

## Immunisation of mice against neosporosis

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### Abstract

In the present study a murine encephalitis model was used to investigate if protection against neosporosis could be achieved by immunisation. Groups of 10 mice were immunised with a sublethal dose of live *Neospora caninum* tachyzoites, *N. caninum* antigens incorporated into iscoms, *N. caninum* lysate mixed with Quil A, or *N. caninum* lysate in PBS. Control mice were given Quil A only. Challenge infection with  $2.5 \times 10^6$  *N. caninum* tachyzoites resulted in clinical symptoms that remained until the end of the experiment in the controls. In contrast, mice immunised with live parasites or parasite lysate in Quil A only showed mild and transient symptoms. Of nine mice immunised with *N. caninum* iscoms, seven recovered while two died. Most severely affected were the mice immunised with parasite lysate only; all of them died within 28 days post-infection. Histological examination and scoring of brain lesions gave a significantly lower ( $P < 0.0001$ ) lesion score in mice immunised with live parasites than in controls. The groups immunised with iscoms or lysate and Quil A also had reduced lesion scores ( $P < 0.04$  and  $0.07$ , respectively) but not the group given parasite lysate alone. The lesions seen in the latter group differed from those in the other groups. There was less cellular reaction and more tachyzoites indicating an active infection. The *N. caninum* specific antibody responses and cytokine production (IFN- $\gamma$ , IL-4 and IL-5) of splenocytes were analysed at the time of challenge infection. The results suggest a correlation between protection and high levels of IFN- $\gamma$ . Also, the immune responses recorded in mice immunised with parasite lysate without adjuvant were relatively weak and more towards the Th2 type, when compared with the other immunisation schedules. This is consistent with the weaker inflammatory response observed in the brains of these mice. © 2002 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

**Keywords:** *Neospora caninum*; Encephalitis; Immunity; Cellular immune response; Cytokines; Antibody response

### 1. Introduction

*Neospora caninum* is an intracellular protozoan parasite closely related to *Toxoplasma gondii* (Ellis et al., 1994; Holmdahl et al., 1994). It was first identified as a cause of neuromuscular disease in dogs (Dubey et al., 1988a) but is now recognised to infect a wide range of animal species and to be an important cause of abortion in cattle. The infection is transmitted transplacentally from mother to foetus (Dubey, 1999). Experimental infection can also be transmitted through ingestion of cysts in the tissues of persistently infected mice, or following oral administration of oocysts shed in the faeces of dogs (McAllister et al., 1998; De Marez et al., 1999). Thus the life cycle is similar to that of *T. gondii*, an important pathogen of humans and animals (Tenter et al., 2000).

In seeking to develop a laboratory animal model for neosporosis it was found that while most mouse strains are highly resistant, inbred BALB/c mice develop clinical neosporosis after inoculation with the parasite (Lindsay et al., 1995). Typical symptoms usually appear after 2–3 weeks and include head tilting, circling, hind limb paralysis and progressive weakness. The main pathological finding is a multifocal meningoencephalitis. This murine model offers the means to investigate whether immunisation against neosporosis is possible. Such work is of great interest since vaccination has been suggested as a strategy to prevent neosporosis in cattle and the considerable economic losses that the infection is causing the farming industry (Dubey, 1999; Trees et al., 1999).

The aim of the present study was to investigate the possibility of inducing protection against *N. caninum* infection either by inoculation of mice with live parasites or by immunisation with parasite antigens. The antigens were either incorporated in immune stimulating complexes (iscoms; Morein et al., 1984) or mixed with the adjuvant Quil A, both of which have previously been found effective for

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induction of protective immunity to *T. gondii* (Kahn et al., 1991; Lundén et al., 1993). The immune responses induced in vaccinated mice were characterised with regards to both antibody and T-cell mediated immunity.

## 2. Material and methods

### 2.1. Parasites

The NC-1 isolate of *N. caninum* (Dubey et al., 1988b) was used for the inoculation of mice and to prepare the different antigen preparations used for immunisation and analysis of the immune response. The parasites were propagated in Vero cells as previously described (Lundén et al., 1998) and were harvested by scraping off the cell monolayers 3–4 days after they had been infected. The resulting parasite suspensions contained <5% Vero cells.

### 2.2. Preparation of parasite lysate

Water soluble parasite antigens were prepared as previously described (Lundén et al., 1998) and used for immunisation of mice and in vitro stimulation of lymphocytes.

### 2.3. Production of iscoms

Iscoms were prepared as previously described (Björkman and Lundén, 1998) by mixing Quil A, cholesterol, phosphatidylcholine and *N. caninum* antigens extracted from tachyzoites by a detergent. The detergent was removed by extensive dialysis, and unincorporated proteins and excess lipids were removed by centrifugation through a sucrose gradient.

### 2.4. Animals and immunisations

Female, 12-weeks-old BALB/c mice were housed in groups of no more than 10 and fed a proprietary rodent mix and given clean water daily ad libitum.

