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The SnSAG merozoite surface antigens of *Sarcocystis neurona* are expressed differentially during the bradyzoite and sporozoite life cycle stages

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ABSTRACT

Sarcocystis neurona is a two-host coccidian parasite whose complex life cycle progresses through multiple developmental stages differing at morphological and molecular levels. The *S. neurona* merozoite surface is covered by multiple, related glycosylphosphatidylinositol-linked proteins, which are orthologous to the surface antigen (SAG)/SAG1-related sequence (SRS) gene family of *Toxoplasma gondii*. Expression of the SAG/SRS proteins in *T. gondii* and another related parasite *Neospora caninum* is life-cycle stage specific and seems necessary for parasite transmission and persistence of infection. In the present study, the expression of *S. neurona* merozoite surface antigens (SnSAGs) was evaluated in the sporozoite and bradyzoite stages. Western blot analysis was used to compare SnSAG expression in merozoites versus sporozoites, while immunocytochemistry was performed to examine expression of the SnSAGs in merozoites versus bradyzoites. These analyses revealed that SnSAG2, SnSAG3 and SnSAG4 are expressed in sporozoites, while SnSAG5 was appeared to be downregulated in this life cycle stage. In *S. neurona* bradyzoites, it was found that SnSAG2, SnSAG3, SnSAG4 and SnSAG5 were either absent or expression was greatly reduced. As shown for *T. gondii*, stage-specific expression of the SnSAGs may be important for the parasite to progress through its developmental stages and complete its life cycle successfully. Thus, it is possible that the SAG switching mechanism by these parasites could be exploited as a point of intervention. As well, the alterations in surface antigen expression during different life cycle stages may need to be considered when designing prospective approaches for protective vaccination.

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 causing an estimated annual loss of more than 100 million dollars in the United States (Dubey et al., 2001b). In addition to causing problems in horses, neurologic disease in sea mammals has been attributed to *S. neurona* (Rosonke et al., 1999; Lindsay et al., 2000; Dubey et al., 2001c; Miller et al., 2001; Thomas et al., 2007). The natural life cycle of this parasite alternates between the definitive host, the opossum (Fenger et al., 1995), and small mammal intermediate hosts such as skunks (Cheadle et al., 2001b), raccoons (Dubey et al., 2001d), armadillos (Cheadle et al., 2001a), sea otters (Dubey et al., 2001c), and cats (Dubey et al., 2000). Sporulated oocysts containing infectious sporozoites are a product of sexual reproduction, which takes place in

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the intestine of the opossum definitive host. This environmentally resistant cyst stage is shed in the opossum feces and serves as the source of infection for intermediate hosts. Asexual reproduction of *S. neurona* in the intermediate hosts is characterized by two developmental stages called merozoites and bradyzoites. Merozoites propagate rapidly in a variety of cell types through a process called endopolygeny, while bradyzoites are a much slower-growing stage that form sarcocysts, most commonly in muscle tissue (Dubey et al., 2001a; Speer and Dubey, 2001)

The cell surface of *S. neurona* merozoites is covered with an array of paralogous glycosylphosphatidylinositol (GPI)-anchored surface antigens called SnSAGs (Howe et al., 2005). These surface proteins were identified based on their homology to the gene family of TgSAGs and SAG1-related sequences (SRSs) in the related parasite *Toxoplasma gondii* (Lekutis et al., 2001). A total of six SnSAGs have been described in *S. neurona*, with only a subset of these SnSAGs expressed by individual parasite strains (Howe et al., 2005; Crowdus et al., 2008; Wendte et al., 2010). In *T. gondii*, a search of the parasite genome has revealed a superfamily of 161 SAG/SRS proteins (Jung et al., 2004). The functional role of these surface antigens has not been fully defined, but there is evidence to suggest that the SAG proteins are involved in host cell invasion, immune modulation and/or virulence attenuation (Mineo and Kasper, 1994; Grimwood and Smith, 1996; Kim and Boothroyd, 2005; Saeij et al., 2008).

