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Short communication

Sarcocystis neurona infection in gamma interferon gene knockout (KO) mice: Comparative infectivity of sporocysts in two strains of KO mice, effect of trypsin digestion on merozoite viability, and infectivity of bradyzoites to KO mice and cell culture

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ABSTRACT

The protozoan *Sarcocystis neurona* is the primary cause of Equine Protozoal Myeloencephalitis (EPM). EPM or EPM-like illness has been reported in horses, sea otters, and several other mammals. The gamma interferon gene knockout (KO) mouse is often used as a model to study biology and discovery of new therapies against *S. neurona* because it is difficult to induce clinical EPM in other hosts, including horses. In the present study, infectivity of three life cycle stages (merozoites, bradyzoites, sporozoites) to KO mice and cell culture was studied. Two strains of KO mice (C57-black, and BALB/c-derived, referred here as black or white) were inoculated orally graded doses of *S. neurona* sporocysts; 12 sporocysts were infective to both strains of mice and all infected mice died or became ill within 70 days post-inoculation. Although there was no difference in infectivity of sporocysts to the two strains of KO mice, the disease was more severe in black mice. *S. neurona* bradyzoites were not infectious to KO mice and cell culture. *S. neurona* merozoites survived 120 min incubation in 0.25% trypsin, indicating that trypsin digestion can be used to recover *S. neurona* from tissues of acutely infected animals.

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1. Introduction

Sarcocystis neurona is a coccidian that is the primary cause of Equine Protozoal Myeloencephalitis (EPM). EPM is the most commonly diagnosed neurological disease of horses in the United States (Dubey et al., 2001a). *Sarcocystis neurona* has a complex life cycle (Dubey et al., 2001a; Lindsay et al., 2004). Opossums (*Didelphis* spp.) are its only known definitive host and several other hosts are its intermediate or aberrant hosts. Domestic cats, skunks, raccoons, armadillos, sea otters are confirmed intermediate hosts (Cheadle et al., 2001a, 2001b; Dubey et al., 2001a, 2001b, 2001c). The intermediate hosts become infected by ingesting food and water contaminated with sporocysts excreted in the feces of opossums. After the ingestion of sporocysts, sporozoites released in the gut lumen invade lamina propria and initiate formation of schizonts in many tissues, preferably in the central nervous system parenchymal cells (Stanek et al., 2002). Merozoites released from schizonts form sarcocysts in muscles. The definitive host, opossum, becomes infected by ingesting mature sarcocysts encysted in tissues of intermediate hosts. The bradyzoites released from sarcocysts penetrate small intestinal lamina propria and transform into gamonts. After fertilization, oocysts are formed and they sporulate in situ. Sporulated oocysts have thin oocyst wall that often ruptures and free sporocysts are released in the gut lumen and passed in feces for months. Addtionally, several other hosts act as aberrant hosts in

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whose tissues only schizonts but, no sarcocysts are formed; severe illness has been reported in aberrant hosts, including horse, ferret, marine mammals, lynx, dogs, and cats (Sellon and Dubey, 2007; Britton et al., 2010).

It is very difficult to reliably induce clinical EPM in horses or other aberrant or intermediate hosts, irrespective of dose or immunosuppression; this has hampered progress on developing effective chemotherapy and immunotherapy (Dubey et al., 2001a). Fortunately, *S. neurona* is infectious to immunodeficient mice. Marsh et al. (1997) first reported *S. neurona* infection with cultured merozoites in the nude mouse. Subsequently, the gamma interferon gene knockout (KO) mouse model was developed to study *S. neurona* biology and chemotherapy (Dubey, 2001; Dubey et al., 2001d, 2001e; Cheadle et al., 2002; Lindsay and Dubey, 2001; Witonsky et al., 2003). KO mice can be infected with sporocysts from opossum feces and merozoites from infected tissues or cell culture (Dubey, 2001). Thus, KO mice have been used in bioassay (Elitsur et al., 2007). KO mice develop neurologic illness simulating EPM, and all infected mice die within 70 days post inoculation (p.i.). It is not known if KO mice or cell culture can be used as a bioassay to detect sarcocysts in infected intermediate hosts because infectivity of bradyzoites for cell culture or KO mice is unknown. It is also unknown if there are differences in susceptibility of *S. neurona* to different strains of KO mice. The resistance/susceptibility of *S. neurona* in mice is interferon gamma mediated because mice lacking gene for another immune mediator (nitric oxide production) were not susceptible to *S. neurona* (Rosypal et al., 2002).

