Evaluation of Immune Responses in Horses Immunized Using a Killed Sarcocystis neurona Vaccine*

Antoinette E. Marsh, MS, PhD
Jeffrey Lakritz, DVM, PhD, DACVIM
Philip J. Johnson, BVSc(Hons), MS, MRCVS, DACVIM
Margaret A. Miller, DVM, PhD, DACVP
Yu-Wei Chiang, PhD
Hsien-Jue Chu, PhD

*a Department of Veterinary Pathobiology
College of Veterinary Medicine
University of Missouri
Columbia, MO 65211

*b Department of Veterinary Clinical Sciences
The Ohio State University
Columbus, OH 54321

*c Animal Disease Diagnostic Laboratory
Purdue University
406 S. University Street
West Lafayette, IN 47907

*d Department of Veterinary Medicine and Surgery
College of Veterinary Medicine
University of Missouri
Columbia, MO 65211

*e Global Biological R&D
Fort Dodge Animal Health
Fort Dodge, IA 50501

**Funding for this study was provided by Fort Dodge Animal Health, Fort Dodge, IA.

Clinical Relevance
Clinically normal horses developed cellular immunity to Sarcocystis neurona following IM vaccination with a commercial killed S. neurona vaccine, as indicated by the development of measurable anti–S. neurona IgG antibodies and additional intradermal skin testing. Large-scale independent assessments of the vaccine’s performance and safety are in progress under field conditions. The next step in the evaluation of this vaccine would be to attempt experimental challenge after a reproducible reliable equine model of S. neurona encephalitis has been established that allows for reisolation of the pathogen after challenge.

Introduction
Sarcocystis neurona, Neospora spp, and Toxoplasma gondii are related protozoan parasites that can cause encephalomyelitis in livestock and laboratory animals. Equine protozoal myeloencephalitis (EPM) is predominantly caused by S. neurona infection of the central nervous system (CNS).1-4 Approximately 50% of horses
in the United States are seropositive for *S. neurona*. However, only a minority of seropositive horses develop clinical signs of neurologic disease. Onset of the neurologic signs of EPM was more rapid in horses subjected to stress associated with prolonged transport in experimental studies, and immunosuppression with corticosteroids increased the severity and progression of EPM. Because the parasite must penetrate the gastrointestinal tract, migrate to the CNS, and cross the blood–brain barrier, it is likely that exposure to and interaction with the host’s immune system affects the chances that neurologic signs will develop in infected horses. Therefore, stimulation of specific immune effector mechanisms to develop preexisting immunity may be protective for the affected individual horse, particularly since the only clinical indication of infection is CNS signs. The objective of the present study was to determine whether horses vaccinated with a killed *S. neurona* vaccine develop a measurable immune response to *S. neurona* as compared with responses of horses vaccinated with a placebo.

### MATERIALS AND METHODS

#### Horses

Fifty-one horses in the United States were screened for seronegativity to *S. neurona* and *Neospora hughesi*. Ten seronegative horses from this group were selected for the study. An additional three horses from the Banff area of Alberta, Canada, in a geographic location devoid of opossums, were initially screened by indirect fluorescent antibody testing (IFAT) and then exported to the United States to participate in this study. The 13 horses (six mares and seven geldings) ranged from 1.5 to 10 years of age and weighed 360 to 500 kg each. Results of physical examinations, complete blood counts, and serum biochemical tests indicated all horses selected for the study were in good general health. Horses were enrolled in the study in lots of three or four.

On arrival, horses were housed in individual stalls and allowed 1 week to acclimate to the test facility and handling procedures before vaccinations were administered. During the acclimation period and throughout the study, horses were fed alfalfa hay twice daily and water was provided ad libitum. At the end of the acclimation period, horses from each lot were randomly assigned to vaccination with a placebo or a commercially packaged *S. neurona* vaccine.

