl of 1 mg/ml *p*-nitrophenyl-phosphate-disodium in 10% diethanolamine containing 0·5 mM $MgCl_2$, pH 9·8. After allowing the colour to develop for 30 min at 20 °C, the absorbance values (405 nm) were read in a tunable microplate reader (Dynatech, Embrach, Switzerland).

Quantitative real-time PCR using cerebral genomic DNA

The conventional *Neospora*-specific quantitative real-time PCR (Müller *et al.* 2002) from genomic DNA was performed as previously described (Srinivasan *et al.* 2007; Debache *et al.* 2008). Brain DNA extraction was performed using the DNeasy® Blood & Tissue Kit (Quiagen) (Müller *et al.* 2002). The DNA concentrations in all brain samples were determined by UV spectrophotometry, and were adjusted to 100 ng/µl with sterile Dnase-free water. The assessments of *N. caninum* tachyzoite loads were performed using the LightCycler™ Instrument (Roche Diagnostic, Basel, Switzerland). The parasite counts were calculated by interpolation from a standard curve with DNA equivalents from 1000, 100 and 10 parasites included in each run.

Quantitative real-time PCR using cerebral cDNA

To quantify the viable tachyzoites in brain samples, NcGRA2-RT-PCR was performed using the corresponding other hemisphere from each mouse (Strohbusch *et al.* 2008*a*, *b*). Then 200 µl of chloroform was added to all brain samples stored in QIAzol™, and the upper phase, obtained after vigorously vortexing and centrifuging the sample for 2 min at 10 000 ***g***, was transferred to a new tube. Ethanol (70% v/v) was added, the sample mixed by pipetting, and loaded onto RNeasy® mini kit (Qiagen) spin columns and total RNA isolation was performed according to the standard protocol. RNA was eluted in RNase-free water and boiled at 95 °C for 3 min. Using the Omniscript® Reverse Transcription kit (Qiagen) and 0·5 µg random primers (Promega, Wallisellen, Switzerland), cDNA synthesis was performed according to the standard protocol for first-strand cDNA synthesis from 1000 ng of RNA (measured and adjusted by NanoDrop™, Thermo Scientific, Delaware, USA). Finally, cDNA was boiled at 95 °C for 3 min and frozen at −80 °C prior to use. The Nc-GRA2-real time PCR was performed according to Strohbusch *et al.* (2008*a*, *b*). The parasite counts were calculated by interpolation from a standard curve with cDNA equivalents from 1000, 100 and 10 parasites included in each run.

Statistics

The cerebral parasite loads in immunized mice, and the numbers of viable tachyzoites expressing NcGRA2 in the intra-nasally vaccinated groups, were statistically assessed by Kruskal-Wallis multiple comparison, followed by Duncan's multiple range test to compare between 2 particular groups. The differences in survival rates were checked by the Kaplan–Meier survival method followed by log-rank statistical test. The comparison of serological responses between the different treatment groups was done by ANOVA test. When comparing the samples from the same group at different time-points (BI and PI), paired Student's *t*-test, was used, and unpaired Student's *t*-test was used when comparing animals from different groups at the same time-point. All analyses of variances were done using the NCSS Quick Start© 2001 software.

RESULTS

Clinical signs of neosporosis caused by parasite challenge following vaccination via intra-peritoneal and intra-nasal routes

Following vaccination and challenge infection with *N. caninum* tachyzoites, mice were carefully monitored for the occurrence of clinical signs of neospororis such as apathy, ruffled hair, loss of body weight, rounded back and circular movements. All mice in the 2 control groups (treated with PBS only) succumbed to infection latest at day 21 p.i. (Fig. 1). When only the adjuvants (either saponin or cholera toxin) were applied, all mice died latest on days 25 and 27, respectively. Upon immunization by the i.p. route, recNcROP2 and recNcMAG1 provided protection in 7 and 5 out of 10 mice, respectively, that did not develop any clinical signs. In contrast, only 2 mice out of 10 that had been vaccinated by i.p. inoculation with recNcPDI survived until day 28 p.i. (Fig. 1A). Intra-nasal vaccination with recNcROP2 proved to provide a similar degree of protection (6 out of 10 survivors) as vaccination through the intraperitoneal route. However, the situation was different for the other 2 antigens. Only 1 out of 10 recNcMAG1-vaccinated mice survived until 28 days p.i., and when recNcPDI was applied intra-nasally, 9 out of 10 mice survived and only 1 animal succumbed to infection on day 20 (Fig. 1B). No histological assessments were made to assess inflammation within the cerebral tissues of these mice.

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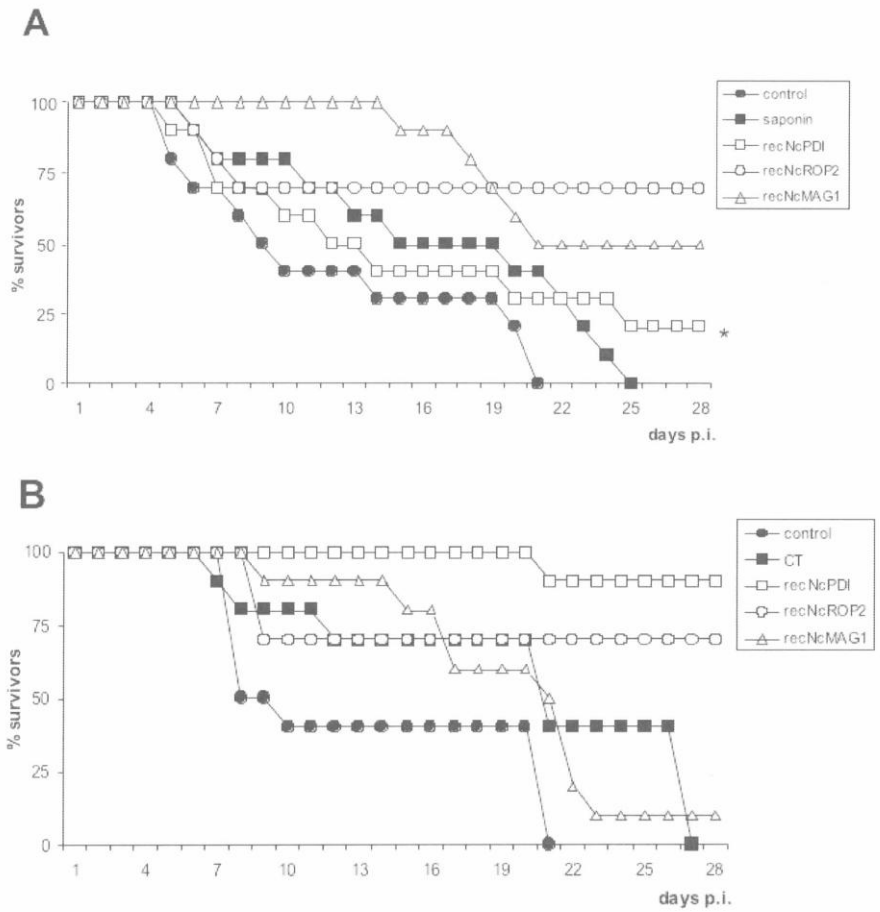

Fig. 1. Survival curves of the different treatment groups following experimental infection with 1×10^6 *Neospora caninum* tachyzoites. (A) Mice were vaccinated intra-peritoneally either with PBS (control), saponin adjuvants, recNcPDI, recNcROP2 or recNcMAG1 in saponin adjuvants. (B) Mice were vaccinated intra-nasally with PBS (control), cholera toxin, recNcPDI, recNcROP2 or recNcMAG1 resuspended in cholera toxin. The asterisk in (A) indicates the statistical difference ($P<0{\cdot}05$) of the recNcPDI-vaccinated group compared with the group vaccinated with recNcROP2 in intraperitoneally vaccinated mice. Note the high protective effect (90%) achieved with the intra-nasally applied recNcPDI vaccine in (B).

Parasite burden in brain tissues of vaccinated mice assessed by N. caninum*-specific real-time PCR*

Cerebral parasite burdens in the different treatment groups were determined by quantitative real-time PCR (Fig. 2). There was no significant difference in parasite load between intra-peritoneal and intra-nasal application of either PBS or adjuvants in the 2 control groups. These values were comparable to respective parasite loads obtained in the recNcMAG1-vaccinated mice. In contrast, parasite load in recNcROP2-vaccinated mice was significantly lower ($P<0{\cdot}05$) compared to the adjuvant control groups by both, intraperitoneal and intra-nasal application.

Brain tissues of recNcPDI-vaccinated mice that had been immunized intraperitoneally did not exhibit any differences in parasite load compared to the PBS-treated and saponin-treated control groups, but brain tissues of mice that had been vaccinated with recNcPDI through the intra-nasal route exhibited values that were in the similar lower range as for the 2 recNcROP2-vaccinated groups ($P<0{\cdot}01$). Thus, recNcPDI has a protective effect when applied intra-nasally, but not when applied intraperitoneally.

Quantification of viable N. caninum *tachyzoites in infected brain hemispheres by GRA2-specific RT real-time PCR*

In order to further study the effects of intra-nasal vaccination on parasite viability, we employed a

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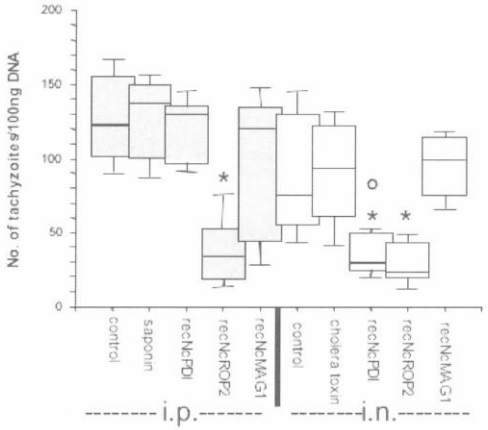

Fig. 2. Cerebral parasite load in the different treatment groups as assessed by real-time PCR (amplification of genomic DNA). The corresponding experimental groups of intra-peritoneally (i.p.) and intransally (i.n.) vaccinated mice are directly compared. Statistically significant differences are indicated. * = $P<0{\cdot}05$ as compared to the corresponding adjuvant-treated group, ° = $P<0{\cdot}05$, as compared to the recNcPDI-treated group of intraperitoneally vaccinated mice. Note the strongly reduced cerebral parasite burden in the group receiving the intra-nasally applied recNcPDI vaccine. Error bars indicate S.D.

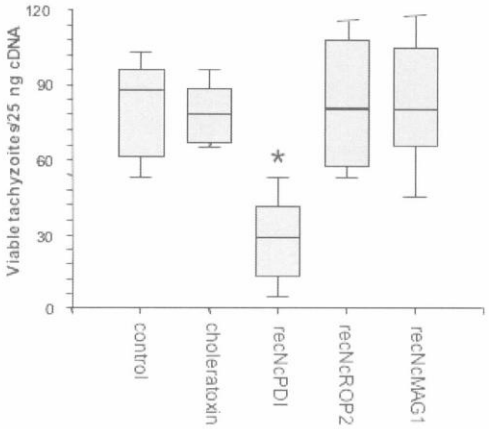

Fig. 3. Assessment of tachyzoite viability by detection and quantification of NcGRA2 transcripts in the brain tissues of the different treatment groups vaccinated by intra-nasal immunization with cholera toxin adjuvant. * = $P<0{\cdot}05$ as compared to the cholera toxin-treated group. Error bars indicate S.D. Note the strongly reduced parasite viability detected in the recNcPDI vaccinated group.

recently developed NcGRA2-specific RT real-time PCR (Strohbusch *et al.* 2008*a*, *b*), which allows quantification of the number of viable tachyzoites in brain tissue samples of the different experimental groups. The rational behind this assay is that NcGRA2-mRNA will only be detected when viable parasites are present, since mRNA is rapidly degraded upon cell death. In contrast, genomic DNA is inherently more stable, thus, in the case where non-viable organisms are present in the brain tissue, these might still be picked up by conventional real-time PCR. We found that the numbers of viable tachyzoites were similar in controls and recNcROP2 and recNcMAG1 vaccinated groups, but significantly decreased in mice vaccinated with recNcPDI ($P<0{\cdot}05$) (Fig. 3).

