Neuritogenic Peptides Derived from Equine Myelin P_2 Basic Protein Detect Circulating Antibodies in Ataxic Horses

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KEY WORDS: Need Keywords

ABSTRACT

Polyneuritis equi is an immune-mediated neurodegenerative condition in horses that is related to circulating demyelinating antibodies against equine myelin basic protein 2 (MP_2). The present study examined the presence of circulating demyelinating antibodies against neuritogenic peptides of MP_2 in sera from horses suspected of equine protozoal encephalomyelitis (EPM), a neurodegenerative condition in horses that may be immune-mediated. The goals of this study were to develop serum ELISA tests that may identify neuroinflammatory conditions in horses with EPM and indirectly relate the pathogenesis of inflammation to IL6 by serum C-reactive protein (CRP) concentration. Circulating antibodies against MPP, recombinant equine myelin P_2 protein (rMP2), and CRP were assayed in 52 neurologically abnormal horses by ELISA in this retrospective study. We detected antibodies against MPP (40%), rMP2 (33%), and CRP (65%) in these horses that had a presumptive diagnosis of EPM. No cross-reactivity between the antigens was observed. An evaluation of the agreement between the assays (McNemar's test) suggests as CRP values increase, the likelihood of a positive MPP ELISA also increases. Clinical signs of EPM may be due to an immune-mediated polyneuropathy that involves complex *in vivo* interactions with the IL6 pathway because MPP antibodies and elevated CRP concentrations were detected in some horses with S. neurona sarcocystosis.

INTRODUCTION

Polyneuritis equi is a neurodegenerative condition in horses that is related to circulating demyelinating antibodies against equine myelin basic protein 2 (MP_2). The clinical signs of polyneuritis equi (PE) are similar to equine protozoal myeloencephalitis (EPM).[1,2,3,4] Equine protozoal encephalomyelitis (EPM) is also a neurodegenerative condition in horses that may be due to an immune-mediated pathogenesis of disease as indicated by infection studies.[5]Muscle wasting, an abnormal gait, and paralysis/paresis of cranial nerves are signs associated with both neurodegenerative diseases. The

etiology of PE is unknown but is generally considered an immune-mediated peripheral polyneuritis. The clinical signs associated with EPM are due to inflammation associated with infections by pathogenic protozoa, in most cases *Sarcocystis neurona*. The histopathological lesions of both diseases have been described.[1,4] It was suggested that alterations in cytokine levels may contribute to neuropathology seen in horses with EPM, particularly T cell-mediated immunity.[5] It is possible that both diseases are linked by a common disease mechanism that stimulates proinflammatory pathways via the IL6 cytokine.

Clinical signs and histopathological lesions that are similar to PE can be experimentally induced in rats.[5] Induction of experimental autoimmune neuritis (EAN) in susceptible animals is accomplished by injection of MP_2 or neuritogenic peptides (MPP) derived from MP_2, or by passive transfer of lymphocytes from syngeneic animals sensitized to MP_2. The neuritogenic site for experimental induction of EAN is located on 26 amino acids of MP2. Induction of disease in rats by adoptive transfer into naive rats suggested that the antigenic site for the disease process may due to at least one T cell epitope identified on MPP.6 The neuritogenic peptide, MPP, contains a putative T cell epitope that corresponds to equine cytokine IL6. Equine IL6 shares amino acid sequence homology with residues within the EAN-inducing site of MPP.[6] Cytokine IL6 is a proinflammatory cytokine related to the pathology associated with adoptive transfer of EAN that can be inhibited by anti-IL6 *in vivo*. Levamisole HCl decreases the synthesis of IL6 in dogs and rats.[9,10] Levamisole HCl was shown to alleviate clinical signs of ataxia in horses with suspected EPM and suggests the pathogenesis of disease may be IL6 related if the mechanism of action of levamisole HCl in horses also blocks IL6 production.[6,7] Antibody reactivity in an ELISA that uses MPP as antigen is not pathogen specific, but may indicate the mechanism of disease is attributable to cytokine IL6 pro-inflammatory pathways. A BLAST (NCBI) analysis reveals that there are amino acid homologies between the neuritogenic peptide MPP contained within PE and some *S neurona* surface antigens. *Sarcocystis neurona* SAG 4 and SAG 5 share homology with MPP residues that are in the non-disease producing regions of the peptide. However, the *S neurona* SAG's 6 and 1 have sequence homology in the neuritogenic region of the MPP peptide.