#### Vaccinations

Nine horses received killed *S. neurona* vaccine, and four were vaccinated with placebo. Mares and geldings were represented in both groups. Each horse received 1 ml of the assigned material by IM injection in the neck on Day 0 (the same day for all horses). The placebo vaccine contained only adjuvant (Meta-Stim; Fort Dodge Animal Health) and cell culture medium used for *S. neurona* in vitro propagation. The placebo vaccine was handled and packaged in the identical manner as the commercial vaccine. Vaccination was repeated for each horse with the appropriate material once between Days 21 and 24.

The horses were monitored immediately after injection and daily for the next 2 weeks for adverse reactions or problems associated with vaccination. This study was conducted with the permission and under the supervision of the University of Missouri’s Animal Care and Use Committee.

#### Evaluations

Cellular activation was detected using a modification of previously described procedures. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using approximately 50 ml of venous blood collected in a tube con-
taining sodium citrate. The buffy coat was removed and mixed with an equal volume of sterile phosphate-buffered saline, overlaid on a commercial solution containing polysucrose and sodium diatrizoate adjusted to a density of 1.077 g/ml (Histopaque-1077; Sigma Diagnostics, Inc), and centrifuged at 500 × g. The mononuclear cells were removed, washed twice in Hank's balanced salt solution without calcium and magnesium, and resuspended at 2 × 10^6 cells/ml in Roswell Park Memorial Institute media containing 2 mM l-glutamine, 50 µg penicillin and 100 µg streptomycin/ml, and 10% heat-inactivated equine serum. Duplicate wells of PBMCs were either incubated with media (negative control), *S. neurona* antigen, or a positive assay control T cell mitogen using either concanavalin A or phytohemagglutinin (PHA) for 6 days at 38˚C in a 96-well plate.

The *S. neurona* antigenic stimulus used was the *S. neurona* Sn-UCD1 isolate, which was cultivated as previously described, and the in vitro antigenic stimulus was prepared using a standard published procedure. On Day 6, 1 µCi of 3H-thymidine was added to each well, and the cells were allowed to incubate an additional 18 hours before harvesting using a semiautomated 96-well cell-harvester system onto filter mats. Filters were air-dried, placed into sealed plastic bags with scintillation fluid, enclosed in the 96-well counting cassettes, and analyzed with a liquid scintillation counter and luminometer (Wallac MicroBeta TriLux 1450; PerkinElmer) according to manufacturer’s directions. The cell-mediated immunity (CMI) results were expressed as a stimulation index.

The CMI responses were measured on Day 0 (before immunization 1), Day 21 (before immunization 2), and Day 49 (final).

Serum samples were obtained weekly to biweekly from each horse for the duration of the study starting on Day 0. Samples were stored at −20˚C until tested for antibodies using immunoblot or IFAT for detection of *S. neurona* antibodies. The immunoblot was performed as previously described using an immunoblot apparatus and *S. neurona* (Sn-UCD1) antigen. The IFAT was performed as previously described with a 1:50 initial dilution.

In addition to in vitro peripheral blood CMI evaluations, an intradermal skin test similar to the Mantoux test for tuberculosis, which is used to measure delayed (cellular) reactions to antigens, was performed in five horses by intradermal inoculation at the end of the study (approximately 8 weeks after the initial vaccination). Two other horses that were still available to the investigators were also evaluated by intradermal testing again 11 months following the initial vaccination. For intradermal inoculation, *S. neurona* antigen and equine dermal cells were similarly prepared and used as in the in vitro assay except that 0.1 ml of 50-µg/ml inoculum was used per site. Histamine and PHA were used as positive controls to test for immediate and delayed reactions, respectively. Sterile saline served as a negative control. Two horses not enrolled in the vaccine study were used to titrate the PHA skin-testing antigen (100, 50, 10, or 1 µg/ml) and were inoculated intradermally to determine optimal concentration for use in this assay.