Humoral immune responses

By ELISA, the sera of all mice exhibited a significant increase ($P<0{\cdot}05$) in IgG titres directed against the recombinant proteins these mice had been vaccinated with (data not shown). Fig. 4 shows the antibody responses following vaccination prior to infection (BI) and post-infection prior to euthanasia (PI) as assessed by ELISA employing crude *N. caninum* extract (Nc-antigen). Prior to infection (BI), total IgG reactive with Nc-antigen was significantly ($P<0{\cdot}05$) increased in all vaccinated mice compared to non-infected and adjuvant-control mice, no matter whether intraperitoneal or intra-nasal vaccination took place, and these levels increased significantly ($P<0{\cdot}05$) in all animals following infection. IgG1 and IgG2a levels remained low before infection, but also mostly increased in post-infection sera, with few exceptions. First, in mice vaccinated intraperitoneally with recNcROP2, only IgG1 levels increased significantly ($P<0{\cdot}05$) after infection, indicating that upon infection an IgG1-biased humoral response has taken place. In the corresponding intra-nasally vaccinated mice, both IgG1 and IgG2a levels increased significantly ($P<0{\cdot}05$) (see 1 and 1′ in Fig. 4), resulting in similar protection levels in both cases. Secondly, mice immunized intraperitoneally with recNcMAG1 exhibited a significant ($P<0{\cdot}05$) increase of both IgG1 and IgG2a after challenge, but in intra-nasally vaccinated mice, only IgG2a was significantly ($P<0{\cdot}05$) elevated after infection, and this was associated with a high mortality post-challenge (see 2 and 2′ in Fig. 4). Thirdly, in recNcPDI-vaccinated mice, intraperitoneal as well as intra-nasal immunization and subsequent challenge infection also resulted in significant ($P<0{\cdot}05$) increase of both IgG1 and IgG2a, but in intra-peritoneally vaccinated animals the values of IgG2a were higher than those for IgG1, while in intra-nasally vaccinated mice, IgG1 levels were higher than those for IgG2a. The IgG1-values measured in i.p. vaccinated and infected mice were significantly lower compared to i.n. vaccinated mice ($P<0{\cdot}05$),

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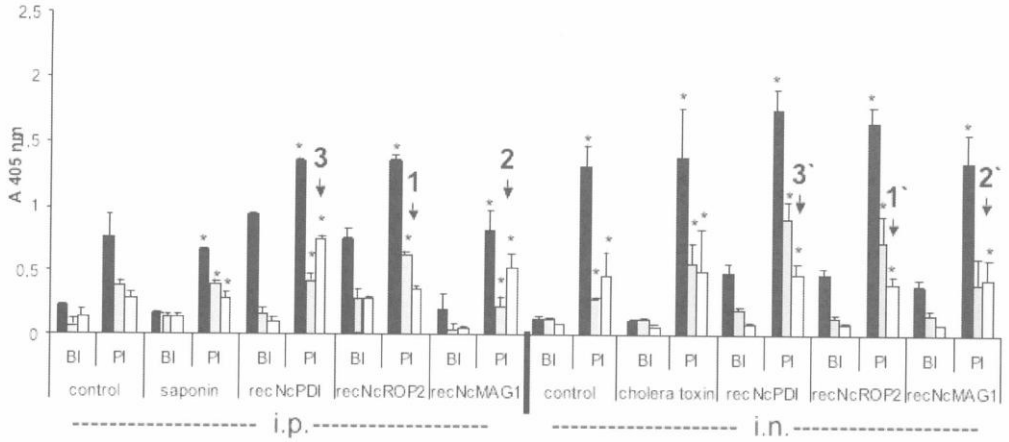

Fig. 4. Serological analysis (total IgG, IgG1, IgG2a) of all mice in all experimental groups. Serum samples were taken either following vaccination before infection (BI), or at the time of death following experimental infection (PI). ELISA wells were coated with Nc-antigen. Mice were vaccinated intraperitoneally (i.p.) with antigens emulsified in saponin, or vaccinated intra-nasally (i.n.) with antigens emulsified in cholera toxin. 1 and 1′ indicate that in mice vaccinated intraperitoneally with recNcROP2, only IgG1- levels increased significantly after infection, while in the corresponding intra-nasally vaccinated mice, both IgG1 and IgG2a levels increased significantly. 2 and 2′ indicate that mice immunized intraperitoneally with recNcMAG1 exhibited a significant increase of both IgG1 and IgG2a after challenge, but in intra-nasally vaccinated mice, only IgG2a was significantly elevated after infection. 3 and 3′ indicate that in recNcPDI-vaccinated mice, intraperitoneal as well as intra-nasal immunization and subsequent challenge infection also resulted in significant increase of both IgG1 and IgG2a, but in intraperitoneally vaccinated animals the values of IgG2a were higher than those for IgG1, while in intra-nasally vaccinated mice, IgG1 levels were higher than those for IgG2a. * = $P<0{\cdot}05$ as compared to BI-sera.

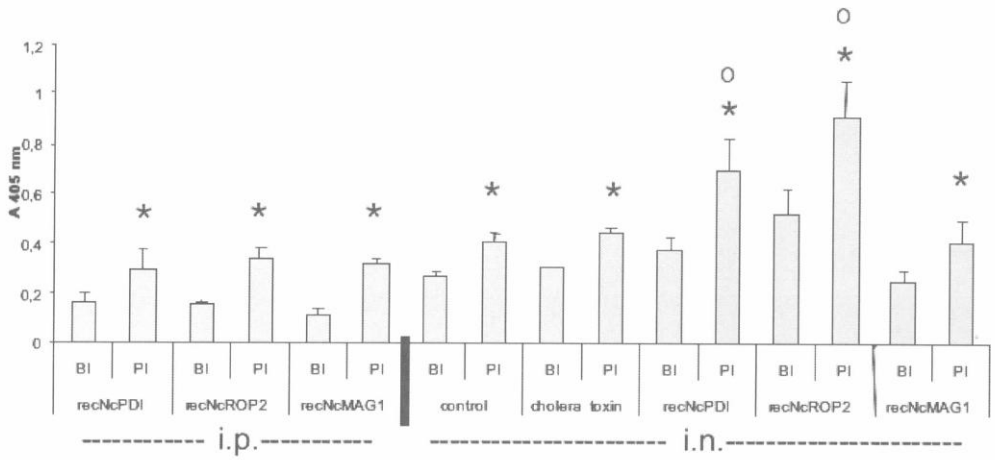

Fig. 5. Detection of IgA reacting with Nc-antigen by ELISA in sera in vaccinated mice; i.p. = intraperitoneal immunization, i.n. = intra-nasal immunization. BI = sera taken before infection, PI sera taken after infection prior to death. Error bars indicate S.D. * = $P<0{\cdot}05$ when comparing the differences between BI and PI sera of each group; ° = $P<0{\cdot}05$ as compared to the cholera toxin-treated group.

and the IgG2a-values in i.p. vaccinated and infected mice were significantly higher ($P<0{\cdot}05$) compared to i.n. vaccinated mice (see 3 and 3′ in Fig. 4).

Sera from immunized and experimentally infected mice were also tested for the presence of IgA reactive with Nc-antigen (Fig. 5) In all groups, IgA levels

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increased following infection, but highly significant increase ($P<0{\cdot}01$) in IgA was only visible in sera of mice vaccinated intra-nasally with NcROP2 and NcPDI.

DISCUSSION

In this study, we compared the efficacy of 3 distinct recombinant *N. caninum* antigens (NcROP2, NcMAG1 and NcPDI) against challenge infection with *N. caninum* tachyzoites in the C57BL/6 mouse cerebral infection model. For intraperitoneal vaccination, antigens were emulsified in saponin, for intra-nasal vaccination, antigens were suspended in cholera toxin. We here demonstrate that the efficacy of recNcROP2 vaccination was not dependent on the route of antigen delivery, while the efficacy of recNcMAG1 deteriorated upon intra-nasal application, and, in contrast, the efficacy of recNcPDI was highly enhanced. This study confirms earlier findings on intraperitoneally applied recNcROP2 that showed protection against both cerebral infection and fetal infection in mice, which was associated with a Th2-dominated immune response (Debache *et al.* 2008, 2009). Protection levels for NcROP2 in these earlier studies were higher compared to the experiments reported herein. On one hand, this could be explained by the slightly different age of the mice at the time-point of infection. On the other hand, there is, due to the nature of the parasite culture system, always the possibility of a certain degree of variation between individual experiments. In our study, we infected mice with *N. caninum* tachyzoites, which were freshly purified from *in vitro*-cultivated Vero cell monolayers, chosen at the moment of their putative highest infective stage (when most tachyzoites were still intracellular). Nevertheless, this time-point can be evaluated only qualitatively and it has been demonstrated earlier, that during extracellular maintenance of *N. caninum* tachyzoites will loose their infectivity within a few hours (Hemphill *et al.* 1996). Thus, a very small qualitative difference could be critical in terms of infectivity.

This study also showed that, in contrast to intraperitoneal delivery, intra-nasal application of recNcPDI resulted in strongly decreased cerebral parasite load, and in decreased numbers of viable tachyzoites in the brain. These effects were associated with an IgG1-biased humoral immune response in intra-nasally vaccinated mice as compared to intra-peritoneally vaccinated mice. This suggests that recNcPDI could represent a valuable vaccine candidate, if applied intra-nasally. Our findings confirm earlier studies on PDI in apicomplexan parasites. In human, natural IgA antibodies targeting *Toxoplasma gondii* PDI have been identified (Meek *et al.* 2002). Moreover, Liao *et al.* (2006) showed that NcPDI was present in excretory-secretory (ES) products, and that IgA antibodies obtained from individual cattle cattle tear samples recognized NcPDI. This suggested that the PDI-specific antibodies may be involved in defence against parasites. In addition, they confirmed earlier results (Naguleswaran *et al.* 2005; Müller *et al.* 2008) showing that PDI-specific inhibitors and NcPDI antiserum showed inhibitory effects on the growth of *N. caninum* tachyzoites *in vitro*, indicating that NcPDI could represent a valuable target for chemotherapy and/or vaccination.

To date, studies on protective immune responses against experimentally induced neosporosis in acute disease mouse models have produced conflicting results. On one hand, a number of investigations have emphasized that the protective effects of vaccines against challenge infection are associated with the development of a Th1-type immune response, dominated by IgG2a antibody production, natural killer (NK) cell proliferation, and increased interferon-gamma production (Khan *et al.* 1997; Baszler *et al.* 1999; Ramamoorthy *et al.* 2007*a*, *b*; Klevar *et al.* 2007; Ellis *et al.* 2008). However, others have shown that both humoral and cell-mediated immune responses were important components of protective immunity against *N. caninum* (reviewed by Hemphill *et al.* 2006; Innes and Vermeulen, 2006; Williams *et al.* 2007), and demonstrated that protective immune responses in mice vaccinated with antigen lysate (Baszler *et al.* 2000) or with purified native NcSRS2 (Haldorson *et al.* 2005) were accompanied by increased IgG1 production, implying the involvement of a Th2-type response. Increased IgG1 antibody responses were also found upon vaccination of mice with recNcMIC3 and recNcMIC1, both of which resulted in reduced cerebral infection rates compared to those in non-vaccinated controls (Cannas *et al.* 2003*a*; Alaeddine *et al.* 2005). Debache *et al.* (2008) showed that vaccination with recNcROP2 protected mice against acute neosporosis no matter whether an IgG1-biased (recNcROP2 emulsified in saponin adjuvants) or IgG2a-biased (recNcROP2 emulsified in Freunds adjuvants) humoral immune response was elicited. In addition, combined recNcROP2-NcMIC1-NcMIC3 vaccination protected mice against fetal infection following challenge during pregnancy, in contrast to vaccination with individual antigens, and this effect was associated with a Th2-biased immune response associated with increased serum IgG1 responses and a high IL-4/interferon-gamma ratio (Debache *et al.* 2009). However, all these studies were carried out by applying vaccine candidates intraperitoneally or subcutaneously.

Another attractive route for vaccine administration is intra-nasal application. The nasal epithelium represents highly vascularized tissue with numerous microvilli, which increase the resorbing surface. There is little proteolytic activity in the intra-nasal cavity, and intra-nasal immunization

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stimulates both humoral and cellular immune responses, mucosally and systemically. Thus, immune responses can be induced also at distant sites owing to the dissemination of effector immune cells (Porgador *et al.* 1997: Arakawa *et al.* 2003, 2005; Hirunpetcharat *et al.* 1998; Pinto *et al.* 2004). Finally, upon intra-nasal vaccination, the antigen is deposited directly to the appropriate immune-competent lymphoid tissues (Partidos *et al.* 2001; Byun, 2001). This results in the activation of T-cells, and the development of B-cells into IgA plasma cells (Partidos *et al.* 2001), resulting in a potentially efficient protection against acquired infection. Intra-nasal vaccination is, as for any immunization regimens, enhanced by the use of an appropriate adjuvant. We have here chosen cholera toxin produced by *V. cholerae*, since it is an effective mucosal adjuvant, which stimulates both Th1- and Th2-type immune responses by co-stimulation of the antigen-primed CD4- and CD8-T cells. In addition, the antigen-specific CD4-T cell-mediated production of IL-2, IL-4 and interferon-gamma is enhanced (Debard *et al.* 1996).

There are different examples of how intra-nasal vaccination has led to protective immune responses against other non-mucosal, penetrative pathogens, including *Plasmodium* and *Leishmania* (Arakawa *et al.* 2003, 2005; Hirunpetcharat *et al.* 1998; Pinto *et al.* 2004). In addition, intra-nasal immunization with *Toxoplasma gondii* TgSAG1 and non-toxic heat-labile enterotoxins protected mice against challenge infection against *T. gondii* (Bonenfant *et al.* 2001), and recently Igarashi *et al.* (2007) showed that intra-nasal immunization in Balb/c mice with recombinant TgROP2, TgGRA5 and TgGRA7 applied with cholera toxin induced partial protection against tissue cyst formation after oral infection with *Toxoplasma* tissue cysts. Others have used intra-nasal immunization using *T. gondii* RNA in mice and demonstrated protection against challenge infection (Dimier-Poisson *et al.* 2006). Intra-nasal vaccination has also been applied in sheep, employing proteins from crude extracts of *T. gondii* encapsulated into poly(D, L-lactide-co-glycolide, resulting in humoral and cell-mediated immunity (Stanley *et al.* 2004). Others have demonstrated protective activity against oocyst shedding in cats vaccinated with crude rhoptry protein preparations via the intra-nasal route (Garcia *et al.* 2007).

