Experimental Infection of Horses with Culture-Derived *Sarcocystis neurona* Merozoites as a Model for Equine Protozoal Myeloencephalitis

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ABSTRACT

A study was designed to develop an experimental model to produce *Sarcocystis neurona* encephalitis in horses. *Sarcocystis neurona*, isolated from the spinal cord of an ataxic horse, was placed in continuous culture using bovine turbinate cells, and the cultured *S. neurona* merozoites were used to infect lymphocytes. A horse that was subsequently experimentally infected with 100,000 of the *S. neurona*-infected lymphocytes developed encephalitis and ataxia. *Sarcocystis neurona* was isolated from the spinal tissues of the infected horse by in vitro culture. Three horses, each infected with a different number of merozoite-infected lymphocytes, were used to estimate an infective dose of parasites needed to induce clinical signs of equine protozoal myeloencephalitis. This is the first report of histologically confirmed experimental infection of horses with culture-derived merozoites of *S. neurona*. Both a hematogenous method of distribution in the host and an intracellular location by the parasite in a lymphocyte is sufficient to produce clinical equine protozoal myeloencephalitis.

INTRODUCTION

The horse is an accidental host for *Sarcocystis neurona*, a parasite of the opossum (*Didelphis virginiana*) that causes equine protozoal myeloencephalitis (EPM).[1] The pathogenesis of *Sarcocystis* infection in some natural intermediate hosts includes excystation of sporocysts in the gut and invasion of intestinal cells with sporozoites that undergo schizogony to produce merozoites.[2–5] The merozoites of some species of *Sarcocystis* disseminate in the intermediate host via the bloodstream.[5–7] Alternatively, merogony occurs in the endothelial cells of blood vessels of the host, in mononuclear cells, or in the vascular endothelium.[1,6,8–10] *S. neurona* merozoites that infect peripheral

blood cells that are capable of crossing the blood–brain barrier would provide a mechanism for central nervous system (CNS) infection. It is likely that immune responses in the CNS, including antibody production, are induced as merozoites pass through the vascular endothelium of the blood–brain barrier and enter the CNS, a location that is sequestered from normal immune defense mechanisms.[3,7,10] The presence of antibodies to *S. neurona* in the cerebrospinal fluid (CSF) of horses has been used as circumstantial evidence that *S. neurona* has crossed the blood–brain barrier; however, Daft and co-workers[11] found that immunoblot tests using CSF could not differentiate infected horses from noninfected horses. The low specificity of Western blot testing of CSF indicated that the test is inappropriate for diagnosing EPM because it frequently gives a false-positive result.[12] The high sensitivity of the test, however, is useful to rule out EPM when the test produces a negative result. Currently, a definitive diagnosis of EPM is made when signs of ataxia are accompanied by gross lesions demonstrating parasites or isolation of merozoites from the CNS.[12]

Sarcocystis spp have been demonstrated in the blood of some intermediate hosts, and infection has been transmitted by blood transfusion, although it was not determined whether the merozoites were free in the blood or in host blood cells.[3–6] *S. neurona* parasitemia has been identified by cell culture in the peripheral blood of a foal affected with severe combined immunodeficiency.[13]

Murine models, using immunodeficient mice, have indicated an immune-mediated resistance to infection.[14,15] Experimental infections in immunocompetent mice suggest that immunity originates from combined increases in humoral and cell-mediated immune responses.[16] The pathogenesis of CNS infection of horses by *S. neurona* merozoites likely involves movement of parasites from the gut into infected lymphocytes that cross the blood–brain barrier. It is hypothesized that factors affecting resistance or susceptibility to infection in horses include the number of parasites successfully entering lymphocytes and subsequent transport of infected lymphocytes across the blood–brain barrier, which is not affected by the presence of peripheral antibodies.

It is further believed that lymphocytes provide both an intracellular location and a hematogenous method of distribution in the host. The purposes of this study were to develop a model of *S. neurona* infection in immunocompetent horses that would result in classic signs of EPM and to estimate the dose of infectious material necessary to induce clinical EPM.

MATERIALS AND METHODS

Lymphocyte infection studies were used to determine that *S. neurona* had invaded and initiated replication in equine lymphocytes in vitro; however, the infection was nonprogressive in these cells. An in vitro 50% tissue culture infective dose ($TCID_{50}$) assay was developed to determine the viability of parasites derived from infected lymphocytes. One horse was used in a pilot study to evaluate the outcome of an experimentally induced parasitemia using infected lymphocytes. Three horses were each injected with a different dose of infected lymphocytes to estimate the dose of infectious material necessary to induce EPM.

