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Research paper

Sarcocystis neurona manipulation using culture-derived merozoites for bradyzoite and sporocyst production

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ABSTRACT

Equine protozoal myeloencephalitis (EPM) remains a significant central nervous system disease of horses in the American continents. *Sarcocystis neurona* is considered the primary causative agent and its intermediate life stages are carried by a wide host-range including raccoons (*Procyon lotor*) in North America. *S. neurona* sarcocysts mature in raccoon skeletal muscle and can produce central nervous system disease in raccoons, mirroring the clinical presentation in horses. The study aimed to develop laboratory tools whereby the life cycle and various life stages of *S. neurona* could be better studied and manipulated using *in vitro* and *in vivo* systems and compare the biology of two independent isolates. This study utilized culture-derived parasites from *S. neurona* strains derived from a raccoon or from a horse to initiate raccoon infections. Raccoon tissues, including fresh and cryopreserved tissues, were used to establish opossum (*Didelphis virginiana*) infections, which then shed sporocysts with retained biological activity to cause encephalitis in mice. These results demonstrate that sarcocysts can be generated using *in vitro*-derived *S. neurona* merozoites, including an isolate originally derived from a naturally infected horse with clinical EPM. This study indicates the life cycle can be significantly manipulated in the laboratory without affecting subsequent stage development, allowing further purification of strains and artificial maintenance of the life cycle.

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1. Introduction

Equine protozoal encephalomyelitis (EPM) can be caused by either apicomplexan parasite *Sarcocystis neurona* or *Neospora hughesi*; however, the majority of cases are attributed to *S. neurona* (Reed et al., 2016). Infection variably results in a devastating neurological disease with a wide variety of presenting clinical signs due to the ability of the organism to infect both grey and white matter of the brain and spinal cord. *S. neurona* is transmitted through fecal-oral transfer of sporocysts excreted by the opossum (*Didelphis virginiana or D. albiventris),* the definitive host (Dubey et al., 2015). Contaminated food or water is ingested by a wide-range of intermediate hosts to complete the life cycle (Dubey et al., 2015).

Clinical disease attributed to *S. neurona* infection has been documented in a number of species such as the horse, cat, dog, skunk (*Mephitis mephitis*), Canada lynx (*Felis canadensis*), sea otter (*Enhydra lutris*), Pacific harbor seal (*Phoca vitulina richardsi*) and California sea lion (*Zalophus californianus*) (Dubey et al., 2015). Marine wildlife infections with *S. neurona*, a terrestrially derived pathogen, are associated with water runoff events, which can impact the health of threatened species such as the Southern sea otter (*Enhydra lutris nereis*) (Shapiro et al., 2012). Recently, vertical transmission of *S. neurona* has been reported in marine mammals (Barbosa et al., 2015). The diversity of affected species by this pathogen and lack of reliable ante-mortem diagnostic and treatment options (Dubey et al., 2015) highlight the need to develop the tools and reagents to more accurately study *S. neurona*.

This study aimed to ascertain if variations in inoculation preparations and parasite strains would impact the parasite's ability to encyst in raccoons in numbers large enough for later sporocyst production in opossums. The results indicate biological differences exist between isolates of *S. neurona*, corroborating results of ear-

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lier studies (Butcher et al., 2002; Dryburgh et al., 2015). Moreover, inoculation methodology impacted tissue cyst numbers, producing many more than previously reported, including the production of a central nervous system sarcocyst. Finally, we detected tissue cysts in a raccoon inoculated with an isolate originally obtained from a horse with EPM. While these latter cysts were smaller and not as numerous, this discovery is the first to demonstrate cysts produced by a clinical EPM-derived *S. neurona* isolate. This work demonstrates the promiscuity of the parasites, their ability to maintain biological capacity for stage development despite cryopreservation, long-term cultivation and manipulation in the laboratory.

2. Materials and methods

2.1. Animal experiments

The Ohio State University's Institutional Animal Care and Use Committee approved all animal husbandry and experimentation (Protocols 2015-76 and 2015-107). Fig. 1 shows the timeline of protocol activities and Fig. 2 diagrams the experimental design.

2.1.1. Raccoon experiments

Commercially reared *S. neurona* sero-negative raccoons (n = 10) were acquired and cared for as previously described (Dryburgh et al., 2015).

2.1.1.1. Raccoon blood sampling and primary mononuclear cell cultures.

Peripheral whole blood was collected with EDTA under injectable anesthesia, ketamine (5–17 mg/kg) (Fort Dodge, Iowa, USA) and xylazine (1–5.3 mg/kg) (Lloyd, Shenandoah, Iowa, USA). The buffy coat layer was overlaid onto 3 mL of Histoplaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) for autologous blood cell cultures. Lymphocytes and mononuclear cells were harvested following the manufacturer's directions, seeded into Nunc EasYFlasks T25 culture flask (Thermo Fisher Scientific, Roskilde, Denmark), and maintained with RPMI-based protozoal media (Davis et al., 1991). At 24 h non-adherent cells were removed and cultures were inoculated with merozoites from the corresponding isolates.

