Neospora caninum is an apicomplexan parasite that was recognized as a cause of neuromuscular disease in dogs in 1988 and subsequently determined to be a major cause of abortions in cattle (see Dubey and Lindsay, 1996). Neospora caninum is also associated with abortions and neonatal paralysis/disease in goats, sheep, black-tailed deer, El d deer, and horses (see Dubey and Lindsay, 1996). Toxoplasma gondii, another important related parasite, is found in many animals that are susceptible to Neospora caninum infection.

We have previously demonstrated that outbred mice vaccinated subcutaneously (s.c.) with the NC-1 or NC-3 strains of N. caninum are not protected from fatal disease when s.c. challenged with tachyzoites of the highly virulent RH strain of T. gondii after vaccination with HBSS, NC-1 tachyzoites, or TS-4 tachyzoites. Mice vaccinated with NC-1 strain tachyzoites survived significantly (P < 0.05) longer than mice given HBSS in experiment 1, but not in experiments 2 and 3. Mice vaccinated with TS-4 strain tachyzoites survived significantly longer than HBSS-vaccinated mice in experiments 1, 2, and 3 and significantly longer than mice vaccinated with the NC-1 strain in experiments 2 and 3. Toxoplasma gondii tissue cyst numbers were significantly lower for mice vaccinated with TS-4 strain tachyzoites than mice vaccinated with HBSS or the NC-1 strain tachyzoites in experiment 1. No difference was observed in tissue cyst numbers in mice vaccinated with HBSS or NC-1 strain tachyzoites in experiment 1. No HBSS-vaccinated mice survived experiment 2, and the numbers of T. gondii tissue cysts were significantly lower for mice vaccinated with the TS-4 strain tachyzoites compared to NC-1 strain tachyzoites. No HBSS- or NC-1-vaccinated mice survived RH strain challenge in experiment 3. Results of these experiments indicate that infection with N. caninum provides some protection against fatal oral infection with T. gondii oocysts of a moderately pathogenic strain but not tachyzoites of a highly pathogenic strain. The protection provided by N. caninum is much less than that provided by previous exposure to T. gondii. The parasite strain would survive a lower s.c. T. gondii infection than in our previous study (Lindsay et al., 1990).
Table I. Protocols for inoculation and challenge of mice and results of mortality and mean survival time postchallenge of mice in experiments 1, 2, and 3.

<table>
<thead>
<tr>
<th>Experiment/ group</th>
<th>First/second vaccination*</th>
<th>Challenge inoculation†</th>
<th>Mean challenge (% mortality)‡</th>
<th>Mean survival (days ± SD)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>HBSS/HBSS</td>
<td>VEG/L</td>
<td>80</td>
<td>21 ± 19.1†</td>
</tr>
<tr>
<td>1/2</td>
<td>NC-1/NC-1</td>
<td>VEG/L</td>
<td>11</td>
<td>54 ± 5.0*</td>
</tr>
<tr>
<td>1/3</td>
<td>TS-4/TS-4</td>
<td>VEG/L</td>
<td>0</td>
<td>56 ± 0.0*</td>
</tr>
<tr>
<td>1/4#</td>
<td>NC-1/NC-1</td>
<td>None</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>2/1</td>
<td>HBSS/HBSS</td>
<td>VEG/H</td>
<td>100</td>
<td>9 ± 1.7†</td>
</tr>
<tr>
<td>2/2</td>
<td>NC-1/NC-1</td>
<td>VEG/H</td>
<td>70</td>
<td>23 ± 22.7*</td>
</tr>
<tr>
<td>2/3</td>
<td>TS-4/TS-4</td>
<td>VEG/H</td>
<td>20</td>
<td>47 ± 19.8*</td>
</tr>
<tr>
<td>3/1</td>
<td>HBSS/HBSS</td>
<td>RH</td>
<td>100</td>
<td>10 ± 1.2†</td>
</tr>
<tr>
<td>3/2</td>
<td>NC-1/NC-1</td>
<td>RH</td>
<td>100</td>
<td>11 ± 1.5†</td>
</tr>
<tr>
<td>3/3</td>
<td>TS-4/TS-4</td>
<td>RH</td>
<td>10</td>
<td>52 ± 11.4*</td>
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</table>