All intradermal injections were administered in the horse’s neck. Skin was prepared by clipping the hair coat, followed by a thorough cleansing and topical application of alcohol. After the skin surface had dried, a grid was drawn on the clipped area using an indelible pen to permit injection sites to be readily identified and observed. Intradermal injections with 0.1 ml of PHA (100, 50, 10, or 1 µg/ml) in saline, histamine (0.1 mg/ml) in saline, and saline were each given with a 25-gauge needle, using individual injection sites that were separated by at least 5 cm. Skin test reactions were examined at 48 and 72 hours. An arbitrary scoring system
was used in which a blinded observer rated any skin reactions in the grid from 0 (no reaction) to 4 (induration >5 mm in diameter).

Following the final reading of the injected sites at 72 hours, each skin site was aseptically prepared and anesthetized using 1.0 ml of mepivicaine hydrochloride SC. Care was taken to ensure that the injected anesthetic solution did not disrupt the skin at the intradermal injection test sites. Skin biopsies were obtained from the PHA, saline, or test antigen sites using a 5-mm biopsy punch and placed into 10% formalin. Following routine histopathologic processing and staining with hematoxylin and eosin, the skin biopsies were evaluated by an unbiased observer with expertise in cuta-

### TABLE 1. Indirect Fluorescent Antibody Test (IFAT) Titers for Horses Receiving Killed Sarcocystis neurona Vaccine or Placebo Days 0 and 49 and Responses to Intradermal Inoculation of S. neurona Antigen

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>IFAT Day 0</th>
<th>IFAT Day 49</th>
<th>S. neurona Antigen Skin Testing Scores 48 hr*</th>
<th>S. neurona Antigen Skin Testing Scores 72 hr*</th>
<th>Biopsy score†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>48 hr*</td>
<td>72 hr*</td>
<td></td>
</tr>
<tr>
<td>S. neurona Vaccine</td>
<td></td>
<td></td>
<td>48 hr*</td>
<td>72 hr*</td>
<td></td>
</tr>
<tr>
<td>V1</td>
<td>&lt;1:50</td>
<td>1:400</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>V2</td>
<td>&lt;1:50</td>
<td>1:100</td>
<td>1</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>V3</td>
<td>&lt;1:50</td>
<td>1:200</td>
<td>1</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>V4</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
<td>1</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>V5</td>
<td>&lt;1:50</td>
<td>1:1600</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>V6</td>
<td>&lt;1:50</td>
<td>1:200</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>V7</td>
<td>&lt;1:50</td>
<td>1:400</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>V8</td>
<td>&lt;1:50</td>
<td>1:400</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>V9</td>
<td>&lt;1:50</td>
<td>1:400</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>V1‡</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>V2‡</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Placebo Vaccine</td>
<td></td>
<td></td>
<td>48 hr*</td>
<td>72 hr*</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>P2</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>P3</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
<td>1</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>P4</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

*Scored from 0 (no reaction) to 4 (induration >5 mm in diameter).
†Microscopic evaluation score 72 hours after inoculation, scored 0 (within normal limits); 1 (one to two layers of lymphocytes around superficial or deep dermal venules, no eosinophils); 2 (two to three layers of lymphocytes with or without rare eosinophils); or 3 (four to five layers of lymphocytes with occasional eosinophils).
‡Intradermal skin testing performed 11 months after initial vaccination.
ND = not done.
neous histopathology. A scoring system of 0 (within normal limits); 1 (one to two layers of lymphocytes around superficial or deep dermal venules, no eosinophils); 2 (two to three layers of lymphocytes with or without rare eosinophils); and 3 (four to five layers of lymphocytes with occasional eosinophils) was used to characterize the microscopic concentration of cellular infiltrate.

**Statistical Analysis**

The stimulation index for each horse on each day (before vaccination and Days 21 and 49) to vaccine antigen was compared between the two groups for normality and equal variance. Because the data were not normally distributed, one-way analysis of variance on ranks was used to compare the stimulation indices between the vaccine and placebo groups at each of the three CMI sampling dates. Significant differences between treatments and among sampling times were further characterized by multiple comparisons using the Dunn test. Significance was assigned when \( P < .05 \) (SigmaStat Statistical Software, Version 3.0; SPSS).