2.1.1.2. Raccoon inoculations with in vitro-derived merozoites.

Raccoons No. 0–7 were inoculated with merozoites of either *S. neurona* isolate SN744 (raccoon derived) or SN-MU1 (equine derived) by intravenous (IV), intramuscular (IM) and subcutaneous (SC) inoculation, under general anesthesia. The strain designations used here are from the original studies describing strain isolation: SN744 (Elitsur et al., 2007) and SN-MU1 (Marsh et al., 2001; Howe et al., 2008). Both isolates were resuscitated from liquid nitrogen cryopreserved material (>10 years) and underwent an unknown number of passages; *S. neurona* grows asynchronously, making the number of replications difficult to determine. The cryopreserved material for isolate SN744, used in this study, was dated within 6 months of original isolation. Frozen cryopreserved material for the SN-MU1 was dated within two years of original isolation. Both isolates were cryopreserved and stored by the original investigator (AEM). Equine dermal cells and bovine turbinate cells were used to cultivate parasites for inoculation, and the inoculums were prepared as previously described (Marsh et al., 1997). Approximately 70% of the adherent monolayer contained parasite stages. A subset of the raccoons also received *S. neurona in vitro* infected raccoon autologous primary mononuclear cell cultures. Table 1 provides the enumerated merozoites and dosing protocol. The number of merozoites and schizonts were not enumerated in the primary mononuclear cells. The infected primary cells were harvested at 24 h after merozoite addition (inoculation 1 preparation) and at 6 days after merozoite addition (inoculation 2 preparation). The primary mononuclear cells were monocyte derived based on positive staining by α-naphthyl butyrate esterase kit (Sigma-Aldrich, St. Louis, USA). The mononuclear culture supernatant and scraped mononuclear cells were centrifuged with the resultant pellet re-suspended in Hanks' balanced salt solution (0.5 mL/raccoon inoculation). Raccoons No. 8 and 9 remained as negative controls, receiving no inoculations.

2.1.1.3. Masseter muscle biopsy.

All raccoons underwent transcutaneous masseter muscle biopsies (5 mm punch) on 148 days post infection (DPI) except for raccoon 6, which was euthanized earlier. The biopsy site was surgically closed and pain management provided with lidocaine (1.5 mg/kg) (Vedco, St. Joseph, MO, USA) at the surgical site and meloxicam (Boehringer Ingelheim Vetmedica, Fort Dodge, IA, USA) (5 mg/ml, SC). Fresh biopsy samples were split for evaluation: 1) direct microscopy of squash preparation and 2) fixation in 10% neutral buffered formalin (NBF), paraffin embedding, sectioning and hematoxylin and eosin (H&E) staining.

2.1.1.4. Raccoon serology for S. neurona and Toxoplasma gondii.

An indirect ELISA was performed using the rSnSAG2/4/3 in carbonate-bicarbonate buffer (pH 9.6) as the antigen (Yeargan et al., 2015). Following overnight plate coating, the plates were rinsed with Tris buffered saline with 0.1% Tween 20 (TBS-T). Superblock T20 (Thermo Scientific, Waltham, MA, USA) was applied to the wells for 1 h. Raccoon pre-inoculation plasma and terminal sera were diluted 1:500 in 1% bovine serum albumin in TBS-T and applied in duplicate wells for 45 min. Wells were extensively washed with TBS-T followed by the addition of diluted horseradish peroxidase-conjugated secondary antibody against raccoon IgG (H&L) (KPL, Gaitherburg, MD, USA) in 1% bovine serum albumin in TBS-T. SureBlue TMB Microwell Peroxidate Substrate 1 component (KPL) was used for color development. The reaction was stopped with the addition of 0.1 N HCl at 15 min. Absorption was measured at 450 nm. The pre-inoculation and terminal samples were tested along with a naturally infected raccoon and TBS-T serving as positive and negative controls, respectively.

Retrospectively, the terminal bleeds of raccoon No. 7 and 3 were also tested for antibodies to *Toxoplasma gondii* using the modified agglutination test (University of Tennessee College of Veterinary Medicine) as these two animals represented animals from the two

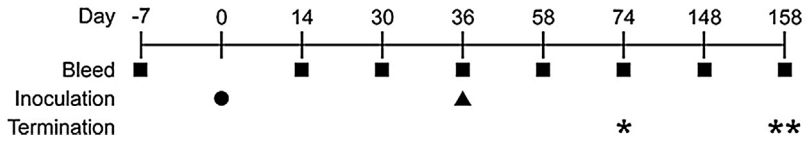

Fig 1. Raccoon experimental timeline. Raccoons were monitored for evidence of infection by obtaining blood samples for serology at various time points during the course of infection (■). For raccoons that were inoculated twice, the second parasite dose was administered 36 days (▲) after initial inoculation on day 0 (●). A single animal was euthanized on day 74 (*) to acquire more detailed knowledge about the state of infection. All remaining raccoons inoculated were euthanized and the negative controls were bled on day 158-post inoculation (**).