In contrast *to T. gondii*, the mouse is not a natural host for *N. caninum*, and experimental infection does not mimic the natural infection route. Thus, when using the Nc-1 isolate, tissue cyst formation in mice has not been observed. The Nc-1-isolate will also not readily form tissue cysts *in vitro* (Vonlaufen *et al.* 2004; Risco-Castillo *et al.* 2004; Aguado-Martinez *et al.* 2009), and only rarely in inbred and outbred mice (Dubey and Lindsay, 1996), thus it is very likely that tachyzoite-to-bradyzoite stage conversion has not taken place in these mice. Therefore, the outcome of infection in our mouse model is most likely related to the viability status of tachyzoite-stage parasites. In this respect, we could demonstrate not only a reduced overall cerebral parasite burden in mice vaccinated intra-nasally with recNcPDI, but also a significantly reduced number of viable tachyzoites in that group.

The latter is intriguing: surprisingly, mice vaccinated with recNcROP2 exhibited a reduced overall cerebral parasite burden, but not a reduced number of viable parasites in the CNS compared with the control groups. These relative differences between overall parasite numbers and numbers of viable parasites in the different treatment groups can be explained by the extensive proliferation of tachyzoites in the CNS of unvaccinated control-group mice, which lead to an increased proportion of non-viable parasite organisms, and also resulted in death of all animals. In contrast, intra-nasal vaccination with recNcROP2 and recNcPDI kept the overall cerebral parasite burden at a lower level, but in the case of recNcROP2-vaccinated mice, those parasites reaching the brain tissue remained largely viable, while in the case of recNcPDI-vaccinated mice, the parasites could still be largely eliminated, once they had crossed the blood-brain barrier.

It is not known why intra-nasal application of recNcPDI induced protection, and application of the same protein via the intra-peritoneal route did not. Most likely though, the marked switch from an IgG2-dominated to a more prominent IgG1 isotype response against Nc-antigen in intraperitoneally versus intra-nasally vaccinated animals could be detrimental to parasite survival, since a Th2-type response implies a lower expression of pro-inflammatory cytokines, and thus less immunopathology, and a higher chance for the host to clear or limit the infection by other immunological or physiological means. For recNcMAG1, the opposite effect could be noted, with intra-nasally vaccinated mice producing a significantly increased IgG2a response, which is indicative for a Th1-biased immune response, and this was associated with enhanced mortality in that group.

Clearly, further studies will be necessary to dissect these immunological and physiological events. However, our results are in line with those reported by Hiroi *et al* (1998), which showed that the nasal-associated lymphoid tissue is characterized by a Th0 environment, that can form a Th1 and/or Th2 phenotype, but the nasal passage was considered to be a Th2-dominant site with some Th1-type T cells, and humoral immunity leading to IgA production. Thus, in addition to a predominantly IgG1 response against Nc-antigen, serum-IgA antibodies were also significantly elevated in the intra-nasally NcROP2 and NcPDI-vaccinated mice following challenge infection, but not in mice vaccinated with NcMAG1.

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It is conceivable that these additional antibodies could cause a more pronounced inhibition of parasite host cell invasion or replication. In addition, the close proximity of the intra-nasal cavity to the cerebral tissue might also provide additional protection against cerebral infection. All these factors, together with, most likely, a number of unknown parameters in the intra-nasally vaccinated mice, resulted in significantly decreased cerebral infection rates.

The fact that fewer viable tachyzoites could be detected in the brain tissues of mice vaccinated with recNcPDI indicates that parasites, once they have crossed the blood-brain barrier, could still be eliminated.

In conclusion, this report represents the first study on intra-nasal vaccination against *N. caninum* infection in mice, and our findings suggest that recNcPDI is a promising vaccine candidate and should be further considered for studies where the potential of this vaccine formulation for protection against fetal infection will be investigated.

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2.4.Incorporation of recombinant *Neospora caninum* protein disulfide isomerase into chitosan nanogels for intraperitoneal or intranasal antigen delivery. Karim Debache, Christian Kropf, Catherine Schütz, Lisa J. Harwood, Peter Käuper, Thierry Monney, Carsten Laue, Kenneth C. McCullough, Andrew Hemphill. (2010)

Incorporation of recombinant *Neospora caninum* protein disulfide isomerase into chitosan nanogels for intraperitoneal or intranasal antigen delivery

Karim Debache[1], Christian Kropf[1], Catherine Schütz[2], Lisa J. Harwood[3], Peter Käuper[2], Thierry Monney[1], Carsten Laue[2], Kenneth C. McCullough[3], Andrew Hemphill[1]

[1]*Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland*

[2]*Medipol SA, PSE-B, CH-1015, Lausanne*

[3]*Institute for Virology and Immunoprophylaxis, Sensemattestrasse 293,CH-3147 Mittelhäusern, Switzerland*

Corresponding authors: Andrew Hemphill, Karim Debache
Institute of Parasitology, Vetsuisse Faculty
University of Berne
Länggass-Strasse 122
CH-3012 Berne, Switzerland
hemphill@ipa.unibe.ch; karim.debache@ipa.unibe.ch

Abstract

We have recently shown that the protection against challenge infection with *N. caninum* tachyzoites mediated by recombinant *N. caninum* protein disulfide isomerase (recNcPDI) in a murine disease model is dependent on the route of antigen delivery: intranasal vaccination employing cholera toxin adjuvant was highly protective, while intraperitoneal application of the antigen emulsified in saponin was not. In this study, we employed the two routes of antigen delivery and recNcPDI as a model antigen, to investigate the effects of nano-encapsulation of recNcPDI upon challenge infection of female Balb/c mice with *N. caninum* tachyzoites. The antigen was incorporated into alginate chitosan, or alginate-mannose-coated chitosan nanospheres. As a control, purified recNcPDI was applied without nano-encapsulation, emulsified as for the nanospheres, either in saponin (for intraperitoneal inoculation) or cholera toxin (for intranasal application). Experimental groups consisted of 10 mice each. Intranasal application of recNcPDI, followed by challenge infection, resulted in protection against disease in 9 out of 10 mice, while in the group receiving recNcPDI intraperitoneally, none of the 10 mice survived. Incorporation of recNcPDI into nanospheres significantly improved survival of intraperitoneally vaccinated mice (60-80% survivors), but the same effects were achieved with nanospheres without recNcPDI. Similar protection levels against *N. caninum* infection (80%) were achieved with nanospheres inoculated through the intranasal route, however, incorporation of recNcPDI into alginate-chitosan-coated particles resulted in 100% protection against disease. Quantification of the cerebral parasite burden in intranasally vaccinated experimental groups showed a significant reduction in parasite numbers in brain tissues of mice vaccinated with recNcPDI, recNcPDI-alginate-chitosan nanospheres, or alginate-chitosan nanospheres alone, but not in the group vaccinated with alginate-chitosan-mannose particles with or without recNcPDI. In intraperitoneally vaccinated groups, no significant differences in cerebral infections densities were measured, but there was a reduction in cerebral parasite burden in the groups vaccinated with recNcPDI conjugated to both types of nanospheres. Analysis of the humoral immune responses (IgG, IgG1, IgG2a, IgGA) prior and post-challenge, and cytokine mRNA expression profiles in spleen of control and experimental groups after challenge infection, indicated that incorporation of recNcPDI altered the antibody isotype responses and cytokine patterns in challenged animals without having a significant impact on protection.

Introduction

Neospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) is an obligate intracellular parasite which was first reported as an unidentified protozoan in dogs with encephalomyelitis and myositis (1). Later, the parasite was described and named by Dubey et al. (2) after demonstrating that dogs presenting severe neuromuscular symptoms were *T. gondii* sero-negative. However, *N. caninum* is, in some aspects closely related to *T. gondii*, in that it has a similar ultrastructure, expresses homologous antigens, can be cultured *in vitro* using similar techniques, will infect many different cell types, undergoes similar stages in its life cycle, and forms tissue cysts which allow the parasite to persist within its host for extended periods of time. On the other hand, there are clear differences in antigenicity, host spectrum, epidemiology, pathology and the final host (3). Meanwhile, *N. caninum* has been reported in various species of livestock including cattle, sheep, goats, horses and deer (4,5,6). At the present time *N. caninum* is not known to infect humans, and no clinical consequences have been reported, but it can cause serious disease mostly in cattle. Thus, this parasite has emerged as a significant veterinary public health problem, as it represents the most important abortion-causing pathogen, and is responsible for severe economic losses in both dairy and beef cattle throughout the world (7,8,9). Besides the loss caused by the abortion itself, reduced milk yield, premature culling, and reduced post-weaning weight gain in beef calves have to be considered (6). *N. caninum* may be transmitted to cattle following ingestion of oocysts via contaminated feed or water, or the parasite may be passed vertically from mother to foetus via the placenta. Oocysts can be shed in the faeces of acutely infected dogs or coyotes that acquired the parasite following the consumption of infected bovine tissue (7,8).

The economic importance of neosporosis in cattle has been the driving force for the development of strategies to prevent or control this disease. To date, different strategies, including selective culling, vaccination and chemotherapy have been proposed as countermeasures against abortion caused by *N. caninum* (9). Despite the fact that protective measures to prevent infection have been sought, there is no non-live vaccine capable of inducing complete protective immunity against neosporosis in cattle. In contrast, live vaccines such as attenuated strains of *N. caninum* (e.g. Nc Nowra) have been shown to elicit protective immunity in cattle and mice (10,11). Despite this efficacy, live vaccines are generally not considered to be an economically viable option, due to logistic problems in production and handling, short shelf life, and the risk of reverting to a more virulent phenotype. The commercialized non-viable vaccine (Neoguard™) based on tachyzoite extracts, which is

currently marketed in the Unites States of America, exhibits ambiguous results (12,13), and was shown to provide only partial protectivity (14). This could be due to the fact that a crude lysate contains immuno-protective, but possibly also immuno-modulatory or even immunosuppressive, components. Thus, other approaches have been focussing on recombinant antigens, which allow researchers to work with defined subsets of proteins that represent interesting targets. Most of the research on *Neospora* vaccines has been carried out employing murine models of cerebral infection (acute disease model) or models of fetal infection during pregnancy

One group of proteins/targets that is of interest for the development of a *N. caninum* vaccine are proteins that are secreted by the parasite at the onset of host cell adhesion and/or invasion. By targeting such molecules, one could possibly interfere in the vital mechanisms that lead to increased replication of the parasite within infected tissues. One of these antigens, NcPDI (protein disulfide isomerase), a protein found in the endoplasmatic reticulum and functioning as a chaperone by interacting with S-S bridges and/or thiol groups and thus ensuring proper protein folding, was also localized in the parasite micronemes and the parasite surface, and was earlier shown to be involved in the interactions between *N. caninum* tachyzoites and host cells (15). NcPDI was also found to represent a target for a group of drugs, thiazolides, which have a profound impact on *N. caninum* tachyzoites by chemical means (16,17,18). Subsequently, Debache et al (19) found that vaccination with recombinant NcPDI (recNcPDI) protected mice from cerebral infection by experimental *N. caninum* infection when applied intranasally, but not when applied intraperitoneally.

We have here exploited these unusal features of recNcPDI and used this protein as a model antigen to investigate the properties of a novel nanosized delivery system, consisting of chitosan-based nanogels, composed of chitosan nanospheres coated with alginate or with alginate-mannose, respectively. Chitosan, a copolymer of D-glucosamine and *N*-acetyl-D-glucosamine is a derivative of chitin, one of the most abundant polysaccharides in nature. This biodegradable biopolymer showed a high ability to traverse the mucosal barrier, to release antigens and to stimulate immunity (20). Nishimura et al. (21) and Shibata et al. (22) demonstrated the capacity of chitosan to up-regulate, to some extent, a number of macrophage functions. The presence of chitosan in a dendritic cell culture induced the expression levels of the co-stimulatory molecules CD86, CD40 and HLA-DQ V (23). The properties of chitosan polymers were intensely investigated in vaccination studies, however to the author's knowledge, there are no reports of intraperitoneal application. Chitosan nanosphere systems can promote entrapment and retention of antigens in local lymph nodes and might protect the

antigens from the adverse environment such as hydrolytic enzymes or low pH (24), an aspect that is particularly important in the enzyme-rich intestinal mucosa (25). The chitosan delivery system can present multiple copies of the antigen of interest on the surface, an effect which has been shown to be optimal for B cell activation (26). In a very recent study, a chitosan solution was explored as an adjuvant for subcutaneous vaccination of mice with a model antigen. It was found that chitosan enhanced the antigen-specific antibody titers over five-fold and antigen-specific $CD4^+$ lymphocytes proliferation over six-fold. Mechanistic studies performed by the same authors revealed that the antigen depot and a transient cellular expansion in draining lymph nodes induced by chitosan may explain its adjuvant properties (27). Chitosan nanoparticles have been used for numerous vaccine applications. This includes the delivery of nanocapsulated meningococcal C conjugate (28), diphtheria toxin (29) and tetanus toxoid (30,31) or chitosan was used by suspending the bulk powder in a solution of the meningococcal C conjugate vaccine (32), influenza vaccine (33,34) or was applied to surface modified PLGA microspheres containing hepatitis B vaccine for intranasal immunization (35).