* Vaccinations were subcutaneous (s.c.) and given on days 0 and 14 postinoculation (PI). HBSS = Hank's balanced salt solution; NC-1 = 1 X 10⁵ tachyzoites of the NC-1 strain of Neospora caninum; TS-4 = 1 x 10⁵ tachyzoites of the TS-4 strain of Toxoplasma gondii.
† Challenges were given orally (VEG/L and VEG/H) or s.c. (RH) on day 28 PI. VEG/L = 1 X 10³ VEG strain T. gondii oocysts; none = nothing given; VEG/H = 5 x 10³ VEG strain T. gondii oocysts; RH = 2.5 x 10³ RH strain T. gondii tachyzoites.
‡ Percentage of mice that died during 56-day postchallenge (PC) period except for experiment 1, group 4, which was 64 days PC.
§ Mean number of days that mice survived PC ± standard deviation. Significant differences (P < 0.05) exist for groups with different alphabetical superscripts within an experiment.
# Mice in this group were examined at necropsy 64 days PC. NA = not applicable.
bovine serum albumin (BSA) in TBS (BSA/TBS) for 2 hr at room temperature (21–23 C). The filters were then placed in a multiscreen apparatus (BioRad) and incubated for 1 hr at room temperature in mouse serum diluted in TBS. The mouse serum was used at a dilution equivalent to its IFA titer. Filters were washed once for 5 min in the apparatus in TBS and then removed and washed 3 times in 0.2% (w/v) BSA/TBS for 5 min each. The filters were then incubated in alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:3,000 in TBS and once in alkaline phosphatase buffer before the reactive bands were visualized by development in nitroblue tetrazolium/BCIP buffer. After the bands became visible, the reaction was stopped by addition of acetic acid to 10% (v/v) and the filters washed in water and air dried before examination.

RESULTS

Experiment 1

The mice inoculated s.c. with HBSS (group 1-1) or TS-4 tachyzoites (group 1-3) did not develop clinical signs prior to oocyst challenge. One mouse in group 2 inoculated with NC-1 tachyzoites developed a head tilt on day 27 PI. Another mouse in group 2 had a swelling on its dorsal surface (indicative of abscess formation) first noticed 28 days PI. This mouse died 4 days PC with oocysts and was too autolytic for examination. It was concluded the mouse died from bacterial infection, and it was removed from the study. Therefore, only 9 mice were included in the analysis of data from group 1-2 mice. None of the mice inoculated with NC-1 strain tachyzoites in group 4 developed clinical signs. Eight of the group 1-1 HBSS-inoculated mice died between 8 and 12 days PC; 1 mouse in group 1-2 NC-1 inoculated died 41 days PC, and none of the mice in groups 1-3 or 1-4 died during the 56-day PC period. Stages of T. gondii were observed in smears from all mice that died and were examined. Results of mean survival days are presented in Table I. Challenged mice in groups 1-2 and 1-3 survived significantly longer than challenged mice in group 1-1.