To test whether prior infection or immunisation would be protective against a challenge infection 60 mice were allocated into six groups each consisting of 10 mice (Table 1). Group 1 mice were each inoculated i.p. with  $10^6$  NC-1 tachyzoites and Group 2 each with  $10^4$  tachyzoites. Group 3 mice were each immunised s.c. with *N. caninum* iscoms (2 µg protein/dose), Group 4 mice each with 2 µg *N. caninum* lysate mixed with 10 µg Quil A (Spikoside, Advet AB) and Group 5 each with 2 µg *N. caninum* lysate in phosphate buffered saline (PBS). Each mouse in the sixth group was given 10 µg Quil A. After 42 days, the immunisations with non-living antigen (Groups 3–6) were repeated when the iscom dose was increased to 6 µg protein (9.6 µg Quil A), and the *N. caninum* lysate dose to 6 µg protein.

In addition, another six groups each of nine mice (Groups 1a–6a, respectively) were used for examination of the cellular immune responses induced by the different immunisations. Five of these groups (1a–5a, respectively) were immunised in an identical manner and with the same parasite preparations as Groups 1–5, respectively, described above, while Group 6a was left untreated. An additional experiment included five mice similarly administered 10 µg Quil A and five untreated control mice. Since it has been previously shown that the structure of the iscom particles without any antigen do not induce immunological memory (Fossum et al., 1990), this control was judged as sufficient to control for non-specific cellular responses induced by Quil A in Groups 3a and 4a.

### 2.5. Challenge infection

Fifty-six days after the initial immunisation, all mice in

Table 1  
Mortality and pathological changes in the brains of mice<sup>a</sup>

Group	Immunisation	Accumulated mortality		Encephalitis <sup>b</sup>		Relative severity of pathological changes			Brain lesion score	
		n	%	n	%	No lesions	Mild lesions	Severe lesions	Mean ± SD	P <sup>c</sup>
						n	n	n		
1	10 <sup>6</sup> live NC-1 <sup>d</sup>	0/10	0	9/10	90	1	9	0	0.9 ± 0.3	0.0001
2	10 <sup>4</sup> live NC-1 <sup>d</sup>	0/10	0	3/9	33	6	3	0	0.3 ± 0.5	0.0001
3	Nc iscom <sup>e</sup>	2/9	22	4/8	50	4	0	4	1.0 ± 1.1	0.0413
4	Nc lysate + Quil A <sup>c</sup>	0/9	0	6/9	67	3	1	5	1.2 ± 1.0	0.0723
5	Nc lysate <sup>e</sup>	9/9	100	6/7	86	1	0	6	1.7 ± 0.8	0.7275
6	Control (Quil A <sup>c</sup> )	1/10	10	10/10	100	0	1	9	1.9 ± 0.3	-

<sup>a</sup> The mice were immunised with different *N. caninum* preparations as shown and on day 56 all groups were inoculated with  $2.5 \times 10^6$  *N. caninum* tachyzoites of the NC-1 isolate.

<sup>b</sup> Day 37 p.i. all surviving mice were killed and their brains examined, when possible the brains from mice that died or had to be killed before day 37 p.i. were also examined.

<sup>c</sup> Compared with Group 6.

<sup>d</sup> Day 0.

<sup>e</sup> Days 0 and 42.

Groups 1–6 were each inoculated i.p. with  $2.5 \times 10^6$  freshly harvested NC-1 tachyzoites in 0.1 ml cell culture medium. Thereafter the mice were observed daily and clinical symptoms recorded. Mice that showed significant symptoms were killed to limit unnecessary suffering. Thirty-seven days after the challenge infection all surviving mice were killed.

### 2.6. Serology

Five mice from each of Groups 1–6 were bled from the tail vein on days 21, 42 and 56 after the first immunisation, and the serum separated and stored at  $-20^\circ\text{C}$ . Individual sera were analysed for *N. caninum* specific IgG1 and IgG2a by enzyme-linked immunosorbent assay (ELISA) essentially as described by Sjölander et al. (1996).

### 2.7. Immunoblotting analyses

Sera collected at the day of challenge infection was analysed by immunoblotting as previously described (Lundén et al., 1993; Björkman et al., 1994). To aid the identification of antigens, strips were also probed with the mAbs NcMab-4, -7, -10 and -13 (Björkman and Hemphill, 1998). HRP-conjugated rabbit anti-mouse immunoglobulin (Dako) was used as secondary antibody and the enzyme was visualised by ECL Western blotting detection reagents (Amersham Life Science). Prestained protein markers (New England Biolabs Inc.) were used for the estimation of molecular weight.

### 2.8. Lymphocyte stimulation assay

Three mice from each of Groups 1a–6a were killed on days 14, 41 and 55 after the first immunisation. The spleens were removed and single cell suspensions were prepared from each mouse. As culture medium, IMDM supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin and 50  $\mu\text{M}$  2-mercaptoethanol, was used. The cells were dispensed into 96-well flat-bottomed microtitre plates at a concentration of  $2.5 \times 10^5$  cells/well with concanavalin A (Con A; 5  $\mu\text{g/ml}$ ) or *N. caninum* lysate (1, 3 and 10  $\mu\text{g/ml}$ ). Unstimulated control cultures were included and, as a further control, cells from mice immunised with iscoms or *N. caninum* lysate were set up in cultures with a Vero cell lysate prepared similarly to the parasite lysate. Each culture was set up in triplicate. After 96 h incubation at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , 0.5  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine was added to each well and the plates were incubated for further 16 h. Cultures were harvested onto filters and the amount of incorporated thymidine was analysed using a gas proportional counter (Canberra Packard).