In this study, recNcPDI was incorporated into these chitosan nanoparticles, and nano-encapsulated recNcPDI, non-encapsulated recNcPDI, and nanoparticles alone were applied intraperitoneally or intranasally prior to challenge infection with *N. caninum* tachyzoites. Analysis of the humoral immune responses prior and post-challenge, and cytokine expression in spleen of control and experimental groups after challenge infection, indicated that the nanoparticle-incorporation of a given antigen can alter the antibody isotype response and cytokine pattern in challenged animals without having a significant impact on protection.

2. Materials and methods

2.1. Culture and purification of *N. caninum*

Unless otherwise stated, all cell culture reagents were supplied by Gibco-BRL (Zurich, Switzerland) and chemicals were purchased from Sigma (St. Louis, MO, USA). *N. caninum* tachyzoites of the Nc1 strain (2) were maintained by serial passages in Vero cells during which time FCS was replaced with 5% immunoglobulin G (IgG)-free horse serum (HS). Likewise, Vero cells were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U of penicillin/ml and 50 ug of streptomycin/ml at 37°C/5% CO_2 in tissue culture flasks. Cultures were passaged at least once per week.

Parasites were harvested as described previously by Hemphill et al. (36). Infected cells were trypsinized, washed twice in cold RPMI 1640 medium, and the resulting pellet was resuspended in 2 ml cold RPMI 1640 medium. Cells were repeatedly passed through a 25 G-needle and liberated tachyzoites were purified by passage through Sephadex-G25 columns (Amersham Biosciences, Otelfingen, Switzerland), previously equilibrated with cold RPMI 1640 medium. The eluted parasites were centrifuged at 600g/10 min/4°C, resuspended in cold RPMI 1640 medium, and the parasite concentration was determined using a Neubauer chamber.

2.2. Expression and purification of recombinant protein disulfide isomerase (PDI)

Recombinant protein disulfide isomerase was cloned into the His-tag expression vector pET151 and expressed in *Escherichia coli* BL21 Star (Invitrogen, Carlsbad, Canada) as previously described (18,19). Purification of recombinant His-tagged PDI protein was performed under non-denaturing conditions using Protino Ni-IDA columns (Macherey-Nagel, Düren, Germany), as recommended by the manufacturer. The obtained recombinant protein was analysed by SDS-PAGE and Western blotting, and the protein concentration was measured employing the Bio-Rad protein assay using acetylated BSA as a standard. Following dialysis into PBS, the recombinant protein was stored at -20°C prior to use.

2.3. Nanogel vaccine preparation

2.3.1. Chitosan nanogel preparation

Chitosan nanoshperes were prepared by the ionic gelation of middle viscous chitosan with penta sodium triphosphate (TPP) (Fluka Sigma-Aldrich Ltd., Buchs, Switzerland). Briefly, one volume of a freshly prepared solution of 0,1% penta sodium triphosphate (TPP) (Fluka Sigma-Aldrich Ltd., Buchs, Switzerland) was filtered through a hydrophilic membrane (0.2 μm) (Sartorius AG, Sartorius, Göttingen, Germany), and was added under mild agitation at room temperature into nine volumes of filtered (0,1 μm) chitosan (0,1%, pH 4), resulting in spontaneous chitosan nanoparticle formation. Obtained nanospheres were stirred for 2h at room temperature, filtered through a membrane of 1,2 μm pore size and stored at 4°C until application.

2.3.2. Alginate-chitosan nanogel preparation and conjugation with recNcPDI

Alginate-coated chitosan nanospheres were obtained by mixing the chitosan particles 1:1 with demineralized water and dropwise, under mild agitation, adding the mixture into 0.1% alginic acid sodium salt (Medipol SA., Lausanne Switzerland). Further, the suspension was filtered (1,2 μm pore size) and stored at 4°C. Loading of alginate-chitosan nanopaticles with recNcPDI was performed by incubating a solution of recNcPDI with an alginate-chitosan nanoparticle suspension under mild agitation at room temperature for 2 hours. The resulting suspension was then filtered, concentrated to 58 μg/ml by nitrogen flow and stored at 4 °C.

2.3.3. Mannose-alginate-chitosan nanospheres and conjugation with recNcPDI

The coupling of polyethylene glycol (PEG; Iris Biotech GmbH, Marktredwitz, Germany) to the alginate necessary for further mannose linking was achieved by stirring of one volume 2% alginate-chitosan suspension for 20 min with one half volume of 0,1% EDC-HCl and 3% sulfo-NHS solution. The resulting mixture was incubated for 16 hours at room temperature with one volume of alfa-t-butyloxycarbonylamino-omega-amino poly (ethylene glycol) PEG-WM 5000 Da. After dialysis using a tubular membrane (Spectra/Por® Biotech Cellulose Ester; Spectrum Ls Europe B.V., Breda, The Netherlands) against 500 volume of demineralized water, the product was freeze-dried, weighed and placed in flat bottom beaker to be completely covered for 40 min at room temperature by TFA, thereafter TFA was removed under a nitrogen flow. The alginate-chitosan nanoparticle-PEG conjugate was suspended in sodium carbonate 0.1M pH 9 to 0,2% and added dropwise into 0,045 volume of DMSO containing 1 mg/ml alfa-D-mannopyranosyl-phenyl isothiocyanate (Fluka Sigma-Aldrich Ltd., Buchs, Switzerland) under mild agitation for 30 min. After an incubation for 16 hours at 4°C, the suspension was dialysed against 300 volumes of demineralized water. The product was filtered (0,2 μm pore size), freeze-dried and stored at 4°C. The loading of the nanoparticle alginate-mannose conjugate with recNcPDI was performed by incubation of a solution containing recNcPDI with a suspension of alginate-mannose-chitosan nanoparticles under mild agitation at room temperature during 2 hours. The resulting preparation was then filtered (1,2 μm pore size), concentrated to 54 μg/ml (protein concentration?) by nitrogen flow and stored at 4°C.

2.4. Evaluation antigen loading by SDS-PAGE and immunoblotting

The loading of recNcPDI antigen onto nanoparticles was evaluated by 12,5 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by silver staining and western blotting as previously described (37). For immunoblotting, rat anti-recNcPDI (18) diluted 1:1000 in PBS containing 0,3% BSA was used. The secondary antibody was an anti-rat IgG alkaline phosphatase conjugate (Promega, Madison, USA), which was applied according to the instructions provided by the manufacturer.

2.5. Vaccination and challenge of female Balb/c mice

130 female Balb/c mice (6 weeks of age) were purchased from Charles River Laboratories (Sulzheim, Germany) and were housed under conventional day/night conditions according to the standards set up by the animal welfare legislation of the Swiss Veterinary Office. On reaching 8-9 weeks of age, mice were checked serologically for the absence of anti-*N. caninum* immunoglobulins (pre-immune sera) and were randomly divided into 13 groups of 10 animals each. The mice were then vaccinated using two antigen delivery modes, namely intraperitoneal (i.p.) and intra-nasal (i.n.) application, as described below:

I.p. injection (100µl per mouse) was used for mice in groups 1-6. Group 1 was treated with saponin adjuvant (SAP; 100 µg/ml). Formulations of groups 2-6 were emulsified in SAP. Group 2 was immunized with recNcPDI (100µg/ml; 10PDI-SAP); group 3 was injected with alginate-chitosan nanospheres in (Alg-SAP;); group 4 was immunized with alginate-chitosan nanoparticles containing 100µg/ml recNcPDI (10PDI-Alg-SAP); group 5 was vaccinated with alginate-chitosan-mannose nanoparticles (Man-SAP; group 6 was vaccinated with alginate-chitosan-mannose nanospheres with incorporated recNcPDI (100µg/ml) (10PDI-Man-SAP)

I.n. delivery through the nares (20 µl) was performed for mice in groups 7-13 under mild isoflurane anaesthesia (19): group 7 received cholera toxin adjuvant (CT) at 250 µg/ml. Groups 8-13 received formulations emulsified in CT. Group 8 was immunized with recNcPDI (100 µg/ml; 10PDI-CT); group 9 was vaccinated with recNcPDI (10µg/ml; 1PDI-CT); group 10 was treated with alginate-chitosan nanoparticle suspension (Alg-CT); group 11 was immunized with alginate-chitosan nanoparticles containing recNcPDI (50µg/ml; (1PDI-Alg-CT); group 12 received alginate-mannose-chitosan nanoparticles (Man-), group 13 was vaccinated with recNcPDI (50 µg/ml) incorporated into alginate-mannose chitosan nanoparticles (1PDI-Man-CT).

These procedures were carried out on days 1, 15 and 30. On day 46 all animals were challenged by i.p. inoculation of $1x10^6$ freshly purified *N. caninum* tachyzoites. Monitoring of body weight was carried out in 3 days intervals from three days before challenge until the time of euthanasia. No non-vaccinated or non-treated groups were included, since in several similar vaccine trials carried out to date no spontaneous deaths of mice occurred under the conditions used (38,39,40,37,41). On day 84, the experiment was terminated, and all animals were sacrificed by CO_2-euthanasia. Animals exhibiting clinical signs of neosporosis (ruffled coat, apathy, hind limb paralysis) were euthanized at the onset of these clinical signs.

2.6. Sample collection

Pre-immune (PrI) and post vaccination blood (BI) were collected on days 0 and 44, respectively, by tail bleeding. On day 86, blood was drawn from the heart by cardiac puncture (post-infection; PI). The blood cells were centrifuged and sera were stored at -20°C until further analysis. Brains were dissected under aseptic conditions and stored at -20°C. The spleen of all mice was also frozen at -20°C in RNAlater reagent (Qiagen) for subsequently measurement of cytokines expression levels.

2.7. Quantitative real-time PCR using genomic brain DNA

Neospora-specific quantitative real-time PCR to determine the number of tachyzoites that had reached the cerebral tissue was performed as previously described (42,37,41). DNA extraction was performed using the DNeasy® Blood & Tissue Kit (Qiagen) (43). The DNA concentrations in all brain samples were determined by UV spectrophotometry (NanoDrop™, Thermo Scientific, Delaware, US), and were adjusted to 100 ng/ml with sterile Dnase-free water. The assessments of *N. caninum* tachyzoite loads were performed using the Rotor-Gene 6000 real-time PCR machine (Corbett Research, Qiagen). The parasite counts were calculated by interpolation from a standard curve with DNA equivalents from 1000, 100 and 10 parasites included in each run.

2.8. Assessment of antibody responses

To evaluate the humoral immune response, PrI, BI and PI serum samples were analysed by enzyme-linked immunosorbent assay (ELISA) as previously described (40,37,19). On one

hand, ELISA wells were coated with somatic *N. caninum* antigen-extract for the detection of *N. caninum*-specific immunoglobulin G (IgG), IgG1, IgG2a and IgA responses. On the other hand, antibody responses against recNcPDI (IgG), IgG1, and IgG2a, were assessed.

2.9. Assessment of cytokine expression levels in spleens

RNA from spleen was isolated using the RNeasy® mini kit (Qiagen), then the isolated RNA was incubated at 95°C for 3 min, and was converted to cDNA using the Omniscript® Reverse Transcription kit (Qiagen). DNA fragments of mouse glutamate dehydrogenase (GDH) and of four different cytokines (IL-4, IL-10, IL-12 and IFN-γ) were amplified from each cDNA using QuantiTec™SYBR®Green PCR kit (Qiagen) and primer pairs previously designed by Overberg *et al*. (45). The quantitative PCR was performed on a Rotor-Gene 6000 real-time PCR machine (Corbett Research, Qiagen). 4 µl of 1:10 diluted cDNA and 0.5 µM of forward and reverse primer were supplemented with 3 mM $MgCl_2$, yielding a final volume of 10µl. PCR was started by initiating the Hot-Start DNA polymerase reaction at 95 °C (15 min), followed by 50 cycles of DNA amplification (denaturation: 95°C, 0 sec; annealing: 60°C, 5 sec; extension: 72°C, 20 sec). Fluorescence was measured after each cycle at 80°C. To calculate the slope and the efficacy of the PCR, serial 10-fold dilutions of probes were included for each primer pair and a standard curve was generated. In order to compensate for variation in mRNA amounts, expression of the housekeeping gene glutamate dehydrogenase (GDH) was evaluated. Respective mean values from triplicate determinations were taken for the calculation of relative cytokine mRNA levels (cytokine mRNA level/GDH mRNA level) and given in arbitrary values.

2.10. Statistical analysis

Cerebral parasite burdens in different treatment and control groups were statistically assessed by Kruskal-Wallis multiple comparison, followed by Duncan's multiple range test to compare between 2 particular groups (P<0,05). Antibodies responses at different time-points (BI and PI) and cytokine expression levels were statistically evaluated by Student's t-test using the Microsoft Excel program and a value of P<0.05 was considered statistically significant. All analyses of variances were done using the NCSS Quick Start 2001 software.

