Vaccination of mice against experimental *Neospora caninum* infection using NcSAG1- and NcSRS2-based recombinant antigens and DNA vaccines

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SUMMARY

NcSAG1 and NcSRS2, the two major immunodominant tachyzoite surface antigens of the apicomplexan parasite *Neospora caninum*, were investigated for their potential as vaccine candidates in mice. Recombinant recNcSRS2 and recNcSAG1 were expressed in *Escherichia coli* as poly-histidine-tagged fusion proteins. Separate groups of mice were immunized with purified recNcSAG1, recNcSRS2, or a combination of both, and were then challenged with *N. caninum* tachyzoites. Subsequent experiments included intramuscular vaccination of mice with the eukaryotic expression plasmid pcDNA3 containing either NcSRS2 or NcSAG1 cDNA inserts, followed by a single booster with the corresponding recombinant antigens. Immunization with a crude somatic antigen (NC1-extract) was included in the experiments. Following challenge, the presence of the parasite in the different organs was assessed by a *N. caninum*-specific PCR, while the parasite burden in infected brain tissue was assessed by quantitative real-time PCR. Immunization of mice employing individual recombinant antigens, or combined recNcSAG1/recNcSRS2, resulted in a lower degree of protection against cerebral infection, when compared to combined DNA/recombinant antigen vaccination. Serological analysis showed that this protective effect was associated with the occurrence of antibodies directed against native parasite antigens in those animals receiving combined DNA/recombinant antigen vaccination. Conversely, mice immunized with recombinant antigens alone generated antibodies recognizing only the recombinant antigens. Mice experiencing clinical signs such as walking disorders, rounded back, apathy and paralysis were observed only in the untreated positive control groups, but never in the vaccinated groups. Our results suggest that a combined DNA/recombinant antigen-vaccine, based on NcSAG1 and NcSRS2, respectively, exhibited a highly significant protective effect against experimentally induced cerebral neosporosis in mice.

Key words: *Neospora caninum*, neosporosis, surface antigens, vaccination, quantitative PCR, serology.

INTRODUCTION

Neospora caninum, an obligate intracellular parasite belonging to the phylum Apicomplexa, was originally isolated from brain tissue of a dog with hind limb paralysis and was identified as a *Toxoplasma gondii*-like organism (Dubey *et al*. 1988). It is now considered as an independent species, which causes neosporosis, namely neuromuscular disease in dogs and abortion and stillbirth in cattle. The low host-specificity is a common feature of *N. caninum* and *T. gondii*, whereas extensive research at the molecular level showed that the 2 organisms are antigenically and phylogenetically clearly distinct (reviewed by Hemphill, 1999). The worldwide economical impact of this parasite, with great importance to the dairy industry, has given a great impulse to its study in order to find means of therapy and prevention (Hemphill & Gottstein, 2000).

Different strains of mice have been used in the past for the study of *N. caninum* infection in mammals as an experimental model for cerebral infection (Lindsay & Dubey, 1989; Lindsay *et al*. 1995; Eperon *et al*. 1999; Nishikawa *et al*. 2001), to produce tissue cysts in brains (McGuire *et al*. 1997), to compare parasite load, lesions and cytokine responses (Kahn *et al*. 1997; Long, Baszler & Mathison, 1998), to evaluate the effect of infection in pregnant mice (Long & Baszler, 1996) and for *in vivo* assessment of the efficacy of substances considered for chemotherapeutical treatment (Gottstein *et al*. 2001). Besides chemotherapy, vaccination is also being seriously considered as an approach to interfere with dissemination of the parasite (Nishikawa *et al*. 2001; Lunden *et al*. 2002), and to block the transplacental passage during pregnancy which results in foetal infection (Cole *et al*. 1995; Liddell *et al*. 1999).

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N. caninum infection in mice has been associated with acute primary pneumonia, myositis, encephalitis, ganglioradiculoneuritis, and pancreatitis (Dubey & Lindsay, 1996), with particular mouse strains appearing more susceptible to infection of the CNS than others (Long *et al*. 1998). A higher number of *N. caninum* tachyzoites is necessary for infection when compared to certain virulent strains of *T. gondii* such as RH, which are highly lethal at very small inoculum doses.

Experimental infection of B-cells deficient (μMT) mice and corresponding parental C57BL/6 mice had demonstrated the dramatically increased susceptibility of antibody deficient mice to *N. caninum* challenge, and pointed towards the important functional role of antibodies in protective immunity against *N. caninum* infections (Eperon *et al*. 1999). Vertical transfer of *N. caninum* in BALB/c mice could be prevented by vaccination with crude *N. caninum* antigen (Liddell *et al*. 1999). However, the preparation of crude antigen depends on fresh parasite supply and its composition may differ from one batch to the other and can therefore not be accurately controlled and standardized. Furthermore, its handling may be critical, mostly due to proteolytic activity found in such antigen extracts (Louie & Conrad, 1999). Therefore, a native crude extract of *N. caninum* does not appear ideal for field vaccination, and defined recombinant proteins and/or DNA vaccines based on *N. caninum* antigens would be preferred as potential tools for vaccination.

Antigens to be considered as potential vaccine candidates are proteins which are either constitutively expressed on the surface of the parasite such as the major immunodominant surface antigens NcSAG1 and NcSRS2 (Hemphill *et al*. 1997; Howe *et al*. 1998; Sonda *et al*. 1998), or secreted antigens which appear transiently on the surface (Lally *et al*. 1997; Hemphill *et al*. 1998; Liddell *et al*. 1998; Sonda *et al*. 2000). Many of these proteins have been suggested, at least *in vitro*, to be involved in the direct physical interaction between parasites and host cells. For instance, antibodies directed against NcSAG1 and NcSRS2 block adhesion to, and invasion of, host cells (Hemphill, 1996; Nishikawa *et al*. 2000). More recently, NcSRS2, expressed in the vaccinia virus system, has been shown to have a protective effect against challenge infection of mice with *N. caninum* tachyzoites. This effect was shown to be based on a high level of respective IgG_1 synthesis at the early stage of infection and on the presence of CD4+ and CD8+ T cell subsets at the late stage of infection (Nishikawa *et al*. 2001). In this study we report on the protective potential of vaccination in mice using recombinant NcSAG1 (recNcSAG1), NcSRS2 (recNcSRS2), and a combined DNA/recombinant antigen vaccination protocol, against experimental challenge infection with *N. caninum* tachyzoites.

MATERIALS AND METHODS

If not indicated otherwise, all biochemical reagents were purchased from Sigma Chemical Co (MO, USA).