### 2.9. Cytokine analysis

Cells were cultured with *N. caninum* lysate, Vero cell lysate (10 mg/ml) or without antigen in duplicate wells as

described for the proliferation assay. Cell culture supernatants were collected after 96 h and analysed for IFN- $\gamma$  and IL-5 by a commercial ELISA (MiniKit, Endogen) according to instructions from the manufacturer. IL-4 was measured by a bioassay based on the IL-2/IL-4 responsive NK cell line (Swain et al., 1981) as previously described (Allen and MacDonald, 1998). Detection limits for the cytokine assays were: IFN- $\gamma$ , 2 ng/ml; IL-5, 60 pg/ml; IL-4, 80 pg/ml.

### 2.10. Histopathological examinations

The brain, left lung and heart were removed from each mouse and placed in 10% formol saline for a period of between 1 and 2 weeks. Coronal blocks of brain were cut from the anterior cerebrum, the mid cerebrum at the level of the thalamus, the posterior cerebrum at a level of the hippocampus, the midbrain, the cerebellum and the pons and were processed through graded alcohols to paraffin wax. Sections 6- $\mu\text{m}$  thick were cut and stained with H & E. Two blocks of lung tissue from the left lobe and a transverse block of heart were similarly processed. Pathological findings in the brain were scored as follows: no lesions = 0; mild lesions = 1; severe lesions = 2.

### 2.11. Immunohistochemistry

Selected sections of brain were also treated with an immunohistochemical method to demonstrate the presence of *N. caninum* antigen, as already described (Buxton et al., 1997).

### 2.12. Statistical analyses

The non-parametric Kruskal–Wallis and Mann–Whitney *U*-tests were used to compare proliferative responses, cytokine levels and brain lesion scores.

## 3. Results

### 3.1. Clinical signs and mortality

None of the mice showed any clinical symptoms following injection with live parasite or the other preparations, but one mouse was found dead in each of Groups 3, 4 and 5 on days 9, 23 and 27, respectively, after immunisation. These mice were discarded.

Four days after administering the challenge infection the hair coat of all mice became ruffled, suggesting fever, but the mice were not severely ill. The control mice remained affected until the end of the experiment. They had ruffled coats and remained active, but two of them progressively lost weight and one had to be killed on day 32 p.i. In contrast, all mice immunised with live parasites or *N. caninum* lysate with Quil A recovered after 4–5 days and remained clinically normal for the remainder of the observation period. Of the nine mice immunised with iscoms, seven recovered, one remained affected and died on day

10 p.i., and one appeared to recover but then developed hind leg paralysis and was killed 21 days p.i. The most severely affected were those given *N. caninum* lysate without adjuvant. They became hunched and reluctant to move, and some of them developed central nervous system (CNS) symptoms such as walking in circles and hind leg paralysis. All died, or had to be killed, by 28 days p.i. (Table 1).

### 3.2. Humoral immune responses

Twenty-one, 42 and 56 days after the primary immunisation, *N. caninum*-specific antibodies were detected in all sera tested from mice immunised with live parasites, iscoms or *N. caninum* lysate with Quil A. The IgG1 and IgG2a titres at the day of challenge infection (day 56) are shown in Fig. 1. In mice given the highest number of parasites, the levels of IgG1 and IgG2a were equally high, while mice inoculated with the lower dose had higher IgG2a than IgG1 titres. High levels of both subclasses were also induced by *N. caninum* lysate mixed with Quil A. The highest titres were detected in sera from iscom immunised mice, and they had somewhat higher IgG1 than IgG2a titres. In the group immunised with *N. caninum* lysate alone, only three out of five mice had seroconverted at day 56. IgG1 dominated in these sera, with low levels of IgG2a detected. When tested 21 and 42 days after the first immunisation, only one of the five mice from this group had antibodies to *N. caninum* and only of the IgG1 subclass. All sera from the control group were negative.

Immunoblotting analysis revealed that sera from mice

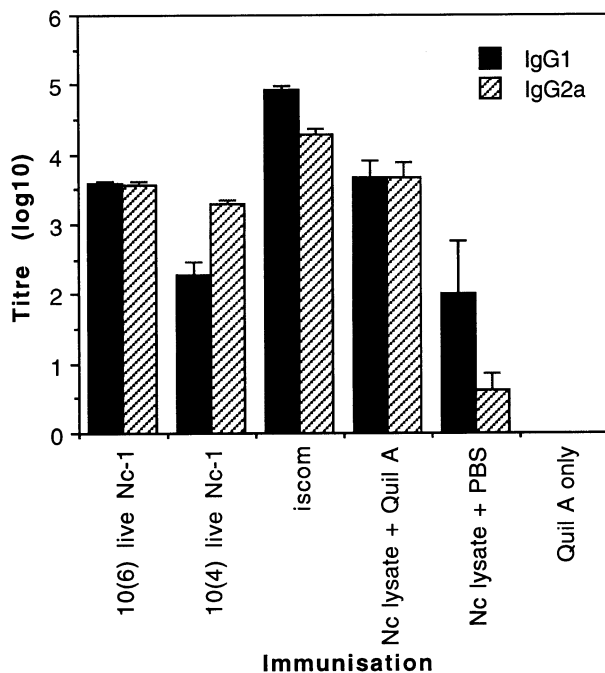


Fig. 1. IgG1 and IgG2a titres in sera from mice immunised once with live *N. caninum* tachyzoites 56 days previously, or twice with *N. caninum* antigens in iscoms, with Quil A or without any adjuvant 56 and 14 days ago. Data are presented as geometrical means  $\pm$  SEM for five mice per group.

