Evidence that Surface Proteins Sn14 and Sn16 of Sarcocystis neurona Merozoites Are Involved in Infection and Immunity†

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Received 7 July 1997/Returned for modification 26 August 1997/Accepted 29 January 1998

Sarcocystis neurona is the etiologic agent of equine protozoal myeloencephalitis (EPM). Based on an analysis of 25,000 equine serum and cerebrospinal fluid (CSF) samples, including samples from horses with neurologic signs typical of EPM or with histologically or parasitologically confirmed EPM, four major immunoblot band patterns have been identified. Twenty-three serum and CSF samples representing each of the four immunoblot patterns were selected from 220 samples from horses with neurologic signs resembling EPM and examined for inhibitory effects on the infectivity of S. neurona by an in vitro neutralization assay. A high correlation between immunoblot band pattern and neutralizing activity was detected. Two proteins, Sn14 and Sn16 (14 and 16 kDa, respectively), appeared to be important for in vitro infection. A combination of the results of surface protein labeling, immunoprecipitation, Western blotting, and trypsin digestion suggests that these molecules are surface proteins and may be useful components of a vaccine against S. neurona infection. Although S. neurona is an obligate intracellular parasite, it is potentially a target for specific antibodies which may lyse merozoites via complement or inhibit their attachment and penetration to host cells.

The apicomplexan Sarcocystis neurona is the causative agent of equine protozoal myeloencephalitis (14), a progressive disease affecting the central nervous system (4, 7, 13). Cases of equine protozoal myeloencephalitis (EPM) have been reported among native horses in North, Central, and South America (3, 10, 11, 15, 16, 26). Serological testing based on immunoblot patterns in Kentucky, Ohio, Pennsylvania, and Oregon detected an average S. neurona exposure rate of 45% (5, 6, 18, 32). The New York State Veterinary College at Cornell University reported that 25% of equine neurologic disease accessions were due to EPM in 1978 (19). The number of cases diagnosed at necropsy at the Livestock Disease Diagnostic Center at the University of Kentucky increased from approximately 8% of all neurological accessions during 1988 to 1990 to 15% during 1991 to 1992 (19).

Although no successful vaccine against related apicomplexan parasites has been widely used, there are encouraging signs that such a vaccine is possible. Surface antigens of coccidia have been shown to be involved in interactions with the host cell membrane during invasion (9, 24), and apical complex proteins of some coccidia have been found to be targets of protective antibodies (24, 28, 33, 34). Apical complex organelles of sporozoites appear to secrete their contents during host cell attachment and formation of the parasitophorous vacuole (21, 30, 35).

Although the pathogenesis of EPM is not fully known, the following events are believed to occur. S. neurona sporozoites penetrate the horse’s intestinal tract, enter vascular endothelial cells, and complete at least one merogonous generation. As immune responses, including antibody production are induced, merozoites may pass through the vascular endothelium of the blood-brain barrier into the immune privileged central nervous system, where they survive. The high rate of exposure to S. neurona and the relatively low incidence of clinical EPM indicate that most horses develop effective immunity that may prevent entry into the central nervous system (5, 6, 18, 32).

Since 1991, approximately 25,000 equine serum and cerebrospinal fluid (CSF) samples, including samples from horses with neurologic signs and with histologically or parasitologically confirmed EPM, have been tested for specific antibody to S. neurona at the University of Kentucky. Four immunoblot band patterns could be consistently identified in these samples. The objective of this study was to attempt to correlate immunoblot band patterns with in vitro neutralizing activity of the serum and CSF. Twenty-three serum and CSF samples, each from a different horse and representative of each of the four band patterns, were selected from a set of samples from 220 horses with a clinical diagnosis of a neurologic disorder resembling EPM and tested for inhibitory activities on parasite infection by an in vitro neutralization assay. Antibodies to two surface polypeptides were correlated with in vitro neutralizing activity.

MATERIALS AND METHODS

Parasite. S. neurona SN3 was originally isolated from the spinal cord of a horse with histologically confirmed EPM (16).