Vero cell and parasite culture, and isolation of N. caninum *tachyzoites*

Vero cells were maintained in 75 cm^2 tissue culture flasks in 20 ml of RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% FCS (Gibco BRL, Life Technologies). The *N. caninum* NC1 isolate (Dubey *et al*. 1988), was cultured in 75 or 175 cm^2 culture flasks within Vero cell monolayers using the same medium but replacing FCS with 10% γ-globulin-free horse serum (Gibco). Isolation and purification of parasites were done as previously described (Hemphill, 1996). Briefly, Vero cells infected with *N. caninum* were passaged through a 25G 5/8 needle, washed and run on PD-10 Sephadex G-25M columns. Eluted tachyzoites were counted in a Neubauer chamber and tested for viability by trypan blue exclusion.

Preparation of recombinant antigens recNcSAG1 and recNcSRS2 and crude N. caninum *extract*

The cDNA fragments coding for the surface antigens NcSAG1 and NcSRS2 were obtained as previously described (Hemphill *et al*. 1997; Sonda *et al*. 1998) and were expressed in *E. coli* strain XL1-blue under the control of the IPTG-inducible Ptac promoter as C-terminal fusions to a peptide containing a poly-histidine (6 × His) stretch. Bacteria expressing the recombinant proteins recNcSAG1 and recNcSRS2, respectively, were harvested by centrifugation. The expressed proteins were affinity-purified on a cobalt column (Novagen, Clontech) under denaturing (recNcSAG1) or non-denaturing (recNcSRS2) conditions according to the instructions provided by the manufacturer. Both recombinant protein fractions were precipitated with methanol/chloroform (Wessel & Flügge, 1988), resuspended in PBS and stored at −80 °C prior to use.

For the preparation of crude somatic antigens from *N. caninum* (NC1-antigen), tachyzoites were treated by 3 freezing-thawing cycles followed by 2 sonications of 30 sec at 57 W in a sonifier cell disruptor B-12 (Bransonic Power Company). Cell debris was removed by centrifugation at 10 000 ***g*** for 30 min at 4 °C, and a sample was collected for SDS–PAGE and immunoblotting as described (Sonda *et al*. 2000). NC1-extract was stored at −80 °C prior to use.

Protein concentration was measured employing the protein assay dye reagent concentrate (Bio-Rad Laboratories) using the bovine plasma γ-globulin standard (Bio-Rad).

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Cloning of NcSRS2 and NcSAG1-cDNA into the pcDNA3 expression vector

The eukaryotic expression plasmid pcDNA3 was obtained from Invitrogen. The NcSAG1 and NcSRS2 cDNA fragments were those originally pulled out of *N. caninum* λgt22 cDNA expression library (Hemphill *et al.* 1997; Sonda *et al.* 1998). The NcSAG1-fragment was obtained through PCR using the forward primer VACp36K 5′-GCG GTA CCA TGG ACC CAC GCG TCC GC-3′ and the reverse primer VACp36N 5′-GCG CGG CCG CTC ACG CGA CGC CAG-3′. The NcSRS2-fragment was amplified using forward primer VACp43K 5′-GCG GTA CCA TGG ACC CAC GCG TCC GG-3′ and the reverse primer VACp43N 5′-GCG CGG CCG CTT AAC ACG GGG GAA TCG. The *Kpn*I and *Not*I-digested PCR fragments were inserted into *Kpn*I/*Not*I-digested pcDNA3, and the expression of respective fragments were confirmed following transfection of Vero cells and immunostaining with anti-NcSAG1 and anti-NcSRS2-antibodies (data not shown).

Mice, vaccination, challenge and euthanasia

Female C57BL/6 mice were used at 6–8 weeks of age. Prior to any immunization, mice were checked serologically for the absence of anti-*N. caninum* immunoglobulins (pre-immune sera) according to Eperon *et al.* (1999). Groups of 8 mice were used. They were treated as follows.

Vaccination with recombinant antigens. Two experiments were performed. In a first experiment, group 1 was inoculated i.p. with PBS alone and remained uninfected throughout the experiment. Group 2 was treated with PBS and was infected i.p. with *N. caninum* tachyzoites (positive control). Group 3 (adjuvant control) was treated with PBS emulsified in RIBI Adjuvant System (PBS–RAS; RIBI ImmunoChem Research, Inc.) according to the manufacturer's recommendations. Group 4 and group 5 were immunized with recNcSAG1 and recNcSRS2, respectively, emulsified in RAS at 75 μg/ml, and group 6 was vaccinated with NC1 crude extract of somatic antigen, emulsified in RAS at 250 μg/ml. Vaccination was carried out by intraperitoneal (i.p.) inoculation of 200 μl of respective preparations at day 0, and a booster injection was given 21 days later. On day 28 mice were challenged i.p. with 1×10^6 live *N. caninum* tachyzoites suspended in 200 μl of PBS.

In a second experiment, vaccination was performed with combined recNcSRS2/recNcSAG1 antigens. The positive and adjuvant groups were treated as in the previous experiment, group 3 received a combination of recNcSAG1 and recNcSRS2 (75 μg/ml of each recombinant protein) resuspended in PBS, and group 4 was treated with the combination of the 2 recombinant proteins, emulsified in RAS. Vaccination was carried out by intraperitoneal (i.p.) inoculation of 200 μl of respective preparations at day 0, and a booster injection was given 21 days later. On day 28 mice were challenged i.p. with 2×10^6 live *N. caninum* tachyzoites suspended in 200 μl of PBS.

Vaccination trial employing the DNA vaccines pcDNA3–NcSRS2 and pcDNA3–NcSAG1, followed by immunization with respective recombinant antigens. This experiment was performed twice, yielding essentially identical results. The infected positive control group was treated as before. To the mice of the adjuvant control group, 100 μl of the pcDNA3 vector without insert (used at a concentration of 1 mg/ml) was inoculated intramuscularly, with 50 μl (corresponding to 50 μg of DNA) being injected into the right and left hind limb muscle, respectively, on day 0 and on day 28. On day 49, these adjuvant control mice received one single i.p. injection of PBS–RAS as in the previous experiments. Group 3 received similar intramuscular applications of pcDNA3–NcSAG1 at day 0 and day 28, followed by a single i.p. injection of 200 μl of recNcSAG1 (75 μg/ml) emulsified in PBS at day 49. Group 4 received treatment with pcDNA3–NcSRS2 and recNcSRS2 as in group 3. On day 59, all mice were challenged i.p. with 2×10^6 live *N. caninum* tachyzoites suspended in 200 μl of PBS.