3. Results

3.1. Analysis of recNcPDI nanoparticles

RecNcPDI was expressed in *E. coli* and purified by Ni^{2+}-affinity chromatography, yielding a single protein band that, by SDS–PAGE, migrated at approximately 55 kDa (Fig. 1). The loading of recNcPDI antigen was visualized using a polyclonal rat anti-recNcPDI antiserum (Fig. 1B). A major part of recNcPDI was degraded upon incorporation into alginate-chitosan nanospheres, but not when conjugated to alginate chitosan-mannose. The degraded protein was nevertheless recognized by the anti-recNcPDI antiserum.

3.2. Clinical signs

Following infection with *N. caninum* tachyzoites of mice, vaccinated either by i.p. or i.n. delivery, the clinical signs (ruffled coat, hind limb paralysis, circular movements, apathy, and inability to reach up for feeding) were first detected in the saponin control mice (SAP) on day 9 post-infection (p.i.) (see Table 1). From day 12 onwards, clinical signs of neosporosis were also apparent in the CT control group (CT). Subsequently, all mice of groups 1 and 2 (SAP and 10PDI-SAP), which were vaccinated i.p., succumbed to infection prior to termination of the experiment, latest on day 32 p.i., Premature death was also recorded for four mice in the group vaccinated with alginate-chitosan emulsified in saponin (Alg-SAP), three mice each in groups 4 (10PDI-Alg-SAP) and 13 (1PDI-Man-CT), and seven out of ten mice in the cholera toxin control group (CT). No clinical signs could be detected in group 11, vaccinated intranasally with recNcPDI-conjugated alginate-chitosan nanogel (1PDI-Alg-CT; see Table 1). The body weights of all mice were monitored in 3 days intervals, starting at the timepoint of the first vaccination, and they remained similar (at 22 ± 0.5 g), no matter which vaccination procedure was applied (data not shown). This indicated that vaccination had no adverse effects, and suggested that all immunization procedures were safe and did not impose stressful conditions that would interfere in the general metabolic functions of the animals.

3.3. Cerebral parasite loads

Quantitative real-time PCR of cerebral tissues from all animals was performed to investigate the cerebral parasite loads (Fig. 2). While infection of the CNS took place in all groups, there are some distinct differences in the actual infection intensities. In the i.p. vaccinated groups,

no differences were found between those groups receiving the antigen (10PDI-SAP, 10PDI-Alg-SAP, 10PDI-Man-SAP) and those groups receiving only the nanogels (Alg-SAP, Man-SAP; see Fig. 2A). In contrast, the intranasal delivery showed significantly lower (P<0.05) cerebral parasite burdens in the groups receiving recNcPDI (10PDI-CT, 1PDI-CT), no matter whether 1 or 10 µg recNcPDI was applied per mouse, and the group receiving alginate-chitosan or recNcPDI-alginate-chitosan nanospheres (Alg-CT, 1PDI-Alg-CT; see Fig. 2B). A slight, but non-significant, reduction in cerebral parasite burden was observed for the group vaccinated with recNcPDI incorporated into alginate-chitosan (1PDI-Alg-CT) compared to the group immunized with nanospheres alone (Alg-CT). No differences in cerebral parasite loads were obtained in mice vaccinated with recNcPDI incorporated into alginate-chitosan-mannose nanogel (1PDI-Man-CT), compared to the alginate-chitosan-mannose groups (Man-CT) or to the cholera toxin control group (CT).

3.4. Serology

Serological reactivities against *N.caninum* tachyzoite extract antigen (Nc. extract) and recNcPDI were measured by ELISA. Pre-immune (Pr-I) sera of all mice were negative for Nc. extract and recNcPDI (data not shown). Total IgG and IgG1 and IgG2a of sera taken after vaccination and prior to challenge infection (BI) and after challenge infection prior to euthanasia (PI), reacting with recNcPDI, are shown in Fig. 3, and respective antibodies reacting with crude *N. caninum* extract are shown in Fig. 4.

Serological reactivity against recNcPDI exhibited the following hallmarks (Fig. 3): (i) total IgG (as well as IgG1 and IgG2a) levels in sera taken pior to challenge were generally low in the groups immunized with adjuvants or nanoparticles only, but significant immune responses were elicited in the groups vaccinated with recNcPDI or recNcPDI-nanogel conjugates; (ii) IgG1 and IgG2a were significantly increased in almost all groups following challenge. However, no increase could be measured in both alginate-chitosan control groups (Alg-SAP and Alg-CT) and in the alginate-chitosan-mannose nanogel receiving groups (Man-SAP and Man-CT); (iii) of those groups receiving the antigen, no increase in IgG2a antibodies isotype after challenge infection was noted for the group intranasally vaccinated with 10µg recNcPDI in CT (10PDI-CT: Fig. 5B). Thus, with the exception of that group, the antibody isotype patterns suggest that a mixed Th1/Th2 type immune response has been elicited against recNcPDI.

Serological reactivity against Nc. extract showed the following characteristics (Fig. 4): (i) total IgG (as well as IgG1 and IgG2a) levels in sera taken pior to challenge were generally low in the groups immunized with antigen-free-reagents as well as in the groups immunized with antigen-containing vaccines; (ii) Following *Neospora* challenge, all mice elicited a significantly increased (P<0,05) total IgG response against Nc. extract antigens; (iii) all groups responded with a significant increase in both IgG1 and IgG2a levels (indicative for a mixed Th1/Th2 type response), with the exception of the group vaccinated intranasally with recNcPDI incorporated into alginate-chitosan nanoparticles, whose IgG2a levels did not increase (Fig 4B).

The IgA response was evaluated using an ELISA with crude *N. caninum* extract as antigen. Fig. 5 illustrates that already prior to infection, *Neospora*-specific IgA levels were elevated in all intranasally vaccinated groups compared to intraperitoneally immunized groups (P<0.05), no matter whether animals were vaccinated with recNcPDI, recNcPDI-nanoparticle conjugates, or nanoparticles alone. Following infection with $1x10^6$ *N. caninum* tachyzoites, the IgA levels were highly significantly increased (P<0.01) only in sera from intra-nasally immunized mice.

3.6. Cytokine transcript expression in spleen

Cytokine transcript levels in spleen of all mice were assessed by real-time PCR at the timepoint of euthanasia. This analysis demonstrated that in the groups 1-6 vaccinated intraperitoneally, IL-4 and interferon-gamma transcription occurred at similar levels in the saponin control group (SAP) and all experimental groups, although slightly less IL-4 transcripts were found in the two control groups receiving nanospheres with SAP compared to SAP alone. On the other hand, IL-10 and IL-12 transcription was increased compared to SAP controls in those groups receiving recNcPDI, nanospheres or recNcPDI-conjugated-nanospheres.

In the groups vaccinated intranasally, the cholera toxin control group (CT) exhibited a more pronounced IL-4, IL-10, and IL-12 transcription compared to the SAP group receiving saponin intraperitoneally, and these mRNA levels were similarly high in the other intranasally vaccinated groups, with the exception of IL-10, whose transcripts were less abundant in the two groups receiving alginate-chitosan-mannose (Man-CT) and recNcPDI-alginate-mannose-chitosan particles (1PDI-Man-CT). In addition, IL-4 transcripts were mostly less abundant compared to the CT control group in all mice vaccinated with nanosphere formulations.

Discussion

An efficient vaccine against neosporosis in cattle should sufficiently stimulate humoral and cell-mediated immune responses in order to prevent tachyzoite proliferation, tissue cyst formation, recrudescence, and transplacental transmission to the foetus (13,10). All these tasks can only be carried out by a vaccine that presents the immune system with the relevant targets to be inactivated. To date, this has only been achieved by attenuated *N. caninum* isolates used as live vaccines (10,11). However, application of a live vaccine poses a series of logistic and economical problems, which render inactivated and/or subunit vaccines much more attractive, provided a reasonable degree of protection against infection and disease can be achieved. Several research groups have reported on promising results when using recombinant antigens for vaccination studies, others have reported failures or even anti-protective effects (3,9). This shows that the antigen-repertoire of *N. caninum* contains protective, but also immuno-modulatory and even immuno-suppressive molecules, and these need to be defined and investigated. In addition, changing the route of antigen delivery and the adjuvants can significantly alter the efficacy of a given vaccine candidate (38,40,41).

Infection studies in cattle do not represent a cost-effective system to work with, and only a limited number of research groups have taken up the enormous task to work with cattle directly (12,9). Thus, in order to do studies in a laboratory setting, murine models been extensively used for proof-of-concept studies on how an immune response against a vaccine could limit parasite dissemination and pathology. The currently used experimental murine models include acute disease models, where challenge infections of non-pregnant mice leads to cerebral infection and finally death, and models of fetal infection, where mice are challenged during pregnancy, and the transplacental transmission of *N. caninum* tachyzoites can lead to stillbirth, abortion or birth of infected offspring (9).

In this study, we have employed the cerebral infection (acute disease) model in non-pregnant animals, to investigate the effects of incorporation of recNcPDI into nanogel formulations with respect to its efficacy as a vaccine. RecNcPDI has been previously shown to be ineffective when applied intraperitoneally emulsified in saponin adjuvants, but highly effective and mediating protection against cerebral infection and disease when applied intranasally in the presence of cholera toxin (19). The purpose of the current work was to use chitosan nanospheres, combined with different adjuvants (saponin and cholera toxin), as carriers for *E. coli* expressed recNcPDI antigen, and to investigate whether this would affect

the antigenic characteristics and efficacy as a vaccine upon intraperitoneal and/or intranasal vaccination.

SDS-PAGE and immunoblotting showed that recNcPDI was efficiently conjugated to both types of nanoparticles and retained its reactivity with a polyclonal anti-recNcPDI antiserum. However, a major portion of recNcPDI was degraded upon incorporation into alginate-chitosan nanoparticles (Fig. 1). Chitosan nanospheres of 300 - 600 nm are positively charged. The conjugation of these nanogels to recNcPDI was done by electrostatic interaction between the negatively charged soluble recNcPDI and the positively charged chitosan nanoparticles (46). Thus it is unlikely that modification of the properties of recNcPDI occurred by chemical means during the conjugation process, and therefore limited proteolysis was probably responsible for this effect. Although unlikely, we cannot exclude that this could have altered the antigenic properties or the protein, and this has to be taken into account when analyzing the results obtained with the experimental group vaccinated with recNcPDI-conjugated alginate-chitosan nanoparticles (10PDI-Alg-SAP and 10PDI-Man-SAP).

Protection against cerebral infection and disease in intraperitoneally vaccinated mice

When assessing the protection against cerebral disease in intraperitoneally vaccinated animals, we confirmed the findings that vaccination with recNcPDI antigen emulsified in saponin (3 times 10 µg per mouse) did not confer any protection, with all mice succumbing to disease (see Table 1). However, mice receiving either of the two types of nanogel formulations, or recNcPDI-conjugated nanospheres, exhibited increased survival rates (60-80%). Assessment of cerebral parasite burden by real time PCR (Fig. 2) showed that those groups receiving recNcPDI-coated nanospheres exhibited a lower, albeit not statistically significant, cerebral infection intensity compared to the groups receiving nanospheres without the antigen, which indicates that incorporation of recNcPDI into both types of nanogels could potentially lead to an improved immune response, hence the slight reduction in cerebral invasion by *N. caninum* tachyzoites.

Regarding humoral immunity, upon intraperitoneal vaccination of mice with naked nanospheres no significant IgG, IgG1 and IgG2a responses against recNcPDI (Fig. 3) were measured by ELISA. This shows that the nanospheres and recNcPDI do not share common epitopes. In contrast, substantial antibody responses against recNcPDI were found in mice vaccinated with recNcPDI-conjugated nanogels. Antibody responses against crude *N. caninum* antigen were also analyzed. (Fig. 4), and post-infection sera of mice vaccinated with

nanoparticles alone exhibited similar reactivities in terms of IgG and IgG isotypes as those sera taken from mice vaccinated with recNcPDI-conjugated nanoparticles. Thus, the nanoparticles by themselves acted as non-specific stimulators of the humoral immune response following infection, which is not surprising, and can potentially have some impact on the cerebral parasite burden. However, other studies in both mice and cattle have demonstrated that high antibody titers against *N. caninum* antigens are not necessarily indicators for protection (38,42,10). In any case, the results that (i) intraperitoneal vaccination with nanospheres and nanosphere-recNcPDI conjugates leads to increased survival and (ii) incorporation of recNcPDI into these particles reduces the cerebral parasite load, albeit not significantly, are promising. They warrant further consideration and analysis, especially in relation to optimization of the immunisation protocol and possibly with regard to alterations in the preparation of conjugated nanospheres to improve presentation of the antigen.