Table 1. *Antigen sequence for MPP, SAG 6, SAG 1, and IL6 that are homologous to residues on equine myelin basic protein 2.*

Sequence	Name
GFKNTEISFKLGQEFEETTADN	MPP
EFEETTAD	SAG 6
FEET	SAG 1
TTAD	IL6

Cytokine IL6-dependent stimulation of the acute phase C-reactive protein (CRP) results in detectable serum CRP levels in horses.[7] The IL6 plasma buffering system in horses may preclude sensitive measure of IL6 in plasma, however, the serum CRP concentration may provide an indirect measure of IL6 activity in equine disease.[8] The purpose of this study was to develop a serum ELISA to evaluate the presence of MPP antibodies in horses and implicate the pathogenesis of disease to IL6 pro-inflammation. A serum MPP ELISA may be useful to elucidate the complex *in vivo* interactions associated with neuroinflammation present in PE and EPM.

MATERIALS AND METHODS

Antigens

A peptide (MPP) that corresponds to amino acid residues 57-78 of equine myelin protein was synthesized (United BioSystems, Herndon, VA). Recombinant myelin P2 protein (MP2) was produced in *E coli* (MyBiosource, San Diego, CA). *Sarcocystis neurona* 1, 5, 6 antigens were produced as previously described.[6] The sequence of the

common amino acids are shown in Table 1.

Sera

A positive control *S neurona* SAG 1, 5, 6 serum sample (CP) was obtained from a 12-year-old thoroughbred mare with a 5-week history of severe ataxia (grade 4). Negative control sera was pooled from 10 clinically normal yearling thoroughbred horses that did not have antibodies against S neurona.

Sera from untreated horses (n=52) that had clinical signs of ataxia with or without muscle wasting determined by veterinarians that suspected EPM were analyzed for *S neurona* antibodies and retrospectively assayed for antibodies against MP_2, MPP, and CRP. Antibodies were assayed against *S neurona* SAG 1, 5, 6, and MP_2 in 52 sera. Anti-MPP antibodies and serum concentration of C-reactive protein was determined in 49 sera. Rabbits were immunized with a synthetic 14 amino acid peptide within amino acids 17-77 of human PMP_2 protein, affinity purified, and lyophilized (Antibody Verify; Las Vegas, NV). Rabbit anti-PMP2 antibodies were diluted to a concentration of 1 mg/ml as a stock reagent. Alkaline phosphatase anti-rabbit was obtained from Sigma Aldrich (St. Louis, MO).

Assay

The ELISA was performed in 96 well (HyBind, Greiner, Monroe, NC) microtitration plates coated with approximately 0.1µ grams of protein in carbonate buffer. Plates were read on a Molecular Devices automatic plate reader. Optimum concentrations for antigen coating and the detecting antibodies were determined by standard checker-board titration.

The equine P_2 myelin protein ELISA described by Fordyce was modified by using MPP or MP_2 coated and blocked plates.6 Briefly, the plates were incubated for 40 minutes (MP_2) or 2 hours (MPP) at 37°C with diluted serum. The plates were washed and alkaline phosphatase conjugated anti-horse whole molecule (Sigma, St. Louis MO) was added and incubated for 30 minutes at 37°C. Diluted control sera binding to antigens was detected with alkaline phosphatase conjugated anti-rabbit whole molecule (Sigma, St. Louis MO). The plates were washed and reacted with 4-nitrophenyl phosphate in carbonate buffer (pH 9) for 15 and 30 minutes. A positive result was recorded if the OD was > 0.518. The reciprocal of the last dilution positive (OD 0.518 or greater at 405nm) was recorded as the titer.