In vitro Assay for *S. neurona* in Equine Lymphocytes

Four noninfected horses, including three adult thoroughbred mares and one adult stallion, were used to obtain lymphocytes for an in vitro assay to determine the $TCID_{50}$ of parasite-infected lymphocytes required to destroy monolayers of bovine turbinate cells compared with the $TCID_{50}$ of merozoites inoculated into bovine turbinate cells. Six replicate wells were evaluated for evidence of infection, and the $TCID_{50}$ was determined when three wells showed complete destruction of the monolayer. Multiplication rates and progression of growth after invasion varied according to the host cell type used to culture *S. neurona* merozoites.[17] The $TCID_{50}$ was established for bovine turbinate cells

and the value used as a standard to determine the infectivity of equine lymphocytes.

Blood was collected from the horses by jugular venipuncture into a 16-mL blood collection tube containing EDTA and was centrifuged at 600 ×g for 5 minutes. The buffy coat was removed and suspended in 0.5 mL phosphate-buffered saline (PBS). Lymphocytes were concentrated using a ready-made, sterile, endotoxin-tested solution of Iodixanol, 5,5′-[(2-hydroxy-1-3 propanediyl)-bis(acetylamino)] bis [N,N′-bis(2,3dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide], designed for the in vitro isolation of biological particles (Optiprep; Sigma), according to manufacturer's recommendations. The buffy-coat suspension was layered onto a 0.5-mL cushion of 1.077-buoyant density Optiprep in a microcentrifuge tube. The gradient was over-layered with 250 μL PBS and centrifuged at 200 ×g for 20 minutes at room temperature. The aqueous phase, including cells, was collected and diluted 1:1 with Roswell Park Memorial Institute (RPMI 1640) medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin (2.5 μg/mL), β-mercaptoethanol (0.055 mM), and 10% bovine serum. After centrifugation at 5000 ×g for 5 minutes, the supernate was discarded. The cell pellet was suspended in RPMI 1640 supplemented with antibiotics and serum, and the purified cells were counted using a hemocytometer. The resulting cell suspension was 95% lymphocytes, as determined by a Geimsa-stained thin smear.

S. neurona from the spinal tissues of a thoroughbred horse that had a clinical diagnosis of EPM was isolated by cell culture in bovine turbinate cells. Merozoites from a mature culture of *S. neurona* were collected by scraping the monolayer into a 50-mL conical tube and centrifuged at 600 ×g for 10 minutes at room temperature. The merozoites were counted after resuspension in RPMI 1640 supplemented with antibiotics and bovine serum, as for the aqueous phase. Noninfected equine lymphocytes (n = 24,000) and merozoites (n = 24,000) were added to each of six microcentrifuge tubes. A control tube containing 24,000 merozoites and no equine cells was included. Tubes were incubated for 2 hours at 37°C in a 5% carbon dioxide atmosphere. The number of successfully infected lymphocytes was determined by culturing the lymphocytes on bovine turbinate monolayers. The $TCID_{50}$ of merozoites from infected lymphocytes was determined by observing the dilution at which three of six replicate wells had monolayers destroyed by parasites. The $TCID_{50}$ of merozoites from infected lymphocytes was compared with that of known numbers of *S. neurona* merozoites inoculated into bovine turbinate cells, and this value was used to estimate the number of infectious protozoa. Immuno-affinity beads (Sepharose beads; Amersham Biosciences) coated with monoclonal antibody that binds SnSAG1 (the immunodominant surface antigen of *S. neurona*) were used to remove any infectious parasites that had not entered lymphocytes.

Briefly, 200 μL of a 50% suspension of Sepharose beads was washed three times with carbonate buffer (pH 7.2). The washed beads were suspended in 500 μL buffer with 40 μg of monoclonal antibody to SnSAG1, incubated for 2 hours at room temperature with gentle rocking, gently rewashed in buffer, centrifuged (200 ×g), and suspended in RPMI 1640 with antibiotics. An equal volume of the SAG1 immuno-affinity beads was added to each of the six microcentrifuge tubes containing infected lymphocytes, and the tubes were incubated for 5 hours at 37°C in a 5% carbon dioxide atmosphere. A discontinuous gradient was formed by carefully placing a cushion of 1.077-Optiprep density gradient under the bead suspension using a 16-gauge needle fitted on a 1-mL syringe. Using a micropipette, 250 μL PBS was layered over the surface of the bead suspension. The tubes were centrifuged at 200 ×g for 20 minutes at room temperature. The supernate above the interface was collected and diluted 1:1 with RPMI 1640 with antibiotics followed by centrifugation (5000 ×g) for 5 minutes. Each pellet was suspended in 300 μL