The present study compares infectivity and susceptibility of *S. neurona* sporocysts to two strains of KO mice, tests infectivity of bradyzoites to cell culture and KO mice, and studies the effect of trypsin digestion on viability of merozoites.

2. Materials and methods

2.1. S. neurona isolate

S. neurona strain SN-37R was used in this investigation; its origin and history was described by Sofaly et al. (2002). Laboratory raised raccoons were fed SN-37R sporocysts and euthanized 3 months later at the Ohio State University (OSU).

2.2. Gamma interferon gene knockout (KO) mice

Two strains KO mice (C57BL/6-Ifng, stock #002287, black and BALB/c-Ifng, stock #002286-white) were obtained from The Jackson laboratory (Ben Harbor, ME). They were females and 7–8 weeks old at the time of the experiments.

2.3. Infectivity S. neurona of sporocysts to two strains of knockout mice

Sporocysts were collected from intestinal scrapings of laboratory raised opossums fed with muscles of laboratory raised raccoons that had been fed *S. neurona* sporocysts after eight opossum-raccoon passages (Sofaly et al., 2002). Sporocysts were collected as described by Dubey et al. (1989). Briefly, opossum small intestinal scrapings were homogenized in water, shaken, filtered through gauge, and then through metallic 90 μm, and 45 μm sieves to remove large particles. Sporocysts were stored in antibiotic solution at 4 °C before used in the present experiment. Sporocysts were counted and serially diluted 10-fold so that the last dilution contained <2.5 sporocysts per ml. In total 40 KO mice (20 of each strain) were inoculated orally 0.5 ml of aliquot from each dilution containing 12,500 sporocysts (highest dose) and the lowest <2 sporocysts (last dilution) (Table 1). The experiment was terminated day 74 post-inoculation (p.i.). All mice that died and those euthanized at termination were necropsied. Specimens of most internal organs including the heart, lung, liver, spleen, intestines, kidneys, adrenals tongue, leg muscles, whole brain and eyes were fixed in 10% buffered neutral formalin. Paraffin embedded sections were cut at 5 μm thickness, and examined microscopically after staining with hematoxylin and eosin stain.

2.4. Effect of trypsin digestion on S. neurona merozoites

To study effect of trypsin on *S. neurona* merozoites, whole brain of a KO mouse was used; the mouse had been fed *S. neurona* sporocysts 30 days earlier and had demonstrable merozoites in its cerebellum. The infected brain was homogenized in 0.85% NaCl solution (saline) to make the final volume to 60 ml (suspension A). This suspension was divided equally in to two aliquots (B and C). Suspension B was mixed with 30 ml of 0.5% trypsin (Sigma) in saline and incubated at 37 °C in a water bath; final concentration of trypsin was 0.25%. Suspension C was incubated in saline without trypsin. After intervals of 15, 30, and 60 min, 5 ml of each of suspensions B and C were removed, centrifuged, sediment suspended in saline, centrifuged again and the sediment suspended in 5 ml antibiotic saline (Dubey, 2010) and inoculated subcutaneously into 4 KO mice.

This experiment was repeated a week later with another *S. neurona* infected mouse, with the difference that the incubation in suspensions with or without trypsin was extended to 120 min and only 2 KO mice were used for bioassay at each time point of 15, 30, 60, 120 min incubation period.

2.5. Infectivity of S. neurona bradyzoites to KO mice and cell culture

Bradyzoites were obtained from the sarcocysts in diaphragms of two experimentally infected raccoons .The raccoons were euthanized in good health 3 months after feeding SN-37R sporocysts. Infected diaphragms were shipped cold to the Animal Parasitic Diseases Laboratory, Beltsville, Maryland. Diaphragmatic muscle (20 g) of the first raccoon was ground briefly in 100 ml saline in a blender, and pre-warmed acid-pepsin solution was added and incubated at 37 °C for 15 min with shaking, as described previously for *T. gondii* (Dubey, 2010). The muscle homogenate was centrifuged, neutralized with sodium bicarbonate, washed with saline solution, centrifuged

Table 1
Dose titration of *Sarcocystis neurona* sporocysts in two strains of gamma interferon knockout mice.