The serum concentration of C-reactive

Table 2. *Results showing the reactivity of sera against antigens used in the ELISA. Rabbit α PMP2 was positive against MPP and MP2 and rSAG 1 and rSAG 6, but not rSAG 5. Control positive sera CP bound antigens MPP, MP2, rSAG's 1, 5, and 6.*

	MPP	MP_2	rSAG 1	rSAG 5	rSAG 6
Rabbit α PMP_2	pos	pos	pos	neg	pos
CP	pos	pos	pos	pos	pos

Table 3. *Raw OD 405 showing the binding of rSAG 1, 5, 6, MPP, and MP_2 by S. neurona positive sera (CP) in serial 2 fold dilutions 1:8 through 1:64, wells A-E. The raw OD 405 after reacting rSAG 1 and rSAG 6 wells with rabbit anti-PMP2 followed by ELISA using S. neurona positive control sera, CP, are shown, wells F, G, and H.*

	rSAG 1	rSAG 5	rSAG 6	MPP	rMP_2	rSAG1	rSAG5	rSAG6
	A	B	C	D	E	F	G	H
1:8	1.937	1.944	1.935	1.935	1.921	1.509	1.944	0.841
1:16	1.922	1.929	1.916	1.898	1.890	1.500	1.929	0.680
1:32	1.897	1.896	1.540	1.824	1.865	1.296	1.896	0.753
1:64	1.894	1.893	1.505	1.632	1.640	1.473	1.893	0.619

Table 4. *Results of sera tested by antigen showing the number of sera that were positive and the percent of total sera tested.*

	Number of sera	%
MP2 positive	18	33%
MPP positive	21	40%
S. neurona positive	31	60%
S. neurona SAG 1 positive	29	56%
S. neurona SAG 6 positive	25	48%
CRP positive	32	65%
MPP and MP_2 positive	7	13%
MPP positive and MP_2 negative	14	27%
MPP negative and MP_2 positive	11	21%
MPP negative and MP_2 negative	20	38%
CRP and MPP positive	12	24%
CRP positive and MPP negative	20	41%
CRP negative and MPP positive	7	14%
CRP and MPP negative	10	20%
MP_2 and *S. neurona* positive	17	33%
S. neurona negative	21	40%
SAG 6 and MPP positive	12	23%
SAG 6 positive and MPP negative	13	25%
SAG 6 negative and MPP positive	9	17%
SAG 6 and MPP negative	18	35%
SAG 1 and MPP positive	13	25%
SAG 1 positive and MPP negative	15	29%
SAG 1 negative and MPP positive	8	15%
SAG 1 and MPP negative	16	31%

protein was measured by capture ELISA as per manufacturer recommendations (Genway, San Diego CA).

RESULTS

Rabbit anti-PMP_2 antibody was positive against MPP and MP_2, and rSAG 1 and rSAG 6, but not rSAG 5 (Table 2). Control positive horse sera (CP) was obtained from an ataxic horse with a presumptive diagnosis of EPM, and was positive against MPP, MP2, rSAG's 1, 5, and 6. Negative control sera from horses without disease did not bind any of the antigens MPP, MP_2, rSAG 1, 5, or 6 (data not shown).

Rabbit anti-PMP_2 slightly blocked binding of *S neurona* positive sera to rSAG 1 and significantly blocked binding to rSAG 6 antigens (Table 3). This antibody did not block binding to rSAG 5 antigens.

The results of an indirect ELISA using rMP2, MPP, and *S neurona* SAG's as antigen are shown in Table 4. The serum concentration of C-reactive protein was determined, a positive result was a value >10 µ grams per ml (data not shown). The distribution of seropositive ataxic horses by antigen is shown in Chart 1.