Stage-specific expression of the SAG/SRS proteins has been observed in both *T. gondii* and *Neospora caninum* (Tomavo et al., 1991; Odberg-Ferragut et al., 1996; Fuchs et al., 1998; Knoll and Boothroyd, 1998; Schares et al., 1999; Lekutis et al., 2000; Radke et al., 2004; Fernandez-Garcia et al., 2006; Risco-Castillo et al., 2007; Saeij et al., 2008). It has been proposed that the tachyzoite-specific SAGs are involved in regulation of virulence and elicitation of immune response to give rise to an acute infection, while the bradyzoite-specific SAG molecules may be important for immune evasion and persistence of a chronic infection (Kim and Boothroyd, 2005; Saeij et al., 2008). Less is known of SAG/SRS gene family members expressed during the sporozoite stage. In the current study, we have examined the *S. neurona* SnSAGs that have been identified in merozoites to determine whether these proteins are expressed constitutively or in a stage specific manner. Our analyses demonstrated that these merozoite surface antigens are differentially expressed in the bradyzoite and sporozoite stages of *S. neurona*, consistent with the findings for other coccidians.

2. Materials and methods

2.1. Parasites

S. neurona strain SN138 (Lindsay et al., 2004), which is an independent culture derived from the SN-37R strain (Sofaly et al., 2002), was maintained in bovine turbinate cells, and extracellular merozoites were harvested as described previously (Howe et al., 2005). The SN-37R strain and SN138 sub-strain lack the *SnSAG1* gene and instead express SnSAG5 (Crowdus et al., 2008; Howe et al., 2008). Strain SN-37R sporozoites were produced previously in laboratory-reared opossums (Sofaly et al., 2002). Bradyzoites were recovered from muscles of a raccoon euthanized 3 months after oral inoculation with strain SN-37R sporocysts (Sofaly et al., 2002). Muscle tissue was ground briefly in a blender, and pre-warmed acid-pepsin solution was added and incubated at 37 °C for 10 min with shaking, as described previously (Dubey et al., 1989). The muscle homogenate was centrifuged, washed with saline solution, centrifuged again, and the supernatant and muscle layer were discarded.

2.2. Western blot analysis

Merozoites and sporozoites were subjected to lysis in SDS sample buffer supplemented with 2-mercaptoethanol and a protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, and sodium EDTA (Sigma, St. Louis, MO, USA). For sporozoite protein, 1.5 ml of sporocysts (Sofaly et al., 2002) was pelleted at 5000 rpm for 15 min. The pellet was resuspended in 500 μl of sodium dodecyl sulfate (SDS) sample buffer, and homogenized for 2 min using a Heidolph homogenizer (Sigma–Aldrich). Antigen equaling 5×10^4 culture-derived merozoites per lane was separated on 12% polyacrylamide gels (Laemmli, 1970). The approximate equal amount of sporozoite antigen was determined empirically based on the amount of parasite actin detected by Western blot in the two life cycle stages. The separated proteins were transferred to nitrocellulose membranes by semi-dry electrophoretic transfer in Tris–glycine buffer (pH 8.3). The membranes were blocked for 30 min with phosphate-buffered saline (PBS) containing 5% non-fat dry milk (NFDM), 5% normal goat serum (NGS), and 0.05% Tween 20, followed by primary antibody incubation for 1 h in PBS containing 0.1% NGS, 0.1% NFDM, and 0.05% Triton X-114. Primary antibodies were rabbit monospecific polyclonal antisera that had been produced previously against each recombinant SnSAG (Howe et al., 2005; Crowdus et al., 2008). After multiple washes, the membranes were incubated with peroxidase-conjugated, goat anti-rabbit immunoglobulin G secondary antibody (Jackson Immunoresearch Labs, Inc., West Grove, PA, USA) for 1 h. The membranes were then washed and incubated with Supersignal substrate (Pierce, Rockford, IL, USA) for chemiluminescence detection, and visualized with a FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA, USA).

2.3. Immunocytochemistry

For examination of merozoites, culture-derived parasites were harvested and diluted in PBS. A drop of the parasite suspension was smeared and air dried on electrostatically treated Superfrost Plus slides (Fisher Scientific). The slides were fixed for 10 min in cold acetone kept at −20 °C, followed by washing in deionized H_2O (dH_2O). For examination of bradyzoites, the organisms recovered from raccoon muscle (described above) were smeared on IFA or salinized slides and fixed with cold methanol. The slides were washed in dH_2O for 1 min and then subjected to