RPMI 1640 with antibiotics, and 50 μL was added to each of six wells (columns 1–6) in row A of a 96-well round-bottom plate. The control pellet containing merozoites and no equine cells was resuspended in 300 μL RPMI medium, and 50 μL was added to each of six wells (columns 7–10) of a 96-well plate. Four thousand fresh merozoites were added to columns 11 and 12 of row A and served as controls for the affinity-bead purification. Rows A to H were diluted by serial twofold dilutions using RPMI.

The contents of each well were transferred to a complementary 96-well flat-bottom plate that had been seeded with 960 bovine turbinate cells. The plates were incubated for 7 days at 37°C in a 5% carbon dioxide atmosphere. The media was removed and Geimsa stain was added to each well. The wells were washed and the plates evaluated for monolayer destruction. The $TCID_{50}$ was considered the last dilution in which three of the six replicate monolayers were destroyed. Wells showing no destruction were recorded as having a $TCID_{50}$ of zero. The assay had been calibrated using the known $TCID_{50}$ of this strain of *S. neurona* so that the positive control wells could be evaluated in parallel.

Experimental Infection of Horses with *S. neurona*

Animals

One quarter horse gelding 21 years of age served as an immunocompetent animal and was used as a pilot animal to determine the feasibility of infecting horses with culture-derived merozoites. The horse was identified as Horse 1 and maintained in a small grass paddock and fed 12% protein pellets at 0.5% of body weight twice daily. The diet was supplemented with coastal bermuda grass and alfalfa hay fed at 1% of body weight. Water was provided ad libitum.

Four horses (Horse A: a 5-year-old gelding of mixed breeding, Horse B: a yearling thoroughbred filly, Horse C: a yearling quarter horse filly, and Horse D: a 10-year-old quarter horse mare) were examined and found to be clinically normal and were maintained in a small paddock. Horse A served as an uninfected control. Using nonreducing immunoblot and SAG1 ELISA, the CSF of all horses was determined to be negative for antibodies to *S. neurona*.[18–22] The serum of Horse C had antibodies (SAG1 ELISA) at a 1:50 dilution, and the remaining horses were negative for serum antibodies by both nonreducing immunoblot and SAG1 ELISA. Lymphocytes from horses with serum antibodies of 1:200 could support development of the parasite for 24 hours (S. Ellison, unpublished data, 2004), and it has been determined that a titer of less than 100 is not significant in horses (S. Ellison, unpublished data, 2004).

Animal care protocols were approved and monitored by the animal oversight committee at the testing facility. A minimum of 30 animals per year for 15 years have been housed at this facility with no reported cases of EPM.

Infection

S. neurona was maintained in bovine turbinate cells as previously described.[17] Parasites were freshly harvested and used to infect homologous cells from Horse 1. Briefly, the buffy coat was collected and incubated with freshly harvested *S. neurona*. After incubation without agitation for 5 hours, the infected cells were added to a 6-mL blood collection tube containing peripheral blood from Horse 1 in EDTA. The number of viable parasites in equine lymphocytes was determined by counting 5 μL of infected cells dried on a slide stained with Geimsa. All parasite preparations used to infect horse lymphocytes were tested for the ability to infect in vitro cells by inoculation of a duplicate sample on to bovine turbinate cells. Parasite preparations were tested for the ability to infect equine cells using Geimsa staining and were tested for their ability to maintain their infectivity for bovine turbinate cells in an in vitro viability assay each day of the 15-day infection period.

Horse 1 was given homologous equine lymphocytes that contained 100,000 mero-

zoites on Days 0, 7, 14, and 21 by injecting the infected cells into the left jugular vein. Fresh lymphocytes were obtained from the horse by jugular venipuncture on Days –1, 6, 13, and 20. The lymphocytes were placed in tissue culture overnight and infected with *S. neurona* merozoites. The horse was observed daily and euthanized 34 days after infection. Homologous cells from each of three horses (B, C, and D) were infected as described above. Horse B received 100 merozoites, Horse C received 1,000 merozoites, and Horse D received 10,000 merozoites daily via homologous-infected lymphocytes for 15 days. Horse A served as an uninfected control and received uninfected lymphocytes that were treated as described above but were not incubated with merozoites.