Cell and tissue culture medium. Bovine turbinate (BT) cells were purchased from the American Type Culture Collection (Rockville, Md.). Cells were seeded in 75- or 25-cm² tissue culture flasks (Corning Inc., Corning, N.Y.) and incubated in an atmosphere containing 5% CO₂ and 95% air at 37°C. The cell culture was maintained in RPMI 1640 supplemented with 15% fetal calf serum (FCS), 2 mM sodium pyruvate, 0.075% (wt/vol) sodium bicarbonate, 120 U of penicillin per ml, and 120 μg of streptomycin (BioWhittaker, Walkersville, Md.) per ml. Subconfluent cell culture was used in all of the experiments.

Clinical samples of serum and CSF. Twenty-three serum and CSF samples from different horses were selected to represent each immunoblot pattern from a group of samples from 220 horses with a clinical diagnosis of a neurologic disorder resembling EPM. These samples were originally submitted to our laboratory for serological testing for EPM from throughout the United States.

Immunoblotting. Immunoblotting was performed as previously described (17). Approximately 1.5 × 10⁷ S. neurona merozoites were harvested from BT cell culture and dissolved in an appropriate volume of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (65 mM Tris, 2% SDS, 10% glycerol, 1.5% 2-mercaptoethanol [pH 6.8]). After heating in a boiling water bath for 5 min, the sample was separated in an SDS–10% to 20% linear gradient
polycrylamide gel with a thickness of 0.75 mm, using a discontinuous buffer system (25). Separated proteins were electrotransferred to nitrocellulose (NC; Costar Co., Cambridge, Mass.) in Towbin transfer buffer (37). The blot was blocked in 5% nonfat dry milk and 0.4% Tween 20 in phosphate-buffered saline (PBS; pH 7.2) and then placed in a Miniblotter 45 (Immunetics, Cambridge, Mass.). Serum or CSF diluted 1:10 or 1:2, respectively, with blocking solution, was applied. Biotinylated goat anti-horse immunoglobulin G, followed by streptavidin-peroxidase conjugate (Pierce, Rockford, Ill.) and aminoethyl carbazole-hydrogen peroxide, was added to develop the blot.

Neutralization assay. Serum and CSF samples were filtered through a 0.22-μm pore-size syringe filters (Milan Separations Inc., Westborough, Mass.). FCS was used as a control. Approximately 3.3 × 10^7 S. neurona merozoites freshly isolated from BT cell culture were resuspended in 1.0 ml of serum or CSF and incubated at 37°C for 60 min with occasional shaking. The merozoites were then pelleted by centrifugation at 300 × g for 5 min at 37°C. Each of the treated merozoite samples was seeded into three 25-cm² tissue culture flasks. Two days postinfection, merozoites remaining in the medium were removed and fresh medium was added. On day 5, schizonts in 10 fields (400×) of each flask were counted under a phase-contrast microscope. Merozoites were recovered and counted in a hemacytometer on day 7.

Surface protein labeling. About 8 × 10^7 merozoites freshly harvested from BT culture were washed twice with excess volumes of Na_2CO_3 buffer (50 mM Na_2CO_3, 0.85% NaCl [pH 7.4]) by centrifugation at 300 × g for 10 min at 37°C. The organisms were then resuspended in 1 ml of Na_2CO_3 buffer containing 100 μg Immunopure Sulfo-NHS-Biotin (Pierce), incubated at 37°C for 10 min, and then washed twice in excess volumes of Na_2CO_3 buffer at 4°C. The biotin-labeled merozoites were lysed in 1 ml of lysis buffer (50 mM Tris, 1% Triton X-100, 0.1% SDS, 1 mM EDTA [pH 7.6]) at 4°C for 30 min. The lysate was collected by centrifugation at 3,000 × g for 30 min.

Immunoprecipitation. Preparations for immunoprecipitation were conducted at 4°C. Aliquots of lysate from 8 × 10^7 biotin-labeled merozoites were mixed with 150 μl of positive serum from horse with a histologically diagnosed case of EPM (Fig. 1). Nineteen serum and four CSF samples selected from 220 clinical samples were grouped according to their band patterns (Fig. 1 and 2): group 1, N1 to N7; group 2, 31 to 34; group 3, 41 to 46; and group 4, 51 to 56. Serum or CSF samples of group 1 were not reactive with Sn16, Sn14, or Sn11; sera from groups 2, 3, and 4 consistently reacted with Sn16, Sn14, and Sn11, respectively.