Protection against cerebral infection and disease in intranasally vaccinated mice

Assessments of intranasally vaccinated animals confirmed the earlier findings on the protection achieved with recNcPDI (19). However, we encountered restrictions in terms of how much antigen could actually be administred to the mice, with a limit to 1 µg of protein per injection per mouse. This was due to the substantial limitations in nanoparticle-loading, and the fact that the inoculation volume in mice amounted up to a maximum of 20 µl. However, we found that intranasal vaccination of mice with 1µg of recNcPDI did also confer a substantial protection against cerebral disease (90%), with similarly low cerebral parasite burden (see Table 1 and Fig. 2). Conjugation of recNcPDI to alginate-chitosan- or alginate-chitosan-mannose nanoparticles actually led to 100% and 70% survivors, respectively, however, 80% of the mice survived also by the intranasal application of nanoparticles alone, which speaks in favour of an unspecific adjuvants effect, similar to the groups vaccinated by intraperitoneal inoculation. Quantification of cerebral infection intensities in intranasally vaccinated animals, however, showed that alginate-chitosan nanogels were far more superior in limiting cerebral infection compared to alginate-chitosan-mannose nanospheres (Fig. 2), and a slightly, although not significantly, decreased parasite load was seen in the group vaccinated with recNcPDI-conjugated chitosan-alginate nanoparticles (1PDI-Alg-CT). On the other hand, this effect was not seen for recNcPDI conjugated to chitosan-alginate-mannose nanoparticles (1PDI-Man-CT). This is surprising, since theoretically the incorporation of mannose would lead to a better recognition of the complex by dendritic cells through the C-

type lectin receptor, followed by improved processing, MHC-class II mediated presentation of antigen-peptides on the surface, which in turn should ensure efficient and increased presentation to T lymphocytes. It is possible that the conjugation of recNcPDI has masked the relevant sites on the mannosyl residues, thus limiting the interaction with the C-type-lectin receptor. However, the actual interactions between dendritic cells and these nanoparticles need to be defined in more detail.

In another, recently published study, Nishikawa et al (47) employed a fetal infection model and recombinant NcGRA7 antigen entrapped in liposomes coated with mannotriose as a vaccine, and reported on a strong parasite-specific Th1 type immune response that increased the survival of dams and offspring, while vaccination with non-encapsulated NcGRA 7 had no effect. This is in contrast to our study, where conjugation of recNcPDI to nanoparticles has no obvious advantages when employing intranasal vaccination, and only marginal benefits in terms of limiting invasion of the CNS can be seen upon intraperitoneal administration of the vaccine (see above).

The antibody responses in sera of intranasally vaccinated mice confirmed the earlier finding obtained by Debache et al. (19), which had demonstrated that the immune response against recNcPDI emulsified in CT was characterized by a IgG1-biased (and therefore potentially Th2-dominated) immune response. However, alginate-chitosan nanoparticles and recNcPDI-conjugated nanoparticles exerted similar effect in terms of protection, and the corresponding humoral immune responses showed a mixed IgG isotype pattern. It is likely that the significantly increased IgA responses in intranasally vaccinated mice also contributed to the increased efficacy of vaccination (see Fig. 5). However, in the case of the two groups receiving alginate-chitosan-mannose nanoparticles (Man-CT) and corresponding recNcPDI-conjugated nanoparticles (1PDI-Man-CT) other factors, most likely related to cellular immunity, must account for the high infection intensity in the CNS.

Others have shown earlier that protective immune responses against experimentally induced neosporosis in acute disease mouse models have been mainly associated with the development of a Th1-type immune response, dominated by IgG2a antibody production, and natural killer (NK) cell proliferation with increased IFN-γ production (48,49,47). However, there are also reports on protective effects achieved by Th2-type responses in acute disease (39,40,37,41) and in fetal infection models (50,41). All these observations support the idea that both Th1 and Th2-driven immune mechanisms can limit disease, at least in the mouse model.

Cytokines

In order to obtain more information on the type of immune response (Th1 or Th2) that would lead to protective or non-protective effect in vaccinated and non-vaccinated mice, we analyzed the level of transcription of mRNA in splenic tissue coding for interferon-gamma and IL-12 (Th1 cytokines) and IL-4 and IL-10 (Th2 cytokines) at the timepoint of euthanasia by real time PCR.

In intraperitoneally vaccinated mice, the expression levels of cytokine transcripts clearly indicated that a mixed Th1/Th2 response was mounted upon infection, as the ratio between interferon-gamma and IL-4 transcript levels was similar in all groups, with the exception of the two groups vaccinated with nanoparticles alone, where clearly a significantly lower IL-4 expression was noted in relation to interferon-gamma (Fig. 6). Thus, the presence of recNcPDI in the nanoparticle conjugates led to IL-4 expression levels similar to what was found in spleens of mice vaccinated with recNcPDI and saponin adjuvants alone. However, in relation to the saponin control group, the levels of IL-10 and IL-12 transcripts were increased in all vaccinated groups, with a clear bias for IL-12, a Th1 cytokine, except in the group vaccinated with recNcPDI conjugated to alginate-chitosan-mannose nanoparticles.

In intranasally vaccinated mice, the diminished cerebral infection intensity is also associated with a mixed Th1/Th2 cytokine response. However, in contrast to the intraperitoneal vaccination, where the IL-10 levels are mostly lower than IL-12 transcript levels, intranasal vaccination mostly leads to higher levels of IL-10 compared to IL-12 (Fig. 6), which could indicate that an inherent Th2-biased immune response could be responsible for protection against disease (Fig. 6). This confirms earlier findings by Debache et al (19) who, based on the antibody isotype profile, suggested that a Th2-biased immune response was responsible for the protection mediated by intranasal vaccination with recNcPDI. However, the cholera toxin control group (CT) displays a similar cytokine profile, and no significant protection is achieved with cholera toxin alone. Thus, other factors related to other aspects of the immune response are responsible for the protective effects seen in this study. In contrast, in the two groups vaccinated intranasally with alginate-chitosan-mannose (Man-CT) and the corresponding recNcPDI conjugate (1PDI-Man-CT), both exhibiting high cerebral parasite burdens, the transcription of IL-4 mRNA is almost completely down regulated, the interferon-gamma transcription is at the same level as in the cholera toxin control group, and the IL-10 to IL-12 ratio suggests that a predominant Th1 type cytokine patterns is associated with increased infection intensity.

In conclusion, this paper reports on the use of chitosan-based nanoparticles as a delivery system for the vaccine candidate recNcPDI in an acute disease model for nesosporosis, employing intraperitoneal and intranasal antigen delivery. We showed that upon intraperitoneal vaccination, incorporation of recNcPDI into both types of nanoparticles exhibited a noticeable beneficial effect, in that clinical signs were reduced and the cerebral parasite burden was diminished. Although the reduction in CNS infection intensity was not significant, this could open the way for the development of an injectable formulation of a recNcPDI-based vaccine in the future. On the other hand, upon intranasal application of the nanoparticle bound antigen, only the alginate-chitosan conjugate appeared to have a noticeable effect in terms of reducing cerebral parasite load to a similar level as the antigen itself. Protection was mostly associated with a mixed Th1/Th2, or a Th2-biased, IgG and cytokine profile, but further studies are necessary to elucidate the potential mechanisms that lead to protection. We will explore the use of nanoparticle-based vaccines in the mouse model of fetal infection, and other promising antigens will also be evaluated following conjugation to nanoparticles. Thus, chitosan-based nanoparticles represent an interesting platform for intranasal and intraperitoneal vaccination approaches to limit the disease caused by *N. caninum* infection.

Acknowledgements

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Delivery Mode	Mouse groups	Time of death (Day p. i.)	Number of surviving mice	Tachy. Load	
				CNS neg.	<10 tachy./R
Intra-peritoneal	SAP	9/16/18/20/21/27/30/32/	0	0	0
	10PDI-SAP	12/15/16/16/18/20/21/32/	0	0	0
	Alg-SAP	18/18/21/21/	6	0	2
	10PDI-Alg-SAP	9/21/24/	7	0	3
	Man-SAP	15/21/	8	0	4
	10PDI-Man-SAP	21/24/	7	0	4
Intra-nasal	CT	12/15/18/21/27/	3	0	0
	10PDI-CT	15/22/	8	2	3
	1PDI-CT	22/	9	3	1
	Alg-CT	22/	8	0	0
	1PDI-Alg-CT	-	10	3	0
	Man-CT	22/	8	0	3
	1PDI-Man-CT	15/24/25/	7	1	2

Table 1: Survival of mice and cerebral tachyzoite load in the different experimental groups. Mice were challenged by i.p. inoculation with 1 x 10^6 *N. caninum* tachyzoites, and the experiments was terminated at 40 days p.i. . Abbreviations: Tachy. Load = cerebral parasite burden; CNS neg. = no parasites detected in the CNS by PCR; <10 tachy./R = less than 10 tachyzoites detected per reaction, negative central nervous system (CNS neg.) and less than 10 tachyzoites per reaction detected by real-time PCR.

Figure legends

Fig. 1. SDS-PAGE and immunoblot of recNcPDI and recNcPDI-nanogels. (A) SDS-PAGE stained with silver, showing purified recNcPDI (lane 1), alginate-chitosan nanoparticles (lane 2), recNcPDI integrated into alginate-chitosan nanoparticles (lane 3), alginate-chitosan-mannose nanoparticles (lane 4) and recNcPDI encapsulated into alginate.chitosan-mannose nanoparticles (lane 5) (B) Corresponding Western blot stained with polyclonal rat anti-recNcPDI antibodies. M = pre-stained molecular weight markers PageRuler® (Fermentas, Switzerland).

Fig. 2. Quantification of cerebral parasite burdens by quantitative real time PCR. (A) Box plot of cerebral *N. caninum* infection resulting from intraperitoneal vaccination and (B) from those mice vaccinated through the intranasal route. The numbers on the y-axis correspond to the parasite count per 100 ng of genomic DNA. Marked by * are the significantly (P<0,05) reduced cerebral parasite burdens in those mice as compared to the cholera toxin control group. Error bars indicate ±S.D.

Fig. 3. Assessment of recNcPDI-specific total IgG and subclass (IgG1, IgG2a) responses in mice. Sera are taken after vaccination but prior to infection (BI) and after challenge infection at the timepoint of euthanasia (PI). Sera of mice vaccinated through intraperitoneal route are shown in (A) and sera of mice vaccinated through the intranansal route are shown in (B). ELISA wells were coated with recombinant PDI. Values are indicated as A405 nm ±SD. *P<0.05 as compared to BI sera.

Fig. 4. Total IgG and subclass (IgG1, IgG2a) responses against Nc. extract. Mice were vaccinated either through intraperitoneal (A) or intranasal (B) delivery routes. Serum samples were taken either following vaccination but prior to infection (BI) or following infection prior to euthanasia (PI). ELISA wells were coated with Nc. extract Values are indicated as A405 nm ±SD. *P<0.05 as compared to BI sera.

Fig. 5. Detection of IgA reacting with Nc. extract. i.p. = sera of intraperitoneally vaccinated mice, i.n. = sera of intranasally vaccinated mice. BI = sera taken before infection, PI sera taken after infection prior to euthanasia. Error bars indicate SD. *=P<0.01 when comparing

the differences between BI and PI sera of each group; °=P<0.01 when comparing the difference between BI sera of each mode of antigen delivery; ^=P<0.01 when comparing the difference between PI sera of intranasal and intraperitoneal application.

Fig.6. Cytokine transcript levels in spleens processed at the time point of euthanasia. Spleens were harvested at 40 post-infection days and the expression of IL-4, IL-10, IL-12 and IFN-γ mRNA was assessed by real time PCR. Arbitrary units correspond to the expression levels of the reference gene GDH. Error bars indicate standard errors. Statistical comparisons were made between IL-4 and IFN-γ of the same group (*P<0.05).

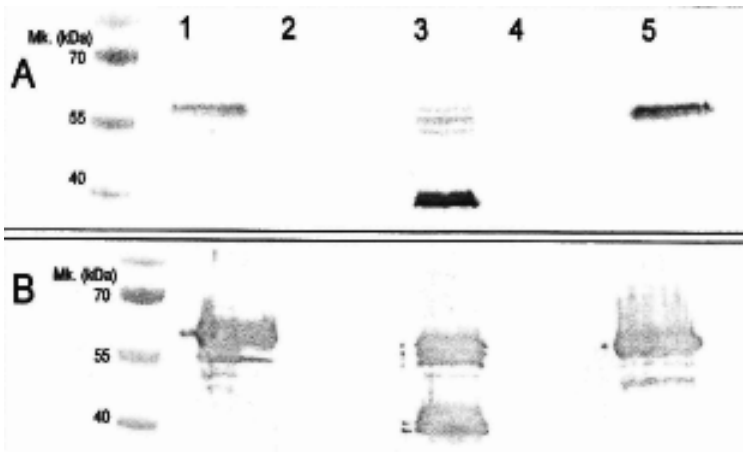

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Figure 1

Delivery Mode	Mouse groups	Time of death (Day p. i.)	N° of surviving mice	Tachy. load	
				CNS neg.	<10 tachy./R
Intra-pritoneal	Saponin (SAP)	9/16/18/20/21/27/30//32/	0	0	0
	10 µg PDI-SAP	12/15/16/18/20/21/32/	0	0	0
	Alg in SAP	18/21/21/	6	0	2
	10 µg PDI-Alg	9/21/24/	7	0	3
	Mann-SAP	15/21/	8	0	4
	10 µg PDI-Mann	21/24/	7	0	4
Intra-nasal	Cholera toxin (CT)	12/15/18/21/27/	3	0	0
	10 µg PDI-CT	15/22/	8	2	3
	1 µg PDI-CT	22/	9	3	1
	Alg in CT	22/	8	0	0
	1 µg PDI-Alg-CT	-	10	3	0
	Mann-CT	22/	8	0	3
	1 µg PDI-Mann-CT	15/24/25	7	1	2

Table 1: Survival of mice and cerebral tachyzoite load in the different experimental groups. Mice were challenged by i.p. inoculation with 1 x 10^6 *N. caninum* tachyzoites, and the experiments was terminated at 40 days p.i.. Abbreviations: tachyzoite load (Tachy. Load), negative central nervous system (CNS neg.) and less than 10 tachyzoites per real-time PCR reaction (<10 tachy./R).