Neurologic and Lameness Examinations

All horses were examined daily for the duration of the study beginning 2 weeks before infection. Appetite, behavior, lameness, ataxia, and neurologic deficits were monitored by observing food consumption, change in behavior, gait, and cranial nerve criteria. The gait examination was performed at a walk, trot, and free in the paddock, and scores were assigned (Grade 0 = normal gait; Grade 1 = minor ataxia detected at a walk; Grade 2 = ataxia easily detected at a walk and exaggerated by backing, turning in tight circles, and walking with head elevated; Grade 3 = prominent ataxia at normal gaits, stumbling, and difficulty maintaining balance). Neurologic status was evaluated once weekly, documented by video camera, and recorded on a form developed for that purpose.

Clinical Pathology Data

Whole-blood samples were collected from the jugular vein of each horse once weekly. Geimsa-stained thin smears of peripheral blood were evaluated daily from Day 0 to Day 7. Weekly serum samples were evaluated for IgG response using an indirect ELISA with recombinant SAG1 as antigen.[18] Reaction of antibodies in serum to *S. neurona* antigens prepared from the same *S. neurona* strain as those used in the infections were determined by standard immunoblot techniques.[22] Additionally, 5 μL of recombinant glutathione S transferase–SAG1 protein diluted to 10 μg/mL was adsorbed to polyvinylidene difluoride membranes followed by blocking with 1% bovine serum albumin in PBS for 1 hour at room temperature. Immunoblots using serum diluted 1:20 were performed using methods previously reported.[22] The horses were euthanized 67 or 68 days after infection. CSF samples were collected from each horse for antigen and antibody determination, and 1 mL of CSF from each horse was submitted to a clinical pathology laboratory for analysis. Each sample was cleared by centrifugation at 600 ×g, and the resulting pellet was cultured on a 60%-confluent monolayer of bovine turbinate cells and observed daily for growth of parasites.

Gross necropsy was performed on Horses A, B, C, and D; samples of liver, lung, spleen, brain, and cervical spinal cord were placed in 10% zinc-buffered formalin for histopathology and immunohistochemistry using monoclonal antibody SAG1. Cervical spinal cord sections were visually examined for areas of discoloration; discolored areas were macerated and placed in cell culture flasks containing bovine turbinate cells. The remaining sections of spinal cord were macerated and the suspensions centrifuged at 200 ×g for 3 minutes. Immuno-affinity beads were used to increase the chance of parasite detection. The supernates from the macerated spinal cord tissues were incubated with 500 μL SAG1 immuno-affinity beads prepared as previously described. The SAG1 immuno-affinity beads and supernate were incubated for 2 hours at 37°C with slight rocking. The beads were collected, washed, and placed on a 1.077-Optiprep density gradient and over-layered with PBS. After centrifugation at 13,000 rpm for 10 minutes, the supernate was removed, placed on a 30%-confluent monolayer of bovine turbinate cells, and incubated in a 5% carbon dioxide atmosphere at 37°C. The cultures

were examined daily for the presence of *S. neurona*. After 10 days, the monolayers were treated with 0.25% trypsin/0.5% EDTA and subcultured into 24-well plates with one cover slip per well. The 24-well plates were incubated in 5% carbon dioxide atmosphere at 37˚C. Three days after subculture, one cover slip was fixed in 10% zinc-buffered formalin or 95% ethyl alcohol, stained with Geimsa, and examined for *S. neurona.* Methanol was not used as a fixative because it tended to remove the monolayers from the cover slip. There were no gross lesions visible in the liver, lung, spleen, or brain tissues, so affinity purification was not performed on these tissues; however, a 0.5-gm sample of fresh tissue from each organ was macerated and placed in culture using bovine turbinate cells. Affinity bead purification was performed on spinal tissues only.

Horse D was pregnant at the time of infection, and a female fetus approximately 85 days through gestation was examined grossly at necropsy. Histopathologic slides of brain, liver, lung, spleen, and cervical spinal cord were prepared for the fetus using hematoxylin–eosin-stained thick sections and serial sections collected on immunohistochemistry slides.