No. of sporocysts inoculated	Knockout mice[b]	
	C57Black-derived	BALB/c-derived
12,500[a]	25 (H, T), 25 (A, H), 27 (H, L), 29 (L)	26 (Lu), 27, 32 (Lu), 32 (Lu)
1250	26 (Lu), 29, 34 (Li), 35 (Lu)	32 (Lu), 33, 34 (Lu), 34 (Lu)
125	32, 33 (Lu), 34, 36 (Lu)	34, 49, 60 (Lu), 69 (Lu)
12.5	49 (H, Lu), S[c], S, S	47, S, S, S
1.2	S, S, S, S	S, S, S, S

[a] Sporocysts were counted, diluted 10-fold, and aliquots (0.5 ml) from each dilution were inoculated orally into 4 knockout mice. The experiment was terminated on day 74.
[b] Day of death or euthanasia. *S. neurona* was demonstrated histologically in the brains of all mice that died or became ill. Additional infected tissues are given in parenthesis. A: adrenal; H: heart; Li: liver; Lu: lung; T: tongue.
[c] S: survived, not infected.

again, and the sediment suspended in tissue culture medium with Hank's balanced salt solution (HBBS). More than 1 million motile bradyzoites were present per ml of the suspension. An aliquot (1 ml) was seeded on to M617 monocyte culture flask. After 1 h incubation, the supernatant was replaced with tissue culture medium. The flask was examined for growth of parasites for two months. Aliquot from the bradyzoite suspension was inoculated subcutaneously into 5 KO mice. The mice were observed for three months for *S. neurona* infection. Smears made from the same preparation of bradyzoites was used to immunologically characterize *S. neurona* bradyzoites (Gautam et al., 2011)

The above experiment was repeated in cell culture, using Percoll-cleaned bradyzoites. For this, 100 g of diaphragm of the second infected raccoon was ground in a blender for few seconds without saline. The ground muscle was incubated in 200 ml of acidic pepsin solution (without salt, used for collecting *Sarcocystis cruzi* bradyzoites, as described in Dubey et al. (1989) at 37 °C in a water bath for on a shaker for 10 min to release bradyzoites from sarcocysts. The homogenate was centrifuged and bradyzoites were collected from the sediment as described (Dubey et al., 1989). The bradyzoite suspension was then mixed with Percoll, and clean bradyzoites were collected. Bradyzoites were washed in HBBS, and suspended in tissue culture medium. Bradyzoites were seeded on to M617 bovine monocytes. After 1 h incubation the supernatant was replaced with fresh medium and the flask incubated for 3 months at 37 °C. The bradyzoites seeded on to flask were motile and appeared intact.

2.6. Immunohistochemical staining

For immunohistochemical (IHC) staining, sections from each mouse were reacted with anti-*S. neurona* serum prepared in a rabbit injected with culture-derived *S. neurona* merozoites as described (Dubey et al., 1999). The specificity of the *S. neurona* antiserum has been described (Dubey and Hamir, 2000).

2.7. Ethics

All experiments were performed in accordance with animal Care Committees of both institutions.

3. Results

3.1. Comparative infectivity of sporocysts to two strains of KO mice

There was no difference with respect to infectivity of *S. neurona* sporocysts to two strains of mice (Table 1). All mice inoculated 12 or more sporocysts died or became ill of *S. neurona* infection between 24 and 69 days p.i. (Table 1). All 8 mice inoculated aliquots from the last dilution containing fewer than two sporocysts remained asymptomatic and *S. neurona* was not demonstrable in their tissues. In general, the black mice died sooner than the white KO mice and in histological sections the neural lesions were more severe in black versus white mice. By IHC, *S. neurona* was demonstrable in brains of all infected mice, especially in the cerebellum. Additional infected tissues in each mouse by IHC are given in Table 1. It is of interest that one white mouse inoculated 125 sporocysts survived past 2 months; it developed head tilt, but otherwise appeared normal. The mouse was euthanized day 69 p.i., and *S. neurona* was found in it's lung and brain.

3.2. Effect of trypsin digestion on S. neurona merozoites

In the first trial all 24 KO mice inoculated with *S. neurona* brain homogenate incubated for 15, 30, and 60 min in saline with or without trypsin died of *S. neurona* encephalitis within 28 days p.i. and merozoites were found in cerebellums of all mice. Similar results were obtained in the repeat trial for incubation periods of 15, 30, and 60 min. However, after incubation period of 120 min, both the KO mice incubated in saline without trypsin became infected whereas only 1 of the 2 mice inoculated with homogenate incubated in trypsin became infected with *S. neurona*.

3.3. Infectivity of S. neurona bradyzoites to KO mice and cell culture

The KO mice inoculated with bradyzoite suspension remained asymptomatic and *S. neurona* was not found in their tissues. *S. neurona* was not seen in the 2 flasks seeded with *S. neurona* bradyzoites; the flasks were not contaminated with bacteria. Bradyzoites remained intact and motile after pepsin digestion.