Results of mean lesion scores and tissue cyst counts for mice in experiment 1 are presented in Table II. The mean lesion scores were significantly higher for mice vaccinated with HBSS or NC-1 strain tachyzoites and challenged with oocysts compared to mice vaccinated with the TS-4 strain and challenged with oocysts. The mean lesion scores of mice vaccinated with the NC-1 strain tachyzoites and not challenged with oocysts were significantly lower than mice vaccinated with HBSS or NC-1 strain tachyzoites and challenged with oocysts but not the TS-4-vaccinated oocyst-challenged mice. All tissue cysts observed in brain homogenates were thin-walled and considered to be T. gondii. All surviving mice in groups 1-1 (HBSS vaccinated) and 1-2 (NC-1 vaccinated) had tissue cysts. No T. gondii tissue cysts were observed in 7 of 10 mice in group 1-3 given TS-4 tachyzoites and fed VEG oocysts. All 7 had antibodies to T. gondii in their sera, and subinoculation of brain homogenate from these 7 mice indicated that all contained T. gondii tissue cysts. The numbers of T. gondii tissue cysts were significantly higher in mice in group 1-1 and group 1-2 than group 1-3. No significant difference was observed in the numbers of T. gondii tissue cysts in mice in groups 1-1 or 1-2. No tissue cysts (N. caninum-like or T. gondii-like) were observed in the 10 mice in group 1-4 given only NC-1 tachyzoites. One mouse died 33 days PI of brain homogenate and had lesions suggestive of neosporosis in tissue sections of brain. Three of the 9 other mice inoculated with brain homogenate were positive for N. caninum antibodies at a 1:50 dilution of serum.

Mice in group 1-1 did not have demonstrable IgG antibodies to T. gondii or N. caninum in the IFAT 28 days PI; the 2 surviving mice had IFAT titers of 1:12,800 and 1:25,600 to T. gondii but did not have IFAT titers to N. caninum 56 days PC. Mice in group 1-2 did not have demonstrable IFAT titers to T. gondii 28 days PC and had IFAT titers of 1:800 to 1:3,200 to N. caninum. The 8 surviving mice had IFAT titers of 1:3,200 to 1:25,600 to T. gondii and 1:200 to 1:800 to N. caninum 56 days PC. Mice in group 1-3 did not have demonstrable IFAT

Table II. Microscopic lesion scores and Toxoplasma gondii tissue cyst counts of mice in experiments 1 and 2.

| Experiment/ group | 1st/2nd vaccination* | Challenge inoculation | No. exam/ no. lesions‡ | Mean lesion score§ | Mean tissue cyst counts ± SD¶ |
|-------------------|
| Experiment 1      |                       |                      |                     |                   |                         |
| 1/1               | HBSS/HBSS             | VEG/L                | 2/2                 | 10.0              | 77 ± 32^               |
| 1/2               | NC/NC                 | VEG/L                | 8/8                 | 8.1              | 74 ± 26^               |
| 1/3               | TS/TS                 | VEG/L                | 10/1b               | 3.3              | 2 ± 3b                 |
| 1/4               | NC/NC                 | None                 | 10/3b               | 4.2              | NA#                    |
| Experiment 2      |                       |                      |                     |                   |                         |
| 2/1               | HBSS/HBSS             | VEG/H                | NA/NA               | NA               | NA                      |
| 2/2               | NC/NC                 | VEG/H                | 3/3a                | 9.0              | 71 ± 31a               |
| 2/3               | TS/TS                 | VEG/H                | 8/3a                | 4.5              | 14 ± 10b               |

* Vaccinations were subcutaneous (s.c.) and given on days 0 and 14 postinoculation (PI). HBSS = Hank's balanced salt solution; NC-1 = 1 × 10^5 tachyzoites of the NC-1 strain of Neospora caninum; TS-4 = 1 × 10^5 tachyzoites of the TS-4 strain of Toxoplasma gondii.
† Challenges were given orally (VEG/L and VEG/H). VEG/L = 1 × 10^5 VEG strain T. gondii oocysts; none = nothing given; VEG/H = 5 × 10^5 VEG strain T. gondii oocysts.
‡ Number of mice examined/number of mice with lesions. Significant differences (P < 0.05) exist for groups with different alphabetical superscripts within an experiment.
§ Mean lesion score. Significant differences (P < 0.05) exist for groups with different alphabetical superscripts within an experiment.
¶ Mean Toxoplasma gondii tissue cyst counts ± standard deviation. Significant differences (P < 0.05) exist for groups with different alphabetical superscripts within an experiment.
# Not applicable.
titers to *N. caninum* 28 days PC and had IFAT titers of 1:800 to 1:12,800 to *T. gondii*. The 10 surviving mice did not have IFAT titers to *N. caninum* and had titers of 1:12,800 to 1:25,600 to *T. gondii* 56 days PC. Mice in group 1-4 did not have IFAT titers to *T. gondii* 28 days PI or 64 days PC and had titers of 1:800 to 1:3,200 to *N. caninum* 28 days PI and titers of 1:200 to 1:1,600 at 64 days PC.