Table 1

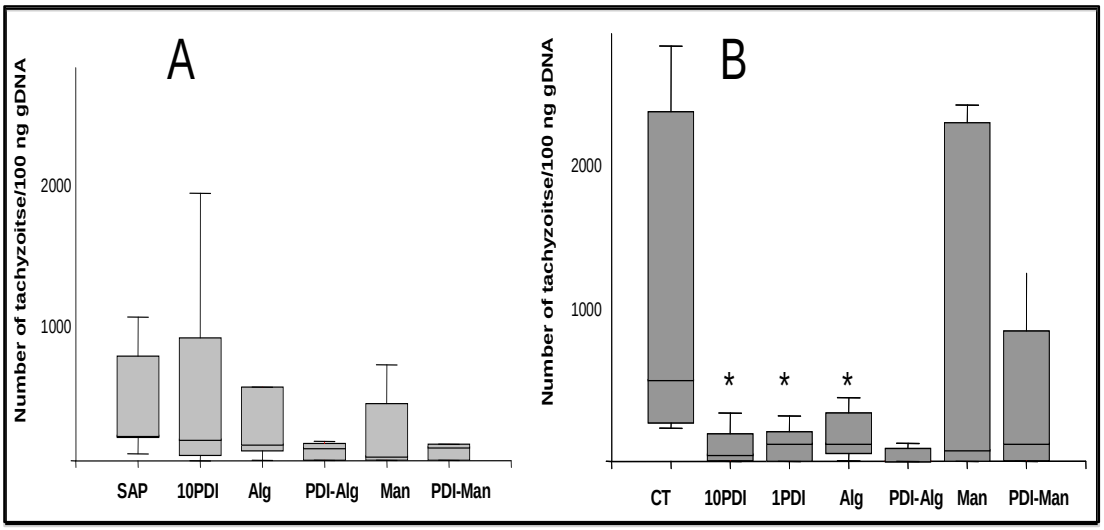

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Figure 2

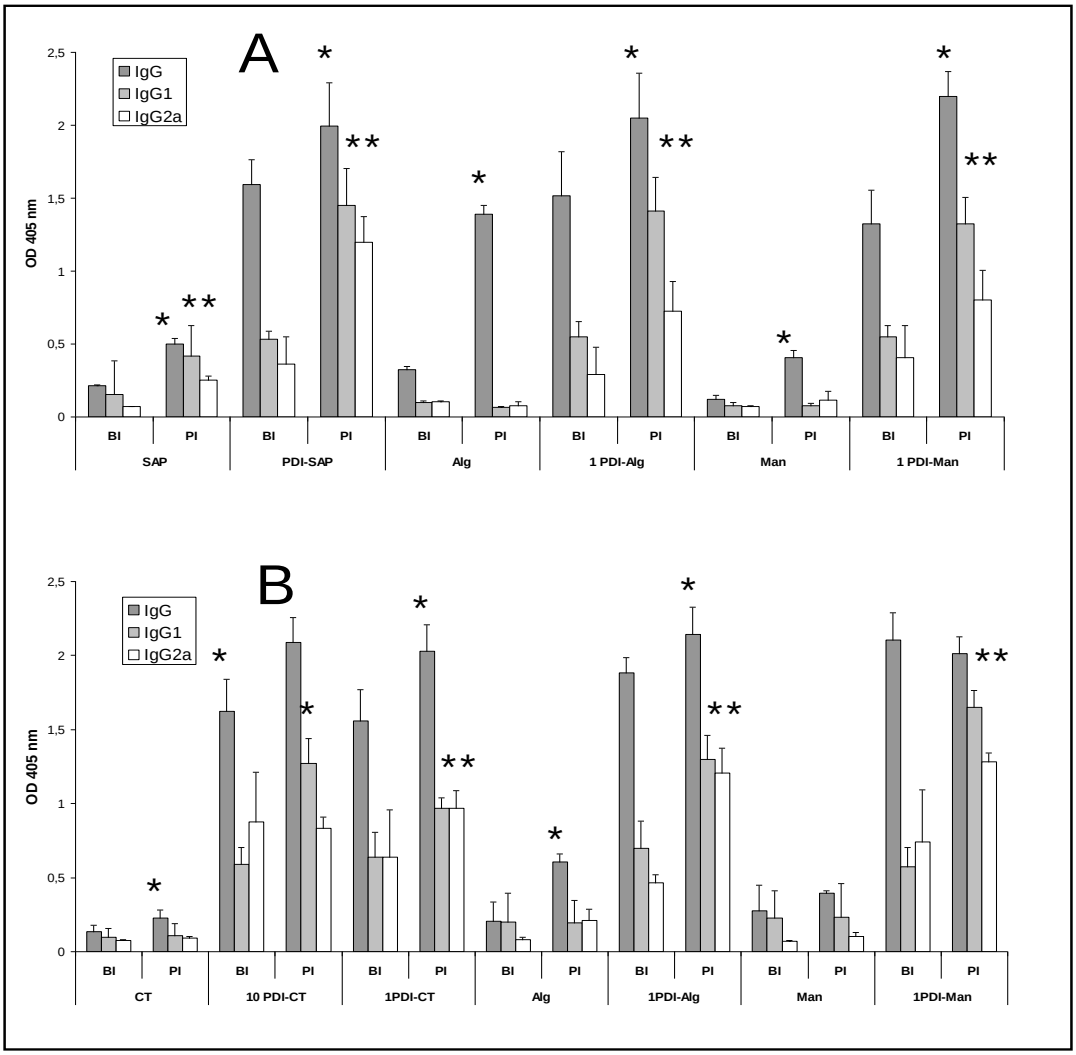

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Figure 3

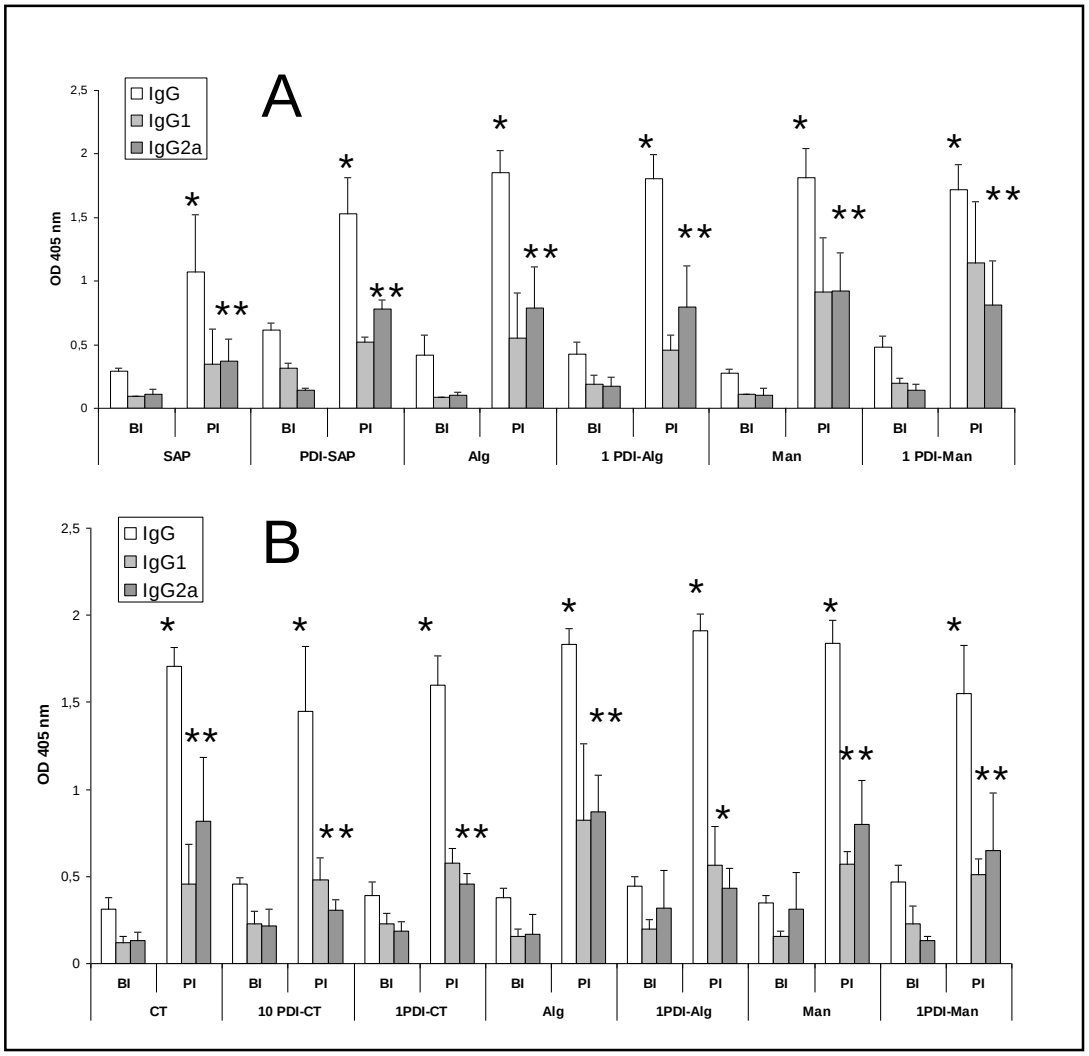

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Figure 4

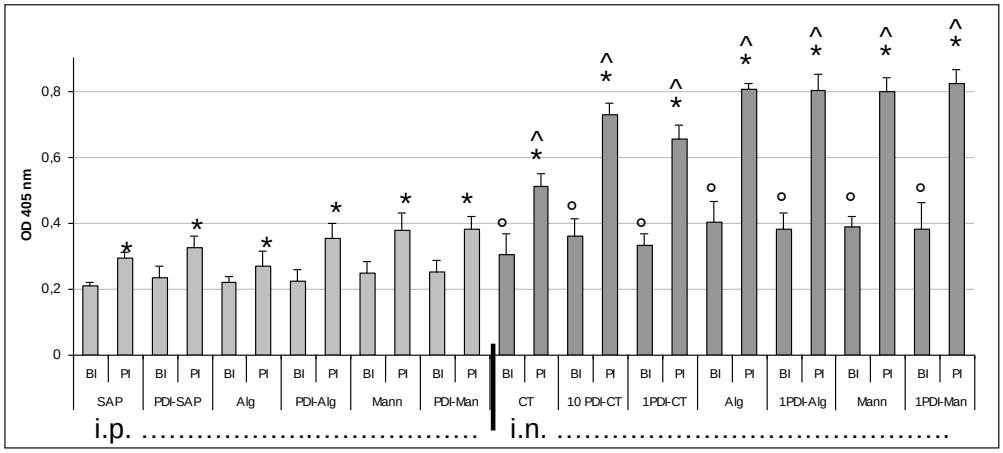

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Figure 5

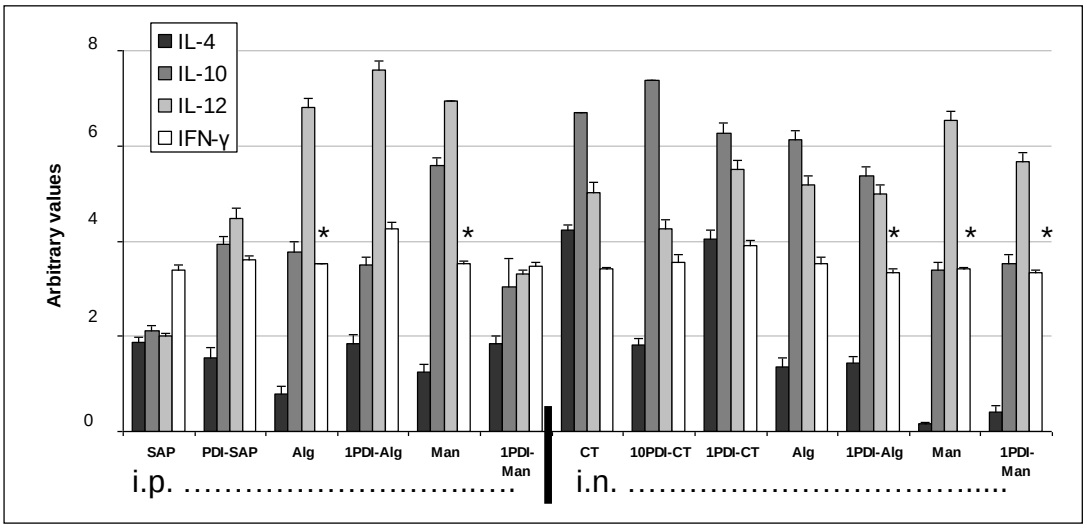

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Figure 6

3. Discussion

Neosporosis is reported as the leading cause of abortion in cattle (Hasler et al., 2006a,b; Hemphill and Gottstein, 2000; Dubey et al., 2007), thus the disease represents an important veterinary health problem and is of high economic significance especially in cattle, as it is also associated with reduced milk yield (Hernandez et al., 2002), premature culling (Thurmondand Hietala, 1996) and reduced post-weaning weight gain in beef calves (Reichel, 2000; Pfeiffer et al., 2002; Hasler et al., 2006a,b). The economic loss has led to research on the development of strategies for prevention of *N. caninum* infection. Vaccination and chemotherapy have been identified as economically promising options (Hasler et al., 2006a,b). However, the disadvantage of the chemical treatment option is that the milk or the meat of the treated animal would probably not be safe or would contain residues of a potential drug (Dubey et al., 2007) whereas the vaccination strategy is cheaper and safer. At the present time however, there is no vaccine capable of inducing complete protective immunity in cattle and the only way to get rid of an infection is culling of infected animals.