All animals were sacrificed by CO_2-euthanasia at day 21 post-infection (p.i.) unless otherwise stated. Blood was drawn by cardiac puncture for serum isolation. The brain, kidney, liver, spleen, lung and heart were obtained by aseptic dissection. One brain hemisphere was fixed in PBS-buffered 4% paraformaldehyde for a maximum of 24 h at 4 °C, and was then processed for immunohistochemistry (Fuchs *et al.* 1998). The other brain hemisphere, as well as pieces of spleen, kidney, liver, lung, and heart were frozen at −80 °C for subsequent PCR analysis.

Serology

For vaccination experiments employing recombinant proteins, sera were analysed by ELISA at 3 timepoints: before immunization (day 0 = pre-immune), 6 days after the final immunization step (post-immune), and on the day of euthanasia (21 days p.i.). For mice treated with combined DNA/recombinant vaccine, sera were analysed prior to immunization, 20 days following DNA vaccination (day 48), 9 days following application of the recombinant antigens (day 58), and 21 days p.i. (day 79). Maxisorp (Nunc) strips were coated overnight at 4 °C with 100 μl of either NC1 extract (5 μg/ml), or purified recNcSRS2 and recNcSAG1 (1 μg/ml), respectively, in 0·1 M $NaHCO_3$–Na_2CO_3, pH 9, washed with 0·3% Tween

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20 in PBS and incubated 30 min with 200 μl of PBS containing 0·05% bovine haemoglobin and 0·3% Tween 20 (HTP). Strips were incubated for 2 h with mouse serum diluted 1 : 100 in HTP. Secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse IgG- (Promega), goat anti-mouse IgG_1, and IgG_{2a}, respectively (Southern Biotechnology Associates) diluted in HTP. Wells were subsequently incubated with 120 μl of 1 mg/ml *p*-nitrophenylphosphate-disodium in 10% diethanolamine containing 0·5 mM $MgCl_2 . 6H_2O$, pH 9·8. The absorbance was read at 405 nm (reference filter at 630 nm) using a Dynatech MR7000 ELISA reader and the corresponding Dynatech Biocalc software (Dynatech, Embrach, Switzerland).

Immunohistochemistry

Immunofluorescence staining of brain tissue sections was performed according to the method described by Fuchs *et al*. (1998). Briefly, paraformaldehyde-fixed brain tissue was embedded in paraffin, and sections were placed onto poly-L-lysine-coated glass slides. Five paraffin sections containing pieces of brain, separated each by 7 sections, were deparaffinized and rehydrated in a graded series of methanol followed by PBS. Non-specific binding sites were blocked in PBS containing 1% BSA and 50 mM glycine (blocking buffer) for 2 h. Subsequently, sections were incubated with a Protein A-purified anti-*N. caninum* antiserum (Hemphill, Gottstein & Kaufmann, 1996) at a dilution of 1 : 250 in blocking buffer for 1 h. Goat anti-rabbit-FITC was applied at 1 : 100 in blocking buffer for 1 h. The preparations were washed, incubated in the fluorescent dye Hoechst 33258 (1 μg/ml in PBS) for 2 min, washed and mounted in Fluoroprep (BioMérieux S. A.). All specimens were viewed on a Leitz Laborlux S fluorescence microscope.

DNA isolation and PCR analyses

For DNA preparation, pieces of tissue were thawed and subsequently prepared with the high pure PCR template preparation kit (Boehringer Mannheim) according to the manufacturer's recommendations. Conventional PCR was performed as previously described (Müller *et al*. 1996). Reactions were done on 1 μl aliquots containing 20 ng of previously denaturated DNA. The 50 μl mixture contained 5 μl of 10 × Gene Amp PCR buffer (Perkin Elmer Cetus), 0·2 mM each dATP, dGTP and dCTP, 0·4 mM dUTP, 20 pmol of *N. caninum*-specific primers Np21plus and Np6plus (Müller *et al*. 1996), 1·25 U of Ampli*Taq* DNA polymerase and 0·5 U of uracyl DNA glycosylase (UDG). Amplification was done in a thermal cycler (Perkin Elmer Cetus) employing 40 cycles for denaturation (94 °C, 1 min) annealing (63 °C, 1 min) and primer extension (74 °C, 3·5 min). At the end, a primer extension was continued for 10 min at 74 °C and 50 μl of chloroform were added to inactivate UDG. False-negative results due to inhibitory compounds were excluded by a duplicate positive control where, in a parallel reaction, each sample was supplemented with an appropriate amount of recombinant inhibition control DNA carrying the primer target sequence. The PCR products were analysed by electrophoresis on 2% agarose gels.

Quantitative PCR

For quantitative PCR, the procedure described by Müller *et al*. (2002) was used. Detection of DNA amplification was achieved using a double fluorescence probe on the LightCycler™ Instrument (Roche Diagnostic, Basel, Switzerland). For detection of amplicons, Nc5-specific 5′-LC-Red 640 labelled Np 5LC detection probe and 3′-fluorescein labelled Np 3FL anchor probe (TIB MOLBIOL, Berlin, Germany) were used. PCR amplification was performed with the LightCycler DNA Master Hybridization Probes™ Kit (Roche Diagnostic, Basel, Switzerland) as previously described. Fluorescence signals from the amplification products were quantitatively assessed by applying the standard software (version 3.5.3) of the LightCycler™ Instrument.

Statistical analysis

The significance of the differences among the control and experimental assays was determined by Student's *t*-test using the Microsoft Excel program. *P* values $<0·05$ were considered statistically significant.

RESULTS

Experimental infection of mice vaccinated with recombinant antigens recNcSAG1 and recNcSRS2

Fig. 1A and B demonstrates the effect of vaccination of mice against challenge infection with *N. caninum* tachyzoites using native NC1 extract and individual recombinant *N. caninum* antigens expressed in *E. coli* and purified by Co^{2+}-affinity chromatography. All 5 groups were challenged with 1×10^6 *N. caninum* tachyzoites by i.p. inoculation. No clinical signs of neosporosis could be detected in any animals in this experiment.