RESULTS

Infection of Lymphocytes and In vitro Assay for *S. neurona*

During the preparatory studies, it was determined that some monocytes contained parasites; however, lymphocytes were the primary cells infected with merozoites. Host cell infection was complete within 2 to 5 hours of incubation (data not shown), thus lymphocytes were used in the equine model. A few monocytes contained more than one merozoite. Merozoites entered host cells within 5 hours after exposure, except when the parasite:host cell ratio was greater than 1, in which case less than 5% of the parasites remained free in the media. The $TCID_{50}$ for *S. neurona* was 350 merozoites in 1,500 bovine turbinate cells. The $TCID_{50}$ for equine lymphocytes was 1,000 merozoites in 1,500 lymphocytes.

Developing schizonts were visible within 24 hours following addition of freshly infected equine lymphocytes to adherent bovine turbinate cells in vitro. Merozoites remained viable when infected equine lymphocytes were incubated in RPMI 1640 with antibiotics for 24 hours and then transferred to monolayers of bovine turbinate cells. However, few live parasites were observed when the infected equine lymphocytes were incubated in RPMI 1640 with antibiotics for 48 hours. The use of Optiprep did not change the infectivity of merozoites for bovine turbinate cells. When the infected equine lymphocytes that were incubated in RPMI 1640 with antibiotics were stained with new methylene blue and observed using a hemocytometer, the lymphocytes were dead. A thin smear of this preparation stained with Geimsa showed that the lymphocytes were ruptured and in close proximity to small (2×3 μm) developing parasites. Immuno-affinity beads were effective at removing parasites that were not contained in cells. The $TCID_{50}$ of supernate from equine lymphocyte suspensions subjected to immunopurification was greater than 6,000, indicating that no monolayer disruption was seen.

Experimental infection of Horse 1

Horse 1 lost weight, drooled, dropped feed, and became ataxic by Day 10. The horse was noticeably lame in the rear, with a spastic movement of the pelvic limbs and was graded 2 for lameness and 3 for ataxia. The lameness plateaued for 7 days but became worse after the third challenge dose (Day 14). Clinical signs progressed to disorientation and moderate, generalized weakness. The horse was euthanized on Day 34. *S. neurona* was recovered from spinal cord cultures within 5 days, suggesting an overwhelming infection of these tissues (Figure 1). Organisms were positive for staining by immunohistochemistry using monoclonal antibody for SnSAG1 and polyclonal rabbit anti-*S. neurona* sera.

Experimental infection of Horses B, C, and D

The uninfected control animal (Horse A) remained clinically normal throughout the
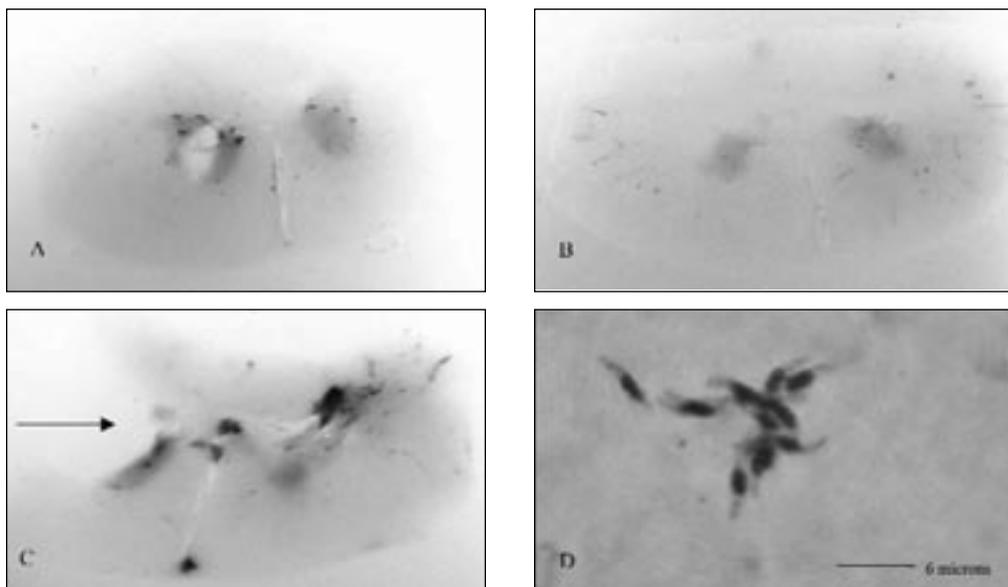
Figure 1. Spinal cord sections from Horse 1 used in pilot study of experimentally induced *Sarcocystis neurona* encephalomyelitis. Unstained tissue in paraffin blocks are shown (**A–C**). The area used to isolate organisms by culture is marked (*arrow*) in Panel **C**. S. neurona merozoites cultured from spinal tissue in Panel **C** are shown in **D** (Geimsa, magnification ×100).

study. Horse B experienced neck pain at Day 13, but clinical signs of inflammation (heat or swelling) were not apparent. The horse was reluctant to bend her neck and was unable to eat from the ground. When asked to turn or trot, there was muscle fasciculation involving muscles surrounding the left eye and left lateral neck. These signs resolved by Day 17. This horse also exhibited an abnormally wide gait Day 14 that was most obvious when trotting. Gait deficits did not progress.