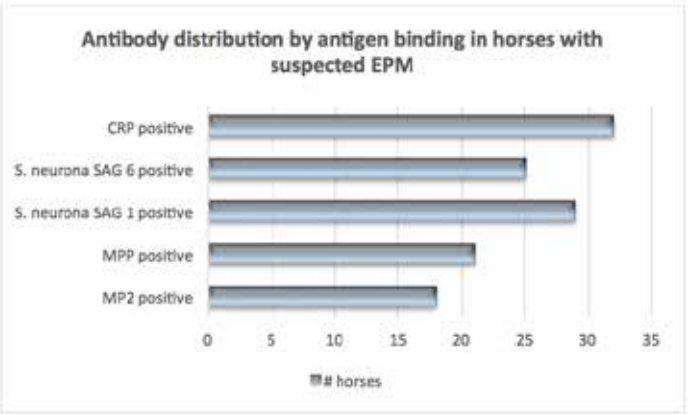
Assuming MPP or MP_2 indicate a neuropathy, a McNemar's test was used to evaluate agreement between the MPP and SAG 1, SAG 5, and SAG 6 results. For each of these comparisons, the P value is not significant, indicating a lack of agreement/lack of cross-reactivity between these antigens. There was no statistically significant relationship between MPP and CRP concentration, however the comparison suggests as CRP values increase, the likelihood of a positive MPP ELISA also increases, Chart 2.

DISCUSSION

The neuritogenic peptide MPP may be useful to detect IL 6-mediated neurological dysfunction in horses with suspected EPM.

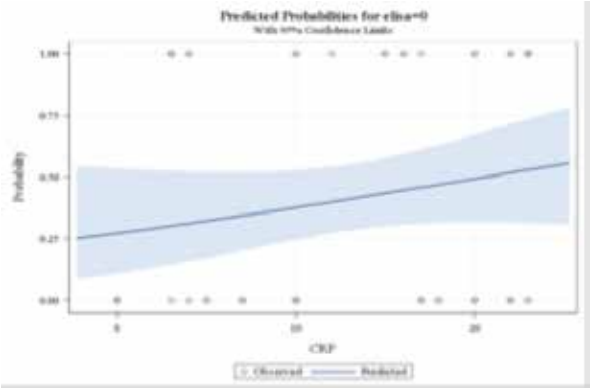
Circulating antibodies against MP_2 and the neuritogenic peptide MPP of MP_2 were detected in 32 of 52 horses with suspected EPM in this study. Not all horses were positive for antibodies against both myelin proteins (Table 4). Continued stimulation with whole myelin protein induces disease tolerance in rats without loss of activity against the neuritogenic peptide MPP. Therefore, chronically diseased animals may be MP_2 negative/MPP positive on antibody tests.5 It was not possible to correlate duration of disease in these horses because onset of disease was not clearly identified.

Chart 1. *Antibody distribution by antigen binding in horses with suspected EPM.*



A diagnostic test that detects MP_2 antibodies in horses with polyneuritis was suggested by Fordyce.[2] Not surprisingly, anti-MP_2 antibody titer was not a useful prognosticator of disease outcome in the Fordyce study. Individual responses to immune stimulation were observed in experimentally induced autoimmune neuritis in rats.[5]

Chart 2. *The relationship between MPP and CRP serum concentration is charted. The line predicting the probablity of an MPP seropositive result indicates that as the serum concentration of CRP increases there is a highr probablity of a positive MPP test indicating that neuroinflammation (MPP positive) may be related to (IL6-induced) CRP.*



Sarcocystis neurona challenged horses also produce antibodies when challenged, however, antibody levels are not directly related to clinical signs or prognosis. It was reported that as clinical disease progressed, the serum titer increased in *S neurona* induced infections.[12] Individual responses to infections are possible with *S neurona* infections and an absence of circulating antibodies can be due to the variable expression of *S neurona* surface antigens.[12,13,14]

The MPP peptide contains a putative T cell epitope that allowed adoptive transfer of experimental neuritis in rats. Homology with equine IL6 amino acids at MPP residues 74-77 may implicate IL6 as a T cell marker for the disease inducing cells in horses. The cytokine IL6 induces the production of CRP by the liver. We found no statistically significant correlation (95% confidence) between MPP seropositive samples and serum CRP concentration in this group of horses. However, the analysis suggested a linear relationship.