The organ distribution and respective dissemination of parasites was analysed by PCR specific for *N. caninum* DNA (Müller *et al*. 1996). The only organ which was found to be infected was the brain. This also accounted for all groups in all subsequent experiments (data not shown). Analysis of the brain tissue of the untreated but infected control group revealed that 7 out of 8 samples were *Neospora*-PCR positive (Fig. 1A). In infected mice treated with

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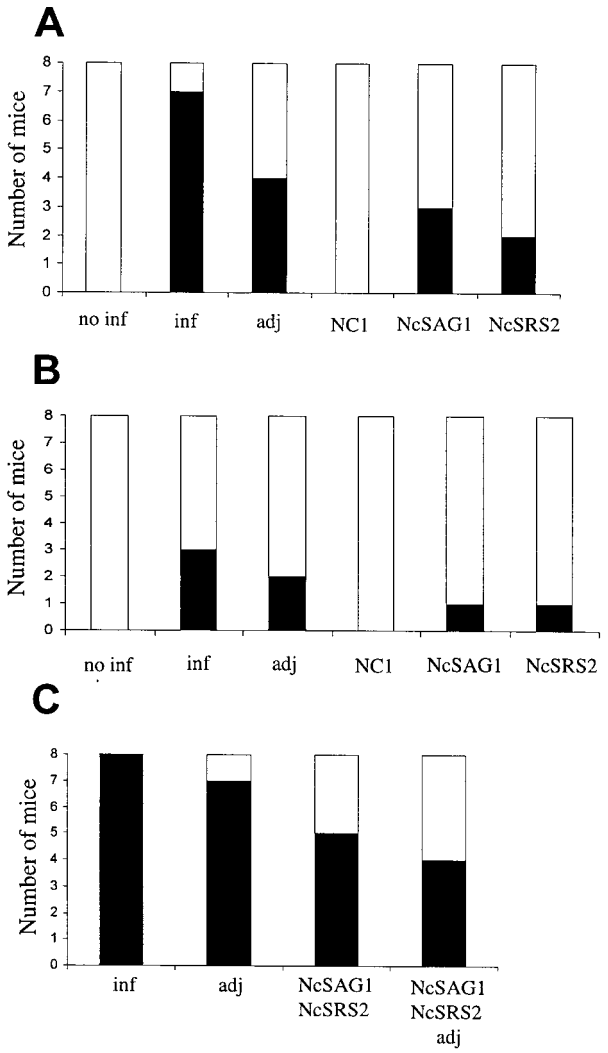

Fig. 1. Effects of vaccination with recombinant antigens on infection of brain tissue of C57BL/6 mice after i.p. challenge with 1×10^6 (A, B) and 2×10^6 *Neospora caninum* tachyzoites (C), as determined by *Neospora*-specific PCR (A, C) and immunohistochemistry (B). no inf = non-treated and non-infected mice; inf = mice remained untreated and were infected; adj = mice treated with PBS–RAS and infected; NC1 = mice vaccinated with NC1-antigen emulsified in RAS; NcSAG1 = mice vaccinated with recNcSAG1 emulsified in RAS; NcSRS2 = mice vaccinated with recNcSRS2 emulsified in RAS; NcSAG1/NcSRS2 = mice vaccinated with recNcSAG1 and recNcSRS2; NcSAG1/NcSRS2adj = mice vaccinated with recNcSAG1 and recNcSRS2 emulsified in RAS. (■) PCR- or IHC-positive samples; (□) negative samples.

PBS–RAS (adjuvant control), the presence of *N. caninum* was confirmed by PCR in 4 out of 8 animals, demonstrating a reduction in the number of infected mice by the administration of the adjuvant alone, although the reduction was not statistically significant ($P = 0{\cdot}12$). Protection against central nervous system infection was found in all 8 animals vaccinated with adjuvants plus NC1-extract ($P = 0{\cdot}019$ when compared with the adjuvant control). In contrast, no significant reduction in the number of PCR-positive mice was achieved upon vaccination prior to infection with PBS–RAS plus recNcSAG1 (5 PCR-negative, 3 positive, $P = 0{\cdot}64$) or with PBS–RAS plus recNcSRS2 (6 PCR-negative, 2 positive, $P = 0{\cdot}33$).

When brain tissue sections of animals were comparatively investigated by immunohistochemistry using a polyclonal anti-*N. caninum* antiserum (Fig. 1B), it was evident that immunohistochemistry was much less sensitive compared to PCR. However, these results confirmed that vaccination of mice with individual recNcSAG1 or recNcSRS2 prior to experimental infection had no significant protective effect.

A second experiment was performed, during which recNcSRS2 and recNcSAG1 were administered as a combined vaccine, resuspended in PBS–RAS. In addition, the role of adjuvant was assessed, in that animals were vaccinated with the mixture of recombinant proteins devoid of adjuvant. In this experiment, mice were challenged with 2×10^6 parasites in order to optimize the infection in control mice. In the infection control group, all 8 animals were PCR positive in the brain, while in the adjuvant control group 1 animal was negative (Fig. 1C). Again, all mice were *Neospora*-PCR negative in lungs, spleen, kidney, heart and liver. In the experimental group vaccinated with recNcSAG1/recNcSRS2 resuspended in PBS devoid of adjuvant, 5 out of 8 mice were PCR-positive ($P = 0{\cdot}06$). In mice treated with PBS–RAS, 7 out of 8 mice were PCR positive in the brain, while vaccination with recNcSRS2/recNcSAG1 emulsified in PBS–RAS resulted in 4 PCR-positive and 4 PCR-negative brain tissue samples ($P = 0{\cdot}12$). Thus, no significant reduction was achieved.

The antibody responses against NC1-antigen extract of the mice in different treatment groups are shown in Fig. 2A. Following vaccination, the sera of NC1-extract-immunized animals exhibited a significantly elevated IgG level, which did not further increase following infection. The anti-NC1 antibodies included both IgG_1 and IgG_{2a} isotypes (data not shown). The IgG concentrations in sera obtained after PBS–RAS treatment alone (adjuvant control) and vaccination with PBS–RAS plus recNcSAG1 and recNcSRS2 were only marginally elevated. However, after infection, antibody concentrations in sera of recNcSAG1- and recNcSRS2-vaccinated animals increased markedly, and reached similar levels as in infection controls (Fig. 2A). Antibody responses directed against recNcSRS2 are shown in Fig. 2B. IgG directed against recNcSRS2 was found in sera from NC1 extract- and recNcSRS2-vaccinated animals already following vaccination (prior to infection), and corresponding antibody levels did not significantly increase following infection. Similar results were obtained for recNcSAG1 (data not shown). Thus, vaccination of mice with

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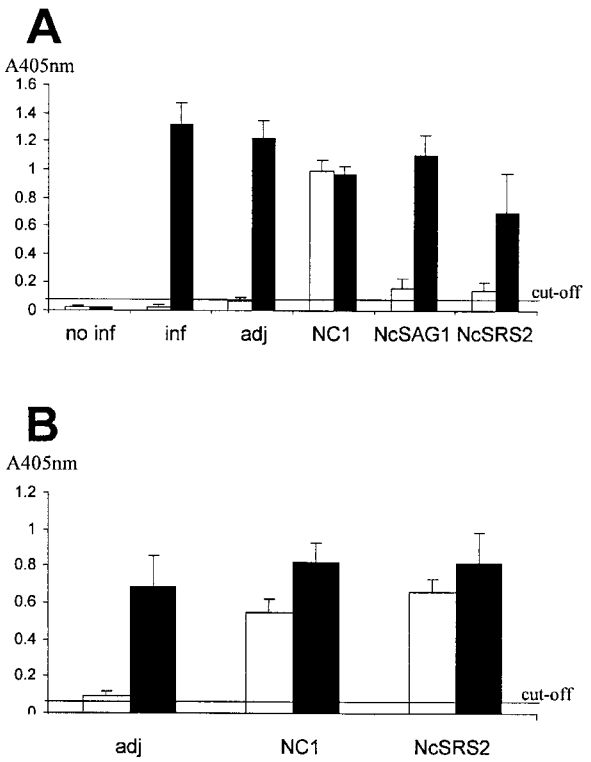