Horse C began dragging the right toe and showed pelvic limb weakness by Day 14; by Day 21, the horse was stumbling and occasionally fell. The deficits progressed to mild rear limb weakness and some thoracic limb weakness by Day 68.

Horse D remained normal until Day 55, at which time the horse was slightly lame at a trot and unable to canter in a tight circle. Horses B, C, and D showed unilateral mild facial paresis by Week 4 of the study. Ataxia was graded 1 for Horses B and D and 2 for Horse C.

S. neurona was isolated from the spinal cord of Horses 1, B, C, and D (Figure 2). The spinal cord cultures were positive for the

growth of *S. neurona* by Day 10 for Horses C and D and by Day 21 for Horse B. No merozoites were recovered from the tissues of Horse A. Parasites were not recovered from lung, liver, muscle, or spleen cultures.

By Day 7 of culture, bacteria overgrew all cultures from the tissues collected from the fetus carried by Horse D, and no parasites were isolated. No bacteria were noted on stained sections; therefore, the contamination most likely occurred postmortem and did not indicate infection of the fetal tissues. Lung sections stained with hematoxylin–eosin contained organisms or artifacts consistent in size and shape with *S. neurona* merozoites (Figure 3). Monoclonal antibodies that bind the SAG1 antigen of *S. neurona* were used in immunohistochemistry stained sections of fetal tissues and antibody binding was observed in a few sites in three serial sections from lung tissues.

At the beginning of the dose titration study, Horses A, B, and D were negative for antibodies to *S. neurona* and Horse C was positive by immunoblot (data not shown) and SAG1 ELISA at a serum dilution of
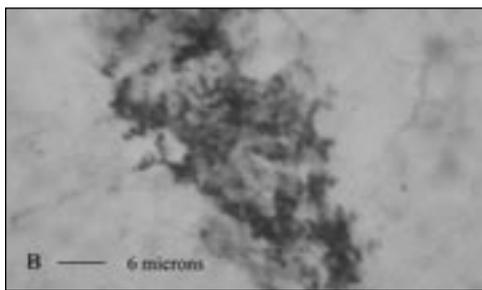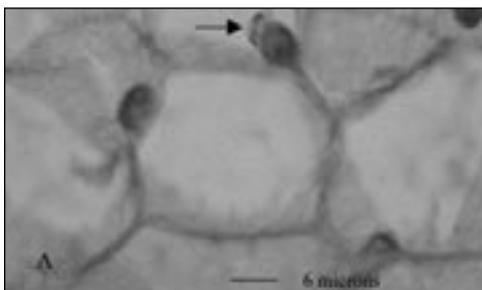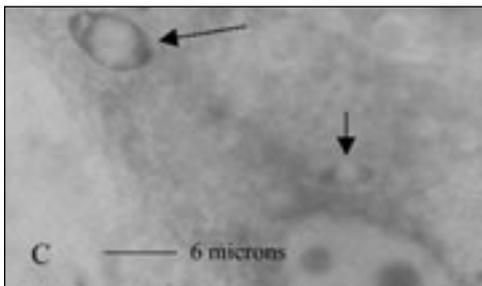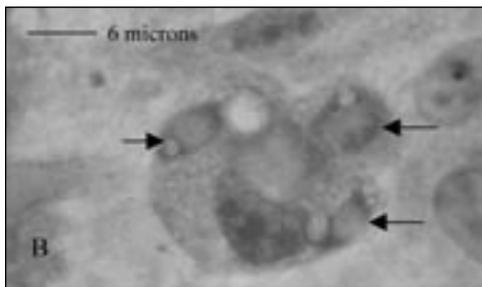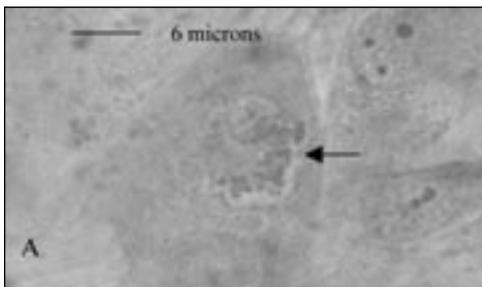
Figure 2. *Sarcocystis neurona* merozoites isolated from three horses and cultured in bovine turbinate monolayers. Panel **A** shows an *S. neurona* schizont (*arrow*) in a host cell from a horse infected with 10,000 merozoites daily at 14 days after culturing. Panel **B** shows three merozoites (*arrows*) in host cells isolated from a horse infected with 1,000 merozoites daily at 21 days after culturing. Panel **C** shows merozoites in host cell isolated from a horse infected with 100 merozoites daily at 21 days after culturing (Geimsa, magnification ×100).