RESULTS

Immunoblot band patterns. The four immunoblot band patterns based on combinations of the Sn30, -16, -14, and -11 proteins (30, 16, 14, and 11 kDa, respectively) are presented (Fig. 1). Nineteen serum and four CSF samples selected from 220 clinical samples were grouped according to their band patterns (Fig. 1 and 2): group 1, N1 to N7; group 2, 31 to 34; group 3, 41 to 46; and group 4, 51 to 56. Serum or CSF samples of group 1 were not reactive with Sn16, Sn14, or Sn11; sera from groups 2, 3, and 4 consistently reacted with Sn16, Sn14, and Sn11, respectively.

Correlation of band patterns with inhibitory activities. The results of neutralization assays are presented in Fig. 2. As expected, serum or CSF samples of the same group showed similar neutralizing activities. Serum and CSF samples of groups 2 and 4 showed approximately equal inhibitory activities, while group 3 sera showed the greatest inhibitory activity of the four
Based on these observations, it appears that Sn16 and Sn14 may have important roles during the initial stage of S. neurona infection and that antibody to Sn14 may be more effective in neutralization than antibody to Sn16. No inhibitory activity correlating with antibody to Sn30 was noted. Interestingly, group 4 sera that contained antibody to both Sn14 and Sn11 had neutralizing activity similar to that of group 2, suggesting that Sn11 antibody in serum may block the neutralizing effect of Sn14 antibody (Fig. 1 and 2).

**Surface protein labeling and immunoprecipitation.** A combination of surface protein labeling, immunoprecipitation, and Western blotting was conducted to determine whether Sn16 and Sn14 are surface proteins. Proteins similar in size to these two proteins were labeled (Fig. 3A, lane b), a result confirmed by immunoprecipitation (Fig. 3A, lane c). Negative serum did not precipitate any Sarcocystis protein (Fig. 3A, lane d).

**Effect of trypsin digestion.** The rapid action of trypsin suggests that these proteins were very accessible to the action of the enzyme and therefore on the cell surface. Within 1 min the Sn14 band was no longer visible, and the Sn16 band showed significantly reduced density at 5 min (Fig. 3B). The density of the Sn30 band was also reduced after 5 min. The trypsin-resistant band between Sn16 and Sn14 in Fig. 3B was recognized by only few equine sera and was apparently not a surface protein, as determined by surface labeling and immunoprecipitation (Fig. 3A). Since trypsin digestion could lyse the parasite, digestion was monitored by counting intact merozoite cells. A significant reduction in merozoite numbers was not observed until after trypsin digestion for 40 min.

**DISCUSSION**

Humoral immunity may play an essential role in clearing S. neurona merozoites at the extracellular stage. Specific antibodies may lyse the merozoites via complement, inhibit their infection by blocking attachment and penetration, or bind to surface receptors and disorder signal transductions. These results suggest that sera containing antibodies specific for Sn16 and Sn14 reduce parasite infection, probably by binding to the merozoite cell surface and blocking attachment and/or penetration. An extensive body of data is available to indicate that antibody to apicomplexan parasites is protective. Treatment of Cryptosporidium-infected immunocompromised patients with hyperimmune bovine colostrum has ameliorated or eliminated clinical symptoms (38), an effect correlated with antibodies specific for sporozoite surface proteins (12, 29, 36). Infection of...
target cells by trypomastigotes of Trypanosoma cruzi is receptor mediated and can be blocked by specific antibodies (1, 39). Yet another example is the penetration-enhancing factor of Toxoplasma gondii that has been identified by using monoclonal antibodies (34).