Fig. 2. Antibody responses of mice towards NC1-antigen (A) and recNcSRS2 (B). Time-points of bleeding were day 0 (prior to vaccination; not shown), day 27 (prior to challenge; □) and day 48 (21 days post-challenge; ■). The arbitrarily selected cut-off represents the mean of the values obtained prior to vaccination plus 3 S.D. (A) The following sera had been tested for antibodies against NC1-extract antigens: sera of mice which had been injected with PBS and not infected (=no inf); injected with PBS and infected (=inf); injected with PBS–RAS (=adj); vaccinated with NC1 extract emulsified in RAS (=NC1); vaccinated with recNcSAG1 (=NcSAG1); vaccinated with recNcSRS2 (=NcSRS2). (B) The following sera were investigated for antibodies against recNcSRS2: sera from mice injected with PBS–RAS (=adj); mice vaccinated with NC1-extract emulsified in RAS (NC1); mice vaccinated with recNcSRS2 emulsified in RAS (NcSRS2). Essentially identical results were obtained for recNcSAG1 and the combined recNcSAG1/recNcSRS2 vaccines (data not shown).

recNcSRS2 and recNcSAG1 elicited a humoral immune response directed against the recombinant proteins, but respective antibodies reacting with the corresponding native antigens could not be detected. The same accounted for the sera obtained from mice which had been vaccinated with a combined recombinant recNcSAG1/recNcSRS2 vaccine (data not shown).

Vaccination trial employing the DNA vaccines pcDNA3–NcSRS2 and pcDNA3–NcSAG1, followed by immunization with respective recombinant antigens

Since immunization of mice with bacterially expressed recombinant NcSRS2 and NcSAG1 induced

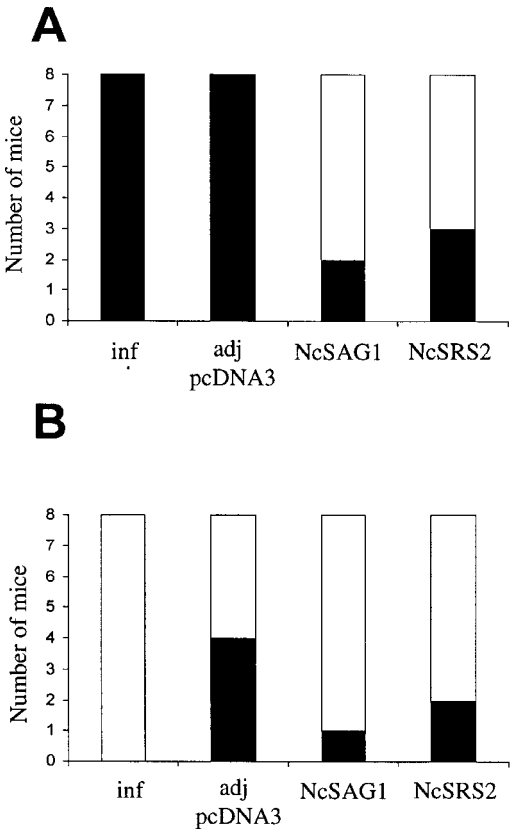

Fig. 3. Effect of vaccination on infection of brain tissue of C57BL/6 mice after i.p. challenge with 2×10^6 *Neospora caninum* tachyzoites, as determined by *Neospora*-specific PCR (A) and immunohistochemistry; (B) inf=mice remained untreated and were infected; adj/pcDNA3=mice immunized with pcDNA3 and PBS–RAS and infected; NcSAG1=mice were immunized with pcDNA3–NcSAG1 plus recNcSAG1; NcSRS2=mice were immunized with pcDNA3–NcSRS2 plus NcSRS2.

no significant protective effect against *N. caninum* challenge infection, another vaccination approach was used. The cDNA fragments corresponding to NcSRS2 and NcSAG1 (Hemphill *et al.* 1997; Sonda *et al.* 1998) were cloned into the eukaryotic expression plasmid pcDNA3. Vero cells were transfected with these plasmids, and immunofluorescence showed that corresponding polypeptides were expressed within these Vero cells (data not shown). Intramuscular DNA vaccination of mice was performed using pcDNA3 (as a negative control), pcDNA3–NcSAG1, and pcDNA3–NcSRS2, respectively. After a second application of DNA vaccines, an antigenic booster was given with the corresponding recombinant antigens emulsified in PBS–RAS. Mice were then experimentally infected by i.p. inoculation of 2×10^6 *N. caninum* tachyzoites. This experiment was performed twice, yielding essentially identical results.

Fig. 3A shows the results with regard to the detection of parasite DNA in brain tissues. All mice in the infection control group treated with PBS suffered from cerebral neosporosis, as they exhibited

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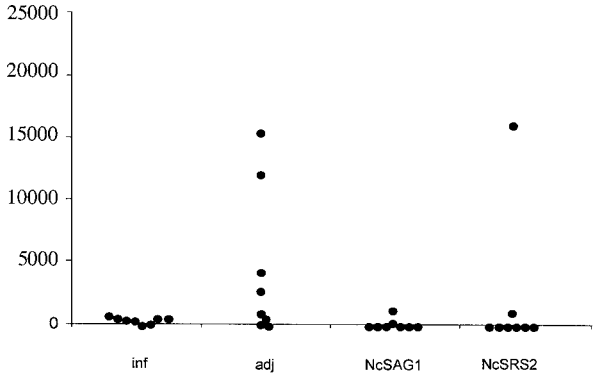

Fig. 4. Infection intensities in brain tissue as determined by quantitative *Neospora*-specific PCR. inf = mice remained untreated and were infected (mice were sacrificed at 10 days p.i.); adj = mice immunized with pcDNA3 and PBS–RAS and infected; NcSAG1 = mice were immunized with pcDNA3–NcSAG1 plus recNcSAG1; NcSRS2 = mice were immunized with pcDNA3–NcSRS2 plus NcSRS2; the numbers correspond to the actual parasite number/brain hemisphere.