The parasite can only survive, proliferate and proceed during most stages of its life cycle as an intracellular parasite, thus the processes which govern host cell invasion and intracellular development are of crucial importance, and they represent potential targets for vaccination and/or chemotherapeutic intervention (Hemphill et al., 2006a, b). Many of these potential targets are located either on the parasite's surface or within secretory organelles such as micronemes, rhoptries and dense granules. So far, a limited number of recombinant proteins have been investigated as vaccine candidates against neosporosis. These include mostly immuno-dominant proteins such as the major surface antigens and proteins localised in secretory organelles. Therefore, the purpose of this study has been to investigate the involvement of different *N. caninum* proteins in the tachyzoite host cell infection process, to enhance the capacity of the post-vaccination immune response, and to evaluate the protective efficacy of bacterially expressed recombinant $(His)_6$-tagged antigens in the murine cerebral infection model with assessment of several vaccine protocols, antigen concentrations, types of adjuvant and routes of administration.

Mice have been widely used as laboratory models for proof-of-concept studies involving vaccine candidates, in spite of the fact that cattle is the natural host to *Neospora* and murines and bovines exhibit somewhat different immunological characteristics (Innes, 2007; Innes and Mattsson, 2007). In fact, various criteria favour murine over other animal models to *N. caninum* in-vivo study. Firstly, the use of mice is cheaper and easier regarding both the daily

monitoring and administration or removal. Secondly, the gestation period of murines (21 days) is short compared with cattle (9 months) which makes congenital transmission experiments easier and it is possible to gain time. Furthermore, it is possible to increase the number of animals per trial to generate data that can be analysed statistically. Furthermore, mouse models mimic acute and chronic infections, and develop different clinical symptoms and particularly the production of tissue cysts (Quinn et al., 2002; Collantes-Fernàndez et al., 2004; Rettigner et al., 2004). However, using murine models for the study of protective immune responses against experimentally induced neosporosis in acute disease has produced conflicting results. On one hand, a number of investigations have emphasized that the protective effects of vaccines against the challenge infection are associated with the development of a Th1-type immune response, dominated by IgG2a antibody production, natural killer cell proliferation, and increased IFNγ production (Khan et al. 1997; Baszler et al. 1999; Ramamoforthy et al. 2007a, b; Klevar et al. 2007; Ellis et al. 2008). However, others have shown that both humoral and cell-mediated immune responses were important in reducing clinical signs of neosporosis and the cerebral parasite burden (Innes and Vermeulen, 2006; Williams et al. 2007), and demonstrated that protective immune responses in mice vaccinated with antigen lysate (Baszler et al. 2000) or with purified native NcSRS2 (Haldorson et al. 2005) were accompanied by increased IgG1 production, implying the involvement of a Th2-type response. Increased IgG1 antibody responses were also found upon vaccination of mice with recNcMIC3 and recNcMIC1, both of which resulted in reduced cerebral infection rates compared to those in non-vaccinated controls (Cannas et al. 2003a; Alaeddine et al. 2005).

In this work, our first finding showed that intraperitoneal vaccination with single recombinant NcROP2 protein which is one of the rhoptry family antigens that are involved in a variety of cellular functions related to host cell invasion, formation of the parasitophorous vacuole and parasite–host cell interplay, can induce protection in mice against acute neosporosis. In previous studies, similar protection was induced in mice vaccinated with plasmid encoding TgROP2 against a lethal challenge with *T. gondii* cysts, in which the number of brain cysts was lowered compared to controls (Vercammen and D'Hooghe, 2000; Leyva et al., 2001). This diminution of the infection rate might be due to the inactivation by anti-NcROP2 antibodies. The protein kinase homologue identified in NcROP2 is known to regulate the majority of cellular pathways, especially those involved in signal transduction. In addition, the protection is achieved no matter whether an IgG1-biased (recNcROP2 emulsified in saponin

adjuvants) or IgG2a-biased (recNcROP2 emulsified in Freunds adjuvants) humoral immune response was elicited.

Secondly, we focussed on the effects of prevention of both foetal *N. caninum* infection and *N. caninum*-induced abortion using intraperitoneal NcROP2 as single vaccine or mixed with microneme proteins, which play a crucial role in the initial host cell interaction / invasion process, where the parasite actively penetrates the host cells by pulling transmembrane micronemes-complexes and/or the moving junction towards its posterior end, and thereby invaginates the host cell surface membrane to create a vacuole. Moreover, Cannas et al. (2003b) and Alaeddine et al. (2005) demonstrated the partial protection against *N. caninum* challenge infection obtained in vaccinated C57BL/6 mice with respectively his-tagged recNcMIC3 and GST-tagged recNcMIC1 micronemes proteins. In this study, we showed that combined recNcROP2-NcMIC1-NcMIC3 vaccination protected mice against foetal infection following challenge during pregnancy. This effect was associated with a Th2-biased immune response related with increased serum IgG1 responses and a high IL-4/interferon-gamma ratio. Our results confirmed the importance of micronemes proteins for the infection process as previously extensively demonstrated. Cerede et al. (2005) showed that single deletion of the TgMIC1 gene decreased invasion of *T. gondii* in fibroblasts in vitro, whereas TgMIC3 deletion had no effect. Individual disruption of TgMIC1 or TgMIC3 genes slightly reduced virulence in mice, whereas double-depleted parasites were severely impaired in virulence. The importance of MIC proteins as vaccine candidates against *T. gondii* infection was also documented by Ismael et al (2003), showing that intramuscular DNA vaccination of mice with TgMIC3 elicited a strong specific immune response and provided effective protection against *T. gondii* infection. In fact, the *T. gondii* MIC1-3 double knock-out mutant was a powerful vaccine and protected mice against chronic and congenital toxoplasmosis (Ismael et al., 2006). However, this protection was achieved in a murine model that was vaccinated and subsequently infected during pregnancy, thus mimicking the exogenous transplacental infection. This situation is less common in the natural transmission of neosporosis in cattle. Further studies of cerebral infection rates and immune responses in endogenous transplacental infection in murine models, are needed. However, the murine model remains an important model for the initial validation of the concept of protection induced by new vaccine candidates and the protection results obtained with this model should be interpreted with the utmost care (Reichel and Ellis. 2009).

As mentioned, the above studies were carried out by applying vaccine candidates intraperitoneally, testing two different adjuvants. Another attractive route for vaccine

administration is intranasal application. This tissue is well known as highly vascularized with numerous microvilli, lower proteolytic activity in the intranasal cavity, and intranasal immunization stimulates both humoral and cellular immune responses, mucosally and systemically (Porgador et al. 1997: Arakawa et al. 2003, 2005; Hirunpetcharat et al. 1998; Pinto et al. 2004). In the third experiment of the present work, this intranasal delivery route was investigated in comparison with the classical intraperitoneal route using protein disulfide isomerase (NcPDI). Naguleswaran et al. (2005) showed earlier the capacity that anti-NcPDI antibodies are able to reduce adhesion of parasites to the host cell surface and inhibition of NcPDI activity either with a number of sulfhydryl blockers or the PDI inhibitor bacitracin had a negative influence on the tachyzoite-host cells interaction (Müller et al. 2008). NcPDI was mixed each time with different appropriate adjuvant formulations. Intraperitoneal vaccination of mice with NcPDI induced a strong humoral immune response but was completely ineffective to impair the cerebral parasite infection. However, when NcPDI, suspended in cholera toxin adjuvants, was administered by intranasal inoculation, there was a pronounced protective effect with 90% survivors, all with significantly decreased cerebral parasite burden (Debache et al., 2009a). Probably different immunological and physiological events related to intranasal application are involved to achieve this decreased cerebral infection rate compared to the intraperitoneal administration such as the highly vascularized tissue, less proteolytic activity and stimulation of both mucosal and systemic immune responses. Moreover, the protection obtained in this delivery route encourages further studies focused on the NcPDI antigen that might modify the cholera toxin adjuvant. The study will be performed with similar experiments but using enzymatically non-active or inactivated NcPDI antigen obtained either by heating or chemical treatment.

Recently, incorporation of recombinant proteins into biodegradable nanoparticles for efficient uptake and processing by dendritic cells has emerged as promising means of vaccinations. In this regard, we performed an experiment using biodegradable alginate-chitosan (D-glucosamine polymer) coated directly with NcPDI or indirectly through mannose. The adsorbed mannose aims to be efficiently recognized by dendritic cells through the C-type lectin receptor which will lead to phagocytosis and proteolytic degradation and MHC-class II mediated presentation of antigen-peptides on the surface, ensuring enhanced and efficient presentation to T cells. This could significantly improve the immune response to a given vaccine candidate, therefore nanoparticle-coupled NcPDI was assessed in the acute disease model. Female Balb/c mice were treated by the following two antigen delivery modes, namely intraperitoneal and intranasal application, by three doses with two-week intervals. Only

NcPDI either suspended with cholera toxin or loaded into alginate nanoparticle (delivered through the intranasal route), showed significant reduction of the parasite infection rate compared with the adjuvant alone. This was accompanied by a Th2-biased immune modulation. The achieved cerebral protection associated with a Th2-dominated immune response is in accordance with the earlier findings on intraperitoneally applied recNcROP2 that was shown to confer protection against both cerebral infection and fetal infection in mice (Debache et al. 2008, 2009b). However, other investigations have emphasized that the protective effects of vaccines against challenged murine models are associated with the development of a Th1-type immune response (Khan et al. 1997; Baszler et al. 1999; Ramamoorthy et al. 2007a,b; Klevar et al. 2007; Ellis et al. 2008).

The conclusions from these studies are the following:

- Either intraperitoneal or intranasal vaccination with NcROP2, an antigen previously shown to be involved in host cell interaction, resulted in protection against cerebral infection.
- For other antigens, but not NcROP2, protection was dependent on the route of antigen delivery. This concerns e.g. NcPDI (intraperitoneal: no protection, intranasal: good protection).
- Vaccines comprised of antigen combinations, most notably the NcROP2-NcMIC1-NcMIC3-combination, induced increased protection in the cerebral and the foetal infection models.
- Alginate-chitosan-based nanogels showed a sufficient capacity to boost the immune response against recNcPDI, and slightly enhanced protection mediated by recNcPDI vaccination through intraperitoneal antigen delivery. However, no increased protection was seen when nanoparticle-bound recNcPDI was applied intranasally.

4. Outlook

The ultimate goal is the development of a safe and efficient vaccine that will protect the foetus in the bovine system or possibly in dogs. Therefore, the better strategy that will be useful for planning future studies may be based onto three major aspects:

(i) Improved murine models mimicking the natural situation: The most important model that would mimic the natural situation is the vertical transmission model, known as recrudescence model, in which a reactivation of *N.caninum* bradyzoites

in chronically infected animals occurs during pregnancy. However, the cerebral infection model (acute form of neosporosis), the fetal infection model (infection during pregnancy) and cell-culture model remain an important tool for the initial validation of the concept of protection.

(ii) Targeting of biological processes: It could be important to target other processes besides adhesion/invasion of parasites. Most notably, the processes of differentiation into the cyst-forming stage, the bradyzoite, could be inhibited by eliciting an immune response against antigens that are expressed during this transformation.

(iii) Careful evaluation of vaccine candidates: Probably poly-valent vaccines including surface, secretory and bradyzoite antigens, either as combination or chimeric proteins, could improve vaccination efficacy, and it would be interesting to test their corresponding protectivity.

Another important point for the vaccine study is the choice of the antigen delivery route. Even though, in the murine acute disease model intranasal application offered several immunogical and physiological advantages compared to the intraperitoneal route, this delivery system will be already practical in cattle. Therefore, further studies should focus on nanoparticle-based delivery systems, which exhibit different advantages, including the ability to present multiple copies of the antigen on the surface, increased B cell activation, enhanced $CD4^+$ lymphocytes proliferation and possibly improved adjuvant properties. However, in the case of chitosan nanogels employed in our study, only soluble antigens are suitable for coating the biopolymers. In the future, this delivery protocol will provide further additional knowledge on *Neospora* control. Moreover, the take up, the antigen presentation process and the expression of different co-stimulators (B7-1, B7-2) of the combined nanoparticle-conjugated vaccine candidates could be assessed in vitro using dendritic cells.

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