**Experiment 2**

No clinical signs were noted in mice in experiment 2 prior to feeding of VEG oocysts. The group 2-1 HBSS control mice died 7–12 days after feeding of oocysts. Seven of 10 group 2-2 NC-1-vaccinated mice died 9–11 days PI and the remaining 3 survived for 56 days. Two of the group 2-3 TS-4-vaccinated mice died 9 days PI and the remaining 8 survived for 56 days. Results of mean survival days are presented in Table I. No difference in survival between mice vaccinated with HBSS or NC-1 strain tachyzoites, but both were significantly lower than the mice vaccinated with the TS-4 strain and challenged with oocysts.

Results of mean lesion scores and tissue cyst counts for mice in experiment 2 are presented in Table II. Lesion scores were not obtained from mice in group 2-1 because they all died before the end of the study. Mice vaccinated with the NC-1 strain tachyzoites and fed oocysts had significantly higher lesion scores compared to mice vaccinated with TS-4 strain tachyzoites and fed oocysts. All tissue cysts observed in brain homogenates were thin-walled and considered to be *T. gondii*. All surviving mice in groups 2-2 (NC-1 vaccinated) and 2-3 (TS-4 vaccinated) had tissue cysts, and the mice in group 2-2 had significantly more tissue cysts than did the mice in group 2-3.

Mice in group 2-1 did not have demonstrable IgG antibodies to *T. gondii* or *N. caninum* 28 days PC. Mice in group 2-2 did not have demonstrable IFAT titers to *T. gondii* 28 days PC and had IFAT titers of 1:100 to 1:1,600 to *N. caninum*. The 3 surviving mice had IFAT titers of 1:1,600 to 1:3,200 to *T. gondii* and 1:400 to 1:800 to *N. caninum* 56 days PC. Mice in group 2-3 did not have demonstrable IFAT titers to *N. caninum* 28 days PC and had titers of 1:100 to 1:400 to *T. gondii*. The 8 surviving mice did not have IFAT titers to *N. caninum* and had IFAT titers of 1:800 to 1:6,400 to *T. gondii* 56 days PC.

**Experiment 3**

No clinical signs were noted in mice in experiment 3 prior to challenge inoculations with RH tachyzoites. The group 3-1 HBSS control mice died 7–11 days PC with tachyzoites. All 10 of the group 3-2 NC-1-vaccinated mice died 9–14 days PC. One of the group 3-3 TS-4-vaccinated mice died 20 days PC and the remaining 9 survived for 56 days. Two of the 9 mice in group 3-3 had lesions and the mean lesion score was 3.8. No tissue cysts were observed in the brains of the 9 surviving mice in group 3-3. Infectious stages of the RH strain of *T. gondii* were present in 6 of 9 of the surviving mice in group 3-3; subinoculation of brain homogenate from these mice produced mortalities in 5 mice 14–27 days PI and 1 mouse survived for 56 days. Tissue cysts were observed in brain smears from this mouse, and a mouse inoculated with brain homogenate from this mouse died from toxoplasmosis 16 days PI. Mice in group 3-1 vaccinated with HBSS did not have IFAT titers to *N. caninum* or *T. gondii* 28 days PI and none survived the challenge with RH strain tachyzoites. Mice in group 3-2 vaccinated with NC-1 strain tachyzoites had IFAT titers of 1:800 to 1:6,400 to *N. caninum* but did not have IFAT titers to *T. gondii* 28 days PI, and none survived the challenge with RH strain tachyzoites. Mice in group 3-3 vaccinated with TS-4 strain tachyzoites did not have IFAT titers to *N. caninum* but had IFAT titers of 1:100 to 1:800 to *T. gondii* 28 days PI. The 9 surviving mice did not have IFAT titers to *N. caninum* but had IFAT titers of 1:3,200 to 1:12,500 to *T. gondii* at 56 days PC.