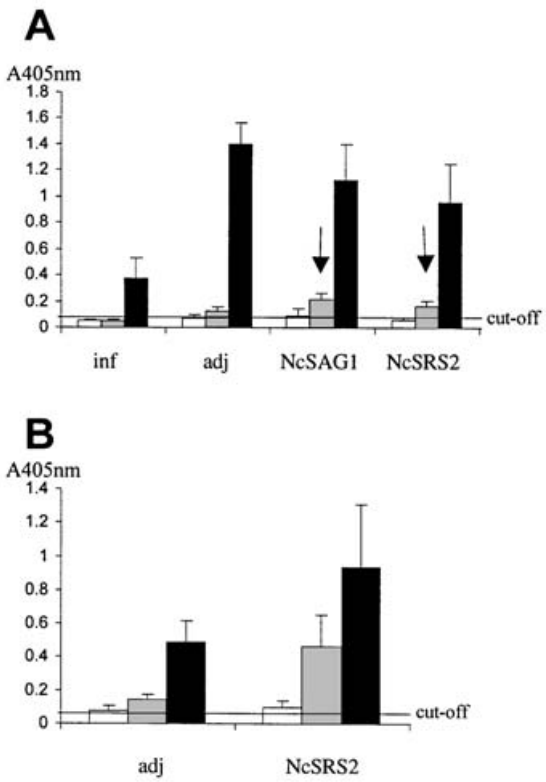

Fig. 5. Antibody response of mice vaccinated with combined DNA/recombinant antigen vaccines towards NC1-antigen (A) and recNcSRS2 (B). Time-points of bleeding were day 0 (prior to vaccination; not shown), day 48 (following DNA vaccination and prior to vaccination with recombinant antigen; □), day 58 (following recombinant antigen booster and prior to challenge; ▒), and the day of euthanasia (21 days post-challenge; ■). The arbitrarily selected cut-off represents the mean of the values obtained prior to vaccination plus 3 S.D. (A) The following sera were investigated for antibodies against NC1 antigen: inf = mice remained untreated and were infected; adj = mice immunized with pcDNA3 and PBS–RAS and infected; NcSAG1 = mice were immunized with pcDNA3–NcSAG1 plus recNcSAG1; NcSRS2 = mice were immunized with pcDNA3–NcSRS2 plus NcSRS2. The increase in antibody values after vaccination with NcSAG1 and NcSRS2 is significant and marked with arrows. ($P = 0{\cdot}0007$ for NcSAG1; $P = 0{\cdot}004$ for NcSRS2). (B) The following sera were investigated for antibodies against recNcSRS2: sera from mice injected with PBS–RAS (= adj); mice vaccinated with pcDNA3–NcSRS2 plus recNcSRS2. Similar results were obtained for NcSAG1-based vaccine (data not shown).

clinical signs such as tilted heads and distorted walking, as well as apathy. These mice had to be euthanized already after 10 days p.i. As expected, *N. caninum* DNA was detected by PCR in the brain of all 8 animals. In the second control group, treated with pcDNA3 and PBS–RAS (adjuvant control group), none of the animals exhibited clinical signs until day 21 p.i., however, parasite DNA was detected in the brain of all 8 mice in this group. In contrast, a combined pcDNA3–NcSAG1/recNcSAG1 vaccination approach conferred resistance to cerebral *N. caninum* infection in 6 out of 8 mice ($P = 0{\cdot}0004$), and 5 out of 8 mice were protected by applying pcDNA3–NcSRS2/recNcSRS2 vaccination ($P = 0{\cdot}004$). None of the animals in the 2 experimental vaccine groups exhibited any clinical symptoms. Immunohistochemistry also indicated, that the number of infected animals in vaccinated groups was reduced (Fig. 3B).

A quantitative assessment of infection intensities in brain tissues was performed using a recently developed LightCycler-based *Neospora*-PCR approach (Müller *et al.* 2002). This assay, shown here for 1 representative experiment in Fig. 4, revealed that the parasite burden within the brain tissue was extremely variable. The number of parasites in the brain of those mice vaccinated with pcDNA3–NcSAG1/recNcSAG1 was reduced compare to brain tissue of pcDNA3-adjuvant control mice. However, statistical analysis demonstrated that the difference was not significant ($P = 0{\cdot}065$). In addition, one of the animals in the NcSRS2-vaccinated group which was positive by conventional PCR, was negative by quantitative PCR, demonstrating the slightly lower sensitivity of the quantitative PCR assay. The infection intensity in the brain tissue of the positive control group was low but these mice were sacrificed on day 10 p.i. (Fig. 4).

Measurement of IgG directed against NC1 extract antigen was performed, and showed that DNA vaccination alone did not elicit a measurable humoral immune response (Fig. 5). However, the additional booster with recombinant antigens emulsified in PBS–RAS resulted in the production of antibodies directed against epitopes present on native parasite antigens, most significantly for NcSAG1

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($P=0{\cdot}0007$), but also for NcSRS2 ($P=0{\cdot}0224$). Following infection, IgG titres were high in all experimental groups as well as in the adjuvant control group, but lower in the positive control group treated with PBS only. Thus, the low anti-*N. caninum* antibody response correlated with the occurrence of clinical signs of neosporosis (Fig. 5A).

DISCUSSION

During the acute phase of neosporosis, tachyzoites invade their host cells, divide rapidly, form pseudocysts which rupture, and released tachyzoites infect neighbouring cells. This process triggers an efficient immune response in the immune competent host, and is followed by the formation of intracellular tissue cysts containing the bradyzoite stage of the parasite, surrounded by a cyst wall, eventually leading to chronic infection. The current evidence suggests that *N. caninum* tissue cysts are located predominantly in the CNS. Pregnancy can lead to reactivation of parasites followed by vertical transmission. The invasion of the foetal CNS by *N. caninum* tachyzoites, and the subsequent damage to it, eventually gives rise to frequent abortion in cattle or causes neurological manifestations as they have been observed in infected calves and dogs (e.g. reviewed by Dubey & Lindsay, 1996; Hemphill, 1999). It has been concluded that immunity generated during previous *N. caninum* infection, unlike the situation in human toxoplasmosis, is not sufficient to prevent vertical transmission.