Figure 3. Histopathologic sections of lung tissue, showing a possible merozoite (arrow), from a fetus recovered from a horse infected with 10,000 merozoites daily for 15 days (**A**) and a Immunohistochemistry-stained section of lung tissue with an area that bound monoclonal antibody to the surface antigen of S. neurona (**B**) (hematoxylin–eosin, magnification × 100).

1:50. A positive test result for serum at a screening dilution of 1:50 was not considered indicative of disease because the CSF was negative for anti-*S. neurona* antibodies in these horses using the same procedure. By Day 7, Horses B and D had detectable serum IgG to *S. neurona* determined by immunoblot. All three infected horses continued to have serum antibodies to *S. neurona* for the duration of the study. Antibodies to *S. neurona* also were detected in the CSF of all three horses by immunoblot by Day 7. The control horse remained negative for antibodies to *S. neurona* in both the serum and CSF for the duration of the study.

Results of the animal titration studies showed that autologous cells infected with either 1,000 or 10,000 organisms daily for 7 days induced marked changes in the CNS and allowed recovery of the *S. neurona* organism in vitro by Day 7. At these doses, a parasitemia was demonstrated throughout the infection period from thin smears prepared daily and stained by Geimsa. A parasitemia was detected for 4 days after the last day of challenge by Geimsa-stained thin smears of peripheral blood taken from the opposite side of the neck used for challenge inoculations (data not shown).

DISCUSSION

The IV administration of *S. neurona*-infected lymphocytes to infect horses resulted in

the development of clinical signs and histopathologic lesions compatible with EPM, *S. neurona*-specific antibodies in CSF, and isolation of the organism. The control animal that received noninfected homologous lymphocytes remained clinically and neurologically normal. Other horses present in the facility but not included in the study also remained normal.

Equine infections by *S. neurona* may involve a hematogenous phase as indicated in *Sarcocystis* infections in other species. The successful use of a daily challenge method may indicate that a sustained challenge is required for infection. However, the lowest cumulative dose of parasites successfully used in the current report was 1,500, which is far less than any other challenge dose reported in the literature. In addition, Horse 1 had clinical signs of encephalomyelitis before the second injection, indicating that severity of signs may be related to dose and not the number of consecutive days of challenge. Four challenge doses were given to Horse 1 to increase the potential for parasite recovery from the CNS. A 15-day consecutive parasite challenge was chosen to develop a model to test prophylactic drug therapies.

It is possible that the methods used here released free merozoites from the infected lymphocytes into the horse, or perhaps, the merozoites infected vascular tissues at the site of repeated injection in the jugular vein. A free merozoite control would have been useful but was not included in this study because IV injection of free merozoites was unsuccessful for other researchers.[19] It is also possible that merozoites introduced in infected lymphocytes were able to invade somatic tissues outside the CNS because a parasitemia was sustained for 4 days after challenge in two horses. The location of infection following the challenge was undetermined; the amount of tissue examined for lung, liver, or spleen was not sufficient to rule these tissues out. A strong possibility is lymphatic tissue. It is possible that *S. neurona* can cross the blood–brain barrier independent of host cell participation as free

merozoites if they are freshly released from host cells. The determination of extra-CNS replication of *S. neurona* in horses was beyond the scope of this project but would allow sufficient numbers of freshly released parasites available for infection of the CNS.

In a previous study,[19] the introduction of culture-derived merozoites directly into the CNS was unsuccessful. Various experimental methods to induce clinical infections with recovery of *S. neurona* from the CNS in horses have been unsuccessful, despite high doses and multiple challenges.[19,21,23–26] The authors believe *S. neurona* is an obligate intracellular parasite in all growth stages, and the period of time that allows host cell invasion is short. The methods used here satisfy the hypothesis that lymphocytes provide both an intracellular location for the parasite and a hematogenous method of distribution in the host.