**RESULTS**

The horses exhibited no detectable adverse reactions at any time during the study. In vitro mononuclear cell proliferation in response to *S. neurona* antigens was significantly \((P < .05)\) elevated 21 days after vaccination in comparison with prevaccination (Day 0) indices (Figure 1). By Day 49, proliferation had declined to a level that was not different from the indices on Day 0. There was no significant difference between groups for the in vitro response to the *S. neurona* antigen at any sampling time. There was no significant difference between the vaccine and placebo groups when either the mitogen or media controls were compared.

All *S. neurona*-vaccinated horses seroconverted based on results of serum immunoblot analysis; however, the intensity of the immunoblot reaction varied among these horses (Figure 2). The IFAT titers varied among the vaccinated horses from negative \(<1:50\) to 1:1,600 (Table 1). There was no apparent correlation in the intensity of the immunoblot reactivity and the IFAT titer as indicated by the horse having the highest IFAT titer (1:1,600) that did not have the strongest immunoblot reaction, whereas two horses with different IFAT titers (1:400 and 1:200) had similar reactivity by immunoblot (Figure 2, lanes 4 and 5). The placebo-vaccinated horses, which were seronegative at the start of the study, remained seronegative on immunoblot and IFAT testing throughout the study.

Results from skin testing and microscopic
than to the saline negative control. The five vaccinated horses also developed inflammation in response to skin testing with equine dermal cells (scores ranged from 1 to 3).

**DISCUSSION**

The prevalence of horses in the United States that are seropositive to *S. neurona* has been reported to range from 0% to 54%, depending on the region and the test used. Therefore, it was not surprising that a large number of horses were required for screening in the midwestern part of the country to identify horses that were seronegative to *S. neurona* by immunoblot testing. Horses experimentally exposed to opossum feces containing *S. neurona* sporocysts have been shown to seroconvert as soon as 2 weeks after experimental exposure.

Cell-mediated immunity is an important mechanism for control of intracellular parasites. Mice strains lacking functional immune components such as interferon-γ or T and B cells (nude mice) developed encephalitis associated with *S. neurona*, which suggests that CMI responses play a protective role against this parasite. Also, the genetic background and major histocompatibility of the hosts play a role in their susceptibility to protozoal infections, and this information is not well defined for *S. neurona* or for horses. In previous studies, horses with *S. neurona*-associated encephalitis (as detected by immunohistochemical demonstration of organisms within the CNS) had detectable anti-*S. neurona* IgG antibodies, and these horses still developed clinical signs, including a large number of CNS-infiltrating mononuclear cells. There is a paucity of

**Figure 2.** An immunoblot of Sarcocystis neurona merozoite nonreduced antigen probed with sera from horses vaccinated with placebo (lanes 1–3) or *S. neurona* vaccine (lanes 4–7) before Day 0 and after vaccination (Week 4). Lane 8 is blank and lane 9 is a positive control sample from an *S. neurona*-infected horse.

analysis of biopsy samples are summarized in Table 1. The horses used to titrate the PHA had a detectable reaction with the 100-, 50-, and 10-µg/ml concentrations. The cellular induration extended more than 12 mm from the injection site with the 100 µg/ml PHA. At 50 µg/ml, PHA elicited a readily visible (>5 mm diameter) and palpable reaction (score = 4) with histologically evident cellular infiltration (score = 3), whereas the saline control elicited no visible, palpable, or microscopic reaction. The histamine gave an immediate response, which declined to the background appearance by 48 hours.

Skin reactivity was evident (>5 mm induration) 48 hours after intradermal inoculation of *S. neurona* antigen in seven of the nine horses that received *S. neurona* vaccine. One of the four horses vaccinated with placebo reacted to *S. neurona* antigen injection. Histologically, all horses in both groups had a greater dermal inflammatory response to *S. neurona* antigen (scores ranged from 1 to 3) than to the saline negative control. The five vaccinated horses also developed inflammation in response to skin testing with equine dermal cells (scores ranged from 1 to 3).
published data on the mechanism of pathogenesis of *S. neurona* and the immune response of the horse to infection. One method to evaluate pathogenesis, immune response, or vaccine efficacy is through experimental challenge models; however, the equine model of EPM that uses transport stress is still undergoing refinement. In addition, horses have highly variable genetic backgrounds, adding additional levels of interanimal variability. Another method for vaccine evaluation may include measuring vaccine antigen-specific CMI and antibody responses in normal horses immunized without performing an infectious challenge.