In this study, as CRP value increased, the probability that the sample is MPP seropositive increased, Chart 2. Antibodies (against MPP) that cross-react or bind IL6 may be expected to reduce

the cytokine dependent production of CRP. That was not seen. Cytokine IL6 is a multifunctional cytokine that regulates pleiotropic roles in inflammation.16 The soluble IL6 receptor (sIL6r) is an IL6 agonist, and the control of many IL6 mediated events are regulated via sIL6r. Possibly CRP affects IL6 mediated inflammatory events by releasing sIL6r into the plasma.[7] If this is true, one would expect to measure IL6 in the plasma in acute disease. The elevated CRP values in the MPP seropositive horses may indicate that the neuritis is acute, and is mediated through the IL6 cytokine pathways. However, we did not measure IL6 in this study. A sustained elevation in anti-MPP in a horse that is CRP negative may indicate a chronic neuritis as seen in the hyper-immunized EAN rats.

The EPM-suspect horses were seropositive against antibodies to *S neurona* SAG 1, 5, or 6. There is amino acid sequence homology between *S neurona* SAG's 1, 4, 5, and 6 and MPP.[6] In order to investigate the proteomics of SAG proteins in the neuroinflammatory disease process, we identified sequence homology between *S neurona* SAG's and the neuritogenic peptide MPP and MP_2. We determined the binding of equine sera to the MP_2 and MPP proteins and the relationship to SAG 1, 5, and 6. Control horse sera was seropositive for all the proteins used in this study. A monospecific polyclonal antibody against amino acid residues was specific to MP_2 bound MPP, rSAG 1, and rSAG 6, but not rSAG 5 antigens. Based on structural and antibody binding data, anti-PMP_2 antibodies presumably reacted with the neuritogenic peptide MPP and not the non-disease producing residues of the equine myelin basic protein 2.

We showed that anti-PMP_2 could partially block binding of CP sera to rSAG 1 and rSAG 6, but not rSAG 5. Blocking the binding of CP sera to antigens that had homology in the disease-producing regions of MPP, but not the antigens with homology to the non-disease producing regions of MPP, may support that anti-PMP_2 binds the active disease producing residues of the antigen. The anti-PMP_2 sera did not completely block binding of CP sera to MPP, suggesting anti-PMP_2 does not bind the same regions on MPP as the sera from a diseased horse but may react with multiple epitopes of MPP that include the non-disease producing residues of MPP or additional antigens of MP_2.

Anti-PMP_2 antibody did not completely block CP binding to rSAG 6, indicating that the antibodies anti-PMP_2 and CP bind different epitopes of rSAG 6 that may include the neuritogenic region of MPP at residues EFEETTAD in this assay. The test results using sera from these clinically ill horses were evaluated by McNemar's test and showed insignificance between MPP or MP2 ELISA and SAG 1, 5, or 6 ELISA test results. The lack of agreement between The ELISA tests indicate the antigens *S neurona* SAG 1, 5, or 6 and MPP are not cross-reactive.

IL6-mediated disease can be assayed using MPP and MP_2. Assays that use MPP and MP_2 may be useful to elucidate the pathogenesis of disease associated with EPM, but not the etiology. Possibly, early infections with *S neurona* induces peripheral polyneuritis in horses that confounds a diagnosis of EPM. It is possible that the proteomics of parasites antigens facilitate inflammatory disease. However, the SAG 1, 5, 6 ELISA can indicate the etiology is due to *S neurona* because these assays measure different antigens. If the inflammatory component of EPM is IL6-mediated, blocking IL6 is a viable therapeutic strategy for diseased horses.

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