inoculated with live parasites reacted with 10–20 antigen bands in reduced *N. caninum* whole tachyzoite antigen. The reactions were stronger and more bands were visible on strips incubated with sera from mice given the highest dose compared with those mice given the lower dose. Sera from mice immunised with *N. caninum* lysate in Quil A recognised three different antigens of approximately 52, 44, and 34 kDa that appeared to correspond to those recognised by mAbs NcMab-13, -10, -4, and a fourth antigen of approximately 16 kDa. A very faint band of approximately 28 kDa was also seen. Sera from iscom immunised mice reacted with the same antigens, but the reactions were much stronger. In addition, a strong double band around 58–56 kDa and two faint bands around 68, and 23 kDa could be seen. Immunisation with *N. caninum* lysate alone induced antibodies recognising only two bands of about 34 and 44 kDa that appeared to correspond to the antigens recognised by NcMab-4 and 10 (Fig. 2). Immunoblotting with non-reduced *N. caninum* antigen revealed that the 16 kDa band mentioned above corresponded to the band recognised by NcMab-7 (not shown).

### 3.3. Cellular immune responses

The proliferative responses and the concentrations of cytokines in supernatants from cell cultures set up at the time of challenge infection (day 55) are presented in Fig. 3A–D.

Cells from all infected or immunised mice proliferated in a dose-dependent manner when stimulated in vitro with *N. caninum* lysate. At days 14 and 55 there was no significant difference between the groups, but at day 41 the mice immunised with *N. caninum* lysate, alone or with Quil A, responded significantly less ( $P < 0.0019$ ) than animals immunised with live parasites or iscoms. In cultures from

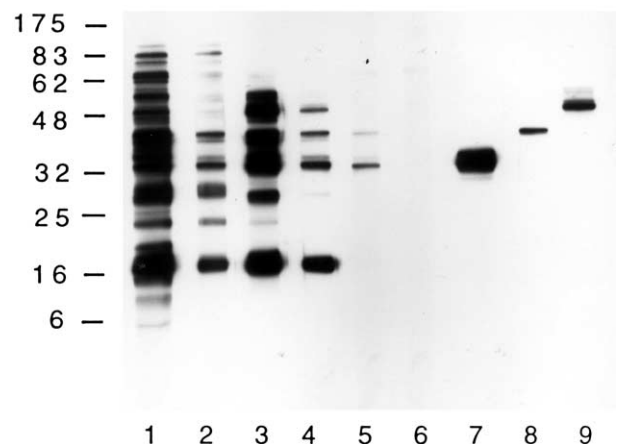


Fig. 2. Immunoblotting analysis of antibody responses. Strips with *N. caninum* tachyzoite antigens separated under reducing conditions were incubated with sera from mice immunised with  $10^6$  (1) or  $10^4$  (2) live *N. caninum* tachyzoites, *N. caninum* iscoms (3), *N. caninum* lysate with Quil A (4), *N. caninum* lysate without any adjuvant (5), or Quil A only (6). Strips with *N. caninum* antigen were also incubated with the monoclonal antibodies NcMab-4 (7), NcMab-10 (8) and NcMab-13 (9).