Dubey and coworkers[20] have reported that *S. neurona* lost virulence for interferon-γ gene knockout mice when maintained in continuous culture. The use of avirulent merozoites may explain failed attempts to produce EPM using culture-derived merozoites. The strain of *S. neurona* used in this study was passed continuously in bovine turbinate cells and maintained the ability to induce disease for more than 24 months. It is suggested that the pathogenicity of *S. neurona* for horses is maintained if the merozoite infects lymphocytes. Once infected, these cells can be transported across the blood–brain barrier. Perhaps the ability to cross the blood–brain barrier is simply a "Trojan horse" mechanism used by the parasite. A slight variation existed in the $TCID_{50}$ for equine lymphocytes derived from individual horses (data not shown). It is unknown what factors contribute to resistance of infection by individual horses; however, these differences may be independent of anti-*S. neurona* antibody status since Horse C (with anti-*S. neurona* antibodies) became infected.

One previous study[13] demonstrated parasitemia in a foal with severe combined immunodeficiency following oral challenge

with *S. neurona* sporocysts. That immuno-compromised foal did not have clinical encephalomyelitis, and no *S. neurona* was recovered of from the CNS. This is supportive data that functional lymphocytes are required for *S. neurona* to cross the blood–brain barrier in horses. Experimental autoimmune encephalomyelitis studies in laboratory animals require a state of activation for encephalitogenic T cells to cross the endothelial blood–brain barrier, whereas activated T lymphocytes can transverse the blood–brain barrier irrespective of their antigen specificity.[27] In EPM, an antigen-primed lymphocyte or a lymphocyte carrying a viable parasite could provide the activated state necessary to enter the CNS.

It has been established that *Plasmodium* spp sporozoites migrate through multiple hepatocytes before reaching their final host, and it has been shown that *Toxoplasma gondii* tachyzoites migrate through epithelial barriers. It is undetermined how *S. neurona* migrates to the CNS naturally in horses; however, based on data from this study, lymphocyte transport is a possible mechanism.

The pathogenesis of *Sarcocystis* infections in other species includes a parasitemia, and this may be the case for *S. neurona* in horses. The parasite may reside briefly inside the host's lymphocyte cells, evade the host's immune system, and passively enter the CNS. In this model of *S. neurona* infection, a relationship existed between clinical EPM and infected lymphocytes. Successful entry into the CNS was shown by isolation of *S. neurona* by in vitro culture.

Viable parasites were not recovered following rupture of lymphocytes after 48 hours of in vitro incubation in culture, suggesting these cells are not competent host cells and may be a dead end for the parasite. Infected lymphocytes harbored infectious parasites for 24 hours, and the parasites replicated when subcultured onto bovine turbinate cells. Size or metabolic constraints of the lymphocyte may allow the parasite to suspend development until a more hospitable environment is encountered. This is compatible with knowledge that some *Sarcocystis* spp become dormant while awaiting ingestion by a proper host. Data generated in this study suggest that merozoites may cross the equine blood–brain barrier in less than 48 hours. Alternatively, the parasite may become sequestered in an undetermined site in the horse until the parasite can make its way into the CNS.

Early signs of disease in acute EPM coincided with increases in *S. neurona*-specific IgG in the CSF, as measured by a SAG1 ELISA, which has been previously reported.[28] The absence of specific antibodies to SAG1 in the CNS before challenge and immunoconversion in the CSF after infection provide evidence that specific antibodies were induced in the challenged horses but not in the uninfected control. In this model, antibodies to SAG1 determined by ELISA served as an indication of disease before necropsy of the horses.

The time of onset of classical signs of EPM, such as ataxia and lameness, varied with the individual animal but were apparent to veterinarians skilled in assessing neurologic disease and blinded to the procedures in the study. Early signs were noted as soon as 10 days after the last challenge dose given to Horses B, C, and D. Horse 1 showed signs prior to the second challenge dose, indicating that a single dose challenge may be sufficient; however, the severity of the signs may be dose dependent. The use of this model will allow researchers to begin examining early disease in equine subjects, rather than the chronic syndrome upon which our current understanding of EPM is based and will enhance our understanding of the pathogenesis of infection in natural disease. This can improve diagnosis and assist researchers in developing rational strategies for the treatment and prevention of EPM in the horse.

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