Since both mature cattle and mice are relatively resistant to disease, but not to initial tachyzoite proliferation and subsequent persistence of the parasite (Long *et al.* 1998), mouse models can be used to perform studies on the pathogenesis of neosporosis and associated immune responses. C57BL/6 mice were chosen in this study, since this mouse strain has been successfully used in previous experiments which demonstrated the dramatically increased susceptibility of antibody-knock-out mice (μMT mice) towards *N. caninum* challenge infection compared to the respective wild-type C57BL/6 mice (Eperon *et al.* 1999). In addition, we previously found that experimental infection of C57BL/6 mice at a dose of $1–2\times10^6$ tachyzoites results in predominantly CNS infection (Eperon *et al.* 1999). These results were confirmed in the present study. Thus, C57BL/6 mice represent a suitable model system to investigate cerebral neosporosis. Three types of vaccines were tested for protective activity against brain infection upon challenge infection with *N. caninum* tachyzoites. These were crude somatic antigens (NC1-extract), recombinant proteins (recNcSAG1, recNcSRS2) expressed in *E. coli*, and a combined DNA/recombinant antigen vaccination protocol.

Vaccination with NC1-extract induced an immune response which was 100% protective with regard to CNS infection. These results are in agreement with earlier studies (Liddell *et al.* 1999), which had shown that vertical transfer of *N. caninum* in BALB/c mice could be prevented by vaccination with crude *N. caninum* antigen. This observation lead to vaccination trials in cattle (Andrianarivo *et al.* 1999). However, *N. caninum* extracts presented under POLYGEN^{TM}-adjuvant formulation failed to prevent foetal infection in pregnant cattle following experimental tachyzoite challenge (Andrianarivo *et al.* 2000). Other adjuvant formulations have also been shown not to be capable of inhibiting placental infection, but they were partially successful in preventing abortion of the foetus (Innes *et al.* 2002).

The two major immunodominant surface antigens, NcSAG1 and NcSRS2, both functionally implicated in playing a role during adhesion and/or invasion of host cells *in vitro* (Hemphill, 1996; Nishikawa *et al.* 2000), were considered as potential vaccine candidates. Our experiments, employing these recombinant antigens either individually or in combination, in the presence or absence of adjuvants, revealed that recombinant antigens conferred no significant protectivity. In the primary experiment, where a lower infection dose of 1×10^6 tachyzoites was used, fewer mice (4 out of 8) were PCR-positive in the adjuvant control compared to the infection control group (7 out of 8). Thus, the non-specific stimulation of the immune system was already sufficient to eliminate the parasites in 4 out of 8 mice. This adjuvant effect was largely absent in the second experiment (with 7 out of 8 PCR-positive adjuvant control mice), where infection was carried out using 2×10^6 *N. caninum* tachyzoites/mouse.

Analysis of IgG production in sera obtained from mice vaccinated with NC-extract, recNcSAG1, recNcSRS2, or the combination of both, could provide a possible explanation for the lack of protection in animals vaccinated with recombinant proteins. Sera generated against the recombinant proteins did not contain IgG capable of recognizing native antigens, but antibodies reacted readily with the corresponding recombinant antigens, demonstrating that either the relevant B-cell epitopes on the recombinant and the native proteins are different, or that the native and recombinant proteins might undergo differential processing by dendritic cells. This lack of functional antibodies could be largely responsible for the lack of protection (Eperon *et al.* 1999).

Application of a combined DNA/recombinant antigen vaccination protocol exhibited a markedly higher degree of protectivity. Immunization of mice with pcDNA3–NcSAG1/recNcSAG1 resulted in only 2 out of 8 mice in which brain tissue was *Neospora*-PCR positive (75% protection), while similar experiments using pcDNA3–NcSRS2/recNcSRS2 resulted in 3 out of 8 PCR-positive mice (62·5% protection). This result was reproducible in 2 independent experiments and is statistically

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significant. Serology revealed that IgG recognizing the native antigens could be detected in the experimental groups only after application of both, DNA-vaccine and recombinant antigens, with the highest efficiency of antibody production noted for NcSAG1. Thus, efficient protection of mice against experimentally induced cerebral *N. caninum* infection is associated with the occurrence of antibodies recognizing native NcSAG1 and NcSRS2.

The parasite burden in infected brain tissues of animals vaccinated with the combined DNA/recombinant antigen protocol was assessed by quantitative *Neospora*-specific PCR (Müller *et al.* 2002). The parasite burden was generally highest in the adjuvant control group, followed by the NcSRS2 and the NcSAG1 group. The parasite burden was lowest in the group immunized with pcDNA3–NcSAG1/recNcSAG1, with the exception of those mice from the positive control group (untreated and infected). All mice from this positive control group showed clinical signs of neosporosis (tilted head, walking disorders, apathy) starting already at day 8 p.i., which made it necessary to euthanize these animals at the latest on day 10 p.i. In contrast, positive control mice infected with the same number of parasites in the previous experiment did not exhibit any clinical signs until day 21 p.i. Thus, there is a 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 timepoint 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 infectivity.

In the combined DNA/recombinant antigen vaccination trials, the parasite burden was found to be lower in the brain tissues of the positive control group mice, suffering from neosporosis than in the adjuvant control group, despite the fact that mice in the adjuvant control group did not experience clinical signs. This effect could be attributed to the administration of PBS–RAS, which is likely to mediate protection against disease via yet unknown unspecific adjuvants effects.

As *N. caninum* is an intracellular parasite, not only the humoral but also the cellular immune response must be considered as an important mediator of resistance or susceptibility. Resistance to infection, either innate or following immunization, seems to depend on a Th1-type cellular immune response. It was previously shown that splenocytes obtained from infected mice proliferated *in vitro* in response to NC1-antigen and expressed significant quantities of IL-12 and IFN-γ (Kahn *et al.* 1997; Eperon *et al.* 1999). Mice treated with anti-IL12 or anti-IFN-γ antibodies were rendered susceptible to infection (Kahn *et al.* 1997). In another study, susceptibility in BALB/c and C57BL/6 mice to *N. caninum* was associated with a low IFN-γ : IL-4 ratio from antigen-stimulated splenocytes, whereas the resistance in B10.D2 mice was associated with a high splenocyte IFN-γ : IL-4 ratio (Long *et al.* 1998). Therefore, our future studies will be directed towards the analysis of the cellular immune mechanisms in animals treated with vaccines based on these immunodominant antigens.

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REFERENCES

ANDRIANARIVO, A. G., CHOROMANSKI, L., McDONOUGH, S. P., PACKHAM, A. E. & CONRAD, P. A. (1999). Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. *International Journal for Parasitology* **29**, 1613–1625.

ANDRIANARIVO, A. G., ROWE, J. D., BARR, B. C., ANDERSON, M. L., PACKHAM, A. E., SVERLOW, K. W., CHOROMANSKI, L., LOUIE, C., GRACE, A. & CONRAD, P. A. (2000). A POLYGEN^{TM}-adjuvanted killed *Neospora caninum* tachyzoite preparation failed to prevent foetal infection in pregnant cattle following i.v/i.m experimental tachyzoite challenge. *International Journal for Parasitology* **30**, 985–990.