Detection of S. neurona infection by demonstration of reactivity of serum and CSF samples with the Sn11, Sn14, and Sn16 antigens has been extensively used as a diagnostic tool (5, 6, 32) and is sensitive (20, 21). However, the test has not yet been fully validated by studies of serum and CSF samples from cases in which S. neurona-like organisms have been detected historically and cultured. Neutralization assays revealing significant differences in inhibitory activities between the groups of serum and CSF samples with different immunoblot band patterns strongly suggest that antibodies specific for Sn14 and Sn16 have protective activity against S. neurona, at least in vitro (Fig. 1 and 2) and support the use of the immunoblot test in diagnosis of EPM. Antibodies to Sn30 are not recognized as specific since a 30-kDa antigen immunoreactive with sera from horses with EPM is found in other Sarcocystis spp.

The serum neutralization data obtained in this study were based mainly on the use of an in vitro bioassay developed in our laboratory. Although assays for other apicomplexan parasites such as Cryptosporidium (12), Plasmodium (24), and Toxoplasma (27) species have been described, this study represents the first application of such an assay to a Sarcocystis sp. Optimum inhibition required sensitization of merozites in serum or CSF for at least 40 min (data not shown), suggesting that maximum inhibition of parasite infection requires saturation with specific antibody. Although serum and CSF samples of the same band pattern group did not have equal antibody activities as estimated by immunoblotting (Fig. 1), all samples saturated their antigens under the assay conditions and gave similar inhibitions in neutralization assays (Fig. 2). This result was supported by serum dilution and time of incubation data (data not shown). Although schizont and merozoite counts under conditions of maximum inhibition were not equal for these two experiments, percent reductions in numbers of schizonts and merozoites were very similar, i.e., 84 and 92% in the serum dilution assay, compared with 81 and 84% in the incubation assay. These small differences in counts resulted from the unequal dosages of merozoites used for infection in the two assays. Reductions in schizont production by group 3 sera ranged from 78% (sample 45) to 80% (sample 42) relative to the FCS control (Fig. 2). These highly consistent results suggest that the assay was valid and that counts of schizonts and merozoites may serve as indicators of inhibitory activity.

Although S. neurona was sensitive to specific antibodies, a 10-min exposure to antisera was required to yield a significant reduction in parasite production (data not shown). This may partially explain why protective antibodies to some apicomplexan parasites are effective in vitro but not in vivo (23). Newly released parasites are exposed to serum for a shorter time in vivo, and the access of neutralization-sensitive epitopes to antibody may be limited (31). Merozoites in vivo may move more directly from cell to cell. However, in the case of EPM, disease occurs only after the merozoite passes through the vascular endothelium of the blood-brain barrier into the central nervous system, and so humoral responses may play an essential role in blocking this migration. Moreover, specific cytotoxic T cells are ineffective in attacking merozoites migrating to the central nervous system in the bloodstream.

When antibodies to Sn11 and Sn14 (group 4) coexisted, the inhibition activity of the serum was reduced to that of sera of group 2 (Fig. 2), suggesting that antibody to Sn11 blocked the effect of Sn14 antibody. Therefore, these two proteins may be located in close proximity on the merozoite surface.

The results of biotin labeling and immunoprecipitation studies (Fig. 3A) are consistent with the hypothesis that the effects of antibodies to Sn14 and Sn16 are mediated via binding to surface antigens. A combination of these techniques has been shown to be effective in the identification of specific surface antigens (8). However, since nonantigenic proteins may be coprecipitated, the results may not be definitive. The results of controlled trypsin digestion were, however, consistent with the conclusion that Sn14 and Sn16 are localized on the surface of the parasite (Fig. 3).

Although monoclonal antibodies are often used to study parasitic proteins, the sera of naturally infected animals have unique advantages in that they can provide important information on protective antigens in the natural host. The parasite may express different proteins at different stages of infection and in vitro development; and some proteins may be expressed and function essentially only in vitro. Such proteins would be inappropriate targets for vaccine development. S. neurona infection of the horse induces production of antibodies to Sn14 and Sn16, indicating that these two proteins are expressed in vivo and are strong immunogens in the horse. Clearly, they warrant further investigation as candidate antigens for inclusion in vaccines against S. neurona infection.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Grayson-Jockey Club Research Foundation and the Abercrombie Foundation.

REFERENCES


Editor: S. H. E. Kaufmann