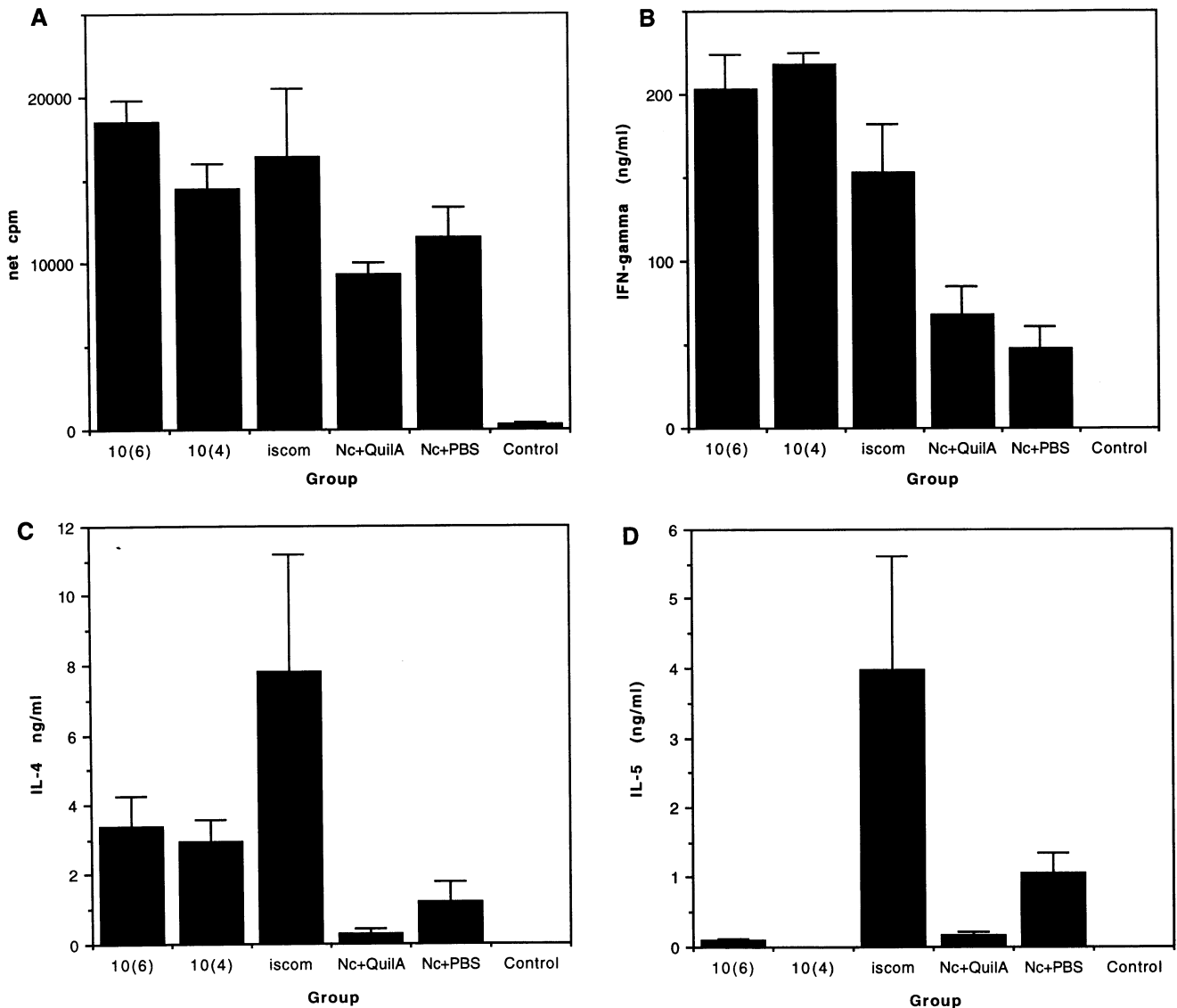


Fig. 3. Proliferation and cytokine production in spleen-cell cultures set up at the time of challenge infection and stimulated in vitro with *N. caninum* lysate. (A) Net cpm; (B) IFN- $\gamma$ ; (C) IL-4; and (D) IL-5. Results from each group of mice are presented as the mean  $\pm$  SEM ( $n = 3$ ).

non-inoculated controls or mice given Quil A only proliferation did not exceed 2.5 times the level of medium control cultures (Fig. 3A). Only low levels (230–1,387 cpm) of spontaneous proliferation were detected in the medium control cultures.

When tested at 55 days after the first immunisation, *N. caninum* lysate induced IFN- $\gamma$  and IL-4 production in cell cultures from all infected or immunised mice. Cells from mice inoculated with live parasites produced significantly more ( $P < 0.0047$ ) IFN- $\gamma$  than cells from mice immunised with different parasite antigen preparations. Culture supernatants from iscom immunised mice contained more IFN- $\gamma$  than those from mice given *N. caninum* lysate in Quil A or PBS, but the difference between the groups was not significant ( $P < 0.0509$ ) (Fig. 3B). Cells from mice inoculated with live parasites produced significantly more IL-4 than

cells from mice given *N. caninum* lysate in Quil A or PBS ( $P < 0.0104$ ). Cultures from mice given iscoms contained very high, but variable, levels of IL-4 (Fig. 3C). IL-5 was detected at very low levels in cultures from mice immunised with live parasites or *N. caninum* lysate with Quil A. In contrast, cells from all three mice immunised with *N. caninum* lysate in PBS produced substantial amounts of IL-5. Even higher, but variable, levels were detected in cultures from mice given iscoms (Fig. 3D).

Analysis for IFN- $\gamma$  in supernatants collected from cultures set up at days 14 and 41 showed a similar pattern with the exception that IFN- $\gamma$  was not detected in cell cultures from all mice given *N. caninum* lysate in PBS. At these time points, IL-4 was only detected in supernatants from infected mice, and IL-5 only in one of two supernatants from mice given *N. caninum* lysate without adjuvant (not shown).

Cytokines were not detected in any cell culture from the non-inoculated control group, from the mice given Quil A only, or in medium control cultures from the other groups. Stimulation with Vero-cell antigen occasionally resulted in proliferation or cytokine production, but this was not a consistent finding and the responses were very weak compared with those induced by parasite antigen (not shown).

### 3.4. Histopathology

#### 3.4.1. Brain

In the control group, nine of the 10 mice showed a severe non-suppurative meningoencephalitis. Scattered throughout the brain there were well-circumscribed multiple foci of intense inflammation in which necrotic neuropil had been replaced by a closely packed, mixed population of mononuclear cells including microglia, macrophages, lymphoid cells and plasma cells (Fig. 4A). In some instances there was