The results of the present study demonstrated that horses immunized with the killed *S. neurona* vaccine have significantly elevated CMI responses to *S. neurona* merozoite antigens compared with responses before vaccination. The highest indices were at the midpoint rather than at the end of the study. Two possible reasons have been proposed for the observed results regarding the elevated response at the midpoint for the group vaccinated with *S. neurona* vaccine and the subsequent decreased response at Day 49. One hypothesis is that the interval between the booster vaccination and the final analysis was 1 week longer than the interval from the initial vaccination and the midpoint analysis. A more likely reason for the difference is that the horses demonstrated a greater response in peripheral blood lymphocytes during the primary exposure to the vaccine than after the booster vaccination. After the second exposure (i.e., the second vaccination), the immune responsiveness, as determined by in vitro proliferation to *S. neurona* antigen, may be much greater if lymphocytes were obtained from regional lymphoid or splenic tissues rather than from peripheral blood. In a mouse model of equine herpesvirus, T cell responses varied in the course of infection and were dependent on the lymphoid tissue and mouse major histocompatibility genetic background used such that T cell responses in the spleen were prolonged in comparison with those in the cervical lymph node.

The antibody responses of the horses in the present study demonstrated that the vaccine induced seroconversion in the horses, which has also been recently reported by Witonsky et al. Others also have shown that horses seroconvert after intrathecal inoculation with live merozoites in as little as 8 days or within 14 days after intragastric administration of sporocysts. The lack of correlation between the IFAT titers and the immunoblot reactivity was not surprising as others have shown a lack of correlation between the *S. neurona* direct agglutination (SAT) and immunoblot analysis for detection of antibodies in normal horses.

The skin testing was used to corroborate the results of the in vitro proliferation assay. Because this study did not include a challenge component, there were limited tests and reagents for evaluating CMI responses to *S. neurona* antigens. There are published methods for quantification of equine interferon-γ, but equine-specific interferon-γ tests and reagents are not commercially available. This study also used flow cytometry for differentiation of peripheral blood lymphocytes with the presence of surface markers for CD4 or CD8; significant differences between the vaccine and the placebo groups were not detected (data not shown). In vivo skin test reaction has been shown to be more sensitive than in vitro lymphocyte transformation or macrophage migration following mycobacteria sensitization using killed mycobacteria. Early work in detection of antibodies in humans to *T. gondii* showed a good correlation with *T. gondii* skin tests; a similar test was used in this study. However, a crude antigen preparation was used in the present study that may have also contained media proteins used for parasite propagation. The placebo-vaccinated horses exhibit-
ed a minimal visible response to the *S. neurona* skin test but had microscopic lesions similar to the horses receiving the *S. neurona* vaccine, which suggests that the placebo-vaccinated horses may have been immunized against these media components when vaccinated with the placebo vaccine containing the media and adjuvant. In the future, either a recombinant protein or highly purified parasite proteins could be used for the dermal testing to ensure the cellular response is restricted to measuring reactivity to the parasite.

This study demonstrated that clinically normal horses develop cellular immunity to *S. neurona* following IM vaccination with a commercial killed *S. neurona* vaccine. Large-scale independent assessments of the vaccine performance and safety are in progress under field conditions. The next step in the evaluation of this vaccine would be to attempt experimental challenge after a reproducible reliable equine model of *S. neurona* encephalitis has been established that allows for reisolation of the pathogen after challenge.

### ACKNOWLEDGMENTS

The authors thank Aislinn Halaney and Mary Cockrell from the University of Missouri for their technical assistance.

### REFERENCES