**Western blot analysis**

Sera from mice not vaccinated did not produce reactions in western blots to tachyzoites of either *N. caninum* or *T. gondii*. Sera from mice vaccinated with *N. caninum* recognized numerous peptides in western blots of *N. caninum* tachyzoites, but no or inconsistent reactivity was observed with tachyzoites of *T. gondii*. Sera from mice vaccinated with *T. gondii* recognized numerous peptides in western blots of *T. gondii* tachyzoites but no or inconsistent reactivity was observed with tachyzoites of *N. caninum*.

**DISCUSSION**

The present study indicates that vaccination with *N. caninum* can increase the survival time for mice fed oocysts of a moderately pathogenic strain of *T. gondii*. The mechanism of this induced protection is not known. Humoral antibodies do not appear to play an important role because none of the NC-1-vaccinated mice had demonstrable serum IgG antibodies to *T. gondii* in the IFAT.

There is little consensus on cross-reactive antigens recognized by *N. caninum* antiserum on *T. gondii* tachyzoites and *T. gondii* antiserum on *N. caninum* tachyzoites in western blot analysis of animal sera (Bjerkås et al., 1994; Björkman et al., 1994; Paré et al., 1995; Baszler et al., 1996). Bjerkås et al. (1994) found that rabbit *N. caninum* antiserum recognized antigens of 45, 66, and 85 kDa on *T. gondii* tachyzoites, whereas rabbit *T. gondii* antiserum recognized antigens of 46, 88, and 97 kDa on *N. caninum* tachyzoites. Björkman et al. (1994) found that rabbit *T. gondii* antiserum recognized a 40-kDa antigen on *N. caninum* tachyzoites. Paré et al. (1995) did not find reactivity of bovine *N. caninum* antiserum with *T. gondii* proteins in western blots. Baszler et al. (1996) found that bovine *T. gondii* antiserum recognized *N. caninum* tachyzoite antigens that ranged from 11 to 80 kDa if a 1:100 serum dilution was used, but that if a 1:10,000 dilution was used, then only *N. caninum* tachyzoite antigens of 14 and 37 kDa were recognized.

Cell-mediated immunity is likely to be involved in the protection observed in *N. caninum*-vaccinated mice and may be stimulated in some nonspecific manner by vaccination. Khan et al. (1997) have shown that splenocytes from mice inoculated with the NC-1 strain of *N. caninum* will undergo proliferation in response to soluble *T. gondii* (strain PLK) lysates. The host response responsible for prolonging the survival of NC-1-vaccinated *T. gondii*-challenged mice does not appear to influence *T. gondii* tissue cyst formation because tissue cyst numbers were not significantly different from nonvaccinated controls.
Mouse and \textit{T. gondii} strain are known to influence tissue cyst production and resistance to primary \textit{T. gondii} infection in inbred mice (Suzuki et al., 1989, 1991, 1993; Brown and McLeod, 1990; Blackwell et al., 1993).

No protection was provided from s.c. challenge with the RH strain of \textit{T. gondii} in \textit{N. caninum}-vaccinated mice. This is identical to our previous findings (Lindsay et al., 1990) and was obtained with a log lower dose of RH strain tachyzoites used as challenge inoculum.

\section*{Acknowledgments}

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