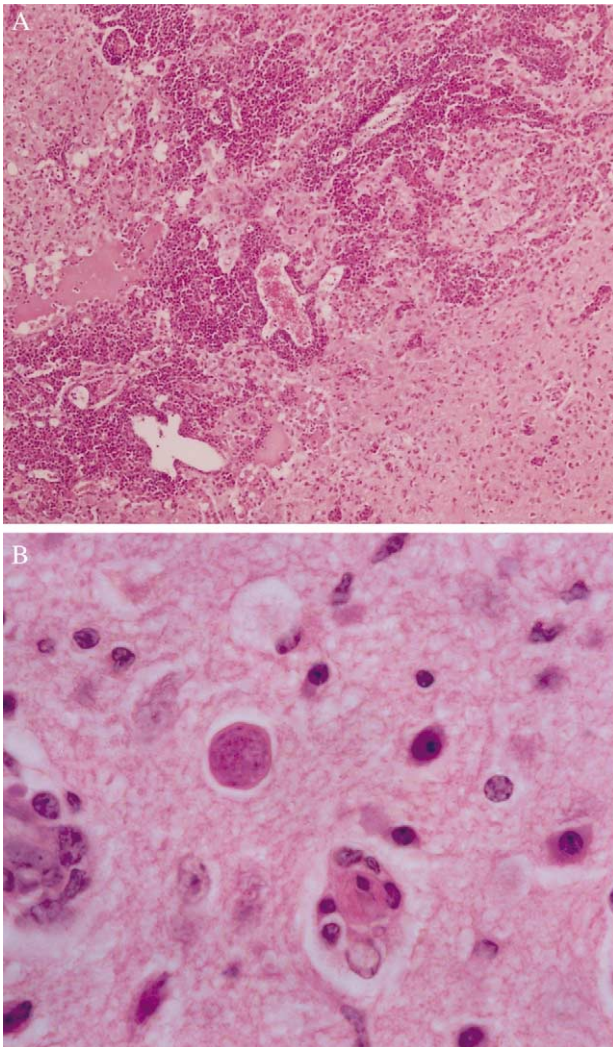


Fig. 4. (A) Focal inflammation in the cerebrum of a mouse from the non-immunised control group (H & E  $\times$  100). (B) A tissue cyst in the cerebrum of a mouse from the same group as in (A) (H & E  $\times$  1,000).

also associated serum leakage. Scattered perivascular cuffs were present in and around these foci as well as in other areas of the neuropil. Cyst-like structures, each with prominent cyst wall, were observed in six cases (Fig. 4B). The meningitis tended to be focal and to be mainly composed of lymphoid cells but in some instances it also contained plasma cells and macrophages. One control mouse showed only mild inflammatory changes with no necrosis (Table 1).

Four mice given iscoms and five mice given *N. caninum* lysate with Quil A showed similar multifocal necrosis and associated inflammation, although tissue cysts were scarce. In a further four and three mice, respectively, no significant changes were observed. One mouse immunised with *N. caninum* lysate in Quil A showed mild inflammatory changes. The mean lesion score for the group given iscoms was significantly lower ( $P < 0.04$ ) than for the control group, while the difference between the group given *N. caninum* lysate with Quil A and the controls was not significant ( $P < 0.07$ ; Table 1).

Nine of the 10 mice inoculated with the highest dose of live parasites and three of the nine inoculated with the lower dose showed mild inflammation while no significant changes were observed in the remaining mice in these groups. The mean lesion score for each of these two groups was significantly lower ( $P < 0.0001$ ) than for the controls (Table 1).

The mild inflammation, that was recorded in mice from four of the six groups, was characterised by multiple foci of microgliosis with associated infiltrating lymphoid cells as well as lymphoid perivascular cuffing. Meningitis was also often present but generally composed of mild, focal and lymphoid cells.

In the group immunised with *N. caninum* lysate without adjuvant, six of the seven mice examined had severe lesions with multifocal necrosis presenting predominantly as cytolysis. Associated inflammation was much less prominent than in the other groups and consisted of relatively mild microgliosis (Fig. 5A). Clusters of parasite-like structures were often present in the necrotic foci, and frequently, in the surrounding tissue, there were lymphoid cuffs, sometimes containing a small number of granular leukocytes. No lesions were found in the seventh mouse in this group. The mean lesion score was not significantly different ( $P < 0.7275$ ; Table 1) to that of the control group.

#### 3.4.2. Lung

Mild to moderate interstitial inflammation was present in eight of the 10 control mice. While mainly consisting of small nodules of lymphoid cells scattered in the parenchyma, sometimes associated with a blood vessel, there were also areas of diffuse non-suppurative interstitial inflammation involving alveolar septa. Very mild interstitial inflammation was also observed in at least half of the mice in Groups 1–4. Due to an error at the time of necropsy no samples of lung were available from Group 5.

### 3.4.3. Heart

No significant histopathological changes were found in any of the samples of heart from all six groups.

### 3.5. Immunohistochemistry

*N. caninum* antigen was observed in four forms; tachyzoites, aggregates of parasites in cyst-like structures, particulate antigen either free or within the cytoplasm of phagocytic cells, and antigen defined as ‘soluble’ which was characterised as diffuse, amorphous labelling of the cytoplasm of certain cells and of necrotic debris.

Antigen was not detected in the brains of mice immunised with live parasites. In the mice with severe focal inflammation (controls and mice given iscoms or *N. caninum* lysate in Quil A) antigen was relatively sparse and was often present as soluble or particulate antigen within inflammatory cells. A few clusters of tachyzoites were also present in these foci

as well as infrequent thick-walled cysts. Relatively, dense aggregates of soluble and particulate antigen were also present in the necrotic tissue at the centre of a few inflammatory foci. In contrast, in the foci of necrosis in mice given *N. caninum* lysate without adjuvant, antigen was abundant and consisted of numerous tachyzoites as well as particulate and soluble antigen (Fig. 5B) and the occasional small cyst-like structure.