COLE, R. A., LINDSAY, D. S., BLAGBURN, B. L. & DUBEY, J. P. (1995). Vertical transmission of *Neospora caninum* in mice. *Journal of Parasitology* **81**, 730–732.

DUBEY, J. P., HATTEL, A. L., LINDSAY, D. S. & TOPPER, M. J. (1988). Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *Journal of the American Veterinary Medical Association* **193**, 1259–1263.

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

EPERON, S., BRÖNNIMANN, K., HEMPHILL, A. & GOTTSTEIN, B. (1999). Susceptibility of B-cell deficient C57BL/6 (μMT) mice to *Neospora caninum* infection. *Parasite Immunology* **21**, 225–236.

FUCHS, N., SONDA, S., GOTTSTEIN, B. & HEMPHILL, A. (1998). Differential expression of cell surface- and dense granule-associated *Neospora caninum* proteins in tachyzoites and bradyzoites. *Journal of Parasitology* **84**, 753–758.

GOTTSTEIN, B., EPERON, S., DAI, W. J., HEMPHILL, A. & GREIF, G. (2001). Efficacy of toltrazuril and ponazuril against experimental *Neospora caninum* infection in mice. *Parasitology Research* **87**, 43–48.

HEMPHILL, A. (1996). Subcellular localization and functional characterisation of Nc-p43, a major *Neospora caninum* tachyzoite surface protein. *Infection and Immunity* **64**, 4279–4287.

HEMPHILL, A. (1999). The host–parasite relationship in neosporosis. *Advances in Parasitology* **43**, 47–104.

HEMPHILL, A. & GOTTSTEIN, B. (2000). A European perspective on *Neospora caninum*. *International Journal for Parasitology* **30**, 877–924.

HEMPHILL, A., GOTTSTEIN, B. & KAUFMANN, H. (1996). Adhesion and invasion of bovine endothelial cells by *Neospora caninum* tachyzoites. *Parasitology* **112**, 183–197.

HEMPHILL, A., FELLEISEN, R., CONNOLLY, B., GOTTSTEIN, B., HENTRICH, B. & MÜLLER, N. (1997). Characterization of a cDNA clone encoding Nc-p43, a major *Neospora caninum* surface protein. *Parasitology* **115**, 581–590.

HOWE, D. K., CRAWFORD, A. C., LINDSAY, D. L. & SIBLEY, L. D. (1998). The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. *Infection and Immunity* **66**, 5322–5328.

INNES, E. A., ANDRIANARIVO, A. G., BJORKMAN, C., WILLIAMS, D. J. & CONRAD, P. A. (2002). Immune responses to *Neospora caninum* and prospects for vaccination. *Trends in Parasitology* **18**, 497–504.

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

LALLY, N., JENKINS, M., LIDELL, S. & DUBEY, J. P. (1997). A dense granule protein (NCDG1) gene from *Neospora caninum*. *Molecular and Biochemical Parasitology* **87**, 239–243.

LIDDELL, S., LALLY, N., JENKINS, M. C. & DUBEY, J. P. (1998). Isolation of the cDNA encoding a dense granule associated antigen (NCDG2) of *Neospora caninum*. *Molecular and Biochemical Parasitology* **93**, 153–158.

LIDDELL, S., JENKINS, M. C., COLLICA, M. C. & DUBEY, J. P. (1999). Prevention of vertical transfer of *Neospora caninum* in BALB/c mice by vaccination. *Journal of Parasitology* **85**, 1072–1075.

LINDSAY, D. S. & DUBEY, J. P. (1989). *Neospora caninum* (Protozoa: Apicomplexa) infections in mice. *Journal of Parasitology* **75**, 772–779.

LINDSAY, D. S., LENZ, S. D., COLE, R. A., DUBEY, J. P. & BLAGBURN, B. L. (1995). Mouse model for central nervous system *Neospora caninum* infections. *Journal of Parasitology* **81**, 313–315.

LONG, M. T. & BASZLER, T. V. (1996). Fetal loss in BALB/c mice infected with *Neospora caninum*. *Journal of Parasitology* **82**, 608–611.

LONG, M. T., BASZLER, T. V. & MATHISON, B. A. (1998). Comparison of intracerebral parasite load, lesion development, and systemic cytokines in mouse strains infected with *Neospora caninum*. *Journal of Parasitology* **84**, 316–320.

LOUIE, K. & CONRAD, P. A. (1999). Characterization of a cDNA encoding a subtilisin-like serine protease (NC-p65) of *Neospora caninum*. *Molecular and Biochemical Parasitology* **103**, 211–223.

MCGUIRE, A. M., MCALLISTER, M. M., JOLEEY, W. R. & ANDERSON SPRECHER, R. C. (1997). A protocol for the production of *Neospora caninum* tissue cysts in mice. *Journal of Parasitology* **83**, 647–651.

MÜLLER, N., ZIMMERMANN, V., HENTRICH, B. & GOTTSTEIN, B. (1996). Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. *Journal of Clinical Microbiology* **34**, 2850–2852.

MÜLLER, N., VONLAUFEN, N., GIANINAZZI, C., LEIB, S. L. & HEMPHILL, A. (2002). Application of real time fluorescent PCR for quantitative assessment of *Neospora caninum* infections in organotypic slice cultures of rat central nervous tissue. *Journal of Clinical Microbiology* **40**, 252–255.

NISHIKAWA, Y., XUAN, X., NAGASAWA, H., IGARASHI, I., FUJISAKA, K., OTSUKA, H. & MIKAMI, T. (2000). Monoclonal antibody inhibition of *Neospora caninum* tachyzoite invasion into host cells. *International Journal for Parasitology* **30**, 51–58.

NISHIKAWA, Y., INOUE, N., XUAN, X., NAGASAWA, H., IGARASHI, I., FIJISAKI, K., OTSUKA, H. & MIKAMI, T. (2001). Protective efficacy of vaccination by recombinant vaccinia virus against *Neospora caninum* infection. *Vaccine* **19**, 1381–1390.

SONDA, S., FUCHS, N., CONNOLLY, B., FERNANDEZ, P., GOTTSTEIN, B. & HEMPHILL, A. (1998). The major 36 kDa *Neospora caninum* tachyzoite surface protein is closely related to the major *Toxoplasma gondii* surface antigen 1. *Molecular and Biochemical Parasitology* **97**, 97–108.

SONDA, S., FUCHS, N., GOTTSTEIN, B. & HEMPHILL, A. (2000). Molecular characterization of a novel microneme antigen in *Neospora caninum*. *Molecular and Biochemical Parasitology* **108**, 39–51.

WESSEL, D. & FLUEGGE, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergent. *Analytical Biochemistry* **138**, 141–143.