4. Discussion

As stated in the introduction, *S. neurona* schizonts parasitize tissues of several hosts. Demonstration of schizonts and merozoites is difficult unless large numbers of organisms are present. Based on experimental studies in animals with the related coccidian, *Toxoplasma gondii*, it is difficult to demonstrate organisms in histologic sections unless 100,000 organisms are present per gram of tissue (Dubey, 2010). Therefore, bioassays (in cell culture or mice) are often used to recover *T. gondii* from infected tissues, because bioassay can detect low concentration of organisms. Digestion of animal tissues with proteolytic enzymes (trypsin, pepsin) for 1–2 h is often used to free *T. gondii* from animal tissues (Dubey, 2010). Pepsin digestion kills tachyzoites, merozoites but not the bradyzoites. Trypsin digestion does not kill *T. gondii* tachyzoites and bradyzoites. *S. neurona* and *T. gondii* are related coccidians but the effect of trypsin digestion on *Sarcocystis* merozoites was unknown. Results of the present study demonstrated that *S. neurona* merozoites also survive trypsin digestion, and the method could be used to recover merozoites for bioassay in mice or cell culture. These results suggest loss of infectivity of merozoites after 120 min trypsin incubation. Therefore, incubation in trypsin for 60 min is recommended for recovery of *S. neurona* merozoites from tissues

Our results indicate that morphologically intact bradyzoites of *S. neurona* can be obtained by digestion in pepsin (with or without NaCl). *Sarcocystis* species bradyzoites can be obtained free of host material by short incubation in pepsin (without NaCl), and by Percoll flotation (Dubey et al., 1989). In the present study the bradyzoites obtained by pepsin digestion were intact and motile, but were not infective to mice or cell culture. The observation made in this study supports the obligatory two-host life cycle of *S. neurona.*

In the present study, infectivity of *S. neurona* to two strains of KO mice was similar but, the C57-derived mice developed clinical signs sooner than BALB/c-derived mice, and results were confirmed by histopathological observations. Therefore we use C57-strain derived KO mice for *S. neurona* infection studies. The lack of infectivity of low numbers of sporocysts was probably related to the storage/dilution effect of sporocysts for 8 months before use in the present.

References

Britton, A.P., Dubey, J.P., Rosenthal, B.M., 2010. Rhinitis and disseminated disease in a ferret (*Mustela putorius furo*) naturally infected with *Sarcocystis neurona*. Vet. Parasitol. 169, 226–231.

Cheadle, M.A., Tanhauser, S.M., Dame, J.B., Sellon, D.C., Hines, M., Ginn, P.E., MacKay, R.J., Greiner, E.C., 2001a. The nine-banded armadillo (*Dasypus novemcinctus*) is an intermediate host for *Sarcocystis neurona*. Int. J. Parasitol. 31, 330–335.

Cheadle, M.A., Yowell, C.A., Sellon, D.C., Hines, M., Ginn, P.E., Marsh, A.E., Dame, J.B., Greiner, E.C., 2001b. The striped skunk (*Mephitis mephitis*) is an intermediate host for *Sarcocystis neurona*. Int. J. Parasitol. 31, 843–849.

Cheadle, M.A., Ginn, P.E., Lindsay, D.S., Greiner, E.C., 2002. Neurologic disease in gamma-interferon gene knockout mice caused by *Sarcocystis neurona* sporocysts collected from opossums fed armadillo muscle. Vet. Parasitol. 103, 65–69.

Dubey, J.P., Speer, C.A., Fayer, R., 1989. Sarcocystosis of Animals and Man. CRC Press Inc., Boca Raton, Florida, pp. 215.

Dubey, J.P., Mattson, D.E., Speer, C.A., Baker, R.J., Mulrooney, D.M., Tornquist, S.J., Hamir, A.N., Gerros, T.C., 1999. Characterization of *Sarcocystis neurona* isolate (SN6) from a naturally infected horse from Oregon. J. Eukaryot. Microbiol. 46, 500–506.

Dubey, J.P., Hamir, A.N., 2000. Immunohistochemical confirmation of *Sarcocystis neurona* infections in raccoons, mink, cat, skunk and pony. J. Parasitol. 86, 1150–1152.

Dubey, J.P., 2001. Migration and development of *Sarcocystis neurona* in tissues of interferon gamma knockout mice fed sporocysts from a naturally infected opossums. Vet. Parasitol. 95, 341–351.

Dubey, J.P., Lindsay, D.S., Saville, W.J.A., Reed, S.M., Granstrom, D.E., Speer, C.A., 2001a. A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). Vet. Parasitol. 95, 89–131.