## 4. Discussion

The results of the present study show that a subclinical *N. caninum* infection can induce protective immunity in mice against challenge with a number of parasites sufficient to cause severe encephalitis in non-immunised controls. While we used different doses of the relatively virulent NC-1 isolate of *N. caninum* for both immunisation and challenge, similar results have been obtained by inoculation with avirulent strains of the parasite. Atkinson et al. (1999) report that BALB/c mice inoculated with the relatively avirulent NC-SweB1 isolate were partially protected against a lethal challenge with the more virulent NC-Liverpool isolate. Similarly, an avirulent, non-persistent, temperature-sensitive strain generated from NC-1 by chemical mutagenesis and selection for growth at 32°C has been used to immunise BALB/c mice. After challenge with the NC-1 isolate the survival rate was higher and the mean brain lesion score significantly lower than in non-immunised controls (Lindsay et al., 1999).

We also found that a lesser degree of protective immunity could be induced by immunisation with parasite antigens together with the adjuvant Quil A. Two different formulations were used, crude parasite lysate mixed with Quil A and parasite antigen incorporated into iscoms, which are submicroscopic particles consisting of Quil A, cholesterol and other lipids (Morein and Lövgren Bengtsson, 1999). Both groups had lower brain lesion scores than the controls given Quil A only, although only the difference between iscoms and controls was statistically significant. All mice given parasite lysate with Quil A survived while two of the nine iscom immunised mice died compared to one of the 10 controls. Judged by clinical symptoms surviving mice in both groups thrived better than the controls.

Other studies in BALB/c mice of the protective effect of *N. caninum* antigens have met with variable success. Immunisation with a crude parasite lysate together with the adjuvant ImmuMAXSR<sup>®</sup> prevented transplacental transmission following challenge infection during pregnancy (Liddell et al., 1999). In contrast, mice administered parasite antigens mixed with Freund’s complete adjuvant or entrapped in non-ionic surfactant vesicles had more brain lesions than the controls. This adverse effect was associated with cytokine production and an antibody subclass profile indicating induction of a type 2 immune response (Baszler et al., 2000).

In general, immunity to intracellular parasites is mainly

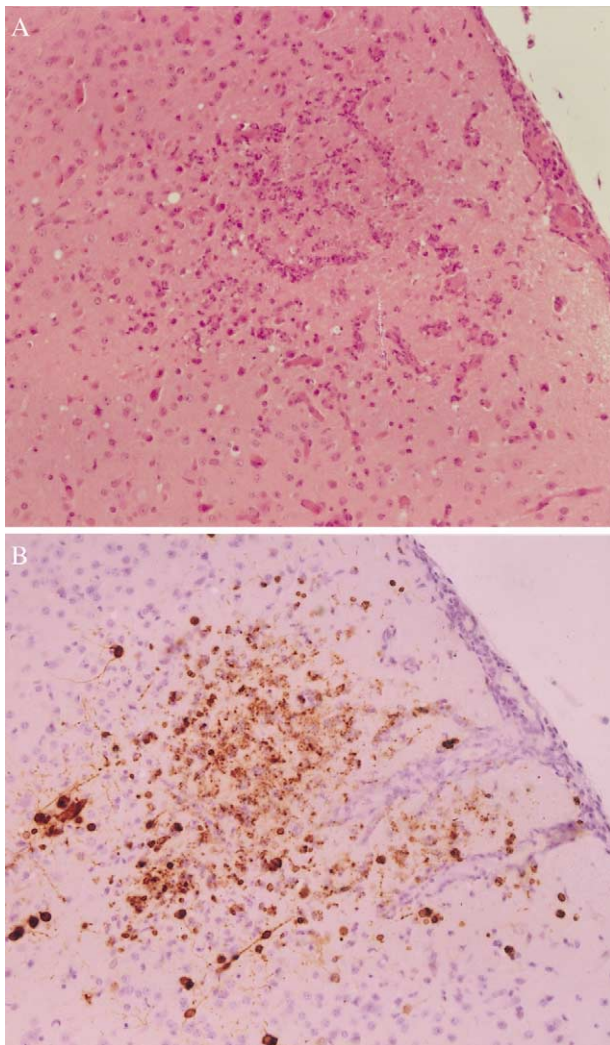


Fig. 5. (A) Focal necrosis with a mild inflammatory response in the cerebrum of a mouse immunised with *N. caninum* lysate without any adjuvant (H & E  $\times 200$ ). (B) Abundant *N. caninum* antigen in the focus of necrosis and inflammation shown in (A) (immunohistochemical staining  $\times 200$ ).

cell mediated and associated with a Th1 response characterised by production of IL-12 and IFN- $\gamma$ , while Th2-type responses and production of IL-4 and IL-10 are regarded as unfavourable and may lead to disease progression. Typically, these two types of response are cross-regulatory and tend to down-regulate each other. However, in most cases induced immunity is not a clear-cut Th1 or Th2 response but involves both sets of cytokines which interact in complex regulatory pathways (Allen and Maizels, 1997). Since Th1 cytokines favour production of IgG2a and Th2 cytokines stimulate IgG1 production, analysis of these two subclasses can be used to assess the *in vivo* production of cytokines.