Dubey, J.P., Rosypal, A.C., Rosenthal, B.M., Thomas, N.J., Lindsay, D.S., Stanek, J.F., Reed, S.M., Saville, W.J.A., 2001b. *Sarcocystis neurona* infections in sea otter (*Enhydra lutris*): evidence for natural infections with sarcocysts and transmission of infection to opossums (*Didelphis virginiana*). J. Parasitol. 87, 1387–1393.

Dubey, J.P., Saville, W.J.A., Stanek, J.F., Lindsay, D.S., Rosenthal, B.M., Oglesbee, M.J., Rosypal, A.C., Njoku, C.J., Stich, R.W., Kwok, O.C.H., Shen, S.K., Hamir, A.N., Reed, S.M., 2001c. *Sarcocystis neurona* infections in raccoons (*Procyon lotor*): evidence for natural infection with sarcocysts, transmission of infection to opossums (*Didelphis virginiana*), and experimental induction of neurologic disease in raccoons. Vet. Parasitol. 100, 117–119.

Dubey, J.P., Fritz, D., Lindsay, D.S., Shen, S.K., Kwok, O.C.H., Thompson, K.C., 2001d. Diclazuril preventive therapy of gamma interferon knockout mice fed *Sarcocystis neurona* sporocysts. Vet. Parasitol. 94, 257–263.

Dubey, J.P., Lindsay, D.S., Kwok, O.C.H., Shen, S.K., 2001e. The gamma interferon knockout mouse model for *Sarcocystis neurona*: comparison of infectivity of sporocysts and merozoites and routes of inoculation. J. Parasitol. 87, 1171–1173.

Dubey, J.P., 2010. Toxoplasmosis of Animals and Humans, 2nd ed. CRC Press, Boca Raton, FL, pp. 1–313.

Elitsur, E., Marsh, A.E., Reed, S.M., Dubey, J.P., Oglesbee, M.J., Murphy, J.E., Saville, W.J.A., 2007. Early migration of *Sarcocystis neurona* in ponies fed sporocysts. J. Parasitol. 93, 1222–1225.

Gautam, A., Dubey, J.P., Saville, W.J., Howe, D.K., 2011. The SnSAG merozoite surface antigens of *Sarcocystis neurona* are expressed differentially during the bradyzoite and sporozoite life cycle stages. Vet. Parasitol. 183, 37–42.

Lindsay, D.S., Dubey, J.P., 2001. Determination of the activity of pyrantel tartrate against *Sarcocystis neurona* in gamma-interferon gene knockout mice. Vet. Parasitol. 97, 141–144.

Lindsay, D.S., Mitchell, S.M., Vianna, M.C., Dubey, J.P., 2004. *Sarcocystis neurona* (protozoa: apicomplexa): description of oocysts, sporocysts, sporozoites, excystation, and early development. J. Parasitol. 90, 461–465.

Marsh, A.E., Barr, B.C., Lakritz, J., Nordhausen, R., Madigan, J.E., Conrad, P.A., 1997. Experimental infection of nude mice as a model for *Sarcocystis neurona*-associated encephalitis. Parasitol. Res. 83, 706–711.

Rosypal, A.C., Lindsay, D.S., Duncan, R., Ahmed, S.A., Zajac, A.M., Dubey, J.P., 2002. Mice lacking the gene for inducible or endothelial nitric oxide are resistant to sporocyst induced *Sarcocystis neurona* infections. Vet. Parasitol. 103, 315–321.

Sellon, D.C., Dubey, J.P., 2007. Equine protozoal myeloencephalitis. In: Sellon, D.C., Long, M.T. (Eds.), Equine Infectious Diseases. Saunders-Elsevier, Philadelphia, pp. 453–464.

Sofaly, C.D., Reed, S.M., Gordon, J.C., Dubey, J.P., Oglesbee, M.J., Njoku, C.J., Grover, D.L., Saville, W.J.A., 2002. Experimental induction of equine protozoal myeloencephalitis (EPM) in the horse: effect of *Sarcocystis neurona* sporocyst inoculation dose on the development of clinical neurologic disease. J. Parasitol. 88, 1164–1170.

Stanek, J.F., Dubey, J.P., Oglesbee, M.J., Reed, S.M., Lindsay, D.S., Capitini, L.A., Njoku, C.J., Vittitow, K.J., Saville, W.J.A., 2002. Life cycle of *Sarcocystis neurona* in its natural intermediate host, the racoon, *Procyon lotor*. J. Parasitol. 88, 1151–1158.

Witonsky, S.G., Gogal, R.M., Duncan, R.B., Lindsay, D.S., 2003. Protective immune response to experimental infection with *Sarcocystis neurona* in C57BL/6 mice. J. Parasitol. 89, 924–931.