It has been shown that IL-12 and IFN- $\gamma$  play an important role in the immune control of *N. caninum* (Kahn et al., 1997). It has also been found that susceptibility is associated with a mixed response characterised by a low IFN- $\gamma$ :IL-4 ratio (Long et al., 1998; Baszler et al., 1999). In the present study, cells from infected mice produced substantial levels of both IFN- $\gamma$  and IL-4 when stimulated with *N. caninum* antigen *in vitro*, and *N. caninum* specific IgG1 and IgG2a were detected at comparable titres. These findings are in accord with previous descriptions of the immune responses to a primary *N. caninum* infection in BALB/c mice (Long et al., 1998; Baszler et al., 1999). From the results of the present study it can be concluded that although BALB/c mice are relatively susceptible to *N. caninum* infection, they have the capacity to mount a protective immune response and that such a response can be protective against a secondary infection despite a relatively strong Th2 component.

Comparison of the immune responses evoked by the different immunisation schedules indicates a possible correlation between protection and the level of IFN- $\gamma$ . The best protection was achieved by inoculation with live parasites and spleen cells from these mice produced significantly more IFN- $\gamma$  than mice immunised with non-living antigen. On the other hand, the dramatic difference in susceptibility between mice given only parasite lysate and those given parasite antigens together with an adjuvant could not be related to any significant difference in IFN- $\gamma$  production. Neither was there any obvious overall correlation between protection and production of IL-4 and IL-5 or the ratio of IL-4 to IFN- $\gamma$ . This was probably a consequence of the striking differences in cytokine production between the two partially protected groups immunised either with iscoms or antigen with Quil A, respectively. In the latter group the response was dominated by IFN- $\gamma$ , although at moderate levels compared with the other groups, and only low levels of IL-4 and IL-5 were detected. In contrast, spleen cells from iscom immunised mice secreted high levels of all three cytokines. This kind of mixed response is often seen after immunisation with iscoms containing different antigens (Sjölander et al., 1997; Morein and Lövgren Bengtsson, 1999). Iscoms containing *T. gondii* antigens also have the capacity to induce partial protection (Uggla et al., 1988; Lundén et al., 1993) despite inducing a similar mixed response (unpublished results).

Mice immunised with parasite lysate alone were not protected, and appeared even more susceptible than the control mice. The lack of protection after immunisation with parasite antigen without adjuvant is in agreement with the findings of Lindsay et al. (1999), who, however, did not record any increased susceptibility when compared with non-immunised controls. However, since we used mice treated with Quil A as controls, and non-specific effects of Quil A cannot be ruled out, further work is necessary to investigate whether this treatment enhanced non-specific immune mechanisms such that immunisation with parasite lysate alone appeared to increase susceptibility. On the other hand this seems unlikely since mice inoculated with Quil A in the form of 'empty' iscoms were not protected against a lethal *T. gondii* infection (Lundén et al., 1993).

The brain lesions in the mice immunised with parasite lysate without any adjuvant differed substantially from those seen in severely affected mice in the other groups. In the latter there was clear evidence of a host cellular immune response in which necrotic neuropil had been replaced by a closely packed mixed population of mononuclear inflammatory cells. Furthermore, relatively few parasites were observed indicating a degree of suppression of parasite multiplication. In mice given lysate without adjuvant, the inflammatory response appeared to be much less developed and numerous tachyzoites were observed, indicating a more active infection consistent with the clinical signs and mortality. Long et al. (1998) reports a similar qualitative difference between brain lesions seen in different mouse strains, with more necrosis and less cellular infiltration in susceptible BALB/c and C57BL/6 mice than in resistant B10.D2 mice. Thus it is concluded that the observed inflammatory response had a role in controlling the infection. Judged by antibody production and the cytokines produced *in vitro*, the immune response induced by immunisation with parasite lysate alone was relatively weak and more towards the Th2 type, when compared with the other immunisation schedules, which is consistent with a weaker inflammatory response (Denkers, 1999).

The antigen specificity of the antibody responses at the time of challenge infection was analysed by immunoblotting. It is notable that some of the antigens recognised by sera from mice immunised with the lowest dose of tachyzoites, which were well protected against the challenge infection, were not recognised by sera from the less well-protected groups, and vice versa. Thus it could be speculated that the antigens seen only in blots from Group 1 and 2, such as the 28 kDa antigen, might have contributed to the induction of protective immunity, and that the 52 kDa antigen recognised by NcMab 13 may not be necessary for protection. This mAb is directed against an intracellular antigen. The other mAbs used in this study are against antigens present both on the tachyzoite surface and on intracellular membranes (Björkman and Hemphill, 1998). These three antigens were recognised by sera from both fully and partially protected groups. Although the identities of

these antigens have not been entirely clarified it appears that NcMab 4 and NcMab 10 are directed against the surface antigens NcSAG1 (or Ncp-36) and NcSRS2 (or Ncp-43), respectively (Hemphill et al., 1997a,b; Howe et al., 1998). These antigens are both likely to play important roles in infection, since antibodies against them can inhibit host cell invasion in vitro (Hemphill, 1996; Nishikawa et al., 2000).

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